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VINEGAR BIOTECHNOLOGY:
NEW TOOLS TO SUPPORT THE INDUSTRIAL RESEARCH

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Ai miei nonni

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Abstract

Acetic acid bacteria (AAB) are versatile organisms converting a number of carbon sources into biomolecules of industrial interest. Such properties make them suitable organisms for food, chemical, medical, pharmaceutical and engineering applications. At current, well-established bioprocesses by AAB are those derived from the oxidative pathway that lead to organic acids synthesis, such as acetic acid and gluconic acid and ketones. Currently, foods and beverages with healthy and functional properties, especially those that claim to prevent chronic diseases, are receiving more and more interest. Vinegar and non-alcoholic fermented beverages have a high potential for growth because is a part of products with enhanced properties. Currently consumers' demand for a healthier and more sustainable diet including fermented beverages and vinegar from lesser known fruits and vegetables. Moreover, grains health-based recommendations include reducing consumption of calories from added sugars, alcohol and foods that contain refined grains with high amounts of added sugars and sodium. Fruits are optimal substrates for microbial transformations intended to produce non-alcoholic beverages and vinegars, due to their high amount of sugar and fermentative feasibility. To contribute to reducing food loss and satisfying new market demand new opportunities open up a great interest in the valorisation of unconventional fruits for non-alcoholic beverages and vinegars. Yeasts and AAB are cooperative microorganisms which, through selective fermentations, can promote the valorisation of fruits by alcoholic fermentation followed by acetic fermentation for producing vinegar and low acetic beverages. Furthermore, AAB through glucose oxidation can provide gluconic acid. This latter metabolic pathway can be exploited for producing new non-alcoholic beverages. The production of both acetic and gluconic acid is a common feature of AAB belonging to *Acetobacter*, *Gluconacetobacter* and *Komagataeibacter* genera.

The research developed in the frame of this PhD project included two main activities: build up the state of the art on fermented vinegars produced from non-conventional raw materials, and evaluate fermentation capacity of fruits and qualitative characteristics of the products obtained. Laboratory and prototypal experiments were conducted at UMCC (Unimore Microbial Culture Collection) laboratory and Ponti S.p.A. Company, respectively. Date juice was chosen as raw material to develop controlled fermentations aimed at producing acetic beverages and vinegars at different acetic acid content, both in static and submerged systems. The production of acetic acid was satisfactory with the ZJ 555 strain (*K. europaeus* species), reaching values of 58.605 g/L and with the DL 13 strain (*A. pasteurianus* species), which produced 59.19 g/L of acetic acid. Moreover, the production of 61.667 g/L of gluconic acid was reached with the bacterial strain ATCC 621H of the *Gluconobacter oxydans* species.

Another interesting part of the research included the evaluation of currant, blackberry and raspberry juices regarding their aptitude to undergo a gluconic fermentation. Blackberry juice (10 and 25°Brix) and raspberry juice (25°Brix) were found to be particularly efficient in the production of gluconic acid. Parameters and conditions have been identified that strongly influence the production of gluconic acid in the various juices, including the initial acidity values, the Brix degrees of the starting juice and the amount of glucose present. These outputs offer the chance to valorise juices by selective fermentations, contributing to development of sustainable bioprocesses and obtaining non-alcoholic drinks with a low acid content. Furthermore, total phenolic compounds and antioxidant activity of vinegar samples produced from juices and fruits was evaluated in order to acquire information on the influence of the kinetics / fermentation regime. Finally, the microflora present at different conditions during industrial vinegar production was studied to the aim to correlate the fermentation parameters to the microbial species.

Key words

Acetic acid bacteria, Vinegar, Acetic acid, Gluconic Acid, Non-alcoholic beverages

Riassunto

I batteri acetici (BA) sono organismi versatili che convertono una serie di fonti di carbonio in biomolecole di interesse industriale. Tali proprietà li rendono organismi adatti per applicazioni alimentari, chimiche, mediche, farmaceutiche e ingegneristiche. Attualmente, i bioprocessi consolidati di BA sono quelli derivati dalla via ossidativa che portano alla sintesi di acidi organici, come acido acetico e acido gluconico e chetoni. Attualmente stanno riscuotendo sempre più interesse gli alimenti e le bevande con proprietà salutari e funzionali, in particolare quelle che aiutano a prevenire le malattie croniche. Tra i prodotti, l'aceto e le bevande fermentate analcoliche hanno un alto potenziale di crescita. L'attuale tendenza a produrre bevande fermentate e aceto, anche da frutta e verdura meno conosciute, si adatta alla domanda dei consumatori di una dieta più sana e sostenibile. Inoltre, le raccomandazioni sulla salute includono la riduzione del consumo di alcol, di zuccheri aggiunti e la limitazione del consumo di alimenti che contengono cereali raffinati, in particolare quelli con elevate quantità di zuccheri aggiunti e sodio. I frutti sono substrati ottimali per le trasformazioni microbiche destinate alla produzione di bevande analcoliche e aceti, grazie alla loro elevata quantità di zucchero e alla fattibilità fermentativa. Oltre alla frutta convenzionale sfruttata per bevande analcoliche e aceti, c'è grande interesse nella valorizzazione di quella non convenzionale, contribuendo ad aprire nuove opportunità per ridurre la perdita di cibo e soddisfare la nuova domanda del mercato. Lieviti e AAB sono microrganismi cooperativi che, attraverso fermentazioni selettive, possono favorire la valorizzazione dei frutti mediante fermentazione alcolica seguita dalla fermentazione acetica per la produzione di aceto e bevande a basso contenuto acetico. Inoltre i BA, in un'unica fase di fermentazione, può fornire acido gluconico dall'ossidazione del glucosio. Quest'ultima via metabolica può essere sfruttata per produrre nuove bevande analcoliche. La produzione di acido acetico e gluconico è una caratteristica comune degli BA appartenenti ai generi *Acetobacter, Gluconacetobacter e Komagataeibacter*.

La ricerca sviluppata nell'ambito di questo progetto di dottorato prevedeva due attività principali: costruire lo stato dell'arte sugli aceti fermentati prodotti da materie prime non convenzionali e valutare la capacità fermentativa dei frutti e le caratteristiche qualitative dei prodotti ottenuti. Esperimenti di laboratorio e prototipi sono stati condotti rispettivamente presso il laboratorio UMCC (UNIMORE Microbial Culture Collection) e la Società Ponti S.p.A..

Il succo di dattero è stato scelto come materia prima per sviluppare fermentazioni controllate finalizzate alla produzione di bevande acetiche e aceti a diverso contenuto di acido acetico, sia in sistemi statici che sommersi. La produzione di acido acetico è stata soddisfacente con il ceppo ZJ 555 (specie *K. europaeus*), raggiungendo valori di 58.605 g/L e con il ceppo DL 13 (specie *A.*

pasteurianus), che ha prodotto 59.19 g/L di acido acetico. Inoltre, la produzione di 61.667 g/L di acido gluconico è stata raggiunta con il ceppo batterico ATCC 621H della specie *Gluconobacter oxydans*. Un'altra parte interessante della ricerca ha riguardato la valutazione dei succhi di ribes, mora e lampone in relazione alla loro attitudine a subire una fermentazione gluconica. Il succo di mora (10 e 25°Brix) e il succo di lampone (25°Brix) sono risultati particolarmente efficaci nella produzione di acido gluconico. Sono stati individuati parametri e condizioni che influenzano fortemente la produzione di acido gluconico nei vari succhi, tra cui i valori di acidità iniziale, i gradi Brix del succo di partenza e la quantità di glucosio presente. Questi risultati offrono la possibilità di valorizzare i succhi mediante fermentazioni selettive, contribuendo allo sviluppo di bioprocessi sostenibili e ottenendo bevande analcoliche a basso contenuto di acidità. Inoltre sono stati valutati i composti fenolici totali e l'attività antiossidante di campioni di aceto prodotti da succhi e frutta al fine di acquisire informazioni sull'influenza del regime cinetico/fermentativo.
Infine, è stata studiata la microflora presente a diverse condizioni durante la produzione di aceto industriale allo scopo di correlare i parametri di fermentazione alle specie microbiche.

Parole chiave
Batteri acetici, Aceto, Acido Acetico, Acido gluconico, Bevande analcoliche

Thesis outline

This thesis work has been carried out in the frame of a collaboration between the Laboratory of Food Microbiology/UMCC (Unimore Microbial Culture Collection) of Department of Life Science, University of Modena and Reggio Emilia, and Ponti SpA, during 2019 and 2022. Laboratory and prototype experiments were conducted at UMCC (Unimore Microbial Culture Collection) laboratory and Ponti S.p.A., respectively.

The project was aimed at evaluating the fermentation capacity of fruits and qualitative characteristics of the products obtained thought selective fermentations. The fruits considered were date, blackcurrant, blackberry and raspberry. During the first year, the research was focused on build up the state of the art on vinegars produced from non-conventional raw materials. A bibliographic search on these fruits or juices, characterisation and potential transformation thought fermentation was considered. After that, a study was conducted on these raw materials for the production, evaluation of total phenolic compounds and antioxidant activity in vinegar samples produced from juices and puree. Finally, to test the suitability of the process at industrial scale, prototype trials were conducted on date to set a working protocol of fermentation.

Chapter 1.0 introduces AAB involved especially in industrial applications. This chapter will give to the readers an overview about the versatility of AAB from industrial point of view. The chapter describes metabolic pathway of AAB, genera of interest for industrial application, focusing on the importance of non-alcoholic fermented beverages and their production system.

Chapter 2.0 is focused on the current trend to produce fermented beverages and vinegar, including those from lesser-known fruits and vegetables, which fits with consumers' demand for a healthier and more sustainable diet. In this project, the first unconventional raw materials considered was date. Date is a versatile raw material rich in carbohydrates and dietary fiber. Dates have total sugars mainly represented by glucose and fructose that are main carbon substrate for alcoholic, acetic and gluconic fermentation. Moreover, the high prevalence of phenolic compounds and vitamin C is correlated to antioxidant activity from date fruits. This peculiar composition and date potential, that match with our production scope, was elaborated in a review titled *Date Fruits as Raw Material for Vinegar and Non-Alcoholic Fermented Beverages*, published in 2022 [1].

Yeasts and AAB are cooperative microorganisms which can promote the valorisation of date fruits by alcoholic fermentation followed by acetic acid fermentation. Moreover, AAB, in a single

fermentation step, can provide gluconic acid (GA) from glucose oxidation. In this study the GA production, was exploited for designing the production of new non-alcoholic beverages or low-alcoholic beverages from all the fruits considered.

Non-alcoholic beverages or low-alcoholic beverages are widely consumed in Asian countries, but they are still less known in western countries. Date juice was chosen as raw material to develop controlled fermentations aimed at producing acetic beverages and vinegars at different acetic acid content, both in static and submerged systems. At UMCC laboratory, the research work was divided in two steps: (1) Gluconic fermentation of diluted date juice to produce a non-alcoholic beverage. In this phase, a high producer strain of GA was used. After a period of adaptation to the substrate, results highlights that the strain belonging to the *Gluconobacter oxydans* species (ATCC 621H) is suitable for the production of GA. (2) Using 5 different AAB strains belonging to *Acetobacter pasterianus* and *Komagateibacter europaeus* species, standard acetic acid fermentation from diluted date alcohol was tested at laboratory scale. Results had signed up to the scientific evidence of the possibility to produce acetic beverages from date juices using selected AAB strains. To test the suitability of the process at industrial scale, prototype trials were conducted. First, the juice was fermented with a *S. cerevisiae* strain and then a static acetic acid fermentation of alcoholic date juice was performed by a mixed AAB starter culture. Then a submerged fermentation in two pilot fermenters was developed. After several tests, we set a scaling up procedure, obtaining date vinegar. Results of this study provide evidence of the feasibility of developing controlled fermentation of date juices for obtaining beverages/vinegars at different acetic acid content, both in static and submerged systems. In order to evaluate the antioxidant capacity of these products start and final concentration of phenolic compounds were tested. The effect of fermentation on phenolic content of vinegar was evaluated in different studies. It was observed a decrease during alcoholic fermentation and a subsequent increase during acetic fermentation. The concentration of total phenolic compounds, in general, did not show significant decreases following the fermentation process. A variability on the base of acidity of medium and different strain is observed. Further studies could be targeted at evaluate different fermentation conditions to identify the best combinations of process parameters that keep the initial concentration unchanged.

In **Chapter 3.0** the research included the evaluation of currant, blackberry and raspberry juices regarding their aptitude to undergo a gluconic fermentation. Initially a characterization of the raw materials was made to know their composition and the chemical parameters of interest. Gluconic fermentations were subsequently developed using *G. oxydans* (ATCC 621H). Specifically, the three different juices at two different concentrations of soluble solids, were inoculated. The results highlight

the suitability of the raw materials for the gluconic fermentation and some indications for the conduction of these types of fermentation processes. As previously, the effect of fermentation on phenolic content of vinegar was evaluated observing a constant concentration of phenolic compounds during the fermentation processes.

These outputs offer the chance to valorise juices by selective fermentations, contributing to development of sustainable bioprocesses and obtaining non-alcoholic drinks with a low acid content. Furthermore, total phenolic compounds and antioxidant activity of vinegar samples produced from juices and fruits was evaluated in order to acquire information on the influence of the kinetics / fermentation regime.

Finally, the microflora present at different conditions during industrial vinegar production was studied to the aim to correlate the fermentation parameters to the microbial species. This part of the research is still ongoing and preliminary output are presented as **Appendix A**.

Chapter 1. General introduction

1.1 Acetic acid bacteria

Acetic acid bacteria (AAB) are gram negative or gram-variable, non-spore forming bacteria, with ellipsoidal cells that can occur in single, pairs or in short chains. They could be motile due to the presence of peritrichous or polar flagella. AAB are catalase positive and oxidase negative and they have an obligate aerobic metabolism, with oxygen as the terminal electron acceptor. The pH of 5 – 6.5 is the optimum for the growth, while they can even grow at lower values. Their optimum temperature is between 25 – 30°C, although some species are recognized as thermotolerant. They can produce some pigments and also different kinds of exopolysaccharides [2]. AAB as part of the *Alphaproteobacteria*, are assigned to the order of *Rhodospirillales* within the family *Acetobacteraceae*. They are represented by the following genera: *Acetobacter, Acidomonas, Ameyamaea, Asaia, Bombella, Commensalibacter, Endobacter, Gluconacetobacter, Gluconobacter, Granulibacter, Komagataeibacter, Kozakia, Neoasaia, Neokomagataea, Nguyenibacter, Saccharibacter, Swaminathania, Swingsia, and Tanticharoenia* [3]. Recently, the new genus *Novacetimonas* has been proposed. Main distinctive traits of AAB genera are reported in **Table 1.1** [2] [4].

Foods are the most known sources of AAB; though, they play role as plant-associated bacteria (N_2 fixing), insects symbiont and human pathogens. In these environments, the metabolic potential of AAB is expressed by the partial oxidation (not oxidize completely to carbon dioxide) of carbohydrates (alcohols, sugars) releasing the corresponding sugar acid products (aldehydes, ketones and other organic acids) into the surrounding media. In the food industry, AAB are being used as main participants in the production of several foods and beverages, such as vinegar, cocoa, kombucha and other similar fermented beverages. However, their presence and activity can easily derive into spoilage of other foods or beverages such as wine, beer, sweet drinks and fruits [5]. Although processes called “oxidative fermentations”, AAB perform specific oxidation reactions and channel the released electrons to molecular oxygen. Due to these abilities, they are known especially for their role in vinegar production: an aqueous solution of acetic acid (AcOH) that is produced by AAB from a dilute ethanol (EtOH) solution. This was the first investigated environment concerning the biological formation of AcOH. Early researches allowed to recognize that “mother of vinegar”, the surface layer during vinegar formation, was a mass of living microorganisms causing AcOH production [2]. These oxidation reactions of sugars and alcohols by AAB are uniquely carried out by membrane-bound dehydrogenases linked to the respiratory chain located in the periplasmic side of the cytoplasmic

membrane of the organisms [6]. Information on the enzyme systems involved in these oxidative fermentations has accumulated and new developments are possible based on these findings.

Characteristics	G.	A.	Ga.	Ac.	As.	K.	Sw.	Sa.	N.	Gr.	Am.	T.	Ne.	*Ko.	Bo.	En.	Ng.	Swi.	Co.
Production of AcOH	+	+	+	+	-	+	+	v(w/-)	+	v(w/-)	+	-	-	+	-	nd	-	v	nd
Oxidation of																			
Acetate to CO_2 and H_2O	-	+	+	+	w	w	W	-	-	w	+	-	-	+	-	-	+	-	nd
Lactate to CO_2 and H_2O	-	+	+	v(-/w)	w	w	W		-	+	w	-	-	+	-	-	-	-	nd
Growth on 0.35 % AcOH (pH 3.5)	+	+	+	+	-	+	+	-	+	nd	+	+	-	nd	nd	nd	W	-	nd
Growth in the presence of 1 % KNO_3	-	-	-	+	-	-	+	nd	-	nd	-	-	-	nd	nd	nd	-	+	nd
Production of keto-D-gluconic acid from D-glucose	v	-	v	-	-	-	nd	nd	nd	nd	-	+	nd	nd	nd	nd	nd	nd	nd
2,5-Diketo-D-gluconic acid	+	v	v	-	+	+	nd	+	+	nd	+	+	nd	-	nd	nd	+	+	nd
5-Keto-D-gluconic acid	+	v	v	-	+	+	nd	+	+	nd	+	+	nd	v	-	nd	-	+	nd
2-Keto-D-gluconic acid	+	v	v	-	v	+	+	-	w	-	w	+	-	v	+	nd	+	+	nd
Production of DHA from glycerol	-	v	-	+	-	-	-	-	-	+	vw	-	nd	+	nd	+	-	+	nd
Growth on methanol as carbon source	v	-	v	-	-	-	+	-	-	nd	-	+	-	nd	nd	nd	nd	nd	nd
Production of c-pyrones from																			
D-glucose	v	-	v	nd	-	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
D-fructose	+	-	-	nd	v(+/w)	v	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Acid production from																			
l-Arabinose	+	v	v	+	+	+	+	+	+	nd	nd	nd	nd	nd	w	nd	nd	nd	-
D-arabinose	+	-	-	v	+	v	nd	-	w	nd	nd	nd	nd	nd	-	nd	nd	nd	-
D-xylose	+	v	v	+	+	+	V	+	+	-	nd	nd	nd	nd	-	+	nd	nd	+
l-Rhamnose	-	-	-	-	v	-	-	-	w	nd	nd	nd	nd	nd	nd	nd	nd	nd	-
D-glucose	+	v	+	+	+	+	+	+	+	w	nd	nd	nd	nd	+	nd	nd	nd	+
D-galactose	+	v	+	+	+	+	+	+	+	nd	nd	nd	nd	nd	+	nd	nd	nd	-
D-mannose	+	v	v	+	+	+	+	+	+	nd	nd	nd	nd	nd	W	nd	nd	nd	-
D-fructose	+	-	+	-	+	-	V	v	+	nd	nd	nd	nd	nd	+	nd	nd	nd	nd
L-Sorbose	+	-	v	nd	+	-	nd	-	-	nd	nd	nd	nd	nd	-	nd	nd	nd	nd
Melibiose	+	-	-	v	+	+	nd	+	+	nd	nd	nd	nd	nd	-	nd	nd	nd	-
Sucrose	+	-	-	-	+	v	nd	+	+	-	nd	nd	nd	nd	+	-	nd	nd	+
Raffinose	-	-	-	-	-	+	nd	-	+	nd	-	w	-	nd	-	nd	w	w	nd
D-mannitol	+	-	v	-	v	-	-	+	w	-	-	-	-	nd	+	-	-	+	nd

D-sorbitol	+	-	-	-	v	-	+	-	+	-	-	-	-	nd	-	-	-	v(w/-)	nd
Dulcitol	-	-	-	-	v	-	V	-	w	-	-	-	-	nd	nd	-	-	v(w/-)	-
Glycerol	+	-	+	+	+	+	+	-	+	v(w/-)	w	+	-	nd	-	+	-	v	+
EtOH	+	+	+	+	-	+	+	-	+	+	+	+	-	+	-	nd	-	-	nd
Production of cellulose	-	-	v	-	-	-	nd	-	nd	nd	nd	nd	nd	V	nd	nd	nd	nd	nd
Production of levan-like mucous substance (s) from sucrose	-	v	-	-	-	+	nd	-	-	nd	-	-	-	nd	nd	nd	+	-	nd
Growth in the presence of 30 % D-glucose	-	v	v	+	+	-	nd	+	+	nd	-	+	+	nd	+	nd	w	+	nd
Motility and flagellation	N-m 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	N	N-m	Sub-p	pe	N	N-m
Major ubiquinone	Q10	Q9	Q10	Q10	Q10	Q10	Q10	Q10	Q10	nd	Q10	Q10	Q10	Q10	Q10	Q10	Q10	Q10	nd
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	55.8-63.4	54.9	60.3	68.1-69.4	46.9-47.3	37

Table 1.1 G., *Gluconobacter*; A., *Acetobacter*; Ga., *Gluconacetobacter*.; Ac., *Acidomonas*; As., *Asaia*; K., *Kozakia*; Sw., *Swaminathania*; Sa., *Saccharibacter*; N., *Neoasaia*; Gr., *Granulibacter*; Am.; *Ameyamaea*; T., *Tanticharoenia*; N., *Neokomagataea*; + positive; - negative; w weak; v variable; nd not determined; N-m non-motile; po polar; pe peritrichous. *) Recently species of the new genus *Komagataeibacter* were reclassified and included in the *Novacetimonas* gen. nov. (Adapted from Mamlouk & Gullo, 2013 [2] and https://www.bacterio.net/ [3])

1.2 Metabolic pathways

1.2.1 Respiratory Chains of AAB

In AAB respiratory chain ubiquinone (UQ) connect principal dehydrogenases eith final ubiquinol oxidase (**Figure 1.1**). The UQ present in the respiratory chain of AAB varies depending on the bacterial species. Mainly, *Acetobacter* species has UQ9 and *Gluconobacter* species and *Gluconacetobacter* have UQ10. *Gluconacetobacter* was later subdivided into two genera (*Gluconoacetobacter* and *Komagataeibacter*) [7]. Recently, phylogenomic and comparative genomic analysis of *Komagataeibacter*, revealed that several type strains formed an independent genomic group as members of a novel genus, hereby termed *Novacetimonas* gen. nov. [8].

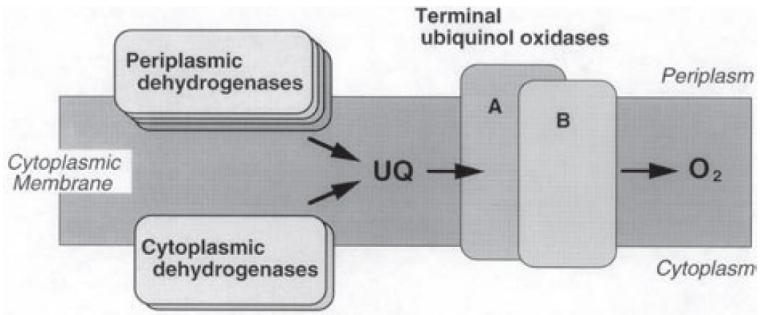

Figure 1.1 Respiratory chain of AAB. (Matsushita et al., 2004) [6]

Although both the primary dehydrogenase and the terminal oxidase parts are very divergent in the respiratory chain of AAB, the diversity of the terminal oxidase is not unusual while the primary dehydrogenase is especially divergent in the respiratory chain. The terminal oxidases accept the electrons from ubiquinone, transferring them to molecular oxygen, the final electron acceptor, forming water. AAB have two terminal ubiquinol oxidases, designated cytochrome *bo3* ubiquinol oxidase and cytochrome *bd* quinol oxidase. Cytochrome *bo3* ubiquinol oxidase catalyzes a reaction, which contributes to the generation of a proton-motive force while cytochrome *bd* quinol oxidase does not. An important function of the latter terminal oxidase is believed to be the re-oxidation of ubiquinol to ubiquinone, thus rapidly regenerating ubiquinone that can contribute to further reactions in the respiratory chain or in the reactions of oxidative fermentation [9]. In AAB, many peculiar periplasmic dehydrogenases are working for the specific alcohol, sugar or sugar alcohol oxidation systems [6]. Several different species of ubiquinol oxidase are known in the respiratory chain of AAB, with respect to the cytochrome species [6].

1.2.2 Carbon Sources

1.2.2.1 Ethanol Oxidation

AAB partially oxidize EtOH by two successive catalytic reactions of the alcohol dehydrogenase (ADH) and a membrane-bound aldehyde dehydrogenase (ALDH) that are bound to the periplasmic side of the cytoplasmic membrane. The complete oxidation of EtOH occurs at cytoplasmic level by a NAD-ADH and NAD-ALDH. The AcOH produced can be further utilized by acetyl CoA synthase and via tricarboxylic acid cycle (TCA cycle). When ADH is disrupted, EtOH is assimilated though NAD-ADH and NAD-ALDH, as shown in **Figure 1.2**.

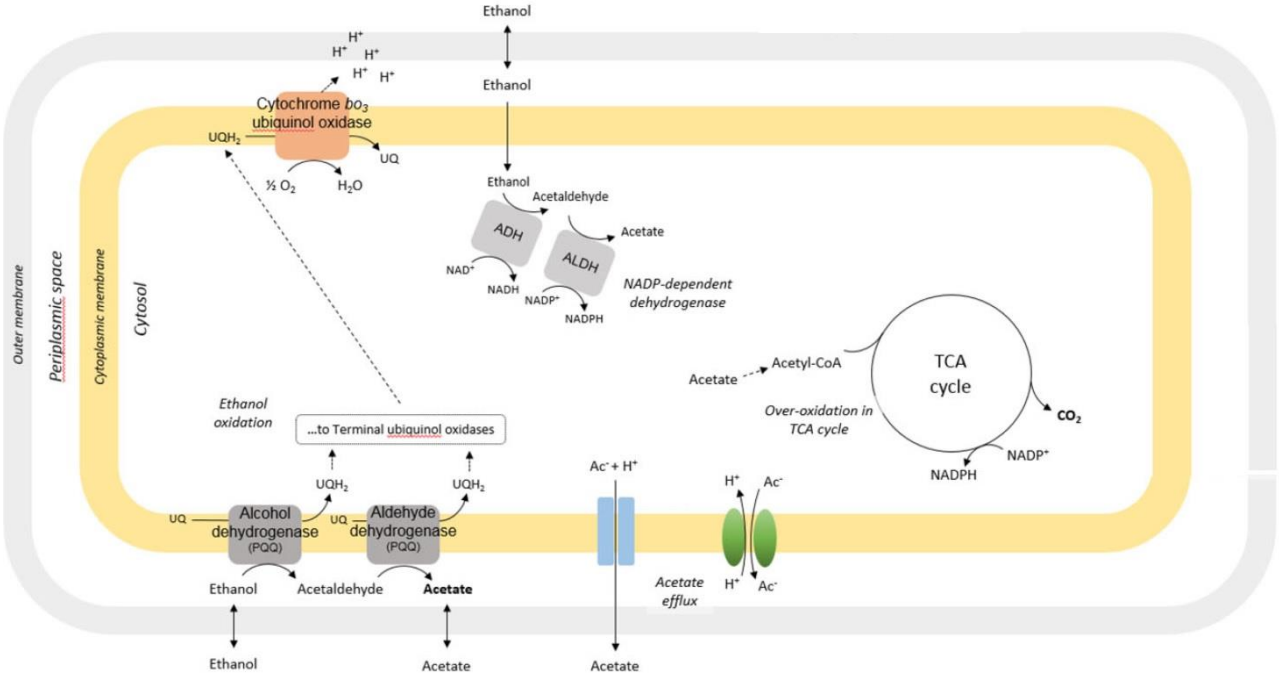

Figure 1.2 Respiratory chain of AAB. (Adapted from Lynch et al., 2019) [9]

The membrane-bound ADH and ALDH complexes are tightly linked to the respiratory chain, which transfers electrons via UQ and a terminal ubiquinol oxidase to oxygen as final electron acceptor. ADH oxidizes EtOH to acetaldehyde, which is further oxidized to AcOH by ALDH as follows [2]:

$$CH_3CH_2OH + PQQ \rightarrow CH_3CHO + PQQH_2 (alcohol\ dehydrogenase)$$
$$CH_3CHO + PQQ + H_2O \rightarrow CH_3COOH + PQQH_2 (aldehyde\ dehydrogenase)$$

ADH of many AAB is composed by three subunits and oxidation of EtOH occurs at pyrroloquinoline quinone (PQQ) site that acts as two-electron redox mediator.

The ALDH complex of AAB is composed of two or three subunits. Its optimum pH is between 4 and 5, although it can catalyze the oxidation of acetaldehyde to acetate at lower pH values. ALDH is sensitive to oxygen concentrations, and when these are low its activity decreases, accumulating acetaldehyde into the media. It is also more sensitive to the presence of EtOH than ADH [2].

1.2.2.2 Sugars Oxidation

AAB are known to have a high oxidative ability against sugars, mainly glucose but also arabinose, fructose, galactose, mannose, ribose, sorbose and xylose. They can catabolize sugars through the cytoplasmic hexose monophosphate pathway (Warburg-Dickens pathway). In *G. oxydans* was reported that the oxidative pentose phosphate pathway (PPP) is the most important route for phosphorylative breakdown of sugars and polyols to CO_2. Therefore, it was expected that *G. oxydans* has the capability to hold and to channel many polyols, sugars and sugar derivatives into the oxidative PPP: polyols are first oxidized by soluble dehydrogenases; these products, other ketoses and aldoses are further modified by isomerases and epimerases. Finally, the compounds are phosphorylated by kinases forming oxidative PPP intermediates [2]. *Acetobacter* species can use sugars through the hexose monophosphate pathway and over the Entner – Doudoroff and Embden – Meyerhof – Parnas pathways. Sugars are further metabolized to CO_2 and H_2O via the TCA pathway, which is not functional in *Gluconobacter*. *Gluconobacter* prefers sugars as carbon source than *Acetobacter* because the species of this genus can obtain energy more proficiently by the metabolisation of the sugars via PPP. The most characteristic reaction is the direct oxidation of glucose into glucono-δ-lactone, which is oxidized into GA.This reaction is particularly active in *Gluconobacter* growing at high concentrations of sugars. Gluconate dehydrogenase (GADH) and 2-keto-D-gluconate dehydrogenase (2KGDH) can oxidase D-gluconate to 2-ketogluconate and 2,5-diketogluconate. These primary dehydrogenases of respiratory chains (GDH, GADH, 2KGDH) are located on the outer surface of the cytoplasmic membrane. On the other hand, for partial assimilation of glucose in the cytoplasm, gluconate and 2-ketogluconate, NAD-dependent glucose dehydrogenase and NADP-dependent 2-ketogluconate reductase are working (**Figure 1.3**)

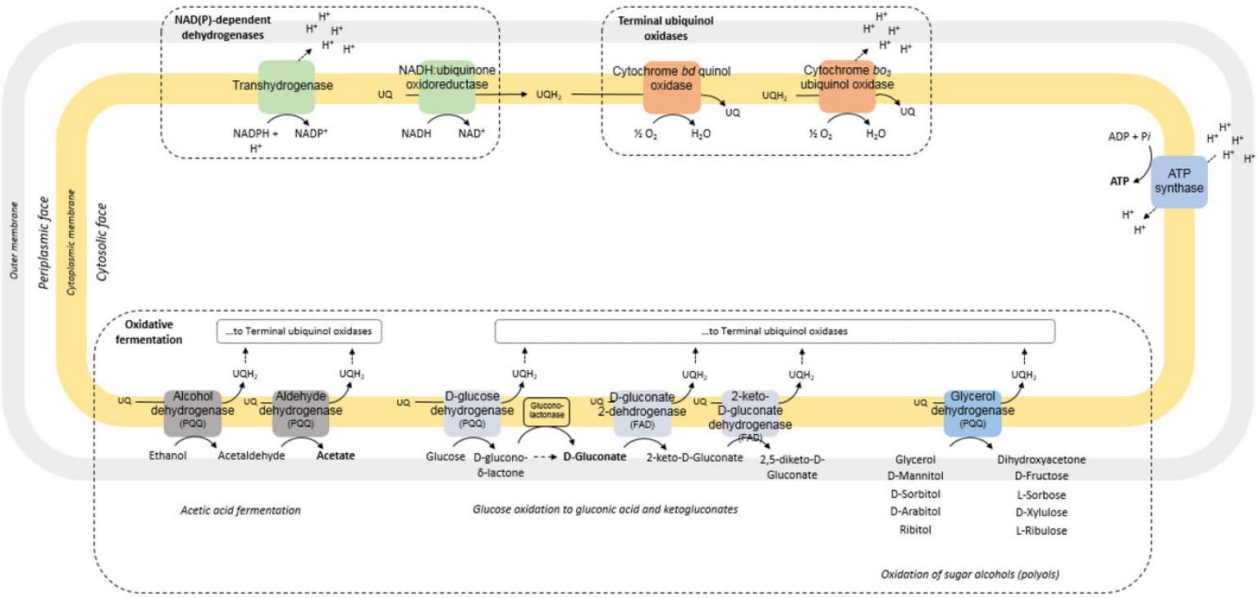

Figure 1.3 Respiratory and oxidative fermentation chains and associated dehydrogenases in AAB. (Adapted from Lynch et al., 2019) [9]

1.2.2.3 Organic Acid Oxidation

The ability of *Acetobacter* and *Komagataeibacter* strains to oxidize AcOH (**Figure 1.2**) generating the so-called "acetate overoxidation" occurs via TCA cycle. Other acids such as citric, fumaric, lactic, malic, pyruvic and succinic are similarly metabolized. In contrast, strains of *Gluconobacter* do not have a functional TCA cycle because of deficiencies in the two key enzymes, alpha-ketoglutarate dehydrogenase and succinate dehydrogenase. Consequently, they are unable to metabolize AcOH and other organic acids. Although the optimum pH for the oxidation of organic acids by AAB is near 6.0, there is evidence that it occur at lower values (3.5 – 4.0) [2].

It is generally recognized that *Acetobacter*, *Gluconacetobacter* and *Komagataeibacter* species exhibit three growth phases in EtOH media. Although the diauxic growth-phase pattern shows some variations from species to species, it can be generally described as follows: first, they perform a rapid oxidation of EtOH to AcOH, which is released from the periplasm into the surrounding environment (EtOH oxidation phase). Then, a stationary phase occurs, resulting in a decrease in viable cell numbers or low growth yields (stationary phase). Finally, there is a second exponential phase (AcOH oxidation phase) in which AcOH is catabolized by soluble ADH and ALDH in the cytoplasm, for both energy generation and carbon assimilation. During EtOH oxidation and stationary phases AAB accumulated AcOH in the environment without utilizing it, while during overoxidation phase (EtOH depleted) they oxidize AcOH to CO_2 and H_2O [10] [6]. The switch from acetate accumulation to acetate oxidation is controlled by changes in the metabolic flow through the TCA cycle.

1.2.2.4 Production of Exopolysaccharides

Cellulose, dextrans and levans are the main exopolysaccharides produced by AAB. *K. xylinus* species have been regarded as model system for the study of biochemistry and genetics of cellulose biogenesis. The rate of cellulose production in *K. xylinus* is proportional to the rate of cell growth, and the yield is dependent on the carbon sources. *K. xylinus* synthesizes a cellulose mat that covers the surface of the growth medium in static cultures, whereas round balls of cellulose are formed in shaking cultures. Several strains of *K. xylinus* elaborate relatively pure cellulose as an extracellular product that is extruded from the surface of the cell [11]. The key enzyme in cellulose synthesis by *K. xylinus* is the membrane bound cellulose synthase which uses UDP-glucose as substrate. The pathway from glucose to cellulose consists of the following four enzymatic steps:

$$Glucose \rightarrow Glucose-6-phodphate \rightarrow Glucose-1-phosphate \rightarrow UDP-glucose \rightarrow Cellulose$$

Cells of cellulose-producing AAB are entrapped in the polymer matrix, supporting the population at the liquid–air interface. This facilitates oxygen and nutrient supply, since the concentration of nutrients in the cellulose matrix is enhanced by its absorptive properties, in contrast to the surrounding aqueous environment. Production of exopolysaccharides especially cellulose from AAB seems to be a promising area of application because of the increasing need of pure cellulose in medical and engineering fields [2]. Instead for industrial applications which exploit the sugar oxidation ability of AAB, the synthesis of cellulose is undesired.

1.2.3 Acetic acid resistance

Under industrial vinegar production, AcOH concentration is a major physiological stressor of cells. Undissociated AcOH can penetrate the cell membrane, disrupting membrane transport processes, and then dissociate inside the cell, resulting in toxic levels of the anion and an associated increase in acidity. Although AAB are tolerant to AcOH at concentrations that are damaging to the majority of microorganisms, significant variation among AAB species exists. Moreover, during the different fermentation phases of submerged fermentation (SF), strains exhibit different degrees of resistance to AcOH. The mechanisms that AAB use to tolerate high concentrations of AcOH can be classified as follows: (1) adaption of and protection of intracellular proteins to and against acid stress, (2) overoxidation, (3) AcOH efflux from the cell, and (4) prevention of AcOH from entering the cell [9]. These resistances are affected by the number and the modality of recursive cultivations in AcOH media, and the time between strain isolation and industrial application. Therefore, the effect of AcOH on AAB growth is a function of the concentrations of substrate (EtOH) and product (AcOH) and of

the growth conditions. For instance, in shaking EtOH cultures of *Acetobacter* strains, it was found that 10 g/L of AcOH has an activating effect on growth, and lower concentrations resulted in a significant decrease in the logarithmic growth phase. Activation and inhibition effects on AAB growth as a function of the total concentration have been observed during the start-up phase in SF. Conversely, during the fermentation step AAB are able to grow at higher concentrations of AcOH. The level of acetate resistance depends on the physiological adaptation under selective pressure due to AcOH content, with significant variation according to species, evolved or wild-type strains [10]. The *aar* genes isolated as "acetate resistance genes" are considered as one of the acetate utilization systems in the overoxidation phase; whereas these acetate utilizing enzymes seem to be suppressed during the EtOH oxidation and the first stationary phases, in which another mechanism must be working to resist a high concentration of AcOH produced by themselves [6]. AcOH resistance is a crucial factor to stably produce large amounts of AcOH by *Acetobacter* species [12].

1.2.4 Growth temperature

AAB are mesophilic bacteria with temperature growth range between 28-30°C.

In industrial conditions temperature control is an important factor. Thermotolerant strains of AAB were isolated in order to serve as the new strains of choice for industrial fermentations, in which the cooling costs for maintaining optimum growth and production temperature in the fermentation vessels could be significantly reduced [13]. For industrial submerged conditions the optimal working temperature is approximately 30°C. Temperatures increase above the optimal value occurs during SF because AcOH fermentation is a thermodynamically favorable aerobic process. A temperature increase induces the denaturation of nucleic acids and proteins and causes cell damage. These reactions, together with the dispersion of cellular compounds due to membrane damage, can irreversibly reduce the metabolic functions of the cells. Additionally, the toxic effects of AcOH concentration in the medium increase the sensitivity of AAB to high temperatures [10].

1.3 Genera of industrial interest

Many applications of AAB have been reported and developed over the past decades. The most important characteristic is still the ability to produce AcOH from EtOH. The main species most used in vinegar production due to their significant abilities to oxidize EtOH to AcOH belong to the genera *Acetobacter*, *Gluconacetobacter*, *Gluconobacter* and *Komagataeibacter* [14]. While AcOH production in European countries is conducted by mesophilic AAB, thermotolerant strains exhibiting the ability to grow at higher temperatures are preferable for production in tropical countries as well as in Africa, in order to reduce cooling cost to control heat generated from fermentation reactions, which would affect bacterial growth and production. Many thermotolerant *Acetobacter* strains with AcOH resistance ability have been isolated from various sources around the world and they were proposed for industrial applications. On the other hand, *Gluconobacter* strains are known as sugar, sugar alcohol, and sugar acid oxidizers, producing a number of commercially valuable compounds. Many strains with features applicable for industrial production of these compounds have been isolated.

1.3.1 *Gluconobacter*

The AAB belonging to the genus *Gluconobacter* exhibit extraordinary uniqueness not only in their biochemistry but also in their growth behavior and response to extreme culture conditions. This uniqueness makes them ideal organisms for microbial process development [15]. The genus *Gluconobacter* is one of the industrially important genera of gram-negative which are placed in the family *Acetobacteraceae*. The genus *Gluconobacter* has the following characteristics: contains straight-chain C_{16} and C_{18} fatty acids; oxidizes EtOH to AcOH, but is unable to oxidize lactate or AcOH completely to CO_2 and water; contains UQ10 as the main quinone compound; possesses an incomplete TCA cycle; carries out single-step oxidations of various polyalcohols to their corresponding ketoses; produces 2-ketogluconic acid from glucose; and has polar multitrichous flagella, when motile [16]. Other characteristic and taxonomic relevant reactions of *Gluconobacter* spp. are the oxidations of sugars to their corresponding sugar acids (**Figure 1.4**). Quinoprotein glycerol dehydrogenase (GLDH) is able to oxidize D-gluconate, D-sorbitol, D-mannitol, D-arabitol, glycerol to 5-ketogluconate, L-sorbose, D-fructose, D-xylulose, and dihydroxyacetone, respectively. D-Gluconate is also oxidized to 2-ketogluconate, then to 2,5-diketogluconate by a flavoprotein GADH and 2KGDH, respectively. L-Sorbose is oxidized to L-sorbosone, then to 2-ketogulonate by a flavoprotein sorbose dehydrogenase (SDH) and an unidentified enzyme sorbosone dehydrogenase (SNDH), respectively. D-Fructose is oxidized to 5-ketofructose by a flavoprotein fructose dehydrogenase (FDH); whereas D-Sorbitol is oxidized to D-fructose by a flavoprotein sorbitol dehydrogenase (SLDH).

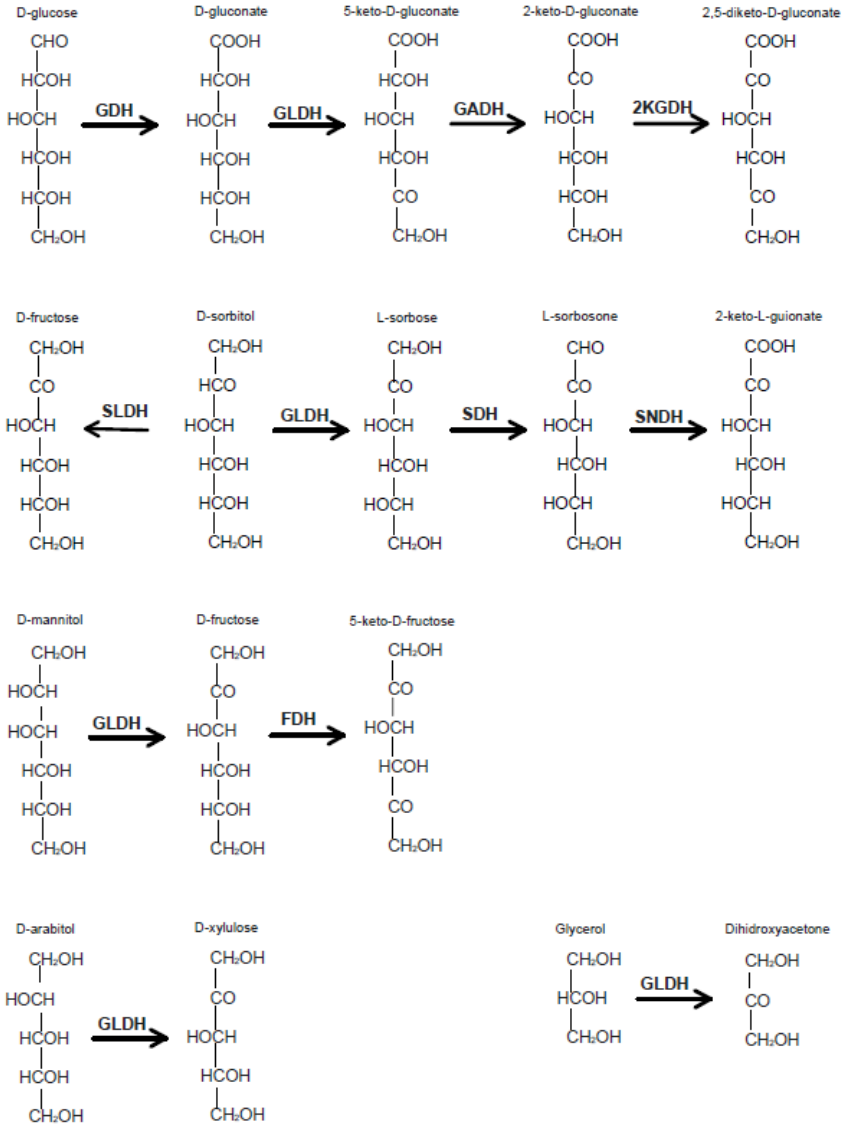

Figure 1.4 Oxidation pathways of several sugar alcohols, sugars, and sugar acids in *Gluconobacter* spp. GDH, glucose dehydrogenase, GLDH, glycerol dehydrogenase; GADH, gluconate dehydrogenase; 2KGDH, 2-ketogluconate dehydrogenase; SDH, sorbose dehydrogenase; SNDH, sorbosone dehydrogenase; FDH, fructose dehydrogenase; SLDH, sorbitol dehydrogenase (From Matsushita et al., 2004) [6]

The first *Gluconobacter* complete genome sequenced was belonging to *G. oxydans* 621H (DSM 2343) [17] [18]. Besides fermented foods, some *Gluconobacter* sp. are used as biocatalysts for the industrial production of a range of compounds, making them important biocatalysts for the development of eco-friendly fermentation processes as an alternative to the chemical synthesis. Relevant *G. oxydans* industrial applications are in the manufacture of L-ascorbic acid (vitamin C), miglitol, GA and its derivatives, and dihydroxyacetone [19]. In particular, strains of *G. oxydans* produce enzymes involved in amino acids synthesis e.g. glutamic and aspartic acids thanks to the incomplete set of TCA cycle enzymes which could function primarily for glutamate, aspartate and succinate

biosynthesis. One of the most important biotechnological application of *G. oxydans* is the production of L-ascorbic acid (vitamin C) precursors such as L-sorbose from D-sorbitol and 2-keto-L-gluconic acid from 2,5-diketo-D-gluconic acid or L-sorbosone [15]. Strains of *G. oxydans* are also exploited for the microbial production of dihydroxyacetone (DHA) that is used in the pharmaceutical. Industry as a cosmetic tanning agent and also as an intermediate for the synthesis of various organic chemicals and surfactants. Among organic acids, GA, used as a bulk chemical in the food, textile, medical and construction industries can be produced by *G. oxydans* which oxidize glucose to gluconate by the membrane bound GDH. Other applications of *G. oxydans* are the production of miglitol's precursors, used as a therapeutic drug for the treatment of non-insulin-dependent diabetes mellitus; D-tagatose, used as a bulking agent in food and a noncalorific sweetener; and shikimate, which is a key intermediate for a number of antibiotics [2 13].

G. oxydans 621H (DSM 2343) is very suitable for biotechnological production because it possesses a high oxidative activity, even under non-growth conditions, and has a poor cell yield. The sequencing of its genome has enabled the characterization of its multiple metabolic pathways and cell physiology [20].

1.3.2 *Acetobacter*

Acetobacter is the oldest recognized genus whitin AAB. Bacteria of the genus *Acetobacter* have been isolated from industrial vinegar fermentation processes and are frequently used as fermentation starter cultures. They also occur in flowers, fruits, palm wine, vinegar, kefir, and fermented foods and can cause infections in grape wine, sake, tequila, cocoa wine, cider, beer, and fermented meat [21]. This genus is differentiated from the other genera by its Q9 UQ system and by the oxidation of acetate and lactate to CO_2 and H_2O. This genus consisted of 34 species. These species were delineated mainly on the basis of DNA-DNA relatedness and phylogenetic relationships [22]. In EtOH culture of *A. aceti*, there are three growth phases; *A. aceti* first grows by oxidizing EtOH completely to AcOH (EtOH oxidation phase), then stops the growth and remains for a long time with the viable cell number being decreased (first stationary phase), and finally starts to grow again by utilizing the accumulated AcOH, the phase of which is called as "overoxidation of acetate". It is thus conceivable that *Acetobacter* species have two different phases related to AcOH resistance, the EtOH oxidation and the first stationary phases where the strains resist against AcOH accumulated in the culture medium without utilizing the acetate, and the overoxidation phase where the strains just utilize the acetate for cell growth [6].

1.3.3 *Komagataeibacter*

The genus *Gluconacetobacter* is divided into two groups ecologically, phylogenetically, and phenotypically: the *Ga. liquefaciens* group and the *Ga. xylinus* group. For the latter group, the genus *Komagataeibacter* is newly introduced, and the type species of the new genus is designated as *K. xylinus*. Twelve species of the *Ga. xylinus* group are transferred to the new genus as new combinations [7]. In the genus *Komagataeibacter* are included species previously accommodated in the genus *Gluconacetobacter*. This genus includes species of relevance for the industrial production of bacterial cellulose and vinegar, such as *K. europaeus* and *K. xylinus*. In particular, in the production of high-acid vinegars using the SF method belong to the species *K. europaeus*. Strains of this species are characterized by the higher ADH stability and activity, and higher AcOH resistance, compared to other AAB [12]. Strains of the genus *Acetobacter* are damaged when AcOH concentration reaches 7-8% [23]. In contrast, the strains from the genus *Komagataeibacter* are able to resist up to 15 – 20% AcOH [24]. *Komagataeibacter* strains are involved in fermentation processes that are carried out by the modern submerged methodology [25] [26].

In spite of the cultivability problems, several strains of the genus *Komagataeibacter* have been isolated during the production of high acid vinegars by the submerged methodology, being *Komagataeibacter europaeus, Komagataeibacter oboediens, Komagataeibacter hansenii* and *Gluconacetobacter. entanii* (species taxonomically included in the genus *Komagataeibacter*) among the most prominent species [27].

1.3.4 *Novacetimonas*

Thanks to genes involved in cellulose biosynthesis (*bcs* genes) and phylogenomic and comparative genomic analysis, was revealed several type strains formed an independent genomic group from those of other *Komagataeibacter*. *Novacetimonas* gen. nov. is a reclassification as members of a novel genus. The results support the reclassification of *K. cocois, K. hansenii, K. maltaceti* and *K. pomaceti* as novel members of the genus *Novacetimonas*. The proposed representative of the novel genus is *N. hansenii* species. Importantly, phylogenetic analysis, showed that the evolutionary history of these genes is closely related to the strain's phylogenomic/taxonomic classification [8].

1.4 Non-alcoholic fermented beverages

To increases nutritional content of food and preservation, fermentation was used since ancient time. In 7000 B.C. several studies have shown evidence of fermented beverages in China, and there is evidence of kombucha tea production and kefir-like milk fermented around 3000 years ago. Fermented beverages have become known for their health-promoting properties in many regions of the world. Innovative efforts have recently been made to develop non-dairy probiotic fermented beverages from a variety of substrates, including cereals, fruit juices, soy milk, vegetables and whey. The fermentation of substrates to produce beverages with these properties is original of many regions of the world, such as Africa, Asia, Europe, Middle East and South America. Modern biotechnological techniques are developing these fermented beverages to improve the next generation of functional fermented beverages, a growing sector of the food industry. Foods that improve well-being and reduce the risk of disease is a growing desire of modern health conscious consumers [28]. An important class of fermented beverages are fermented milk products made with milk and lactic acid bacteria from various sources. On the other hand, non-dairy fermented beverages are important fermentations from cereals. In addition to milk and cereal-based fermentations, there are also other forms of fermented beverages. One example is kombucha, which is a fermented sweetened tea that was originally popular in China but is now enjoyed worldwide. For many of the fermented beverages, it is the strong association between gastrointestinal health and microbial content. Moreover, it has been shown that natural fermented beverages have antihypertensive effects, improve systemic immunity, lower cholesterol and help lower blood pressure. Furthermore, they have been shown to have modulatory effects on the brain and demonstrate anti-cancer potential [28]. Some fermented foods produced depend on defined starter cultures, those produced in Asia and Africa often rely on spontaneous fermentation. Indeed, in these countries, fermented foods are not often commercially produced on an industrial scale. Certainly the introduction of starter culture technology has led to greater consistency, quality and safety [29].

1.4.1 The importance of gluconic acid

Organic acids represent one of the major building blocks obtained through biotechnology processes in the global market of high-volume bulk chemicals. The growing significance and demand for GA have promoted an interest in integrating both issues as a strategy for the revalorization of these resources [30]. The enormous application-dependent growth of GA enhanced the total market value: about US$333 million. The market for GA, also known as pentahydroxycaproic acid ($C_6H_{12}O_7$), is expected to reach USD 1.9 billion in 2028. GA is a common additive used in pharmaceutical, textile, building and, especially, food industries and currently, annual production of GA and its derivatives

is approximately 80.000 tonnes. At present, the production of GA is estimated to amount to approximately 100˙000 ton/year and to be almost exclusively biotechnological, with production costs ranging from 1.20US$/kg for GA to 8.50 US$/kg for calcium gluconate and gluconolactone. Sodium gluconate is the most widely marketed GA derivate, accounting for more than 80% of the world production. GA is commercially available as a 50% aqueous solution of a pH of 1.82 and 1.23g/cm3 of density. GA and its derivatives occur naturally in plants, fruits and other foods such as rice, honey, grapes, apples, meat, wine and vinegar Results from the specific oxidation of the aldehyde group at C1 in D-glucose to a carboxyl group by chemical, electrochemical, or catalytic means or, alternatively, through biotransformation by microorganisms (fungi or bacteria) or their enzymes. In contrast to fungal GA formation which consists of two steps, bacterial GA formation is a one-step process typically catalyzed by a membrane-bound D-glucose dehydrogenase [31]. Among the major agro-food by-products, sugarcane molasses and grape must have high sugar content and can be used for the production of alcohol, which makes them suitable candidates for the glucose acid fermentation process. The alternative carbohydrate sources with the potential to make the GA fermentation process more economical are corn starch, can molasses, grape must, banana must, food processing residues, figs, cheese whey, beet molasses, and saccharified solution of waste paper. GA is usually obtained through biological methods involving the partial oxidation of glucose (**Figure 1.5**).

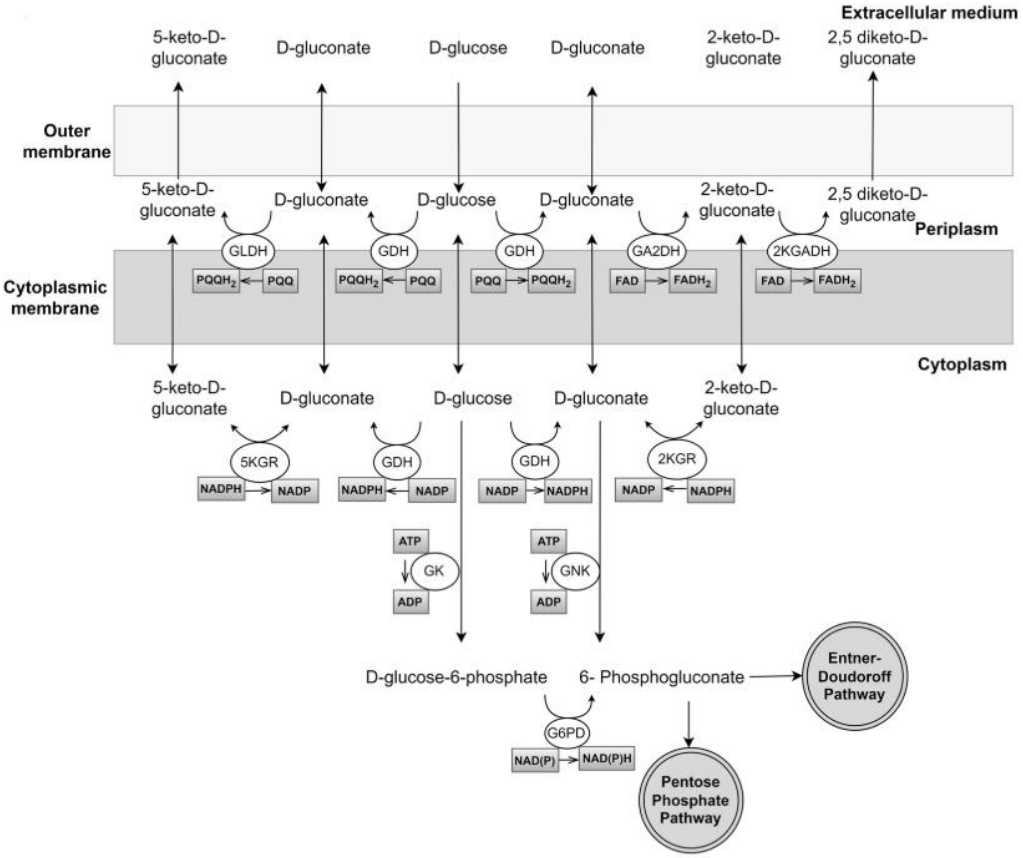

Figure 1.5 Glucose metabolism in *Gluconobacter* spp. (Adapted from Da Silva et al., 2022) [19]

This acid provides an excellent example of how some production wastes and surpluses with high carbohydrate contents can be optimally exploited. GA is a weak acid sugar (K_a= $1.99 \cdot 10^{-4}$ at 25°C) belonging to the aldonic acid family. GA is a weak, non-volatile, harmless (odorless, non-corrosive, non-toxic), easily biodegradable acid that is soluble in water and insoluble in non-polar solvents. Because it is both an acid and an alcohol, GA can undergo 1,5 intramolecular esterification. The process is favored by an acid medium and involves the spontaneous loss of a water molecule to yield intramolecular anhydride gluconolactone, which is acyclic ester. The two molecules are in equilibrium in aqueous solutions. Under typical conditions, the oxidation reaction yields a mixture of 2 and 5-keto-D-gluconate (2-KGA and 5-KGA) and, under extreme conditions, also 2,5-diketo-D-gluconate (2,5-DKGA) in variable proportions. Like many other organic acids, GA is involved in the metabolism of a number of living organisms. The acid and its derivatives have gained increasing interest in food, pharmaceutical, textile and building industries over the past 50 years.

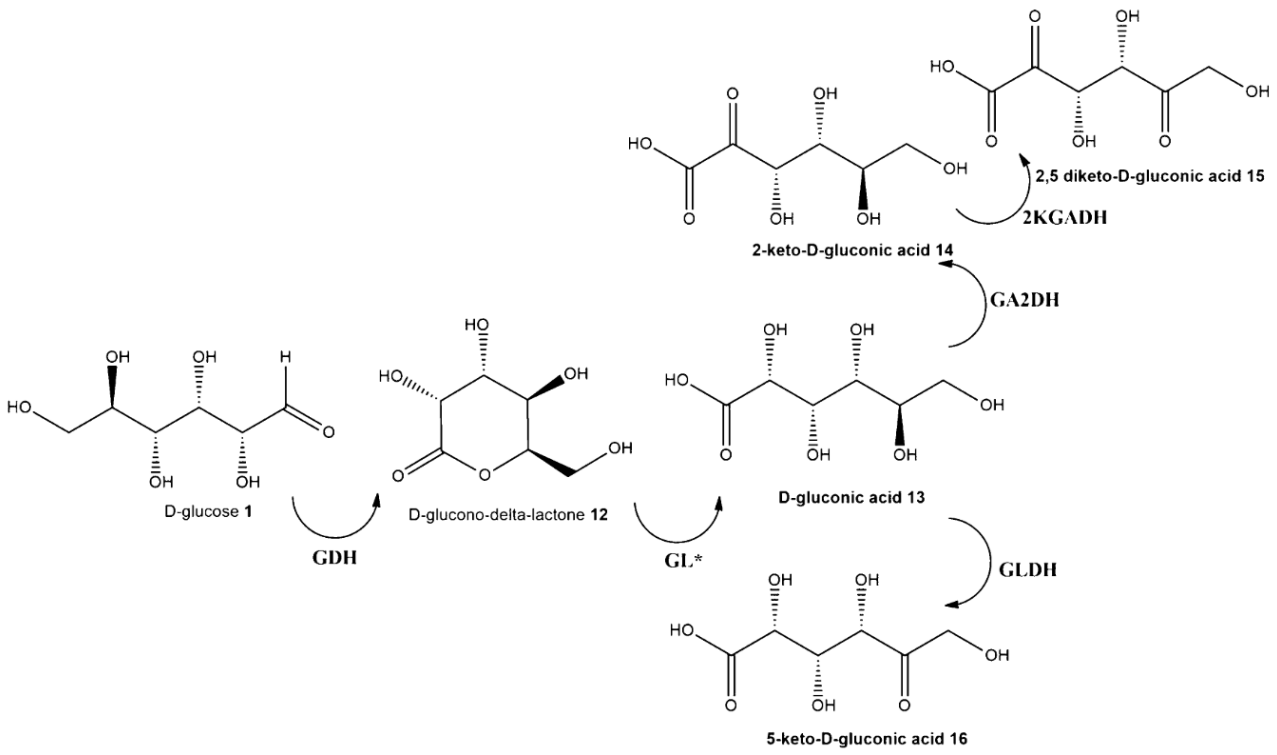

Figure 1.6 GA and ketogluconic acids from glucose oxidation by *Gluconobacter oxydans*; GDH glucose dehydrogenase, GL glucono-δ-lactonase, GA2DH gluconate-2-dehydrogenase, 2KGADH 2-keto-gluconate-dehydrogenase, GLDH glycerol dehydrogenase *The reaction can be catalyzed by GL or occurs spontaneously (Adapted from Da Silva et al., 2022) [19]

Although GA can be obtained using chemical and biotechnological methods, the latter prevails at the industrial scale. Specifically, early methods based on surface fermentation with *Penicillium fungi* have been superseded by the use of submerged cultures of filamentous fungi such as *Aspergillus niger*

or AAB (particularly *G. oxydans*), which are highly selective and technically efficient. Other bacterial strains capable of metabolizing glucose to GA, including *A. diazotrophicus* and *Zymomonas mobilis*, have also been identified. Additionally, new fermentation methods using immobilized cells or enzymes have recently emerged and might become competitive choices for GA production in the future.

The oxidation of glucose to GA by filamentous fungi is an aerobic fermentation process with a high oxygen demand. The process is catalyzed by the enzyme glucose oxidase. This enzyme is a flavoprotein that is predominantly located in the cell wall and the extracellular fluid. Roughly 80% of the total enzyme activity is found to be associated with these two locations in *Aspergillus* and *Penicillium* spp. D-glucose is converted into gluconolactone through a dehydrogenation reaction that also produces H_2O_2; the peroxide is subsequently decomposed into O_2 and H_2O under the action of a catalase. GA is formed by the spontaneous or catalytic hydrolysis of the lactone. The wide variety of bacteria capable of metabolizing glucose to GA includes various AAB genera and strains from other genera such as *Pseudomonas* and *Zymomonas*. The metabolism of most aerobic bacteria involves the thorough oxidation of organic matter to carbon dioxide and water. Only under special growth conditions involving a high nutrient availability, certain microorganisms can lead to incomplete oxidation (the so-called overflow metabolism). As a rule, AAB oxidize their substrates incompletely. AAB have a number of agrifood uses, the most widespread of which is probably vinegar production. Among these, *Acetobacter* sp. and *Gluconobacter* sp. are of high biotechnological interest for GA production. Essentially, the two genera differ in their glagella position and oxidative metabolism. Thus, *Gluconobacter* exhibits polar flagellation but lacks the functional mechanisms of the tricarboxylic acid (TCA) cycle, such that it cannot oxidize acetate (overoxidation). By contrast, *Acetobacter* exhibits peritrichous flagellation and possesses a functional TCA cycle; thus, it can oxidize EtOH to CO_2. Regardless of the bacterial genus, glucose can be oxidized at various cell sites depending on the location of the enzymatic ensemble catalyzing the process. Glucose concentrations above 15 mM and pH values below 3.5 have been found to inhibit the formation of keto-acids. Under optimal industrial conditions, *G. oxydans* shows GA yields within the range of 75–80%, which depend markedly on the pH, the glucose concentration and degree of aeration. The intracellular oxidation of glucose catalyzed by a battery of NADP+-dependent soluble dehydrogenases (GDH and GADH) can proceed through different metabolic pathways that depend on the particular acetic bacterium. *Acetobacter* degrades D-glucose through the PPP and the TCA cycle, whereas *Gluconobacter* uses the PPP and to some extent the Entner–Doudoroff pathway, but lacks a complete TCA (**Figure 1.7**).

The higher production of GA was observed in two strains of *G. oxydans*, however a strain of *Acetobacter* also showed a high production. At industrial scale, massive GA production by *G. oxydans* requires high glucose concentrations, low pH and high aeration rate. The further oxidation to ketogluconic acids is potentially undesirable reaction when using *Gluconobacter* strains for GA production. Suppression of ketogluconates formation has been achieved performing processes at low pH values [2]. The fall in pH, due to the GA production, result in the glucose oxidation stop. The low-pH tolerant strains were also the higher GA producer [32]. *G. oxydans* 621H is very suitable for biotechnological production because it possesses a high oxidative activity, even under non-growth conditions, and has a poor cell yield. The sequencing of its genome has enabled the characterization of its multiple metabolic pathways and cell physiology.

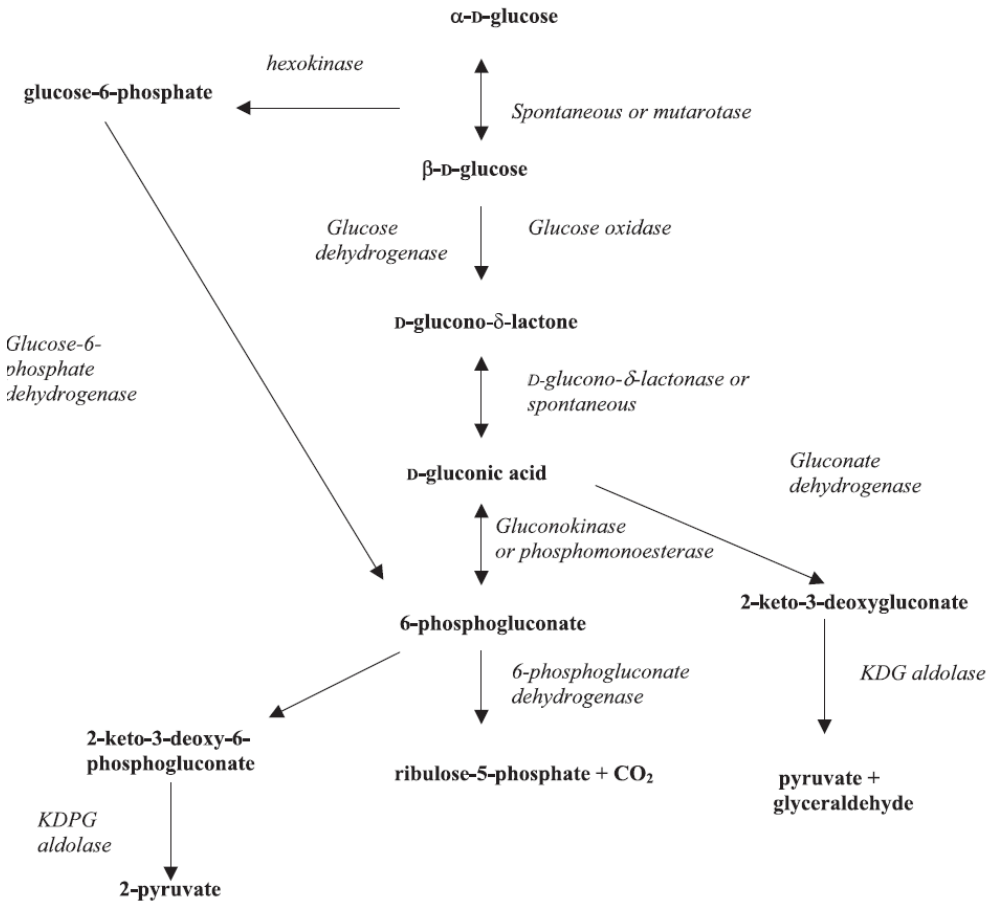

Figure 1.7 General GA pathway (From Gupta et al., 2001) [33]

1.5 Fermentation systems

Strictly aerobic AAB have a long history of use in fermentation processes. In particular, AAB most well-known application is the conversion of EtOH to AcOH for the production of vinegars. Fermentations can be performed by two main systems. The first system is solid-state fermentation (SSF), which uses microorganisms grown on substrates in the absence of free water. The second system is liquid fermentation, which comprises a set of techniques develop, such as submerged system used to produce at industrial scale [10]. A submerged system has several advantages over other techniques, including high yield and process speed.

1.5.1 Solid-state fermentation

For the production of microbial metabolites, SSF is used as a common technique [34]. The process of SSF has the advantages of a high product concentration but a low energy being required and it is performed on a solid substrate with a low humidity content. The substrate in a solid matrix absorbs essential water and offers more advantages for the growth of microorganism for the transfer of oxygen. Several agricultural wastes are being used as substrate for SSF. In Asian countries SSF is largely used to produce vinegars from cereals at small-scale. The basic process steps of these kinds of vinegar are: (1) crushing and steaming of cereals; (2) addition of water and a specific cereal preparation containing moulds, yeasts and bacteria; (3) EtOH fermentation; (4) AcOH oxidation during which cereals hull are mixed with old AcOH fermented product from last batch as seed vinegar [35]. Recently, SSF processes have attracted interest due to its potential not only in vinegar field, but also for the production of food and pharmaceuticals.

1.5.2 Static fermentation

Static fermentation system is an old technique that allow to produce vinegar. In this method the organism is allowed to grow on the surface of a liquid medium without agitation. The bacterial growth is limited to the surface area of the liquid where cells are in contact with air. This method is generally time consuming and needs large, area or space. In static culture, usually cellulose or biofilm pellicle is produced at the gas–liquid interface with a fine three-dimensional structure. The microbial cellulose is produced extracellularly in the form of fibrils that are attached to the bacterial cell [36]. In static culture, the cell density is not high, cells show the lowest viability, the conversion rates of substrates is low [37]. For these reasons, this method is used for home-made production of fermented beverages. Low fermentation yields and high production costs are bottlenecks for industrial applications. Therefore, considering the yield production in term of AcOH, the submerged fermentation (SF) system culture might be a most suitable technique for economical scale production [38 39].

1.5.3 Aerobic submerged fermentation

Industrially, metabolites production is mostly carried out under SF conditions due to the simpler downstreaming process compared to SSF and static one. At the industrial scale, fermentations are mainly conducted by SF. The method of fermentation using submerged culture is based on the presence of a culture of bacteria freely submerged within the liquid to be fermented [37]. SF requires robust AAB strains that are able to oxidize EtOH under selective conditions to produce high-titer AcOH. Currently SF is conducted by unselected AAB cultures, which are derived from previous acetification stocks and maintained by repeated cultivation cycles. Oxygen availability, temperature, AcOH and EtOH content are optimized for increase industrial vinegar production [10].

The basic requirements for SF processes are the availability of suitable AcOH, uninterrupted aeration, tolerant AAB strains for high concentrations of AcOH and EtOH, not sensitive to phage infections and requirement of small quantities of nutrients, to produce high amounts of AcOH. Aeration system is one of the most important features of the bioreactors. This system consists of a hollow body turbine supported by a non-rotating stator. The turbine sucks air from the outside and releases it into radial holes that open in the opposite direction of rotation; the action of turbines results in very fine air bubbles and homogenous air–liquid dispersion. The air–liquid emulsion is pushed upwards and diverted by deflectors. All of the mass is maintained in a constant state of agitation to prevent the formation of low oxygen tension areas, which are unfavorable for the metabolic activity of AAB [10]. The oxygen transfer is influenced by a high number of parameters including physical properties of gas and liquid, operational conditions and geometrical parameters of the bioreactor [10]. In these stirred conditions, the liquid produces foam, which can lead to the formation of a reducing environment and compromise the acetification process. The heat generation during SF is unavoidable because AcOH fermentation is an exothermic reaction, producing approximately 8.4 MJ for every liter of oxidized EtOH. Moreover, during charge of substrate and discharge of product, the temperature can vary greatly. Fermentation breakdown due to temperature variation is generally avoided by heating and cooling systems. SF at the industrial scale is primarily performed in a semi-continuous mode (a repeated fed-batch process). In this operation mode, alcoholic substrates are added after the start of the acetification and then are added intermittently, depending on consumption. Semi-continuous operation is reported to be the most advantageous for vinegar production, partly because it reduces the risk of substrate inhibition and catabolite repression. Moreover, it allows the reuse of the acetifying culture in the subsequent cycle and to obtain products with a wide range of both AcOH and EtOH concentrations [10].

According to previous studies, the indigenous bacterial population, during SF for vinegar production, appears quite homogeneous, as it is mostly composed of the genus *Gluconcetobacter* (now *Komagataeibacter*) and, in some cases, *Acetobacter.* Moreover, the majority of studies identify the establishment of a single strain or only a few strains of the same species, suggesting the formation of a stable environment that exerts a strong selective pressure, due mainly to the presence of AcOH [10].

Rapid loss of productivity could be caused by an oxygen deprivation during SF because AAB are obligate aerobic bacteria. During SF, the level of oxygen consumption is directly related to substrate conversion and growth phase of AAB. The consumed oxygen growing from lag phase to the growth phase of AAB and is proportional to production rate of AcOH. However, little information is available regarding the optimal oxygen levels required by AAB when performing bioprocesses. In SF the optimal rate of oxygen consumption is about 1 mg/L, correspondent to an AcOH production of 45g/L during *A. aceti* continuous culturing in the exponential phase [10]. During SF oxygen is supplied as a mixture of air or sometimes oxygen-rich air. Oxygen-rich air is not used at the industrial scale because of the high cost safety issues for managing. However, it could result in increases in process yield, improve the sensorial characteristics of the vinegar, and reduce the loss of volatile components, including EtOH [10].

The agro-food by-products can be considered as an economical source of carbohydrates sources for GA fermentation. Among the major agro-food by-products, grape must and sugarcane molasses can be used for the production of EtOH because of high sugar content, which makes them suitable candidates for the GA fermentation process. Routine fermentations using conventional microorganisms under submerged conditions led to the development of the SF process for GA production. The SSF was found to be more efficient with 94% yield of GA than any other fermentation types. For the commercial production of biomolecules like 2-keto-L-gluconic acid, D-sorbitol, GA, dihydroxyacetone, aerobic SF are exploited. Mass transfer in submerged fermentation depends upon the rheological properties of fermentation broth. *A. niger*, *G. suboxydans* and *A. methanolicus* have been the main organisms employed for large-scale production through fermentation. However bacteria *G. oxydans* has now gained significant importance [20 30].

1.6 Ponti SpA company

Ponti is an Italian food company that operates in the sectors of wine vinegar, Balsamic Vinegar of Modena P.G.I., apple vinegar, pickles, preserves and condiments for rice and pasta. Active as a family business since 1787, it is the leader in the vinegar market in Italy and exports to over 70 countries around the world

Ponti's core business is wine vinegar, which is produced in the factories of Ghemme (**Figure 1.8a**), Dosson di Casier and Anagni. The Ponti classic wine vinegar is complemented by Aroma Antico, Chianti D.O.C.G. vinegar and Pinot Bianco Oltrepò Pavese D.O.C vinegar [40] (**Figure 1.8b**). In the same plants Ponti apple cider vinegar is produced, using Italian apples from certified supply chain. Instead, pickles and condiments for rice and pasta are produced only in Ghemme plant in a technologically advanced factory. In Vignola plant Balsamic Vinegar of Modena P.G.I. is made from the maturation of wine vinegar and cooked and concentrated grape must in vats, barrels and barriques of different woods, according to the rules of the Production Regulations imposed by the Consortium for the Protection of Balsamic Vinegar of Modena (**Figure 1.8c**). In 2020 Ponti introduced on the labels of all its Balsamic Vinegars of Modena P.G.I. an exclusive classification system certified by SGS that allows the consumer to evaluate and choose the product based on three parameters that influence the taste of the vinegar. These parameters are the maturation period, which gives peculiar aromas and flavours, the quantity of grape must, which gives sweetness and aromas, and the density, which determines the degree of softness of the Balsamic Vinegar and affects its flavour.

Figure 1.8 (a) Overview of the main plant of Ponti SpA in Ghemme (NO) (b) Frings used for the production of vinegar in Ghemme plant (c) Wood vats presents in Vignola (MO) plant for the production of Balsamic Vinegar of Modena P.G.I.

Principally, Ponti produce white and red wine vinegar and apple cider vinegar starting from wine at high amount of EtOH to obtain great amount of AcOH. Through Frings fermentator, vinegars are produced with highest efficiency and quality. The process is conducted in submerged semi-continuous fermentation that can achieve higher production capacities, yields and acidity levels. The standard process is a cyclical production process divided into three sections:

- Charge: new batch of wine or apple cider is pumped into first fermentator. This still contains a residual volume of active AAB from the previous batch.
- Fermentation: the AcOH concentration increases continuously while the EtOH concentration decreases.
- Discharge: when the fermentation reach a defined quantity of EtOH and AcOH, a part of the active fermentation batch is discharged in second fermentator to finish the acetic fermentation as higher AcOH as possible and a minimum EtOH concentration.

These three section are cyclically repeated until the batch of wine or cider vinegar are finished.
The high acidity vinegar obtained is used as a part of the recipes, is sold in bulk or is diluted to bottle it as vinegar for condiment.

Chapter 2. Evaluation of the fermentation capacity of date palm fruits through selective fermentations

2.1 Introduction

Date Fruits as Raw Material for Vinegar and Non-Alcoholic Fermented Beverages

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Abstract

Currently, foods and beverages with healthy and functional properties, especially those that claim to prevent chronic diseases, are receiving more and more interest. As a result, numerous foods and beverages have been launched onto the market. Among the products with enhanced properties, vinegar and fermented beverages have a high potential for growth. Date palm fruits are a versatile raw material rich in sugars, dietary fibers, minerals, vitamins, and phenolic compounds; thus, they are widely used for food production, including date juice, jelly, butter, and fermented beverages, such as wine and vinegar. Furthermore, their composition makes them suitable for the formulation of functional foods and beverages. Microbial transformations of date juice include alcoholic fermentation for producing wine as an end-product, or as a substrate for acetic fermentation. Lactic fermentation is also documented for transforming date juice and syrup. However, in terms of AAB, little evidence is available on the exploitation of date juice by acetic and gluconic fermentation for producing beverages. This review provides an overview of date fruit's composition, the related health benefits for human health, vinegar and date-based fermented non-alcoholic beverages obtained by AAB fermentation.

Keywords: date palm; acetic acid bacteria; vinegar; non-alcoholic beverages

Introduction

The current trend to produce fermented beverages and vinegar, including from lesserknown fruits and vegetables, fits with consumers' demand for a healthier and more sustainable diet. Moreover, health-based recommendations include reducing alcohol consumption, calories from added sugars and limiting the consumption of foods that contain refined grains, especially those with high amounts of added sugars and sodium. The Food and Agriculture Organization of the United Nations (FAO) guidelines for correct nutrition recognized the need to obtain adequate nutrients to reduce the overconsumption of energy, therefore lowering the risk of common chronic diseases, such as diabetes, obesity, cardiovascular disease, and some cancers [1]. Generally, the consumption of foods and beverages with added sugars is discouraged, due to the high calorific content. In addition, the safe use of non-nutritive sweeteners, such as aspartame, is currently under ongoing scientific debate, emphasizing the need to develop natural sweetener products [2]. Due to the above-mentioned trends and health recommendations, non-alcoholic beverages produced without the addition of sugar represent a segment of the food industry that is rapidly growing. The Food and Drug Administration (FDA) considers "non-alcoholic beverages" as all beverages containing less than 0.5% of ethanol by volume [3]. The intake of excessive amounts of ethanol is known to have adverse effects on human

health, which include several acute and chronic illnesses [4–6]. On the other hand, low alcohol intake is associated with beneficial effects on human health [7,8].

Non-alcoholic beverages or any food (other than alcoholic beverages) are further classified by the FDA into two clusters depending on the final pH. Low-acid beverages have a pH greater than 4.6 and a water activity greater than 0.85; whereas acid beverages have a final pH of 4.6 or below and a water activity greater than 0.85, including beverages such as vinegar, kombucha tea, vinegar beverages, and gluconic beverages [3].

Fruits are optimal substrates for microbial transformations intended to produce non-alcoholic beverages and vinegars, due to their high amount of sugar and fermentative feasibility. Aside from conventional fruits exploited for non-alcoholic beverages and vinegars [9–11], there is great interest in the valorization of unconventional ones [12–15], contributing to open up new opportunities for reducing food loss, and satisfying the consumers' demand for sustainable and healthy products.

Date from palm is a versatile raw material, rich in sugar and bioactive compounds, such as phenolic acids, carotenoids, and minerals [16,17]. Due to the peculiar composition, the role of dates and derivatives in reducing the risk of cardiovascular diseases, diabetes mellitus and other illnesses, has been reported [18,19].

However, date production, transformation, and marketing are affected by several issues, such as loss of a high amount of product in field, and no to little available technology for transformation in the production site [20,21]. Previous works highlighted different strategies to valorize date palm fruits and derivative wastes by bioprocesses based on fermentation and enzyme processing. Many added value products can be obtained such as biopolymers, biofuels, or antibiotics [22,23]. In the food industry, the by-products of dates could be used as source of sucrose substitute for the enzymatic synthesis of fructooligosaccharides (FOS), or as raw material to produce high-fructose syrups [24].

Microorganisms that are able to grow and convert date juice into fermented beverages mainly include lactic acid bacteria (LAB), yeasts and AAB. With regard to microbial bioprocessing, some studies have shown the possibility of using LAB to obtain beverages and probiotics from date derivatives. For instance, date syrup has been previously evaluated as a substrate for producing a probiotic beverage by lactic fermentation [25,26] and for probiotics using date powder, as a low-cost carbon source [27]. Moreover, studies have assessed the possibility to use date by-products to produce lactic acid via lactic fermentation and GA without the addition of sugars [21,28].

The alcoholic fermentation of date juice produces wine, as an alcoholic beverage, or as an intermediate product for further fermentations, operated by AAB. Previous studies state the role of Saccharomyces cerevisiae strains as the main yeasts in dates for wine production [29–32]. Further fermentation of date wine into vinegar is also documented [33–35].

Yeasts and AAB are cooperative microorganisms which, through selective fermentations, can promote the valorization of date fruits by alcoholic fermentation followed by acetic fermentation for producing vinegar and low acetic beverages.

Although AAB, in a single fermentation step, can provide GA from glucose oxidation. This latter metabolic pathway can be exploited for producing new non-alcoholic beverages from dates. Although vinegar produced from dates is an existing product, the rational exploitation of AAB for producing both vinegars and low fermented beverages appears underdeveloped. The versatile oxidative metabolism of AAB, and the peculiar traits of species and strains in producing organic acids, mainly acetic and GA, offer different opportunities to valorize date juices (**Figure 2.1**). The production of both acetic and GA is a common feature of AAB belonging to *Acetobacter*, *Gluconacetobacter*, and *Komagataeibacter*; whereas the production of GA as primary metabolite is a peculiar trait of *Gluconobacter* members, especially those of *G. oxydans* species [36].

This review provides an overview of date palm fruit composition, and discusses fermentation strategies, aimed at producing vinegar and low alcoholic fermented beverages. It presents an analysis of existing products and potential new ones, which exploit the oxidative metabolism of AAB.

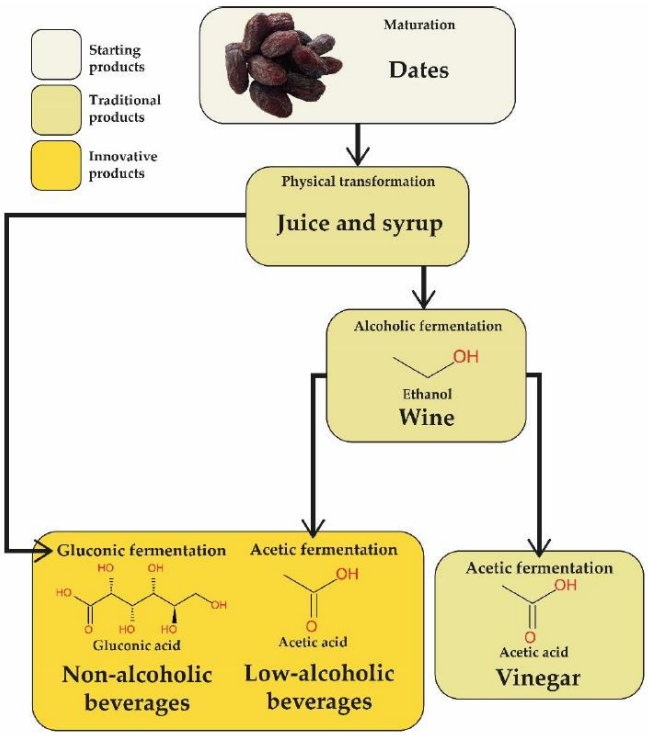

Figure 2.1 Traditional and innovative date fruit products. Box's color indicates the type of products. Chemical compounds and their structure indicate the specific type of beverage.

Date Palm Characteristics

Date palm belongs to the *Arecaceae* family and traditionally it is recognized as a valuable beneficial plant. The genus *Phoenix* is composed of 14 species, including *P. dactylifera*, and has been cultivated in the Middle East for 6000 years [37], and *P. sylvestris*, widely known as the wild date palm [37]. The latter, in turn, is widespread in Bangladesh and India where it is also known as date sugar palm, and silver date palm [38,39]. Fruits of *P. sylvestris* are considered beneficial and they are used for their medicinal properties against hyperthermia, nervous debility, back pain, stomachache, toothache, headache, and arthritis [38,40]. In the European market date varieties are mainly imported from Africa. However, in Eastern countries, many varieties of *Phoenix dactylifera* with great biotechnological potential, such as *Saher,* and *Khadrawi,* are cultivated [20]. Date fruits from P. dactylifera are much appreciated for their flavor, which has been extensively studied [41–43]. The aroma and flavor comes from a complex mixture of volatile compounds. From the volatiloma of 135 varieties of dates, 80 volatile compounds, belonging to acids, alcohols, aldehydes, and esters, were described [41]. The most famous variety of *P. dactylifera* is *Majoul*, which was first imported into the USA from Morocco.

Instead, *Ajwa* variety dates can be distinguished from other date varieties due to their higher nutritional properties. Ancient cultures used every part of the plant, such as trunk, leaves, and fruits and for this reason, date palm has been called “the tree of the life” [44].

Currently, over 2000 varieties of date palms are cultivated all around the world, mainly in the Middle East, North Africa, parts of Central and South America, Southern Europe, India, and Pakistan. In 2019, the production of dates globally reached 9.21 million tons [45]. Date palm has high levels of productivity and adaptability allowing the cultivation in desertic areas, such as Saharan regions. Moreover, its fruits have high nutritional values. All these advantages make date palm essential for farmers’ agricultural incomes. According to FAOSTAT, Egypt was the largest producer of palm dates in 2019 with 1.64 million tons, followed by Saudi Arabia with 1.54 million tons, Iran with 1.31 million tons, Algeria with 1.14 million tons, Iraq with 639,315 tons, and Pakistan with 564,904 tons [45]. Furthermore, date palm is resistant to adverse climatic conditions, withstanding temperatures from−6 ◦C to 50 ◦C and high salinity levels of soil water. The most appropriate areas for the growth of the palm fruit are arid regions with hot and dry climates and limited rainfall [46]. As the trees are tall, microclimates are generated under the top of the date palm, allowing the production of crops and vegetables that are useful for human and animal sustenance [47].

Date palm is marketed as a high-value fruit crop and low-cost food [48]. According to the geographic origin and quality, dates are marketed as fruits and as several derivatives, such as jam, butter, jelly, date syrup, juice, and non-alcoholic fermented beverages [24].

The Composition of Date Palm Fruit and Juice

Date fruits are considered to be a primary source of nutrition and energy since they are rich in arbohydrates, mainly sugars, fatty acids, amino acids and minerals [49] (**Table 2.1**). In addition, they are well known to be an important source of dietary fiber, thus providing health benefits to humans by preventing diseases and increasing gut well-being [50,51].

The maturation stages, from unripe to ripe, are usually described as *kimri* (greenish color, hard texture), *khalal* (yellowish), *rutab* (softer and sweeter), and *tamer* (dark brown color, soft texture, and highest sweetness) [52]. Date fruits are considered ripe at the tamer stage, and they appear dry and firm with a brown/dark color. The nutritional composition of date fruits changes according to the growth stages. For instance, the reduction in phenolic compounds could reach 25% through the ripening stage, represented mainly by carotenoids loss [53,54]. On the other hand, sugar content increases, because of moisture loss during ripening [55]. Total sugar content can reach 81.40% w/w, 83.41% w/w, and 88.30% w/w, in *Barthe*, *Khalas*, and *Deglet Nour* varieties, respectively [56–58].

Sugars are mainly represented by glucose and fructose, while sucrose is present at lower concentrations [19,50,57,59–61]. Glucose and fructose are main carbon substrates for alcoholic, acetic, and gluconic fermentations. However, other reducing sugars, such as mannose and maltose or polysaccharides, such as cellulose and starch, can be found at low concentrations [47].

Concentrations between 2.70 and 20.25 g/100 g dry matter of fiber have been found by Borchani and her co-workers, who tested the fiber extract of 11 date cultivars [57].

In addition, dates can be defined as the richest and most important source of dietary minerals among other common fruits consumed by humans [48]. A 100 g portion of date fruit is enough to provide the 15% of daily recommended minerals [58].

Low levels of sodium and high levels of potassium make date fruits a recommended food for people suffering from hypertension [57]. Furthermore, date fruits contain other minerals, such as iron, calcium, cobalt, copper, magnesium, manganese, phosphorus, and zinc [62].

The vitamins content of dates is reported to be low. Depending on the stage of ripening and production processes, however, they are considered a good source of folate and vitamin C [16,52,58,63].

Dates Composition	Lowest Reported	Highest Reported
	Content [g/100 g]	
Carbohydrates	54.90	88.30
Protein	0.46	3.85
Ash	1.45	2.3
Dietary fiber	2.70	20.25
Fat	0.07	0.57
Amino acids	**Content [mg/100 g]**	
Alanine	8.00	342.00
Arginine	2.00	261.00
Aspartame	230.00	450.00
Aspartic acid	2.00	467.00
Cysteine	11.00	114.00
Glutamic acid	40.00	631.00
Glycine	4.00	349.00
Histidine	0.1	76.00
Isoleucine	0.2	465.00
Leucine	0.5	264.00
Lysine	3.00	282.00
Methionine	0.2	219.00
Phenylalanine	0.8	173.00
Proline	12.00	369.00
Serine	6.00	238.00
Threonine	1.00	264.00
Tryptophan	49.5	100.00
Tyrosine	1.00	181.00
Valine	0.5	271.00
Minerals	**Content [mg/100 g]**	
Potassium	107.40	916.00
Boron	3.30	5.60
Sodium	32.90	131.00
Calcium	9.50	207.00
Magnesium	47.00	215.55
Phosphorus	13.00	63.00
Iron	0.30	32.76
Copper	0.10	2.90
Cobalt	0.41	1.00
Selenium	0.10	0.32
Zinc	0.10	1.80
Manganese	0.21	5.90
Vitamins	**Content [mg/100 g]**	
Folic acid	0.004	0.3
Niacin	0.0004	1.61
Riboflavin (B2)	0.06	0.17
Thiamine (B1)	0.05	0.13
Vitamin C	2.4	17.5

Table 2.1 Composition of date fruits at tamer stage. Values are reported as weight/100 g dry matter. Adapted from [16,50,51,55,57–59,62,63].

Several studies have reported even the presence of numerous bioactive phytochemicals, such as carotenoids, flavonoids, polyphenols, and steroids, in most of the varieties of date fruits [16,17,33,54,62].

Although the maturation stages can negatively affect the number of phenolic compounds and the antioxidant capacity of date fruits, it is estimated that 100 g of dates contains 250–450 mg of total

phenolic compounds (**Table 2.2**) [20,53]. Compared to grapes, dates show a higher phenolic content than white/green grapes but less than dark purple/red ones. However, dates are a richer source of phenolic compounds compared to other fruits (**Table 2.2**).

Fruits	Phenolic Content (mg GAE/100 g Fresh Weight)
Date	326
Green grape	201
Dark purple grape	397
Kiwifruit	112
Orange	243
Plum	311
Apple	100
Pear	125
Raspberry	267

Table 2.2 Total phenolic contents of various fruits estimated by Folin-Ciocalteu method. Adapted from [20,33,53,64–67].

Most of the total phenolic compounds in date fruits are phenolic acids, carotenoids, polyphenols, and phytosterols [54]. Phytosterols are the least present whereas phenolic acids are the most common by far (**Table 2.3**). The latter includes protocatechuic, gallic, caffeic, p-hydroxybenzoic, anillic, ferulic, syringic, p-coumaric, and o-coumaric acid [68].

Bioactive Components	Content [mg/100 g]	
	Lowest Reported	Highest Reported
Phenolic acids	20.24	64.44
Carotenoids	0.03	2.90
Anthocyanins	0.24	1.52

Table 2.3 Bioactive compounds in date fruit at tamer stage (mg/100 g fresh weight). Adapted from [20].

The high prevalence of phenolic compounds and vitamin C is correlated to the strong antioxidant activity of date fruits. Indeed, phenolic acids, anthocyanins, and β-carotene exhibit strong antioxidant potential, playing a key role in the therapeutic effect of date fruits [19]. In addition, such antioxidants could help in reducing chronic inflammation, risk of coronary disease, and evelopment of cancer [17,19,54].

Dates contain a very small quantity of proteins. However, they are rich in aminoacids containing essential (e.g., lysine and leucine) and non-essential (e.g., glycine, aspartic acid, and glutamic acid) ones [16,69]. It has also been reported that the concentrated juice, such as date syrup, has strong antioxidant activity, accordingly to the high total phenolic content. This evidence highlights the potential to produce high-value products, especially date syrup, from dates with high polyphenols content and high antioxidant potential [70].

Fermentation of Date Palm Juice

The feasibility of efficient alcoholic fermentation of date juice is well documented by several studies in which it was intended as single fermentation for obtaining date palm wine and, as the first biological step for obtaining acetic products, such as vinegars.

Palm wine is a generic name for a group of alcoholic beverages obtained from different species of palm, such as *Elaeis guineensis*, *Raphia hookeri*, *Phoenix dactylifera*, *Borassus aethiopum*, and *Cocos nucifera*. Palm wine is the most popular beverage in Africa, and it is claimed that 10 million people in Western Africa consumes it [71]. In Nigeria, palm wine is used during local and traditional events [29].

Saccharomyces cerevisiae has been reported as the principal yeast species in traditional date wines, or the most used in date wine production [29–32]. By using date juice as raw material, wine containing 12% v/v ethanol by inoculating *S. cerevisiae* var. *ellipsoideus* was produced [46]. Authors reported low acidity levels (0.35–0.54% v/v) and a pH ranging between 4.0 and 4.2. Similar results, in terms of acidity and pH, were obtained by Awe and co-workers [72], but with a lower ethanol content (9.2% v/v). Moreover, date wine was richer in vitamin C and protein, compared to a commercial white grape wine [72]. Date extracts were also reported to be suitable for alcoholic and subsequent acetic fermentation for producing vinegar, using a *S. cerevisiae* strain (68 g/L ethanol produced), and a strain of the *Acetobacer aceti* species (45 g/L of acetic acid produced) [73].

In date wine, a decrease in phenolic compounds (e.g. phenolic acids) in juices as a result of the microbial activity is reported. However, date wine is recognized a source of bioactive compounds [74]. In particular, the radical scavenging activity of phenolic compounds present in date wine is associated with a beneficial effect on human health by reducing the risk of coronary disease. Moreover, date wine contains proanthocyanidins, which carry out several advantageous effects on humans such as anticancer, antioxidant, and antidiabetic activity [75,76]. Proanthocyanidins also provides flavor and astringency to beverages [75].

Vinegar

Vinegar is the result of the activity of yeasts and AAB, as the main microbial groups. Once ethanol is obtained by alcoholic fermentation, AAB converts it into acetic acid by an oxidative fermentation. Primarily, partial oxidations of suitable carbon sources are carried out by the activity of membrane-bound dehydrogenases, located in the periplasmic space of the cell membrane. In terms of the production of acetic acid, the aldehyde dehydrogenase (ALDH; EC 1.2.1.-) and alcohol dehydrogenase (ADH; EC 1.1.2.8) are responsible for the conversion of ethanol into acetaldehyde, and then into acetic acid, respectively [36]. The efficiency of this microbial transformation depends

on several factors, such as the raw material composition, the microbial strains, the fermentation regime, and the process parameters [11].

Over time, consumers' conception of vinegar has evolved from a simple ingredient to a condiment, as part of a more sophisticated choice of consumption. Moreover, along with the growth of public interest in vinegar and healthy beverages, as well as the fact that popularity of vinegar drinks that contain phytochemicals found in the given fruit has increased in recent years [77]. Some of these benefits include enhanced immunity, reduction in risk factors for cardiovascular diseases, improved digestion, appetite suppression, reduced fasting blood glucose, reduced blood pressure, and serum cholesterol [78].

The pharmacological potentials of date palm fermented products are also documented. Different studies have shown the anti-hyperlipidemic, anti-obesity, antioxi- dant, and immune-stimulating activities of date vinegar made from date flesh and pits (seeds) [79–82]. Moreover, homemade date vinegar produced from date waste is a traditional product in Iran, widely consumed for its antimicrobial properties [83].

Matloob [35] tested the production of vinegar utilizing *Khistawi* date juice as a raw material. The alcoholic fermentation was carried out by a bakery yeast (S. cerevisiae), while the aerobic oxidation of ethanol to acetic acid by an *Acetobacter* strain. The pH of produced vinegars ranged between 2.40 and 3.26 with a minimum acidity of 4.00%, which is the minimum value required by the North American legislations [84].

The effect of fermentation on phenolic content of vinegar produced from different dates' cultivars was evaluated by Matloob and Balakit, who observed a decrease during alcoholic fermentation from an initial value of 1211.8 mg GAE/L to 1179.8 mg GAE/L (−2.6%). However, the subsequent acetic fermentation provided a significant increase (+20.1%) of total phenolic content in *Khistawi* date vinegar, reaching a peak value of 1453.4 mg GAE/L during 14 days of fermentation [33]. The increase in phenolic com- pounds in vinegar brewing, observed by several authors, could be due to two different reasons [85–87]. First, the acids produced during acetic fermentation could degrade the glucoside bonds of the phenolic compounds, leading to the liberation of compounds with different structure [33,88]. The second reason could be the enzymatic conversion of high molecular weight polyphenol compounds into small molecules having higher biological activity [89–91].

The high content of phenolic compounds confers to date vinegar inhibitory effects against oxidative reactions [80,92–95]. Ali and co-workers [80] tested daily consumption effects of date vinegars on hypercholesterolemic adults. Outcomes showed a positive correlation between date vinegar consumption and the reduction in serum total cholesterol, low-density lipoprotein, and apolipoprotein B (Apo B) concentration. Furthermore, an in- crease in high-density lipoprotein (HDL) concentration

was observed. Health benefits were mainly related to acetic acid, dietary fiber, and phenol compounds concentration. Indeed, acetic acid lowers the levels of the substrate required for serum cholesterol production by suppressing the sterol regulatory element, binding protein mRNA levels. The combination of HDL higher levels and acetic acid effects could lower cardiovascular diseases risk by decreasing Apo B concentration.

Beneficial effects of low acidity date vinegar/beverage supplemented with garlic juice was also stated as a means of lowering the total cholesterol content [79]. Researchers hypothesized that the high intake of bioactive compounds such as carotenoids, fiber and potassium caused an obliteration of intestinal lipid saturation. This evidence highlights the potential to produce functional beverages based on date juice with the added value of benefits for hyperlipidaemic adults.

Date vinegar also contains unsaturated fatty acids, such as oleic, palmitoleic, linoleic, and linolenic acids even though the highest amount is available in date seeds [55].

Exploring Innovative Date Fruits Products by Acetic Acid Bacteria Fermentation

The interest of consumers in vinegars and low acetic acid content beverages, drives the industry to explore unconventional raw materials as having the potential to enhance functional properties of the end-products. Approaches include process improvements by innovative treatment of the raw material to reduce the loss of bioactive compounds. For instance, Siddeeg and co-workers [34] evaluated the influence of ultrasound (US), pulsed electric field (PEF), and the combination of both techniques on the quality of date vinegar compared to untreated ones. Prior to alcoholic fermentation, dates were treated with PEF, US, or PEF + US. Outcomes showed no changes or minor changes in color parameters, pH, total titratable acidity, and residual ethanol concentration. Moreover, PEF + US samples presented a notable increase in total phenolic and flavonoid content, free amino acid content, and volatile components. Date vinegars treated with PEF + US, PEF or US obtained a higher overall acceptability score compared to the untreated samples.

A great opportunity to valorize date juice arises from selected AAB fermentation which, based on the know-how acquired by vinegar production, can drive the development of new processes and products (Figure 1). An optimization strategy, by using full factorial design approach, has been applied for studying traditional date vinegar in Algeria [96]. On the basis of current knowledge, both conventional processes for obtaining vinegar can be modulated to obtain low acetic acid beverages with enhanced properties. Moreover, there is high potential of innovation in terms of GA fermentation. Contrary to vinegar production, non-alcoholic beverages containing GA are produced in one fermentation step process, which consist in the oxidation of glucose to GA. Glucose oxidation leads to the production of glucono-δ-lactone acid in a reaction catalyzed by the membrane-bound

pyrrolo-quinolinequinone-dependent gluconate dehydrogenase (GDH; EC. 1.1.5.2). Glucono-δ-lactone is stable in acid conditions, but it can spontaneously hydrolyze to GA under neutral and alkaline conditions, or can be converted to GA by a membrane-bound gluconolactonase (GNL; EC:3.1.117) [36]. Even though some AAB are able to produce a high amount of GA, this metabolic pathway is not fully exploited for producing acid beverages, yet. Fermented beverages containing GA can take advantage of the "mild" properties of GA, which provide appreciated sensorial properties and, at the same time, preservative properties [97].

As reported in this review, over the past few years, low alcoholic and non-alcoholic beverages with functional properties are emerging products with an outstanding potential. Kombucha tea is an example of a non-alcoholic fermented beverage consumed for its beneficial effects on human health [98–100]. Kombucha tea is produced by fermenting sweetened tea with a microbial community composed of yeasts and AAB, of which most of the strains belong to the genus *Komagataeibacter* [101,102]. Recently, new Kombucha tea-based beverages have appeared on the market. These new products include a number of beverages produced by the basic practice to obtain the original Kombucha tea, but they can be flavored or obtained using raw materials different from tea or tea blended with suitable fermentable juices. Khosravi and co-workers [103] tested the date syrup as a substrate for producing a Kombucha-like beverage. Date syrups with different °Brix values were inoculated with a Kombucha starter culture and final products were compared to a standard Kombucha tea. Outcomes revealed faster changes of fermentation parameters, such as pH and titratable acidity in date syrup Kombucha. During Kombucha tea production, AAB oxidize ethanol to produce acetic acid, whereas glucose and fructose are used to produce GA, glucuronic acid, and bacterial cellulose [36,101,103,104]. Therefore, Khosravi and co-workers' study [103] proved that date syrup is a good raw material for Kombucha-like beverages production stating dates' product as a potential substrate for vinegar beverages and gluconic beverages too.

Conclusions

Among the strategies to valorize fruits and vegetables, microbial fermentation is one of the most valuable tools, helping to provide several sustainable and healthy foods and beverages.

Date fruits and their derivatives are versatile raw materials for microbial fermentation, being rich in fermentable sugars and bioactive compounds. Although they are already on the market as fruits and transformed products, a considerable number of dates are lost annually. On the other hand, efficient microbial transformations could open up the opportunity to satisfy consumers' demand of healthy and sustainable foods, while reducing food loss. When considering the AAB role for valorization of date fruits, both acetic acid and GA fermentations can be exploited for obtaining vinegars, low acetic

and gluconic beverages. Although vinegars from dates are already produced, little evidence is available on low acetic and gluconic beverages, which could be a valuable industrial segment considering the interest in non-alcoholic, non-sugared and functional beverages. The know- how acquired in the vinegar field could drive the rational design of processes aimed at exploiting AAB metabolic potential in performing both acetic and gluconic oxidation.

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2.2 Materials and methods

2.2.1 Laboratory scale experimental set up for date vinegar production

2.2.1.1 Industrial alcoholic fermentation

First a standard oenological yeast strain (*S. cerevisiae var. bayanus*) was inoculated inside a 20° Brix date juice. Alcoholic static fermentation was stopped when an amount of 9.82 g/100g of EtOH was obtained. Different starting batch such as fermenting batch of white wine vinegar and apple cider vinegar present in active Frings fermentation in Ponti SpA and static fermented date vinegar are tested as AAB started culture mixed.

2.2.1.2 Pilot bioreactors

The system consisted of two stirred tank bioreactors 8L each: in tank 1 the substrate (wine with an alcohol content between 10.5 and 11% g/L) was fermented until an optimal balance between the concentration of AcOH and EtOH was reached. In this system, the optimum equilibrium was represented by an AcOH concentration of 8% g/L and an EtOH concentration of 3% g/L. Once this balance had been reached, a part of the fermented AcOH was discharged into tank 2, where the acetic fermentation will end (EtOH < 0.5%) when the pre-established concentration of AcOH was reached, in this case a concentration between 10-11%. Inside bioreactors, there was a Rushton turbine to maintain homogenous conditions and a sparger to provide air insufflation. Typically, the working volume was 80% of total fermenter volume.

2.2.2 Bacterial strains and culture media

AAB strains used in this study belong to the genera *Acetobacter*, *Gluconobacter* and *Komagataeibacter*. Strains, species, sources of isolation and culture medium are listed in **Table 2.4**.

Species	Strain	Isolating source	Growing medium	Reference
G. oxydans	DSM 2343 = ATCC 621H = NCIB 8036	Fruit and flower	GYC	Gupta et al. 2001
A. pasteurianus	AB 0220 = UMCC 1754	Vinegar	GYC	Leibniz Institut DSMZ
A. pasteurianus	DL 13 = UMCC 1716	Chinese cereal vinegar	GYC	Wu et al. 2010
A. pasteurianus	DSM 3509^{T}	Beer	GYC	Leibniz Institut DSMZ
K. europaeus	ZJ 555 = UMCC 1806	Chinese cereal vinegar	GYC	Wu et al. 2010
K. europaeus	DSM 6160^{T}	Vinegar (Germany)	GYC	Leibniz Institut DSMZ

Table 2.4. Microbial strains used in this study

Strains were revitalised following the procedure codified by the UMCC (Unimore Microbial Culture Collection) using liquid GYC (**Table 2.5**). Before use, media were sterilized by autoclaving at 121°C for 15min. Cultures preserved in cryovials at -80 °C were inoculated in 5ml of GYC and incubated for 5 days at 28°C.

Medium composition	Quantity (%wt/v)
Glucose	10
Yeast extract	1
Calcium carbonate ($CaCO_3$)	2

Table 2.5 GYC liquid composition

2.2.3 Cryopreservation of the strains

Cryopreservation is the use of low temperatures to preserve structurally intact living cells. Glycerol solution at 50% was prepared from 99% pure glycerol commercial stock solution and distilled water, sterilized by autoclaving at 121°C for 15min and stored at 4°C until use. When necessary, glycerol solution was dispensed with an appropriate amount of liquid culture to reach an end concentration of 10-15% of glycerol. In particular, cryotubes were filled with 750µl 50% glycerol solution and 300µl liquid culture. After that, the cultures were frozen at -80 °C.

2.2.4 Date juice and puree

Date juice was provided by a supplier in a form of concentrated syrup obtained by the evaporation of water. Date puree was made from fresh fruits grinded, pressed, and sieved.

2.2.5 Gluconic fermentation conditions

The diluted date juice was filtered (0.2 µm). 12 flasks were sterilised for fermentation of the diluted juice. A 5% (v/v) preculture of *G. oxydans* ATCC 621H grown on GYC was used. Culture were incubated at 28°C for 12 days.

2.2.6 Acetic acid fermentation conditions

The filtered (0.2 µm) alcoholic fermented date previously prepared, was the raw material used for the production of vinegar. The alcoholic fermented has been filtered at 0.2 µm in such a way as to eliminate any impurities and microorganisms present inside to obtain a sterile product. For vinegar production five AAB strains were chosen: three of the genus *Acetobacter* (AB 0220, DSM 3509^{T}, DL13) and two of the genus *Komagataeibacter* (DSM 6160^{T}, ZJ 555) as listed in **Table 2.4**. The strains used were taken from -80°C and grown in sterilized tubes containing GYC for 5 days at 28° C. A pre-inoculum was performed sterilising five 100 ml flasks and inoculating bacterial culture, 50% solution of alcoholic fermented date and 50% of GYC medium. For every strain there was three 100 ml flasks containing alcoholic date juice and 5% (v/v) from preinocula, to start acetic fermentation. The tests were carried out in triplicate.

2.2.7 Microbial analysis

Both presence of AAB and yeasts were examined on the alcoholic fermented product supplied by the company. The presence of AAB was checked on GEY, containing glucose, yeast extract, EtOH, peptone and calcium carbonate [41] (**Table 2.6**) supplemented with cycloheximide, a chemical compound which inhibits the synthesis protein in eukaryotic cells by blocking the tRNA attachment and exit from ribosome [42]. Tests were conducted in triplicate.

Once the medium was prepared in a flask, this was sterilized in an autoclave for 15 min. at 121°C.

Medium composition	Quantity (% wt/v)
Glucose	2.0
Yeast extract	0.8
Peptone	0.5
$CaCO_3$	0.3
Agar	0.8
Ethanol	0.5

Table 2.6 Composition of GEY medium

To examine the presence of yeasts, the medium used was Sabouraud (**Table 2.7**). The procedure for seeding is the same used for AAB research. Then, all Sabouraud plates were incubated at 30°C for 3 days.

Medium composition	Quantity (% wt/v)
Pancreatic Casein Peptone	0.5
Meat peptone	0.5
Glucose	4.0
Agar	1.5

Table 2.7 Composition of Sabouraud medium

2.2.8 Analytical determinations

Determination of degree Brix

This Abbe Refractometer 2WAJ (**Figure 2.2**) served for the determination of the refractive index and the color dispersion of liquids, plastic and solid substances. White light was used and in average a temperature of 25°C. In our case, the hand refractometer (Abbe Refractometer 0 – 95 °Brix) is used to measure the total soluble solids of the vinegar and recorded as °Brix. Where one degree Brix is 1 gram of sucrose in 100 grams of solution and represents the strength of the solution as percentage by mass. The calibration was done by distilled water.

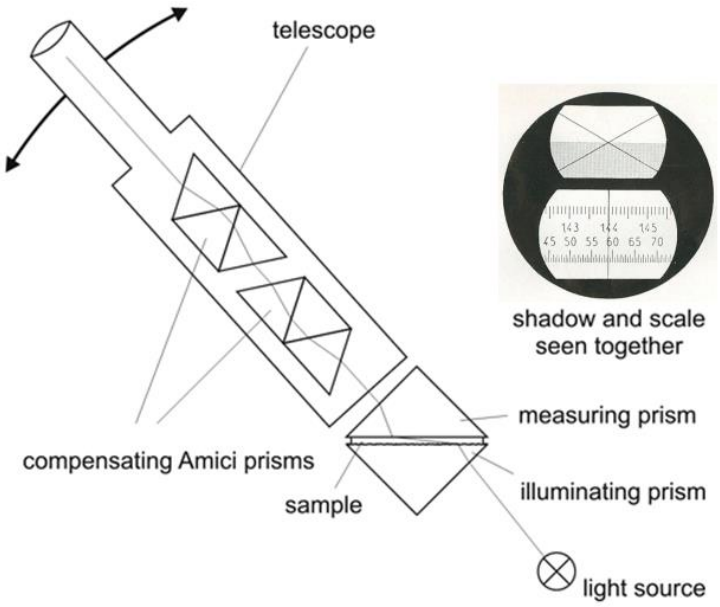


Figure 2.2 Diagram showing principle of Abbe refractometer

Determination of acidity and pH

Measurement of pH and titratable acidity was performed for all samples: diluted juice, concentrated juice, alcoholic fermented beverage and gluconic drink.

Titratable acidity was determined by titration with a basic solution (NaOH 1M or 0.05M). The end point of the titration was pH 7.2. pH-meter Crison 2002 was used. The following specific reaction was used to calculate the AcOH content of the vinegar sample:

$$CH_3COOH\ (aq) + NaOH\ (aq) \rightarrow H_2O(l) + NaCH_2COOH\ (aq).$$

Procedure for NaOH 1M: Sodium hydroxide solution 1M was prepared dissolving 40g of NaOH anhydrous pellets in 1L of distilled water by continuous stirring at room temperature. Vinegar (6mL) was diluted before analysis with freshly distilled water (44mL). The result is expressed in g/100 ml (or %) of AcOH (CH_3COOH, MW=60 g/mol).

Procedure for NaOH 0.05M: Sodium hydroxide solution 0.05M was prepared dissolving 2g of NaOH anhydrous pellets in 1L of distilled water by continuous stirring at room temperature. Vinegar (1mL) was diluted before analysis with freshly distilled water (9mL). The result is expressed in g/100 ml (or %) of AcOH.

$$\%\ \text{Titration acidity} = \frac{V_{NaOH} * M_{NaOH}}{V_{Vinegar} + V_{H_2O}} * MW_{AcOH}$$

pH-meter Crison 2002 was also used to determine pH value.

Determination of Ethanol

Malligand Ebulliometer is a very practical, easy to use measuring instrument to determine the alcohol level in an alcoholic liquid. Precise to 1/10%. The principle of this instrument is based upon the different boiling point of a liquid in a different atmospheric pressure. The boiling point decreases when the alcohol level is increases.

The conical heater was filled with distilled water up to the bottom ring. After that the spirits burner was filled and placed under a ventilation system, the burner was lighted. When the quicksilver in the column stopped the zero-point could be adjusted. This zero-point indicated the boiling point of water at the atmospheric pressure of that moment and could be used for about 2 hours.

Procedure*:* Add the liquid that needed to be tested up to the upper ring. Fill the cooler with cold water. When the quicksilver in the column stopped, the alcohol percentage of the liquid could be read from the graduated ruler.

2.2.9 Enzymatic determinations

Using different assay kit formats (manual, microplate, auto-analyser) available from Megazyme we analysed concentrations of AcOH, EtOH, Fructose, Glucose and GA in vinegar simples.

All enzymatic kits were based on the different ultraviolet absorption spectra between the oxidized and reduced forms of the coenzymes (NAD^+/NADH) (**Figure 2.3**). Both NAD+ and NADH strongly absorb ultraviolet light because of the adenine. Peak absorption of NAD^+ is at a wavelength of 259 nanometers (nm), with an extinction coefficient of 16.900 $M^{-1}cm^{-1}$. NADH absorbs at higher wavelengths, with a second peak in UV absorption at 339 nm with an extinction coefficient of 6.220 $M^{-1}cm^{-1}$. At higher wavelengths makes simple to measure the conversion of one to another in enzyme assays by measuring the amount of UV absorption at 340 nm using a spectrophotometer (JASCO V-550 – UV/VIS Spectrophotometer).

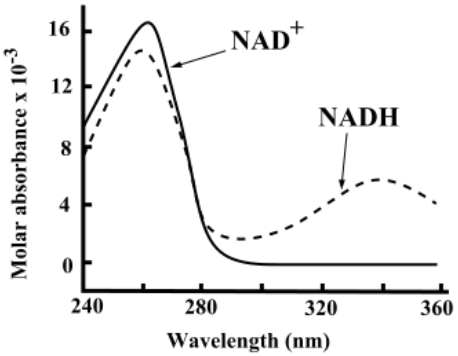


Figure 2.3 UV absorption spectra of NAD+ and NADH.

Acetic Acid [43]

The measurement was based on acetate kinase (AK) that in the presence of ATP converts AcOH into acetyl-phosphate and adenosine-5'-diphosphate (ADP).

$$\text{Acetic acid} + \text{ATP} \xrightarrow{\text{(AK)}} \text{acetyl-phosphate} + \text{ADP}$$

This reaction was significantly accelerated by the rapid conversion of the acetyl phosphate product into acetyl-CoA and inorganic phosphate, by the action of phosphor-transacetylase (PTA) in the presence of coenzyme A (CoA).

$$\text{Acetyl-phosphate} + \text{CoA} \xrightarrow{\text{(PTA)}} \text{acetyl-CoA} + \text{Pi}$$

The ADP formed in the first reaction is reconverted into ATP and pyruvate, by phosphoenolpyruvate (PEP) in the presence of pyruvate kinase (PK).

$$\text{ADP} + \text{PEP} \xrightarrow{\text{(PK)}} \text{pyruvate} + \text{ATP}$$

In the presence of the enzyme D-lactate dehydrogenase (D-LDH), pyruvate was reduced to D-lactate by reduced nicotinamide-adenine dinucleotide (NADH) with the production of NAD+.

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{(D-LDH)}} \text{D-lactic acid} + \text{NAD}^+$$

The amount of NAD+ formed in the above reaction pathway is stoichiometric with the amount of AcOH. It is NADH consumption which was measured by the decrease in absorbance at 340 nm [43].

Reagents

Bottle 1: Buffer (24 mL, pH 7.4) and sodium azide (0.02% w/v) as a preservative.

Bottle 2: NADH, ATP, PEP and PVP. Lyophilised powder.

Bottle 3: CoA, lyophilised powder.

Bottle 4: D-Lactate dehydrogenase, phosphotransacetylase and pyruvate kinase suspension (1.5 mL).

Bottle 5: Acetate kinase suspension (1.5 mL).

Preparation of reagent solutions/suspensions

1. Dissolve the contents of bottle 2 in 15 mL of distilled water. Divide the solution into appropriately sized aliquots and store at -20°C.
2. Dissolve the contents of one of bottle 3 in 0.8 mL of distilled water and store at -20°C.

Sample dilution

The amount of AcOH present in the cuvette should range between 0.3 and 25 µg. The sample solution must therefore be diluted sufficiently to yield an AcOH concentration between 0.03 and 0.25 g/L.

Estimated concentration of AcOH (g/L)	Dilution with water	Dilution factor (F)
<0.25	No dilution required	1
0.25 – 2.5	1 + 9	10
2.5 – 25	1 + 99	100
>25	1 + 999	1000

Table 2.8 Sample dilution to determine AcOH.

Procedure

Wavelength: 340 nm

Cuvette: 1 cm light path (glass or plastic)

Temperature: ~ 25°C

Final volume: 1.33 mL

Sample solution: 0.3-25.0 µg of AcOH per cuvette

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank (mL)	Sample (mL)
Distilled water	1.00	1.00
Sample	-	0.05
Solution 1	0.15	0.15
Solution 2	0.10	0.10
Solution 3	0.01	0.01
Solution 4	0.01	0.01
Mix, read the absorbance of the solutions (A_1) after approx. 2 min and start the reactions immediately by addition of:		
Solution 5	0.01	0.01
Mix, read the absorbances of the solutions (A_2) at the end of the reaction (approx. 4 min).		

Table 2.9 AcOH kit procedure.

Calculation

Determine the absorbance difference (A_1-A_2) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining ΔA_{AcOH}.

The concentration of AcOH can be calculated as follows:

$$c = \frac{V * MW}{\varepsilon * d * v} * \Delta A_{AcOH}$$

where:

V = final volume [mL]

MW = molecular weight of AcOH [g/mol]

ε = extinction coefficient of NADH at 340 nm = 6300 [l x mol-1 x cm-1]

d = light path [cm]

v = sample volume [mL]

Ethanol [44]

The quantification of EtOH requires two enzyme reactions; in the first reaction catalysed by alcohol dehydrogenase (ADH), EtOH was oxidized to acetaldehyde by nicotinamide-adenine dinucleotide (NAD+).

$$\text{Ethanol} + NAD^+ \xrightarrow{(ADH)} \text{acetaldehyde} + NADH + H^+$$

However, since the equilibrium of the first reaction in favour of EtOH and NAD+, a further reaction is required to "trap" the products. This was achieved by the quantitative oxidation of acetaldehyde to AcOH in the presence of aldehyde dehydrogenase (Al-DH) and NAD+.

$$\text{Acetaldehyde} + NAD^+ + H_2O \xrightarrow{\text{(Al-DH)}} \text{acetic acid} + NADH + H^+$$

The amount of NADH formed in this reaction pathway was stoichiometric with twice the amount of EtOH. It was the NADH which was measured by the increase in absorbance at 340 nm [44].

Reagents

Bottle 1: Buffer (15 mL, pH 9.0) and sodium azide (0.02% w/v) as a preservative.

Bottle 2: NAD^+.

Bottle 3: Aldehyde dehydrogenase solution (3.25 mL).

Bottle 4: Alcohol dehydrogenase suspension (1.3 mL).

Preparation of reagent solutions/suspensions

1. Dissolve the contents of bottle 2 in 12.4mL of distilled water. Divide the solution into appropriately sized aliquots and store at -20°C.

Sample dilution

The amount of EtOH present in the cuvette should range between 0.25 and 12 µg. The sample solution must therefore be diluted sufficiently to yield a concentration between 0.01 and 0.12 g/L.

Estimated concentration of EtOH (g/L)	Dilution with water	Dilution factor (F)
<0.12	No dilution required	1
0.12 – 1.2	1 + 9	10
1.20 – 12.0	1 + 99	100
12.0 – 120	1 + 999	1000
>120	1 + 9999	10000

Table 2.10 Sample dilution to determine EtOH.

Procedure

Wavelength: 340 nm

Cuvette: 1 cm light path (glass or plastic)

Temperature: ~ 25°C

Final volume: 1.285 mL

Sample solution: 0.25 – 12.0 µg of EtOH per cuvette

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank (mL)	Sample (mL)
Distilled water	1.00	1.00
Sample	-	0.05
Solution 1	0.10	0.10
Solution 2	0.10	0.10
Solution 3	0.025	0.025
Mix, read the absorbance of the solutions (A_1) after approx. 2 min and start the reactions immediately by addition of:		
Solution 4	0.01	0.01
Mix, read the absorbances of the solutions (A_2) at the end of the reaction (approx. 5 min).		

Table 2.11 EtOH kit procedure.

Calculation

Determine the absorbance difference (A_1-A_2) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining ΔA_{EtOH}.

The concentration of EtOH could be calculated as follows:

$$c = \frac{V * MW}{\varepsilon * d * v * 2} * \Delta A_{EtOH}$$

where:

V = final volume [mL]

MW = molecular weight of EtOH [g/mol]

ε = extinction coefficient of NADH at 340 nm = 6300 [l x mol-1 x cm-1]

d = light path [cm]

v = sample volume [mL]

2 = 2 moles of NADH produced for each mole of EtOH

D-Fructose and D-Glucose [45]

D-Glucose and D-fructose were phosphorylated by the enzyme hexokinase (HK) and adenosine-5'-triphosphate (ATP) to glucose- 6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P) with the simultaneous formation of adenosine-5'-diphosphate (ADP).

$$\text{D-Glucose} + \text{ATP} \xrightarrow{(\text{HK})} \text{G-6-P} + \text{ADP}$$

$$\text{D-Fructose} + \text{ATP} \xrightarrow{(\text{HK})} \text{F-6-P} + \text{ADP}$$

In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P was oxidized by nicotinamide-adenine dinucleotide phosphate (NADP+) to gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH).

$$\text{G-6-P} + \text{NADP}^+ \xrightarrow{(\text{G6P-DH})} \text{gluconate-6-phosphate} + \text{NADPH} + \text{H}^+$$

The amount of NADPH formed in this reaction was stoichiometric with the amount of D-glucose. It is the NADPH which was measured by the increase in absorbance at 340 nm.

On completion of reaction (3), F-6-P was converted to G-6-P by phosphoglucose isomerase (PGI).

$$\text{F-6-P} \xrightleftharpoons{(\text{PGI})} \text{G-6-P}$$

The G-6-P formed reacts in turn with NADP+ forming gluconate-6- phosphate and NADPH, leading to a further rise in absorbance that was stoichiometric with the amount of D-fructose [45].

Reagents

Bottle 1: Buffer (15 mL, pH 7.6) and sodium azide (0.02% w/v) as a preservative.

Bottle 2: NADP+, ATP and PVP.

Bottle 3: Hexokinase plus glucose-6-phosphate dehydrogenase suspension, 2.25 mL.

Bottle 4: Phosphoglucose isomerase suspension (2.25 mL).

<u>Preparation of reagent solutions/suspensions</u>

1. Dissolve the contents of bottle 2 in 12 mL of distilled water. Divide the solution into appropriately sized aliquots and store at -20°C.

Sample dilution

The amount of sugar (D-glucose plus D-fructose) present in the cuvette should range between 4 and 80 μg. The sample solution must therefore be diluted sufficiently to yield a sugar concentration between 0.04 and 0.8 g/L.

Estimated concentration of sugar (g/L)	Dilution with water	Dilution factor (F)
<0.8	No dilution required	1
0.8 – 8.0	1 + 9	10
8.0 – 80	1 + 99	100
>80	1 + 999	1000

Table 2.12 Sample dilution to determine glucose and fructose concentrations

Procedure

Wavelength: 340 nm

Cuvette: 1 cm light path (glass or plastic)

Temperature: ~ 25°C

Final volume: 1.16 mL (D-glucose)

1.17 mL (D-fructose)

Sample solution: 0.4 – 80.0 μg of sugar per cuvette

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank (mL)	Sample (mL)
Distilled water	1.00	1.00
Sample	-	0.05
Solution 1	0.05	0.05
Solution 2	0.05	0.05
Mix, read the absorbance of the solutions (A_1) after approx. 3 min and start the reactions immediately by addition of:		
Solution 3	0.01	0.01
Mix, read the absorbances of the solutions (A_2) at the end of the reaction (approx. 5 min). Then add:		
Solution 4	0.01	0.01
Mix, read the absorbances of the solutions (A_3) at the end of the reaction (approx. 8-10 min).		

Table 2.13 Fructose and glucose kit procedure.

Calculation

Determine the absorbance difference (A_1-A_2) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{D\text{-glucose}}$. Determine the absorbance difference (A3-A2) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{D\text{-fructose}}$. The concentration of sugar can be calculated as follows:

$$c = \frac{V * MW}{\varepsilon * d * v} * \Delta A$$

where:

V = final volume [mL]

MW = molecular weight of sugar [g/mol]

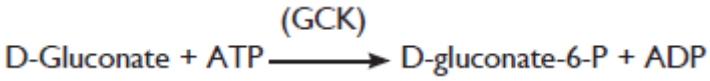
ε = extinction coefficient of NADH at 340 nm = 6300 [l x mol-1 x cm-1]

d = light path [cm]

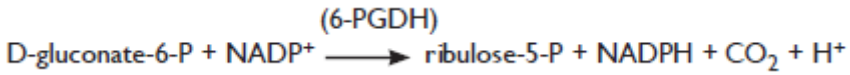
v = sample volume [mL]

D-Gluconic Acid and D-Glucono-δ-lactone [46]

D-Gluconic acid was phosphorylated to D-gluconate- 6-phosphate by adenosine-5'-triphosphate (ATP) and the enzyme gluconate kinase (GCK) with the formation of adenosine-5'- diphosphate (ADP).

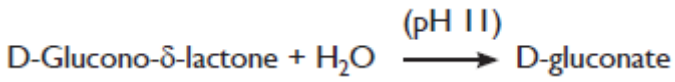


In the presence of nicotinamide-adenine dinucleotide phosphate (NADP+), D-gluconate-6-phosphate was oxidatively decarboxylated by 6-phosphogluconate dehydrogenase (6-PGDH) to ribulose-5-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH).



The amount of NADPH formed in this reaction was stoichiometric with the amount of D-gluconic acid. It was the NADPH which was measured by the increase in absorbance at 340 nm.

D-Glucono-δ-lactone, which was found in association with D-gluconic acid, was determined by the same principle after alkaline hydrolysis [46].



Reagents

Bottle 1: Buffer (12.5 mL, pH 7.6) and sodium azide (0.02% w/v) as a preservative.

Bottle 2: NADP+, ATP.

Bottle 3: 6-Phosphogluconate dehydrogenase suspension (1.25 mL).

Bottle 4: Gluconate kinase suspension (1.25 mL).

Preparation of reagent solutions/suspensions

1. Dissolve the contents of bottle 2 in 12.5 mL of distilled water. Divide the solution into appropriately sized aliquots and store at -20°C.

Sample dilution

The amount of D-gluconic acid and hydrolysed D-glucono-δ-lactone present in the cuvette should range between 0.8 and 50 µg. The sample solution must therefore be diluted sufficiently to yield D-gluconic acid concentration between 0.08 and 0.5 g/L.

Estimated concentration of sugar (g/L)	Dilution with water	Dilution factor (F)
<0.5	No dilution required	1
0.5 – 5.0	1 + 9	10
5.0 – 50	1 + 99	100
>50	1 + 999	1000

Table 2.14 Sample dilution to determine gluconic acid concentrations

Procedure

Wavelength: 340 nm

Cuvette: 1 cm light path (glass or plastic)

Temperature: ~ 25°C

Final volume: 1.27 mL

Sample solution: 0.4 – 80.0 µg of sugar per cuvette

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank (mL)	Sample (mL)
Distilled water	1.00	1.00
Sample	-	0.05
Solution 1	0.10	0.10
Solution 2	0.10	0.10
Solution 3	0.01	0.01
Mix, read the absorbance of the solutions (A_1) after approx. 5 min and start the reactions immediately by addition of:		
Solution 4	0.01	0.01
Mix, read the absorbances of the solutions (A_2) at the end of the reaction (approx. 6 min).		

Table 2.15 Gluconic acid kit procedure.

Calculation

Determine the absorbance difference (A_1-A_2) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{\text{D-gluconic acid}}$.

The concentration of sugar can be calculated as follows:

$$c = \frac{V * MW}{\varepsilon * d * v} * \Delta A_{D-gluconic\ acid}$$

where:

V = final volume [mL]

MW = molecular weight of D-gluconic acid [g/mol]

ε = extinction coefficient of NADH at 340 nm = 6300 [l x mol-1 x cm-1]

d = light path [cm]

v = sample volume [mL]

2.2.10 Total phenolic compounds and antioxidant activity determination

Total phenolic compounds (TPC) were determined by Folin-Ciacolteu method [47] and expressed as milligrams of gallic acid equivalent per litre (mg GAE/L).

Antioxidant activity was determined following the DPPH method, performed as described by Meda et al., 2005 with minor changes [48]. Briefly, 200 μL of sample was added to 2 mL of a 0.1 mmol/L methanolic solution of DPPH. After an incubation of 30 minutes in the dark, samples were read at 517 nm. Data were expressed as mg ascorbic acid equivalent/100 mL of juice.

2.3 Results and discussion

2.3.1 Choice of microbial strains for the production of gluconic beverages and date vinegar

Strain belonging to the *G. oxydans* species was used to make gluconic beverage, while for the production of vinegar five bacterial strains were screened, three belonging to the *A. pasteurianus* species and two to the *K. europaeus* species. ATCC 621H strain belongs to *G. oxydans* species is able to produce high concentrations of GA from glucose, as documented by the scientific literature [49] [50] [33] (**Figure 2.4**). Instead, strains of genus *Acetobacter* and *Komagataeibacter* are used due to high capacity to convert EtOH into AcOH in the presence of oxygen, a characteristic lacking in *Gluconobacter* strains.

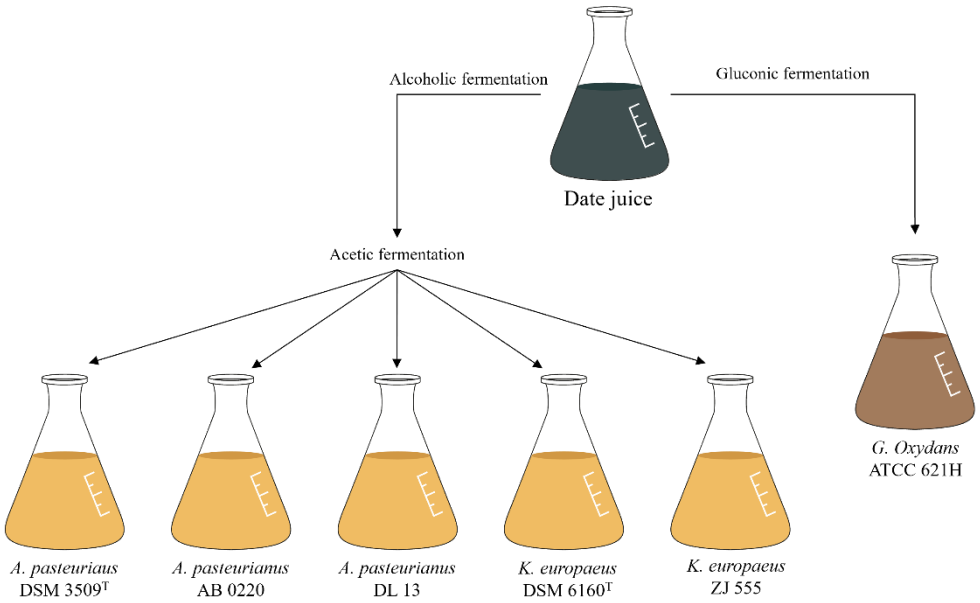


Figure 2.4 Flow chart of experimental plan

2.3.2 Gluconic fermentation: adaptation of the ATCC 621H strain to the substrate

Passmore and Carr, 1975 demonstrated that strains of the genus *Gluconobacter* grow well in sugary niches such as flowers and fruits (ripe grapes, apples and dates) and oxidize large amounts of glucose to GA [51]. GA is a functional additive in many food, pharmaceutical, textile and construction industries and is commonly added to dairy products and soft drinks to preserve and/or enhance their sensory properties. GA is listed as a Generally Permitted Food Additive (E574) by EFSA and as a GRAS (Generally Recognized As Safe) additive by the US FDA [52]. It is usually obtained through biological methods involving the partial oxidation of glucose providing an excellent example of how some waste and production surpluses with a high content of carbohydrates can be exploited in optimal way. GA and its derivatives are found naturally in plants, fruits and other foods such as rice, honey, grapes,

apples, meat, wine and vinegar. Recent research has revealed new potentially beneficial effects of this acid on human and animal health, which have enhanced its use as a prebiotic in food production [20]. The new non-alcoholic fermented drinks containing GA as the main ingredient, can be thus considered a new category of functional beverages. For their production it is important to maintain the nutritional and sensory properties of the fruit as much as possible; one example was the strawberry puree used to produce a naturally sweet non-glucose beverage by converting glucose to GA while retaining the original fructose of the puree. The best inoculation procedures were studied which observed *Gluconobacter* species more prevalently than other bacteria [20]. On the basis of these evidence, in this study, the ability of the ATCC 621H strain to ferment date juice containing a high concentration of glucose for the production of a non-alcoholic beverage was tested.

Before inoculation of the bacterial culture, the diluted date juice (24.27 ± 0.46 °Brix) had the following T_0 starting parameters (**Table 2.16**):

Characteristics	Value
pH	4.03 ± 0.01
Titratable acidity (g/100mL)	0.216 ± 0.023
Glucose (g/L)	292.024 ± 2.495
Fructose (g/L)	306.13 ± 1.756
Gluconic acid (g/L)	0.153 ± 0.009

Table 2.16 Initial composition of diluted date juice with relative standard deviations.

From **Table 2.16** it can be observed that the initial pH of the diluted juice is lower than the optimal pH for AAB growth which is between 5 and 6. However their growth is observed at very low pH values [53]; this is widely documented in vinegars [2], where they are detected at pH values lower than 3. The glucose amount of date juice is also favorable for AAB growth and production of GA, which is optimal in the range of 25-30% [54]. Thus, the growth of *G. oxydans* ATCC 621H was evaluated starting from a 5% v/v inoculum in 250 mL flasks, incubated aerobically at 28 °C. From 0 to 12 days of static fermentation the parameters of: GA, titratable acidity, pH, glucose and fructose were monitored.

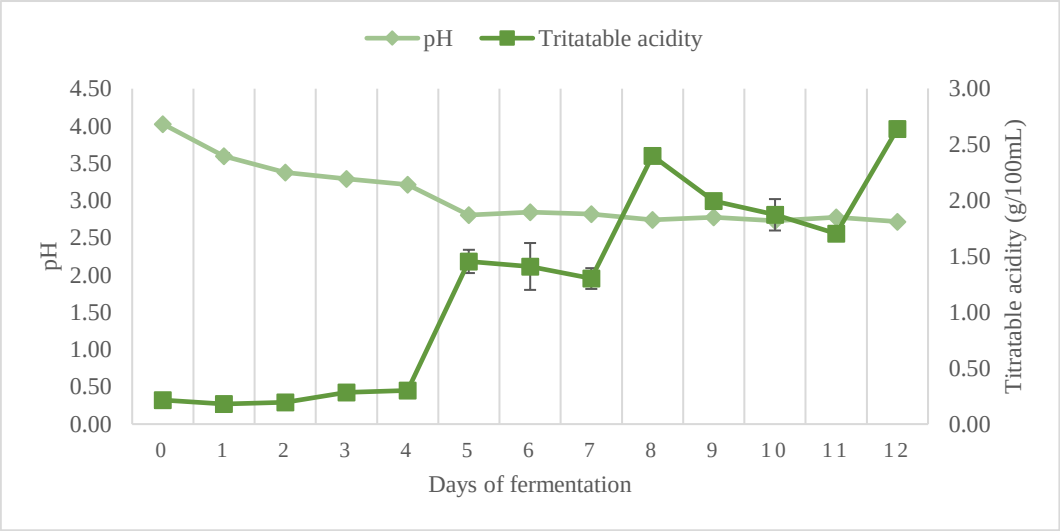


Figure 2.5 Trend of pH and titratable acidity over the 12 days of fermentation with relative standard deviations.

By the trend in **Figure 2.5,** it is possible to observe that the pH during the first 4 days of fermentation is quite linear with the concentration of titratable acidity. After the 4th day and up to the 12th day, the pH of the medium tends to remain constant at 2.7, while the titratable acidity reaches a quantity equal to 2.4% on day 8 and then drops gradually until day 11 and is again on day 12 with a peak of about 2.64%. pH and titratable acidity are two interrelated concepts in food analysis. Titratable acidity deals with measurement of the total acid concentration contained within a food, while the term pH is a mathematical shorthand for expressing this broad continuum of $H3O^{+}$ concentration. In this case there is no direct relationship between titratable acidity and pH, generally pH remain constant while acidity increase.

Determination of GA content

In **Figure 2.6**, it is possible to observe the consumption of glucose and the production of GA during the 12 days of fermentation:

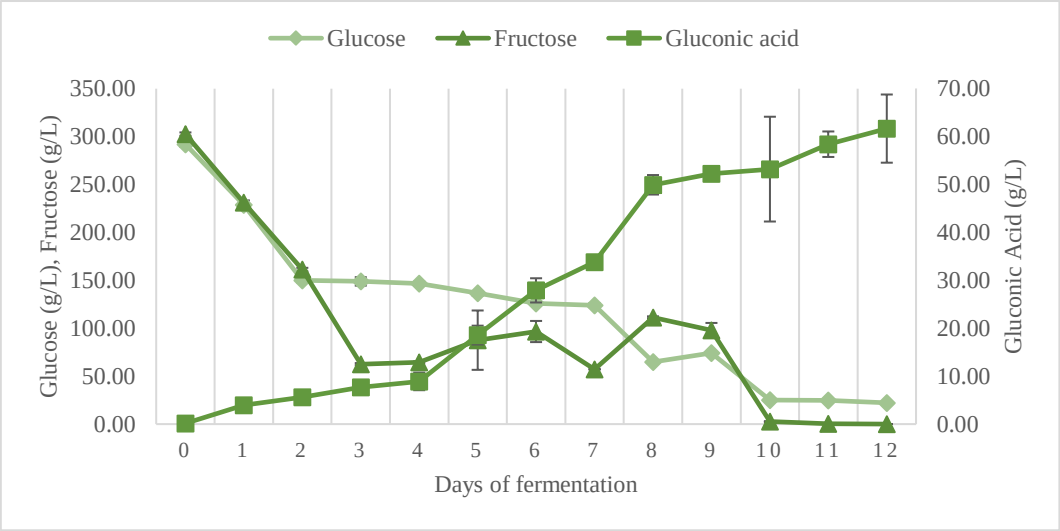


Figure 2.6 Trend of glucose and fructose consumption and production of GA with related standard deviations.

The initial concentration (T_0) of glucose in diluted date juice was 292.024 g/L while that of fructose was 302.613 g/L. During the first four days of fermentation *G.oxydans* 621H oxidizes a greater amount of glucose than the amount of GA produced, respectively 145.481 g/L and 8.907 g/L. This low production of GA could be due to adaptation phase not easy in a new matrix. As regards the consumption of fructose in between, it was evident from the first day, reaching a concentration of 64.523 g/L on the fourth day, only to then run out after 10 days. From day five there is a greater increase in GA, reaching on day eight a quantity of 49.94 g/L and a consumption of glucose equal to 65.429 g/L with consequent lowering of pH (see **Figure 2.5**). From the eighth day to the twelfth day, the production speed and therefore the increase of GA can be defined proportional to the consumption of glucose as shown in **Figure 2.5**, reaching a final concentration of 61.667 g/L while maintaining the pH of the constant medium at 2.7. Cañete-Rodríguez et al., 2016 demonstrated the enzymatic activity of gluconate dehydrogenase (see **Section 1.2.2.2**) is much greater, about 27 times greater, than that of NADP+ in the cytoplasm [55]. These enzymes can rapidly capture and remove glucose and other aldoses from the medium by converting them into organic acids, which are unavailable to many other microorganisms. Moreover, lower pH of the medium, making it a more adverse environment for growth than other microbial populations. As also reported in the literature [49] the production of GA and the consequent consumption of glucose and fructose is depending from the bacterial strain and the medium. Considering that the ATCC 621H strain on day twelve has a concentration of glucose equal to 22.414 g/L, it will be probable a continuous production of GA for other days, reaching a greater quantity of product.

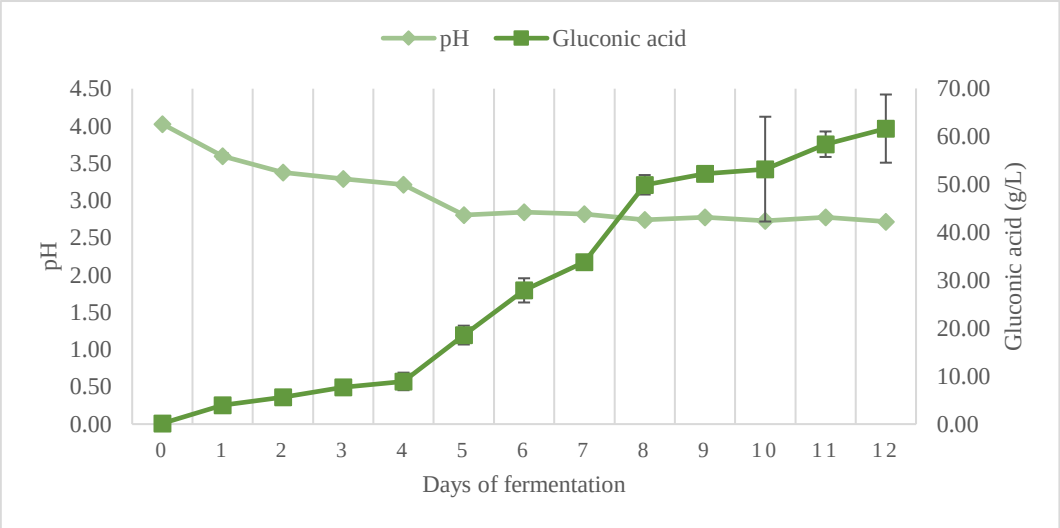


Figure 2.7 pH trend and GA production during the fermentation time with relative standard deviations.

Figure 2.7 shows the pH trend and the production of GA. It should be noted that the pH of the medium was not controlled in order to have a gradual oxidation of glucose and consequent production of GA, but only monitored over the 12 days of fermentation. As the production of GA increases, the pH decreases linearly, stabilizing at 2.7.

Table 2.17 Final parameters of the gluconic beverage on the twelfth day of fermentation.

Characteristics	Value
pH	2.72 ± 0.01
Titratable acidity (g/100mL)	2.64 ± 0.066
Glucose (g/L)	22.414 ± 0.509
Fructose (g/L)	0.028 ± 0
Gluconic acid (g/L)	61.667 ± 7.115

Table 2.17 Final composition of the gluconic drink after 12 days of fermentation with relative standard deviations.

The final production of GA equal to 61.667 g/L (**Table 2.17**) is in line with the production of GA obtained in the study by Cantadori, 2015. The final concentration in the cited study was 60 g/L despite the strain being been inoculated in a matrix different from the date [56]. It could therefore be deduced that, despite being inoculated into a new fermentation matrix, *G.oxydans* 621H had a good response to the production of GA in the medium, reporting a first initial phase of adaptation to the substrate and the subsequent continuous production of GA simultaneous with glucose consumption.

2.3.3 Acetic fermentation: adaptation of the *A. pasteurianus* and *K. europaeus* strains to the substrate

For the production of date vinegar, the alcoholic fermented date was used as a starting material. **Table 2.18** shows the initial characteristics:

Characteristics	Value
pH	4.93 ± 0.04
Titratable acidity (%)	0.565 ± 0.038
Ethanol (g/L)	71.793 ± 0.532
Glucose (g/L)	2.906 ± 0.783
Acetic acid (g/L)	0.520 ± 0.01

Table 2.18 Initial composition of the alcoholic fermented date with relative standard deviations.

The alcoholic fermented product is obtained from the fermentation of sugars by yeasts, generally yeasts of the genus *Saccharomyces* are used [57].

As can be seen from **Table 2.18**, the pH of 4.93 is an optimal value for the growth of AAB. pH value between 4 and 5, or lower values, is optimal for the action of ALDH which it catalyzes the oxidation of acetaldehyde to acetate [2].

2.3.3.1 Microbiological aspects of fermented alcoholic beverages

The alcoholic fermented product received from the Ponti SpA company was subjected to microbiological analyses to examine the presence or absence of yeasts and bacteria.

After incubation at 30°C, to verify the growth or presence of AAB inside the alcoholic fermented product, Petri dishes with GEY medium (see **Section 2.2.6**) did not highlight microbial development and therefore no start of a probable acetic fermentation in progress.

In **Figure 2.8**, instead, Petri dishes with Sabouraud medium (see **Section 2.2.6**) are shown to verify the presence of yeasts.

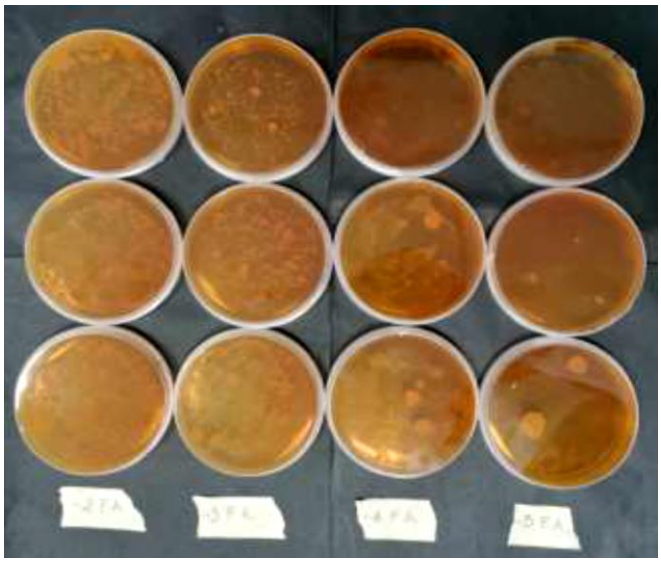


Figure 2.8 Plates with Sabouraud medium for yeast growth after incubation

Unlike the AAB plates, an abundant presence of yeasts can be observed instead. However, the latter was not investigated since was not relevant to proceed to the acetic fermentation. Subsequently, to start the fermentation, the alcoholic fermented product was filtered.

2.3.3.2 Adaptation conditions

Different microbial cultures of AAB belonging to the species *A. pasteurianus* and to the species *K. europaeus* of the UMCC were inoculated in the fermented date (**Table 2.19**). A screening of strains was carried out to see which of the five had better adapted to the starting matrix and therefore had oxidized EtOH with production of AcOH. Indeed, the speed of growth together with the rapid production of AcOH are a very important characteristic for the use of AAB as starters on a large scale. The low cost of the major categories of vinegars is one of the reasons why the industry does not use selected starter cultures for vinegar fermentation. In fact, the use of indigenous cultures of AAB, propagated with a back-slopping procedure, satisfies the main needs of the sector: low production costs, high performance and no specialized skills required to carry out the fermentation because the back-slopping procedure slopping is easily customizable [58]. However, the selection of microbial strains is interesting in order to improve both the characteristics of the products and their diversification.

The work was carried out in triplicate and three different 100 ml flasks were used for each strain.

Strains	Species
AB 0220 = UMCC 1754	*Acetobacter pasteurianus*
DL 13 = UMCC 1716	*Acetobacter pasteurianus*
DSM 3509^{T}	*Acetobacter pasteurianus*
DSM 6160^{T}	*Komagataeibacter europaeus*
ZJ 555 = UMCC 1806	*Komagataeibacter europaeus*

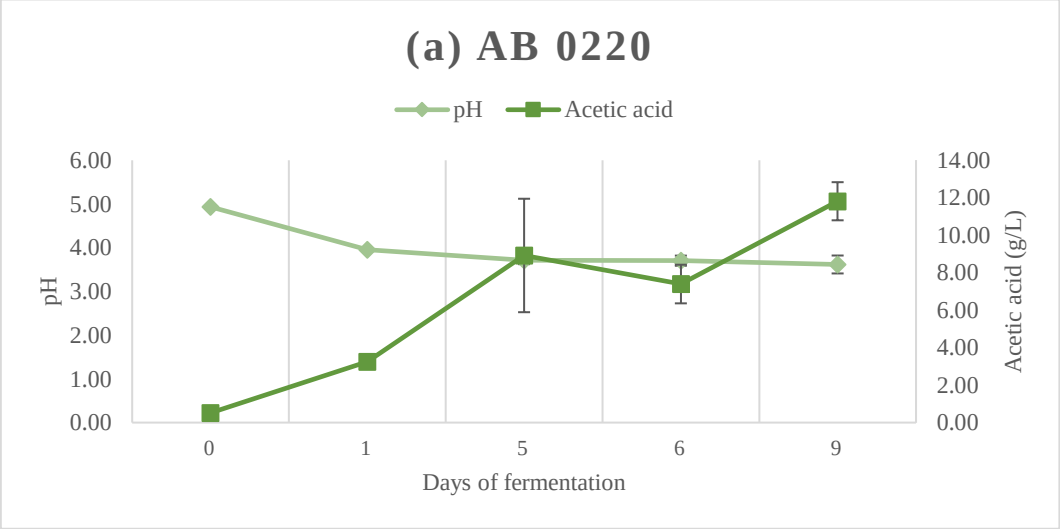
Table 2.19 Strains selected for this study

The monitoring of the acetic fermentation carried out at 1, 5, 6 and 9 days of fermentation for each strain, allowed the observation of the progress of the acidification and therefore the consumption of EtOH and the consequent production of AcOH.

2.3.3.3 Monitoring parameter strains of *A. pasteurianus* species

Three strains of the *A. pasteurianus* species were inoculated into the alcoholic fermented product: AB 0220, DSM 3509^{T} and DL 13.

Figure 2.9, shows the evolution of the production of AcOH determined enzymatically and the changes in pH in different stages of the acetic fermentation of the abovementioned strains:



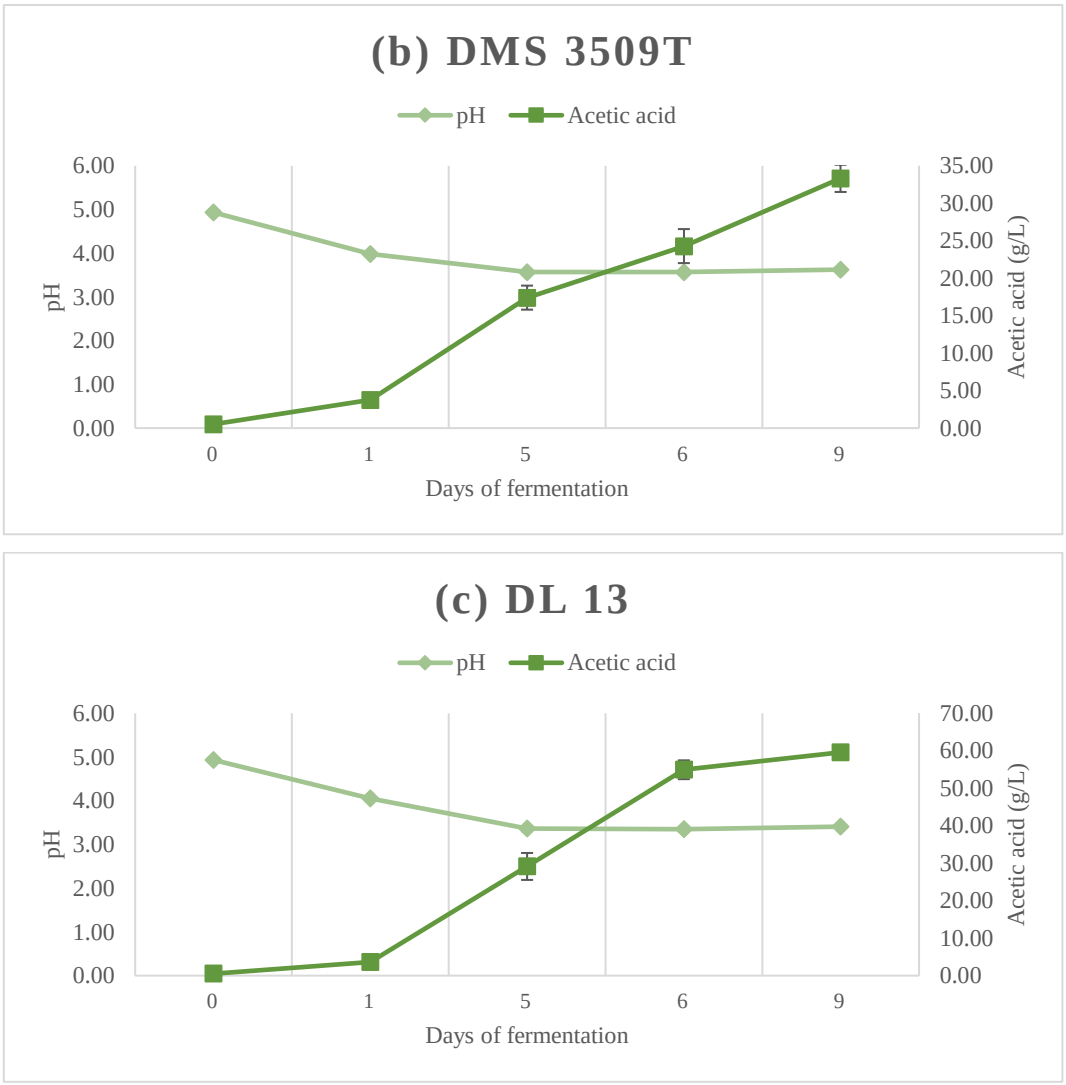


Figure 2.9 pH trend and acetic acid production in strains AB 0220, DSM 3509^T, DL 13 with relative standard deviations.

For all three strains: we start at T_0 with a pH equal to 4.93. The graph of the AB 0220 strain (**Figure 2.9a**) shows that the increase of AcOH corresponds to a non-linear lowering of the pH, up to t_9 in which the strain produces a quantity equal to 11.78 g/L. The graph of the DSM 3509^T strain (**Figure 2.9b**), on the other hand, demonstrates an almost linear increase in the production of AcOH from 0.52 to 33.577 g/L with the lowering of the pH. The trend of both parameters of the DL 13 strain (**Figure 2.9c**) is different: at T_1 the production of AcOH is proportional to the lowering of the pH, until T_6 with high growth of AcOH equal to 57.77 g/L and an unchanged pH. The maximum concentration of ACOH was reached at T_9: 59.189 g/L.

The acidity of the vinegar, in contrast to the lowering of the pH, increases with the duration of the fermentation for all three strains: DSM 3509^T and DL 13 had no difficulty in adaptation to alcoholic fermented dates, unlike AB 0220 which produced 1/3 of AcOH in the same days of fermentation. However, it must be taken into account that the AB 0220 strain was isolated from a surface

acidification system [59], for this reason, because of a completely different matrix, it could have difficulties in starting the fermentation.

2.3.3.4 Monitoring parameter strains of *K. europaeus* species

Two strains of the *K. europaeus* species were inoculated into the alcoholic fermented product: DSM 6160^T and ZJ 555.

In **Figure 2.10,** it is possible to see the evolution of the production of AcOH and the variations in pH at the different stages of the acetic fermentation:

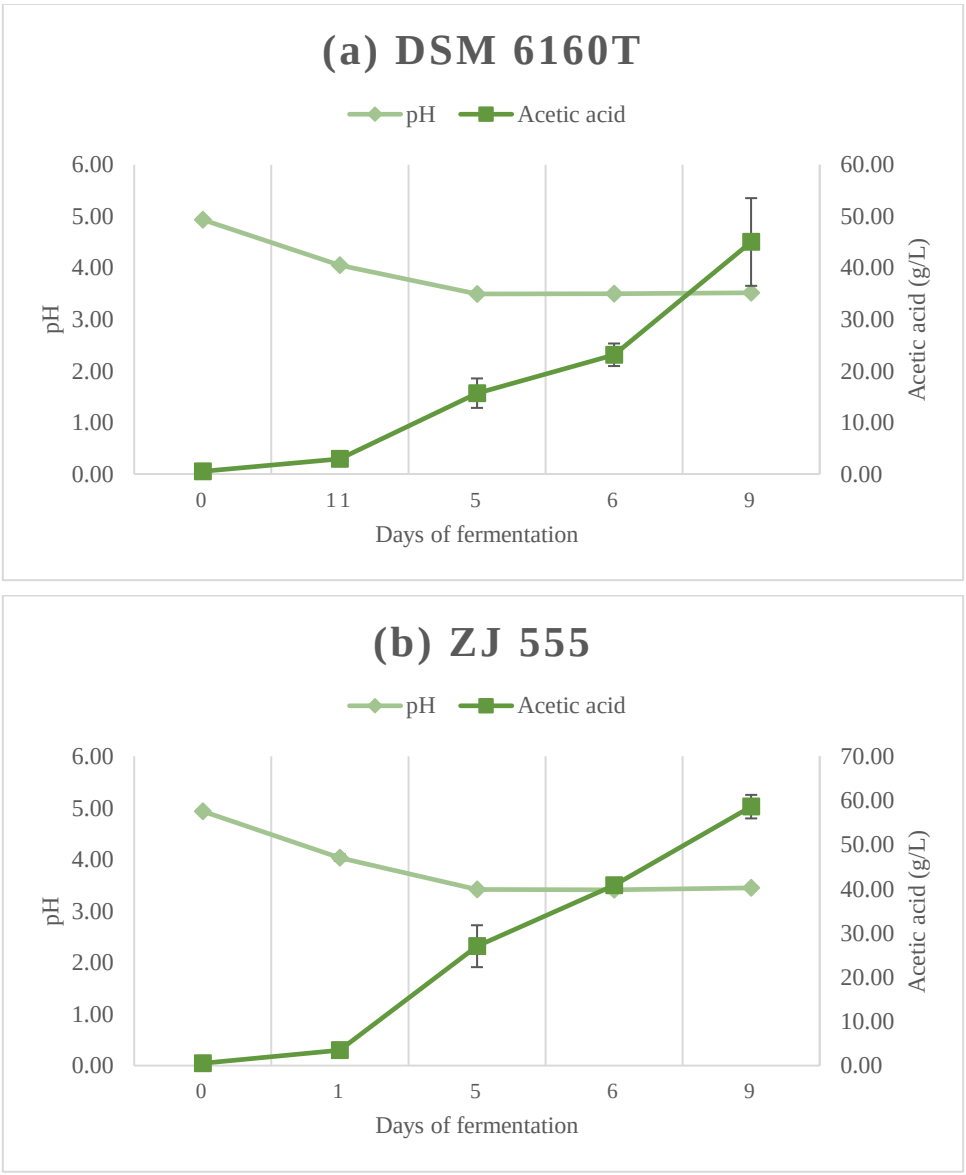


Figure 2.10 Trend of pH and AcOH production in strains DSM 6160^T, ZJ 555 with relative standard deviations.

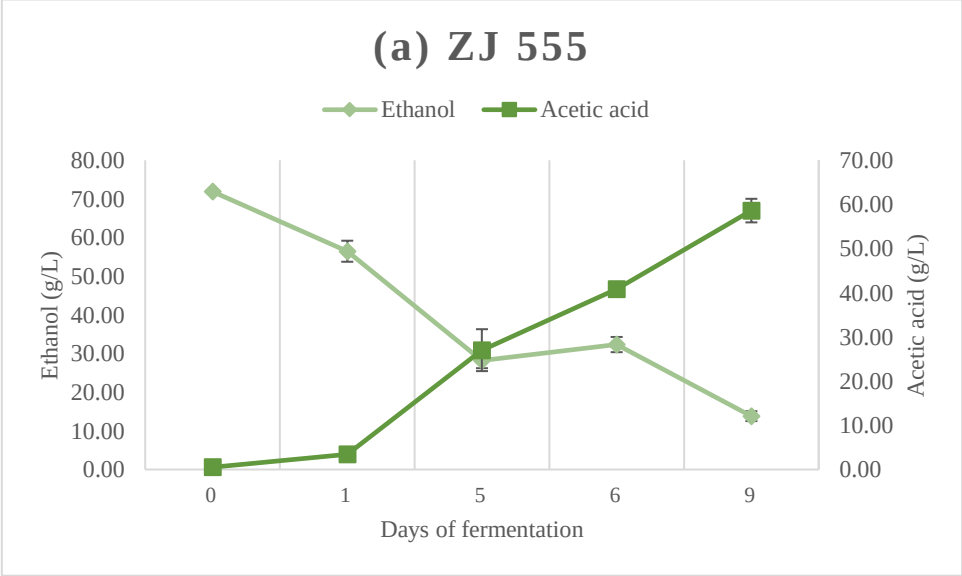
As for the strains of the genus *Acetobacter*, the starting pH of the strains of the genus *K. europaeus* is 4.93. **Figure 2.10a** represents the trend of the DSM 6160^T strain which, as can be seen in the first

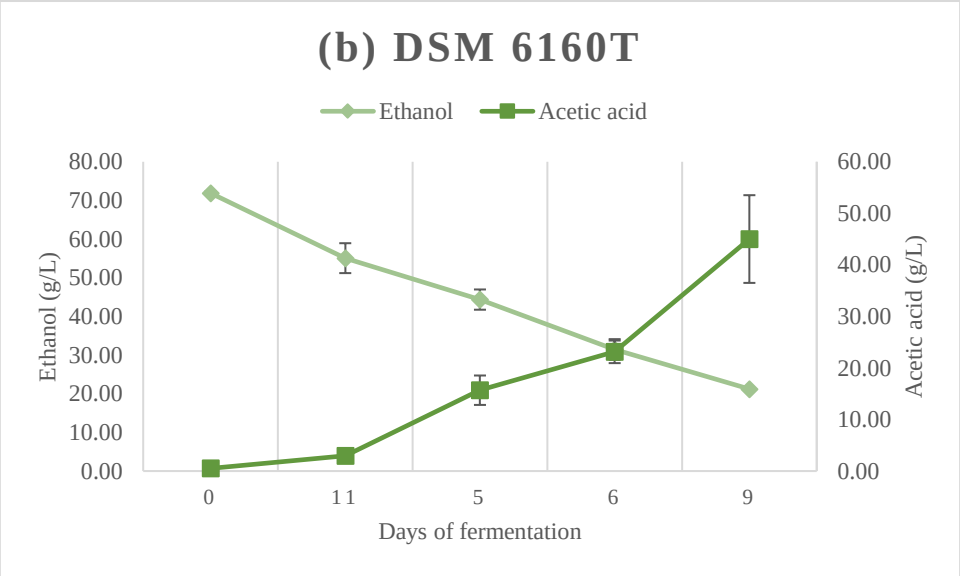
5 days, corresponds to a decrease in pH to the regular production of AcOH. From day 5 to day 9 the pH stabilizes at about 3.50 although there is a continuous production of AcOH and, consequently, one would expect a decrease in the pH level.

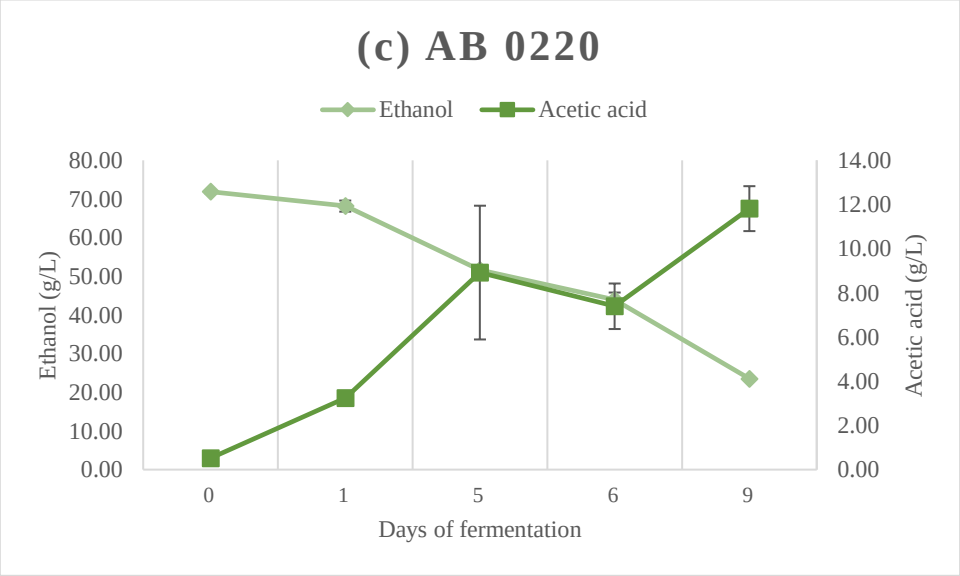
In **Figure 2.10b**, which instead shows the trend of the ZJ 555 strain, the production of AcOH could be defined as almost proportional to the decrease in pH which stops at 3.45 on the 9th day, while the quantity of AcOH produced reaches up to 58.605 g/L.

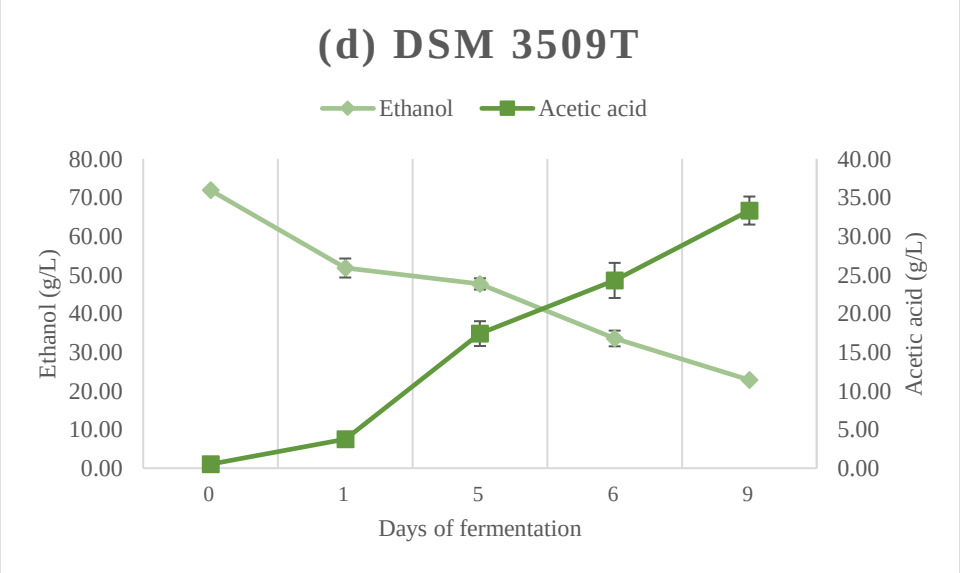
2.3.3.5 Oxidation of EtOH and production of AcOH

An initial EtOH concentration of about 7% as in our case, in conjunction with other parameters, can be considered a suitable factor for the development of AAB. This parameter must be carefully monitored over time as its decrease accompanied by an increase in titratable acidity is an indication of a regular fermentation process [60]. EtOH and AcOH must therefore present a specular trend and when this does not occur it is necessary to investigate thoroughly to identify and correct any anomalies. These trends are shown in **Figure 2.11** where the values of EtOH and AcOH in relation to time are represented.




(b) DSM 6160T
Ethanol
Acetic acid
Ethanol (g/L)
Acetic acid (g/L)
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
0
1 1
5
6
9
Days of fermentation


(c) AB 0220
Ethanol
Acetic acid
Ethanol (g/L)
Acetic acid (g/L)
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
0
1
5
6
9
Days of fermentation


(d) DSM 3509T
Ethanol
Acetic acid
Ethanol (g/L)
Acetic acid (g/L)
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
0
1
5
6
9
Days of fermentation

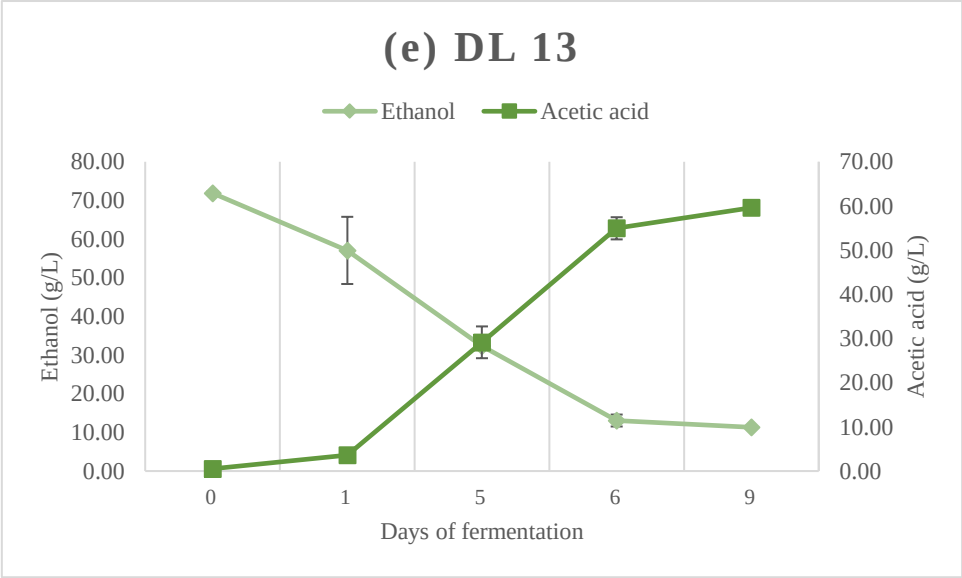


Figure 2.11 Trend of EtOH consumption (g/L) and corresponding production of AcOH (g/L) with relative standard deviations. (a, b) *K. europaeus*; (c, d, e) *A. pasteurianus*.

It is possible to observe in all cases an initial phase (T_1) in which there is an increase in values of AcOH equal to 3.410 g/L and a decrease of EtOH levels by 16.87 g/L for all strains, except strain AB 0220 decreased only to 3.62 g/L.

In the first two graphs represented by the strains of *K. europaeus*, the consumption of EtOH is not perfectly proportional to the production of AcOH. On the other hand, the strains of *A. pasteurianus* confirms what has been reported in the literature: in the presence of EtOH *A. pasteurianus* has an initial rapid oxidation into AcOH, which is then released from the periplasm to the surrounding environment [6].

Following the initial phase and then T_5, strains ZJ 555 and DL13 consumed a quantity of EtOH equal to 24.121 g/L, followed by AB 0220 with a quantity equal to 16.763 g/L and successively from DSM 6160^T, DSM 3509^T strains with an amount of 10.513 g/L and 4.326 g/L, respectively. At t_6, EtOH consumption decreased even more for the DL 13 and DSM 3509^T strains by 19.632 g/L and 13.766 g/L, respectively, followed by the DSM 6160^T strains with consumption equal to 12.908 g/L, AB 0220 with 7.282 g/L and ZJ 555 with a consumption of 4.134 g/L.

After another three days of acetic fermentation and then at t_9, all the strains continued to consume EtOH reaching a quantity of 14.029 g/L for ZJ 555, 10.600 g/L for the DSM 6160^T strain, 20.098 g/L for AB 0220, 10.788 g/L for DSM 3509^T and 2.078 for the DL 13 strain. The results obtained in this study can be compared with those obtained by Matloob, 2014 during the acetic fermentation of Zahdi dates. During his study, the concentration of AcOH produced was equal to 6.62%, in our case instead the strains whose production was higher than the others, showed a production equal to 5.9% [61]. These would be results consistent with those obtained in the previous study, however in the present work

the fermentation times of the strains were shorter. This supports the hypothesis that productivity (g/L days) is higher.

2.3.3.6 Production of AcOH of the different strains examined

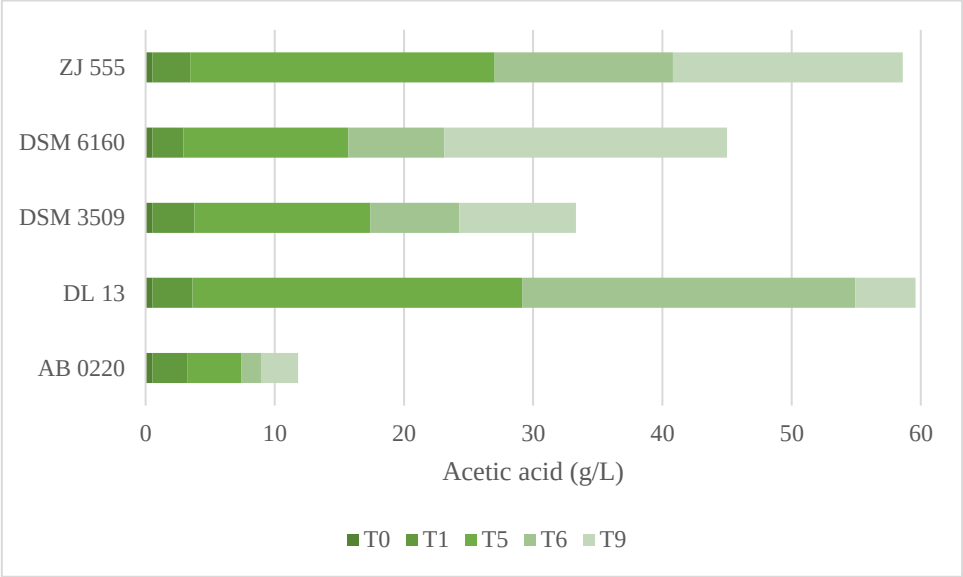


Figure 2.12: AcOH production of the five strains over the nine days of acetic fermentation

Figure 2.12 shows the amount of AcOH (g/L) that each strain produced over the fermentation times. After the first day of fermentation (T_1) it shows a production of AcOH very similar to each other and reach a greater quantity on the fifth day (T_5) where can already see big differences. The DL 13 and ZJ 555 strains produced a similar amount of AcOH (24 g/L), similar to the DSM 6160T and DSM 3509T strains where the amount of AcOH produced was equal to 13 g/L. The only difference was that strain AB 0220 whose production of AcOH in the first five days was only 5.73 g/L. The adaptation to the new matrix is undoubtedly more difficult for the latter strain which continued to have growth difficulties producing only 2.19 g/L on day 9 (T_9).

For the other four strains involved, the production of AcOH in a single day was high, especially for the DL 13 strain which in a single day produced 28.63 g/L, followed by the ZJ 555 strain with 13.81 g/L and from strains DSM 6061^T and DSM 3509^T with approximately 7 g/L. On the ninth and last day of fermentation, the production of AcOH in the various strains decreased for all instead DSM 6160^T which produced a quantity of 21.88 g/L in three days. The DL 13 strain yielded only 1,419 g/L in three days, the ZJ 555 strain 17.778 g/L, and the DSM 3509T 9.297 g/L. Probably the initial adaptation step was easier for all strains except strain AB 0220 and the two strains that were able to adapt better to the matrix were DL 13 and ZJ 555.

	Productivity: Acetic acid (g/L/day)				
Days	**ZJ 555**	**DSM6160^{T}**	**AB 0220**	**DSM 3509^{T}**	**DL 13**
1	3.450 ± 0.270	2.941 ± 0.05	3.250 ± 0.11	3.770 ± 0.25	3.640 ± 0.25
5	5.404 ± 0.890	3.136 ± 0.571	1.796 ± 0.606	3.482 ± 0.321	5.828 ± 0.720
6	6.804 ± 0.074	3.855 ± 0.365	1.598 ± 0.172	4.047 ± 0.378	9.628 ± 0.418
9	6.512 ± 0.296	5.001 ± 0.945	1.309 ± 0.113	3.730 ± 0.201	6.577 ± 0.092

Table 2.20 Daily AcOH production of the different bacterial strains used with relative standard deviations

Table 2.20 shows the daily concentration of AcOH produced by the five strains. And as can be confirmed by the results above, the strains that showed an excellent initial adaptation phase after only 5 days were *K. europaeus* ZJ 555 and *A. pasteurianus* DL 13, with a production peak on the sixth day of 6,804 g respectively /L/day and 9.628 g/L/day.

2.3.3.7 Glucose consumption by the strains used for the study

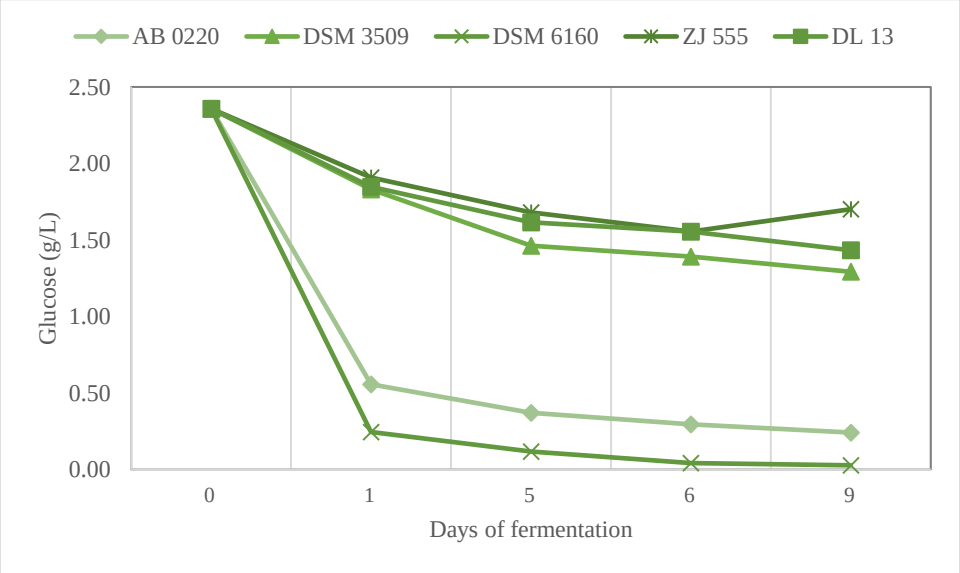


Figure 2.13 Glucose consumption by the strains used for the production of vinegar with relative standard deviations

Figure 2.13 shows the glucose consumption over the 9 days of acetic fermentation of the five strains of AAB. As shown in graphs A and E respectively of the genus *A. pasteurianus* (AB 0220) and *K. europaeus* (DSM 6160^{T}), there was immediately a total consumption of glucose. For the remaining three strains glucose consumption was not significant. AB 0220 and DSM 6160^{T} transformed glucose into GA concomitantly with the oxidation of EtOH and reaching glucose amounts on day 9 of 0.241 g/L for AB 0220 and 0.026 g /L for DSM 6160$^{T.}$ Otherwise, DL 13, DSM 3509^{T} and ZJ 555 strains after oxidized all the EtOH present in the medium, started to gradually consume glucose for the production of GA for their adaptation to the matrix and the continuation of their activity in the vinegar.

Mounir et al., 2016 evaluated the simultaneous ability of selected strains of *A. pasteurianus* for the production of AcOH and GA during acetic fermentation and only one of the two strains used had transformed part of the glucose into GA. However, few studies have reported the simultaneous ability of AAB strains to produce AcOH and GA in the same fermentation cycle [62].

2.3.3.8 Date juice fermented at industrial scale

Vinegar was produced by two stage fermentation processes, the first being the conversion of fermentable sugars into EtOH by yeasts and the second being the oxidation of EtOH by AAB [37]. The genera and species of yeast for the production of vinegars can be traced back to those for wine and the most important species is *Saccharomyces cerevisiae* [59]. At the start, fermentable sugars of concentrate date juice was converted to EtOH by commercial bakery yeast FERMIVIN PDM - *Saccharomyces cerevisiae var. bayanus* [63]. *S. cerevisiae var. bayanus* is one of the best strain to reach higher alcoholic degree, is cryotolerant and isolating from wine. Starting characteristics of concentrated date juice are summarised in **Table 2.21**.

Characteristics	Value
°Brix	67.8 ± 0.1
pH	4.27 ± 0.10
Titratable acidity (g/100mL)	1.35 ± 0.05
Density (g/L)	1.3378 ± 0.0005
Alcohol (g/100g)	0.47 ± 0.08
Fructose (g/100g)	31.50 ± 2.05
Glucose (g/100g)	34.12 ± 2.17

Table 2.21 Initial composition of concentrated date juice.

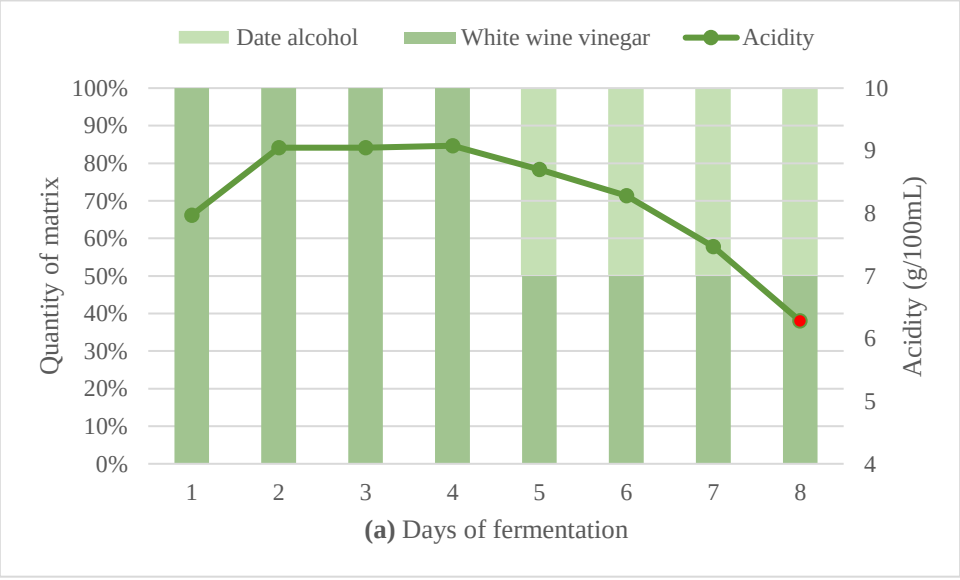
°Brix grades are closely related to alcoholic fermentation, as they reflect the content of sugars, which will determine the alcoholic grade that can be obtained, and this depends on the raw material used as well as on the culture of the microorganisms used for the fermentation process. The medium have also the usual values of pH between 3.5 and 5.5, correct for the cell growth and fermentation efficiency. Because of usual values of sugar content in the medium for a good fermentation rate should not exceed 20%, this concentrated date juice was diluted at 20°Brix, pasteurized and inoculated with bakery yeast (20g/hL). The final target for EtOH was approximately 11%. After 14 days of static fermentation at 20°C the alcoholic date juice had reached following concentrations (**Table 2.22**):

Characteristic	Value
°Brix	5.75 ± 0.1
Alcohol (%)	10.40 ± 0.3
Density (g/L)	1.0227 ± 0.0005

Table 2.22 Composition of alcoholic date juice.

Once the sugar has been transformed into EtOH, the next fermentation that takes place in the process to elaborate fruit vinegars is the acetic fermentation, which consists of the oxidation of the EtOH into AcOH.

Then, we start fermentation with two pilot fermenter of 8 L each inoculated with fermenting batch. We made different test with different starting batch such as fermenting batch of white wine vinegar present in active Frings in Ponti SpA (EtOH 10.71% - AcOH 7.97%) or apple cider vinegar (AcOH 6.58% - EtOH 8.00%). The scope was to find the best starting condition for the bacterial adaptation and growth in submerged pilot system and adding progressively defined amount of alcoholic date. And then operation consists of the development of successive continuous cycles of acetification. At the end of every cycle, a given volume of AcOH is discharged and refilled with alcoholic fuits juice. In both cases fermentations ended after alcoholic date refilling. In **Figure 2.14** was presented the preliminary data from the company.



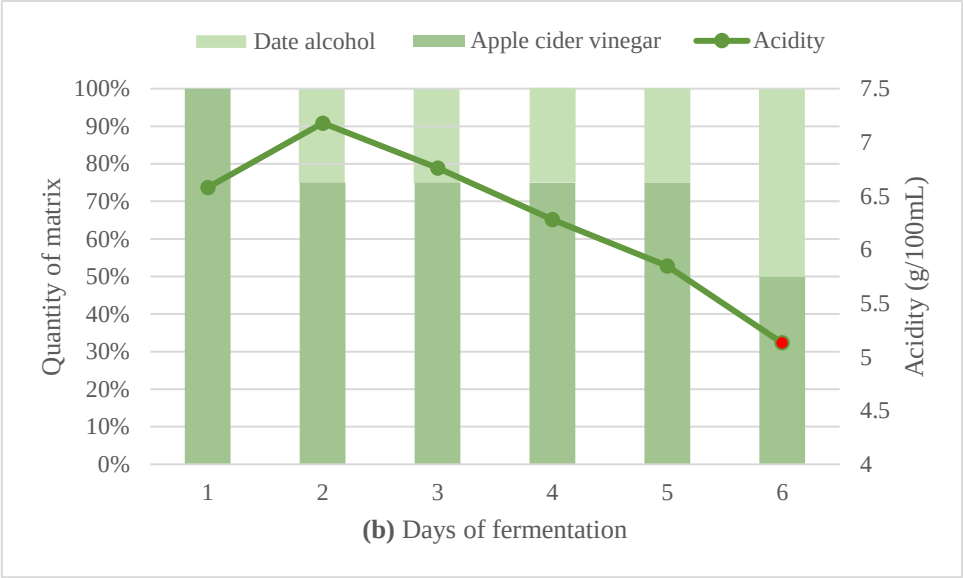
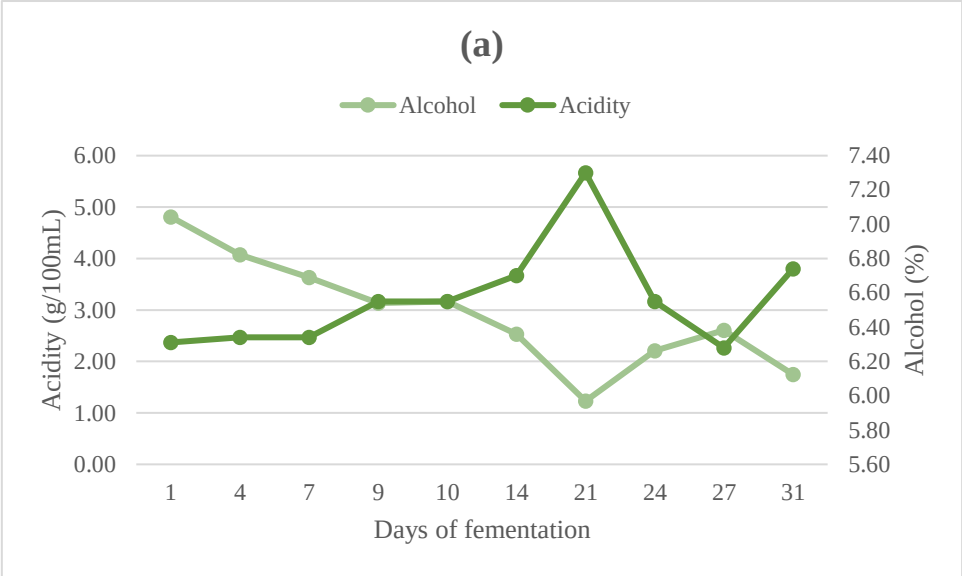


Figure 2.14 First test of pilot fermentation (a) White wine vinegar and (b) Apple cider vinegar mixed with percentage of date alcohol.

Finally, a static AcOH fermentation of alcoholic date juice was performed by a mixed AAB starter culture, then a submerged fermentation in two pilot fermentators (8 L each) was developed. For the static fermentation 30% of date alcohol was replaced with wine vinegar (EtOH 3.25% - AcOH 6.14%) and added to 70% of date alcohol tank, regularly. The static fermentation reach an acidity of 6.74% and a residual EtOH of 1.75% (**Figure 2.15a**). This static fermented batch is used to feed the first pilot fermentator. Cycling the first fermentor was discharged from acidified date alcohol and feed with new date alcohol (EtOH 10.40%). Initially, the air insufflation was led at 30% to permit microorganism adaptation then, was reached the 100% rate of oxygen in order to accelerate the oxidation reaction of EtOH into AcOH [37]. Moreover, the objective was to keep constant 6% of AcOH and 4% of EtOH. In the second pilot fermentor the EtOH concentration was reset and the result was 8L of date vinegar at 6.04% of acidity (**Figure 2.15b**).



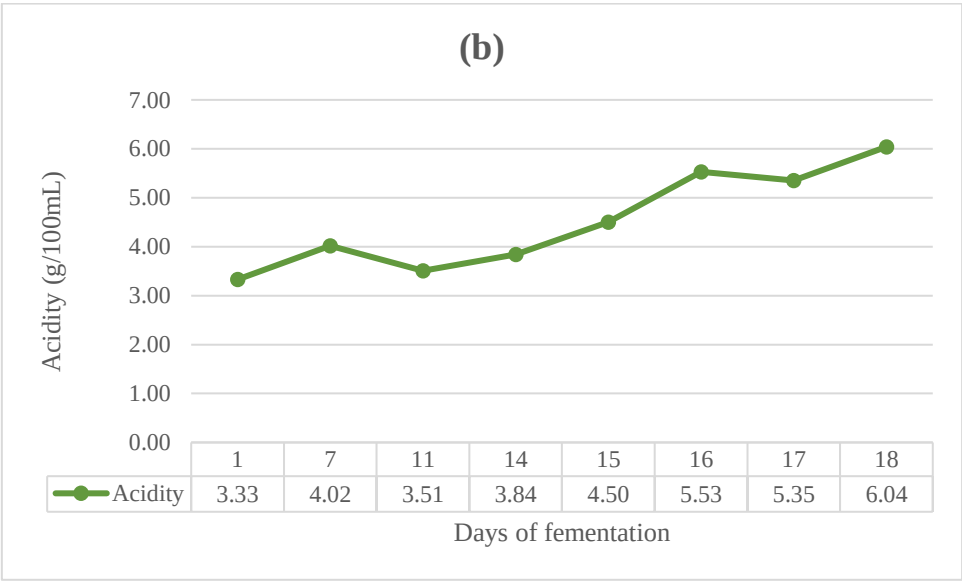


Figure 2.15 Preliminary data form the company (a) static fermentation (b) submerged fermentation

Results of this study provide evidence of the feasibility of developing controlled fermentation of dates juices for obtaining beverages/vinegars at different AcOH content, both in static and submerged systems. These outputs offer the chance to valorise dates juices by selective AcOH fermentations, contributing to development of sustainable bioprocesses.

2.3.4 Evaluation of total phenolic compounds and antioxidant activity in date vinegar samples produced from juices and fruits

Date juice were evaluated for phytochemicals and antioxidants activity. Because of fruits in the form of purees and juices is very popular for many formulations, both starting matrix were considered.

In both cases, juices and purees were pasteurized at 80°C for 20 minutes and subsequently inoculated with oenological strain *S. cerevisiae* UMCC 855. Finally, alcoholic products were fermented for 6 days with a strain of *K. europaeus* ZJ 555 and two strains of *A. pasteurianus* AB 0220 and DL 13.

The concentration of total phenolic compounds, in general, show a slight increase following the fermentation process (**Table 2.23**).

Products	**Start concentration**	***K. europaeus* ZJ 555 After fermentation**	***A. pasteurianus* AB 0220 After fermentation**	***A. pasteurianus* DL 13 After fermentation**
Date juice	$596.800^{a} \pm 11.733$	$482.800^{b} \pm 13.454$	$583.133^{a} \pm 52.814$	$532.133^{ab} \pm 41.296$
Date puree	$86.220^{a} \pm 5.972$	$96.434^{a} \pm 7.817$	$81.124^{a} \pm 6.828$	$99.745^{a} \pm 8.998$

Table 2.23 Total phenolic compound concentration expressed in mg / L of gallic acid equivalent (GAE). Values are reported as average of three replicates ± standard deviation. Significant differences are shown by different letters ($p \leq 0.05$).

Two different reasons could explain the increase or the constancy in phenolic compounds in vinegar brewing. First, glucoside bonds of the phenolic compounds could be degraded from acid during acetic fermentation, causing the liberation of compounds with different structure. The second reason could be the enzymatic conversion into small molecular weight polyphenol compounds having higher biological activity [1]. The present study confirmed that fermentation has great potential for the production of antioxidant active compounds from natural sources. Moreover, fermented fruit juices had higher concentration of phenolic compound as a result of destruction of cellular wall that improve total phenolic compounds release.

Regarding antioxidant activity expressed as an equivalent amount of vitamin C per 100 mL of sample, the analyses, carried out on the raw materials and the fermented products obtained from them, show

tendency to a slight decrease in vitamin C. The data in **Table 2.24** are preliminary and believed interesting to set up further fermentation tests by varying the fermentation conditions in order to acquire information on the influence of the kinetics / fermentation regime on the vitamin C content.

Products	**Start concentration**	***K. europaeus* ZJ 555 After fermentation**	***A. pasteurianus* AB 0220 After fermentation**	***A. pasteurianus* DL 13 After fermentation**
Date juice	$8.00^{a} \pm 0.21$	$6.74^{b} \pm 0.33$	$5.48^{c} \pm 0.12$	$6.74^{b} \pm 0.44$

Table 2.24 Antioxidant activity expressed as an equivalent amount of vitamin C per 100 mL of sample (mg vit C/100 mL). Significant differences are shown by different letters ($p \leq 0.05$).

2.4 Conclusions

The date fruit is a key resource for its cultivation lands and it requires intense efforts for valorisation. Despite the high production in fact, dates They are underutilized and there is a need to focus more research to add value to this cultivation. But, constant research and development is required to achieve this. In this regard, the purpose of this experimental thesis was precisely to enhance the date fruit as a raw material for the production of vinegars and non-alcoholic beverages with the use of specific bacterial cultures and monitoring the pH parameters, glucose consumption, GA production, EtOH utilization and AcOH production. The scope was evaluate the efficiency of the different microbial starters and their adaptation to date juice as a starting matrix.

The strain used for the study of the non-alcoholic beverages, *G. oxydans* 621H, adapted well to the date matrix producing at the end of fermentation a quantity of GA equal to 61.667 g/L which, comparing it with the literature, confirmed its high capacity to consume glucose to produce a large amount of GA. The parameter which, during the gluconic fermentation, showed some doubts about its progress, was the pH which from the sixth and up to the twelfth day maintained a value of 2.7. This may be due to the fact that it was only monitored but not controlled over the twelve days and that value suggest a continuous production of GA. In future studies it will also be interesting to research the reason for the consumption of fructose, trying to lengthen the fermentation times and lowering the Brix degrees values.

For the production of date vinegar, the results obtained from the screening carried out on the strains of the two species of acetic bacteria, *A. pasteurianus* and *K. europaeus*, showed a good general adaptation to the matrix. An exception for the AB 0220 strain (*A. pasteurianus*): minimum quantity of AcOH 11.78 g/L was produced during fermentation days due to the adaptation to the new matrix. The greatest production instead occurred from the ZJ 555 strain, of the *K. europaeus* species, reporting a concentration of AcOH produced equal to 58.605 g/L and from the DL 13, of the A. *pasteurianus*, which reached a final production of 59.19 g/L. The obtained results are linear with those researched in the literature. With the selection of the two strains it is possible to think of producing date vinegar even on a large scale.

Finally, prototypal trials were conducted to test the suitability of the process at industrial scale. Results of this study provide evidence of the feasibility of developing controlled fermentation of dates juices for obtaining beverages at different AcOH content, both in static and submerged systems. The

fermentation was stopped al 6.04% of AcOH content and the process was well underway during the 18 days of fermentation.

In conclusion, considering the rich content in essential nutrients, carbohydrates, fatty acids, proteins, vitamins and mineral salts, the valorisation of date fruits could start from the production of gluconic beverages, non-alcoholic beverages and vinegars using increasingly innovative technologies. These outputs offer the chance to valorise dates juices by selective AcOH fermentations, contributing to development of sustainable bioprocesses.

Chapter 3. Blackcurrant, blackberry and raspberry juices as models for gluconic and acetic fermentation

3.1 Valorisation of non-conventional fruits for beverages and vinegar

Since ancient times, fermented foods and beverages are a part of human nutrition. Their production was originally performed to improve the shelf-life of perishable raw materials from agriculture and animal husbandry origin [64]. However, the various microbial and enzymatic processes that are responsible for the making of fermented foods were unknown for several years [65]. Nowadays, the use of microorganisms and their enzymes, through fermentation (alcoholic, lactic, and acetic fermentations), amino acid conversions, and proteolysis is widely established to make products with desirable quality characteristics, mostly regarding shelf-life, taste, texture, mouthfeel, flavour, and colour [64].

High content of sugars, in particular glucose and fructose, made fruits suitable as raw materials for fermentation. It has been reported that fermentation enhances the nutritive value of the final products. These products are generally distinguished from others by great antioxidant content and lower amount of glucose. Besides, fermented beverages are accepted by consumers for a variety of sensory attributes including refreshing sour and sweet tastes [66] and really important healthy and nutritional aspects. Microorganisms like yeasts and bacteria are the main protagonists of the fermentation process.

The selection of suitable yeast strains for the production of alcoholic beverages is important not only to maximize the fermentation process but also to maintain beverage sensory quality. However, health-based recommendations include the reduction of alcohol consumption and calories [67]. For this reason, soft and low-alcohol drinks sweetened by the fruit's sugar content without added sugars are gaining increasing popularity among consumers. Regarding bacteria, some AAB like those belonging to the genus *Gluconobacter* can partially oxidase glucose into GA. GA is a common additive and is a weak, non-volatile, harmless (odourless, non-corrosive, non-toxic), easily biodegradable acid that is soluble in water and insoluble in non-polar solvents. This acid provides an excellent example of how some production wastes and surpluses with high carbohydrate contents can be optimally exploited.

For instance, date fruits and their derivatives are versatile raw materials for microbial fermentation, being rich in fermentable sugars and bioactive compounds. Although vinegars from dates are already produced, little evidence is available on low acetic and gluconic beverages, which could be a valuable industrial segment considering the interest in non-alcoholic, non-sugared and functional beverages [1]. Among the different kinds of fruits, red fruits, with their intense flavour, are appreciated by almost all consumers. Several studies have stated their phytochemical composition, which includes antioxidant compounds able to exert a protective effect against many chronic diseases[68]. Polyphenols

and carotenoids are the two main kinds of antioxidant phytochemicals. Nevertheless, the profile and concentration of anthocyanins are different depending on the fruit used; therefore, the colour and health benefits of the juices also show differences. Some red fruits, for example strawberries, have lower concentrations of anthocyanins, and others, like black currants, have higher concentrations [69].
In this chapter, the most recent findings in the area of fermented beverages from non-conventional raw materials are discussed with a special focus on the biotechnological transformation of raspberry, blackcurrant and blackberry starting from fruits, juices, and fermented beverages.

3.1.1 Red Fruits features

Blackberry fruit (Rubus subg. rubus)

Blackberry is a worldwide consumed fruit, mostly produced in North America, Europe, and Asia [70]. At the industrial level blackberries are used for several productions, such as dietary supplements or jams. However, they are mostly consumed as fresh fruits or sold as individually quick-frozen packs. Although blackberries' chemical composition is strictly dependent on several factors such as the variety or the stage of ripeness, generally they are rich in sugars, minerals, and phenolic compounds [70]. Total sugars, soluble solids and total anthocyanin increase as the fruit ripens. On the other hand, proteins content, total phenolic compounds significantly decrease [71]. Glucose and fructose are the main sugars, with sucrose being present in traces. Potassium and magnesium are the main minerals detected in blackberries, followed by calcium and manganese [71] (**Table 3.1**).
Blackberry contains a high amount of citric and malic acid. In addition, various studies reported the presence of shikimic, fumaric, and succinic acid. Organic acids content is of fundamental importance for evaluating fruits' quality levels since they act as a stabilizer for anthocyanins. The health benefits of blackberries are associated with anthocyanins and other phenolic compounds such as ellagitannis, flavonols, and procyanidins [70] [72]. Anthocyanins are pigments belonging to the phenolic group, responsible for the characteristic colour of blackberry [73]. In addition, anthocyanins are strong antioxidant compounds with potential antidiabetic, anticancer, anti-inflammatory, antimicrobial, and anti-obesity effects, as well as prevention of cardiovascular diseases (CVDs) [74].

Characteristics		Vitamin content		Mineral content	
Water (g)	88.20	Total ascorbic acid (mg)	21.00	Calcium (mg)	8.1-29.0
Energy (kcal)	43.00	Thiamin (mg)	0.02	Iron (mg)	0.6-0.7
Protein (g)	1.39	Riboflavin (mg)	0.03	Magnesium (mg)	20.0-29.0
Total lipids (g)	0.49	Niacin (mg)	0.65	Phosphorus (mg)	22.0
Ash (g)	0.37	Pantothenic acid (mg)	0.28	Potassium (mg)	137.5-162.0
Carbohydrate (g)	9.61	Vitamin b6 (mg)	0.03	Sodium (mg)	1.0
Total fiber (g)	5.30	Total folate (μg)	25.00	Zinc (mg)	0.3-0.5
Total sugars (g)	4.88	Vitamin b12 (μg)	Nd[1]	Copper (mg)	0.1-0.2
Sucrose (g)	0.07	Vitamin a (iu)	214.00	Manganese (mg)	0.5-0.7
Glucose (g)	2.31	A-tocopherol (mg)	1.17	Selenium (mg)	0.4
Fructose (g)	2.40	B-tocopherol (mg)	0.04		
Maltose (g)	0.07	Γ-tocopherol (mg)	1.34		
Galactose (g)	0.03				

Table 3.1 Chemical composition of blackberries (adapted from Costa et al., 2013) [75].

Raspberries (Rubus idaeus)

Raspberries hold a special position due to their culinary versatility, ideal nutritional profile of low calories, saturated fats, high fiber, mineral, potassium, sodium, vitamins, presence of several essential micronutrients, and phytochemical composition [76] (**Table 3.2**). The main forms of processed red raspberries available are individually quick frozen (unsweetened or sweetened), juice concentrates (single or concentrated strengths), and puree (seedless or seeded) [77]. Worldwide 822,493 tonnes of raspberry are produced per year. Mexico, Serbia and Russian Federation produce together more than 50 % of the world's total raspberry globally. The major raspberry producer in the world is Russian Federation with 174,000 tonnes of production per year, Mexico comes second with 128,848 tonnes of yearly production, and the third largest producer of raspberry with 120,058 tonnes of production per year is Serbia [68].

Raspberries are a good source of phenolic compounds and many nutrients such as vitamins, minerals, and fatty acids [78]. **Table 3.2** shows the nutrient profile of raspberry fruits. Raspberries are also rich in fructose and contain small amounts of glucose and sucrose. Among vitamins, vitamin C is the most abundant, followed by riboflavin, folic acid, and niacin. It's worth noting that a 100g portion of raspberries provides 50% of the recommended intake of vitamin C [77]. Raspberries are also a good source of manganese, potassium, copper, and iron. The nutrient profile of raspberry potentially helps

regulate blood sugar levels by slowing digestion and contributes to a satiety effect (given by the high fiber content).

Likewise, other red fruits, raspberries contain high levels of anthocyanins and ellagitannins. Ellagic acid and ellagitannins exhibit a wide range of beneficial effects on human health such as antioxidant, antimutagenic, anticarcinogenic, and antiviral. Besides anthocyanins and ellagitannins, raspberries contain other phenolic compounds including quercetin, kaempferol, and gallic acid, reaching a total phenolic content between 160-645 mg/100g of fresh fruit [79] [80] [81].

Characteristics		Vitamin content		Mineral content	
Water (g)	87.75	Total ascorbic acid (mg)	26.2	Calcium (mg)	25
Energy (kcal)	52	Thiamin (mg)	0.032	Iron (mg)	0.69
Protein (g)	1.2	Riboflavin (mg)	0.038	Magnesium (mg)	22
Total lipids (g)	0.65	Niacin (mg)	0.598	Phosphorus (mg)	29
Carbohydrate (g)		Pantothenic acid (mg)	0.329	Potassium (mg)	151
Dietary fiber (g)	11.94	Vitamin B6 (mg)	0.055	Sodium (mg)	1
Total sugars (g)	6.5	Total folate (μg)	21	Zinc (mg)	0.42
Sucrose (g)	4.42	Choline (mg)	12.3	Copper (mg)	0.09
Glucose (g)	0.2	Vitamin A, RAE (μg)	2	Manganese (mg)	0.67
Fructose (g)	1.86	Lutein – zeaxanthin (μg)	136	Selenium (mg)	0.2
		Vitamin E, A-tocopherol (mg)	0.87		
		Vitamin K (μg)	7.38		

Table 3.2 Nutrient composition of fresh red raspberries per 100 g (adapted from Rao and Snyder 2010) [77].

Blackcurrants (Ribes nigrum)

Germany, Poland, and the United Kingdom contribute to about 80% of the total production of blackcurrant. The total world black currant production averaged about 205,150 tonnes.

Blackcurrant is widely recognized for containing high concentrations of phenolic compounds (125–151 mg/100 g fresh weight), especially of proanthocyanins and anthocyanins which combined constitute 80% of total phenolics [82] [78]. Furthermore, blackcurrant contains a high amount of vitamin C, ranging from 121 to 230 mg/100 g of fresh fruit, covering 100% of recommended daily intake with a portion of just 25g. High levels of vitamin C, anthocyanins, and phenolic compounds suggest that blackcurrant can be used as a potential nutraceutical ingredient. Therefore, the phytochemicals present in blackcurrant have been extensively studied for their antioxidant activity [83], anti-inflammation activity [84]), neuroprotective actions [85], anti-obesity properties [84] [86], and anti-cancer properties [87].

The phytochemicals, especially anthocyanins existing in blackcurrant berry fruit, have been extensively studied by the scientific communities for their various medicinal values, such as These. Therefore, blackcurrant-sourced food products are trending worldwide as potential functional foods consumed to prevent diseases [82].

Blackcurrants contain high levels of antioxidants with an average content of ascorbic acid 125–151 mg/100 g fresh weight [78], and phenolic compounds in particular anthocyanins. Furthermore, blackcurrants contain 2000 mg/kg of vitamin C, about five times of oranges content [73], high minerals (Potassium, calcium, magnesium, and sodium), and monosaccharides [70]. Total phenolics and anthocyanins contents decreased during the processing of berry fruits to juices, and the reduction of anthocyanins was more pronounced, 12%–80%. The content of total phenolics in berries increased during 1 year of storage by 46.09%–171.76% and in juices by even 107.58%, while the content of total anthocyanins in berries and juices decrease by 5.63%–52.76% and 13.04%–36.82%, respectively. When berries are frozen, chemical changes can occur, including the concentration of solutes and chemicals, oxidative reactions, and enzyme activity. Pectinolytic enzymes are used in industrial juice production to increase the juice yield and production efficiency and these treatments decrease the viscosity of the juice, and significantly increase the extraction of bioactive compounds such as anthocyanins and other phenolic compounds and their related nutritional value. A possible explanation for this phenomenon is that bioactive compounds, which are trapped in the networks of the pectins, are liberated with the effects of the enzymes. However, the use of enzymes in juice processing also results in increased astringency and loss of fresh blackcurrant aroma [88 89].

3.1.2 Exploring red fruits for fermented milk and yogurts

Fermented milk and yogurts are foods widely consumed worldwide due to their excellent flavour, functional properties, and probiotic characteristics. During fermentation, microbial enzymes produce new derived compounds with an impact on aroma and functionality, reduce sugar content, improve nutritional value, and extend the shelf-life of fruit-based beverages [90]. On the other hand, these probiotic products were unsuitable for some people because of the existence of allergens-casein and β-Lactoglobulin. So, food industries have started to search some alternative substrates for probiotic delivery. Blackberry and blueberry are particularly rich in anthocyanins and phenolic acids. To produce low-alcoholic or non-alcoholic berry beverages, various *Lactobacillus* strains have been used in berry fermentation. Fermentation driven by lactic acid bacteria adds health benefits to the final products increasing the bioactive compound [91]. The microbial counts of three strains in blueberry and blackberry juices increased during fermentation and reached the recommended level for potential probiotic foods while anthocyanins had a decreasing trend. After 48-h fermentation, blackberry and

blueberry juices fermented by *L. plantarum* contained higher contents of lactic acid (around 4000 mg/L) and syringic acid (around 4 mg/L). The stronger capacity for the metabolism of phenolics and organic acids contributed to a higher increment of ABTS radical scavenging activity in fermented blackberry and blueberry juices respectively increased by 38.4 mmol Trolox/L and 28.6 mmol Trolox/L. Interestingly, the produced acids by *L. plantarum* enhanced the acceptability of consumers of fermented blueberry juices but decreased the acceptability of fermented blackberry juices [92].

3.1.3 Exploring red fruits for non-alcoholic and refreshing vinegar-based drink

Along with the growth of public interest in vinegar, the popularity of vinegar drinks that contain the phytochemicals found in the given fruit has increased in recent years. The health benefits of natural vinegar are well known. Some of these benefits include enhanced immunity, reduction of risk factors for cardiovascular diseases, improved digestion, appetite suppression, and reduced fasting blood glucose, blood pressure, and serum cholesterol. For instance, all parts and products of date palm for example, such as fruits, seeds, pollen, leaves, and syrup, have beneficial applications for both humans and animals. This attracts researchers to study the different pharmacological potentials of date palm products. Several studies showed the anti-hyperlipidemic, anti-obesity, antioxidant, and immune-stimulating activities of dates vinegar made from date flesh and pits (seeds) [82 93 94 95].

At the industrial scale, vinegar is mainly produced by submerged fermentation with unselected AAB cultures [82] and emerging applications include biopolymers, such as bacterial cellulose and fructans, which are getting increasing interest in the biotechnological industry [96]. AAB drive an acidification of the environment, thereby preventing the growth of competitors, while the producing cells possess several mechanisms to tolerate the acidity. In addition, they can utilize the accumulated organic acids later to further sustain their growth. AAB cells capable of cellulose production form biofilms that allow their retention on the culture surface, which is favourable for the survival of these strictly aerobic bacteria [64]. All these physiological features explain their occurrence and underline their functional role in the production of diverse fermented foods and beverages.

AAB could allow obtaining natural fermented food and beverages, able to provide health and nutrition benefits, preventing the loss of many species and strains useful for human health [97].

Mainly in China, Japan, and other Asian countries, an age-old and traditional process, known as solid-state fermentation, is largely used to produce vinegar from cereals at a small-scale [35].

Thanks to the recent popularity of kombucha (a fermented tea beverage), switchels, shrubs, and drinking vinegars, consumers' palates are changing, and vinegar-based drinks are increasing in popularity once again.

Kombucha is a sweet and tangy beverage of probable Manchurian origins obtained from fermented green or black tea with sugar by a microbial consortium composed of several AAB and yeasts [36]. This mixed consortium forms a powerful symbiosis capable of inhibiting the growth of potentially contaminating bacteria. The fermentation process also leads to the formation of a polymeric cellulose pellicle due to the activity of certain strains of *Komagataeibacter* sp. [87] Actual food trends toward minimally processed products, without additives, high nutritional value and with health benefits have increased with consumer awareness. In this context, traditional Kombucha tea has recently captured the attention of researchers and consumers because of its probiotic characteristics.

Switchels is a non-alcoholic and refreshing vinegar-based drink. The drink is traditionally a mixture of ginger, vinegar and water. Sometimes a sweetener such as honey, maple syrup, molasses is added or sugar., more beverage companies are introducing their own prebottled switchel to be marketed as an alternative energy drink or cocktail mixer but creating their own switchel is a popular option.

Shrubs is a concentrated syrup that is made by mixing fruit, vinegar (usually apple cider vinegar), and sugar. Once the syrup is created, it can be diluted with water, sparkling water, or other mixers to be used in a cocktail or refreshing non-alcoholic beverage.

Numerous studies about berries have shown that they are rich in antioxidant phytochemicals that have a protective role associated with their antioxidant activity since overproduction of oxidants in the human body is involved in the pathogenesis of many chronic diseases [69]. The beneficial effects of vinegar might be due to bioactive substances such as amino acids, organic acids, or phenolic compounds derived from its raw materials. Moreover, the bioactive compounds in vinegars can be produced and/or increased through the overall vinegar fermentation process, where phenolic compounds are transformed into new antioxidative molecules. Owing to their excellent sensorial properties and nutritional compositions having different health-promoting properties, mainly from the antioxidant activities, berries are an appealing ingredient for the production of vinegar. These vinegars were produced via a 2-stage (alcoholic and acetous) fermentation process. The initial soluble solid contents in the blackberry and raspberry juices were adjusted to 22 ºBrix before the fermentation. Alcoholic fermentation was conducted using *Saccharomyces cerevisiae* as the inoculant while *Acetobacter pasteurianus* was used for acetous fermentation.

Notably, the wine produced by the ‘Blackberry’ species exhibited the highest levels of alcohol (11.73 %) while the ‘Raspberry’ species vinegar exhibited the highest levels of antioxidant activity (74.43 %). Regarding the acetous fermentation, at the end of a 15-day acetification process, AcOH content was found to range from 3.96 % to 5.01 % [98].

Another example was blackberry vinegar produced in successive acetification cycles and evaluated the content of total phenolics, anthocyanins, and antioxidant activity along the production. Appreciable contents of anthocyanins, polyphenolic compounds, and high antioxidant activity were observed in the raw materials, wine and vinegar obtained. The antioxidant potential, total phenolics, and anthocyanins were maintained along the cycles, but AcOH transformation led to small reductions compared to alcoholic fermentation [99]. Anthocyanins in blackcurrant appear to regulate postprandial hyperglycaemia and reduce the associated risk of developing type -2 diabetes [100]. Specifically, blackberry vinegar has the highest total anthocyanin content (13.21 mg %), antioxidants activity (10.98 %), total polyphenol content (87.25 mg/100 mL), and flavonoid value (51.12 mg/100mL) [101]. The fermentation by *Gluconobacter* sp. which transforms the glucose content of the fruit into GA to keep the fructose as a sweetener is an innovative trend. The study of Hornedo-Ortega et al. [102] demonstrate that alcoholic fermentation of strawberry purees decreased the anthocyanin content while gluconic fermentation preserved these compounds, which is an advantage of this last process.
The physicochemical properties of various commercially available vinegar drinks consumed in the Korean market, including their pH, acidity, sugar, total soluble sugar, total acid, total amino acid content, and antioxidant capacity have been investigated by Kim et al., 2012 [101]. This research supports that some food must be a part of a healthy diet.

Thanks to the recent popularity of kombucha tea, vinegar, gluconic acid, and lactic acid beverages-based the drink industry is still wide-open for innovation, as it is a newer and growing market. The production of fruit vinegars is commonly used option as an alternative for the exploitation of existing fruit surpluses. In particular, red fruit juices are appreciated fruit juices for almost all consumers, due to their flavour, intense red colour and interesting physiochemical characteristics. Furthermore, the different fermentation tools help to provide several sustainable and healthy food because the type of microorganism used affects the final characteristics of the vinegar produced.

In this thesis blackcurrant, blackberry and raspberry juices was evaluated regarding their aptitude to undergo acetic and gluconic fermentation. Initially a characterization of the raw materials was made to know their composition and the chemical parameters of interest. Then, acetic and gluconic fermentation was conducted to know their suitability of raw material for these processes.

3.2 Materials and methods

3.2.1 Bacterial strains and culture media

The strain used for the following study was ATCC 621H, belonging to the *G. oxydans* species, a gram-negative bacterium consisting of rod-shaped ellipsoidal cells, an obligate aerobe, belonging to the *Acetobacteraceae* family and the *Gluconobacter* genus. Microorganisms belonging to this genus carry out an incomplete oxidation of sugars, alcohols and acids. This microorganism is mainly capable of oxidizing glucose into GA and under certain standard conditions, at controlled pH, there is oxidation of GA with the production of ketogluconates [103].

The medium chosen for the growth and revitalization of the ATCC 621H strain is GYC, a medium based on glucose, yeast extract and calcium carbonate (GYC) (see **Section 2.2.2**). ATCC 621H was cultivated in tube and incubated for 5 days at 28°C. Following the incubation period, an aliquot from each tube was inoculated into 100 mL Erlenmeyer flasks. The inoculum was 5% (v/v) in liquid GYC broth, the incubation time and temperature were the same as in the previous phase. Media and materials were sterilized by autoclaving at 121°C for 15 min.

3.2.2 Fruits juice and puree

Fruit juices was provided by a supplier in a form of concentrated syrup obtained by the evaporation of water. Fruits puree was made from fresh fruits grinded, pressed, and sieved.

3.2.3 Fermentation conditions

Following the growth of the bacterial culture, an aliquot was taken from each flask and inoculated into the different juices, with an inoculum at 5% v/v, to start the fermentation. Fermentation took place under same conditions for all the juices tested, in an incubator at a constant temperature of 28°C for 12 days (see **Sections 2.2.5**).

3.2.4 Samples preparation

To carry out the microbiological analysis, serial dilutions on a decimal basis were made for the juice of blackcurrant, blackberry and raspberry. To carry out the serial dilutions, tubes were prepared with saline solution, autoclaved for 15 minutes at 121°C. Subsequently, under sterile conditions, 1 mL of the concentrated juice was added to the first tube and then serial dilution was carried out in the subsequent tubes, up to a 10^{-3} dilution.

The microbiological analysis was performed in Petri dishes, in which the three culture media were added to evaluate the presence of microorganisms: plate count agar (PCA) for the total mesophilic

load, sabouraud dextrose agar (SDA) for the yeasts and a medium containing glucose, yeast extract, peptone, EtOH and calcium carbonate (GEY) for AAB (See **Section 2.2.7**).

3.2.5 Chemical-physical analyses

The physical-chemical analyses performed were: Brix degrees, pH value, titratable acidity, amount of glucose and GA (see **Sections 2.2.8-2.2.9**). All analyses were performed in triplicate on the initial matrix and on fermented beverages at different fermentation times. Before inoculating the bacterial strain and starting the fermentation process, the initial juices were previously diluted and then filtered with a disposable vacuum filter system, with a pore size of 0.20 μm. Prior to the filtration of the juices, an initial characterization of the starting matrix was carried out in order to know its composition and the physio-chemical parameters of interest. Subsequently, the bacterial strain was inoculated and the fermentation process started, and at each different fermentation time, an aliquot was taken in a falcon test tube for each fermenting juices, and analyses were carried out.

3.2.6 Total phenolic compounds and antioxidant activity determination

Total phenolic compounds (TPC) were determined by Folin-Ciacolteu method [47] and expressed as milligrams of gallic acid equivalent per litre (mg GAE/L).

Antioxidant activity was determined following the DPPH method, performed as described by Meda et al., 2005 with minor changes [48]. Briefly, 200 μL of sample was added to 2 mL of a 0.1 mmol/L methanolic solution of DPPH. After an incubation of 30 minutes in the dark, samples were read at 517 nm. Data were expressed as mg ascorbic acid equivalent/100 mL of juice.

3.3 Results and discussion

3.3.1 Choice of microbial strain

The choice of using the ATCC *G. oxydans* 621H bacterial strain is due to its ability to incompletely oxidize glucose and produce GA in the medium [104]. Furthermore, previous studies highlight the suitability of the strain to grow at high sugar content and low pH values [15], indicating the juice of blackcurrants, blackberries and raspberries as matrices with interesting characteristics for undergoing gluconic fermentation (**Figure 3.1**).

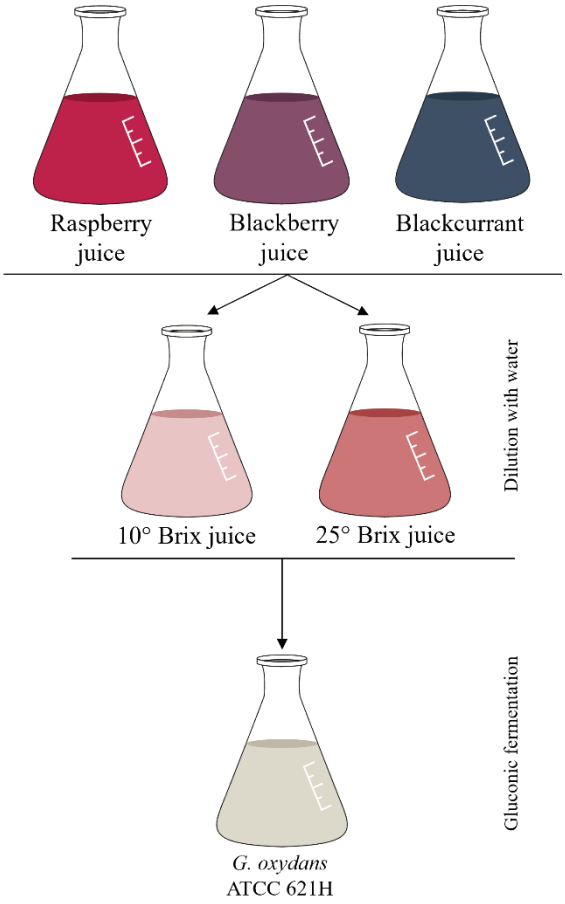


Figure 3.1 Flow chart of experimental plan

3.3.2 Microbiological analysis

In order to evaluate the microbiological suitability of the raw materials under study, analyses were conducted on the three juices. The presence of microorganisms checked on GEY, PCA and SDA was not found and therefore the raw materials were found to be suitable for fermentation (**Figure 3.2**). Despite the absence of microbial growth of the initial matrices, they were filtered to eliminate any residues and favour the adaptation and growth of the bacterial strain.

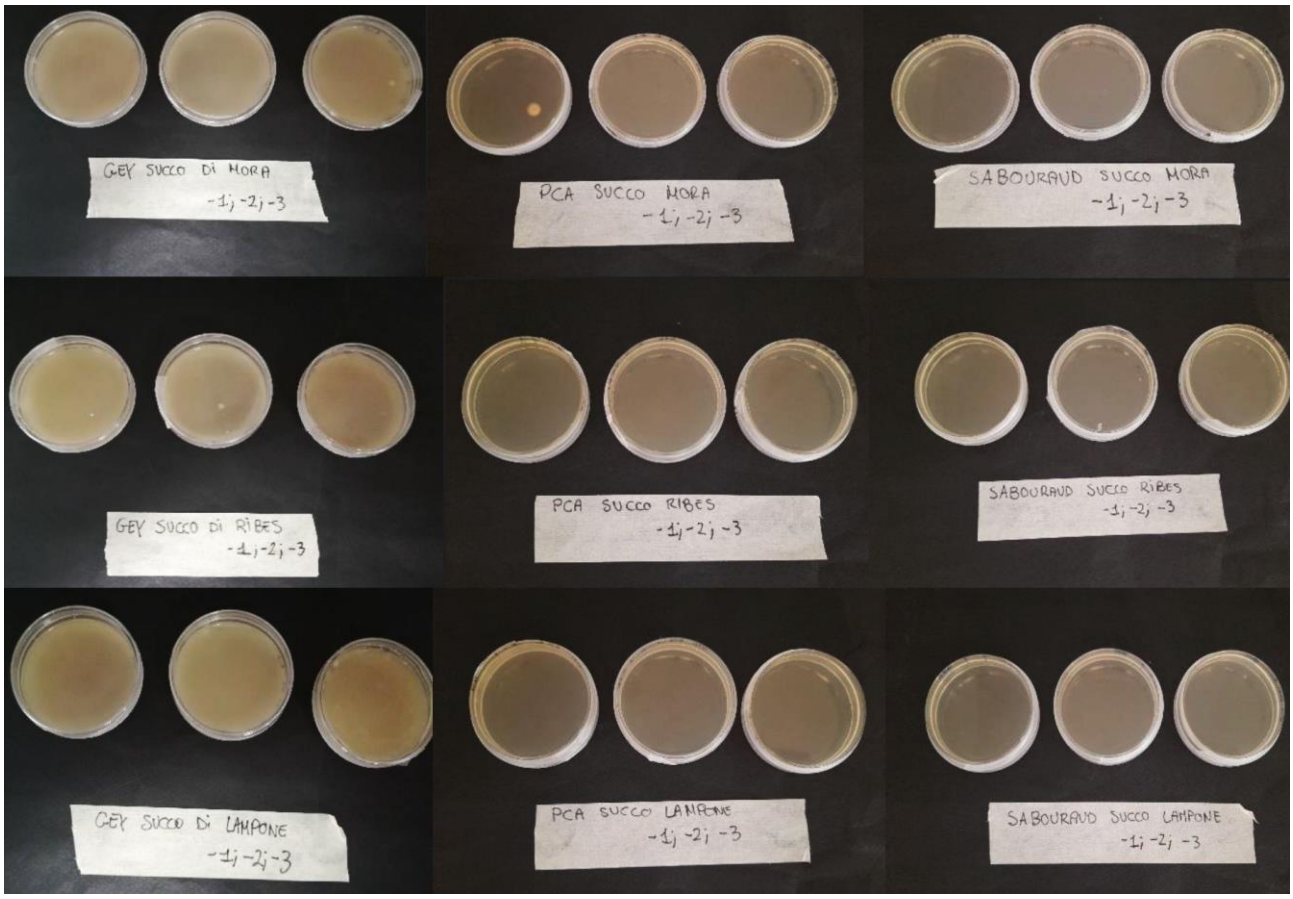


Figure 3.2 Results of microbiological analysis on raw materials of GEY, PCA and SDA growth media.

3.3.3 Chemical-physical characterization of the initial matrices at 25° Brix

The determination of the different chemical-physical parameters analysed in the three matrices at 25° Brix, highlighted different characteristics in the three starting juices. **Table 3.3** shows the general framework of the parameters of the three initial matrices.

	pH	Titratable acidity (g/100mL)	Glucose (g/L)	Gluconic acid (g/L)
Blackberry	$3.37^{a} \pm 0.02$	$5.39^{c} \pm 0.22$	$168.6857^{a} \pm 4.0144$	$0.2083^{a} \pm 0.0006$
Raspberry	$3.25^{b} \pm 0.02$	$6.90^{b} \pm 0.16$	$168.3867^{a} \pm 4.7103$	$0.1613^{b} \pm 0.0514$
Blackcurrant	$3.09^{c} \pm 0.01$	$9.84^{a} \pm 0.39$	$144.2370^{b} \pm 1.4453$	$0.1059^{ab} \pm 0.0186$

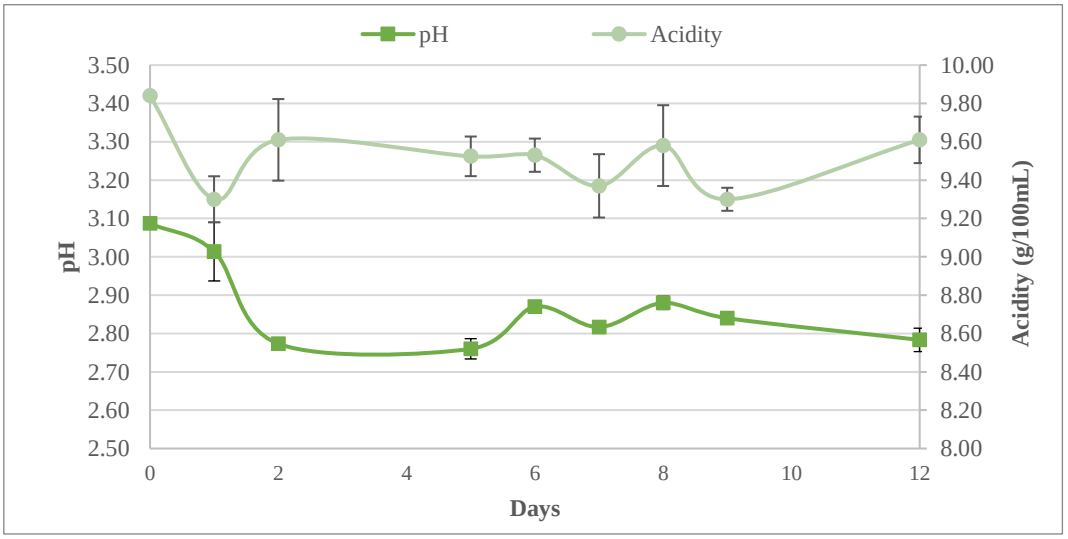
Table 3.3 pH values, titratable acidity, glucose and GA of the three initial matrices at 25° Brix. Values are reported as average of three replicates ± standard deviation. Significant differences among initial matrixes are shown by different letters ($p \leq 0.05$).

The initial parameters indicate suitable conditions for AAB growth, including the ability to survive in a medium with low pH values between 2.5 and 4.0 [104].

As can be seen from **Table 3.3**, blackcurrant juice has lower pH values, higher titratable acidity and a slightly lower initial glucose amount than raspberry and blackberry juices. The least stressful conditions for the survival and growth of the bacterial strain are in the blackberry and raspberry matrix, having more favourable pH and titratable acidity values for the adaptation and growth of ATCC *G. oxydans* 621H, since the blackcurrant juices is very acidic. As expected, the amount of initial GA is practically close to zero.

3.3.3.1 Evaluation of parameters of juices at 25° Brix during fermentation

The initial characteristics of *blackcurrant juice* at 25° Brix are low pH values and high titratable acidity. During fermentation, a slight decrease in pH was noted, while the titratable acidity was found to be almost the same (**Figure 3.3a**). A non-linear trend can be seen, with decreases and increases in non-regular values, which do not follow a defined trend. The values indicate an unfavourable environment for the adaptation and growth of the bacterial strain, which remains in a latent phase as confirmed by the low production of GA (**Figure 3.3b**). Despite sufficient initial glucose to undergo gluconic fermentation, the acidic environment made it difficult for the strain to grow, resulting in low GA production.



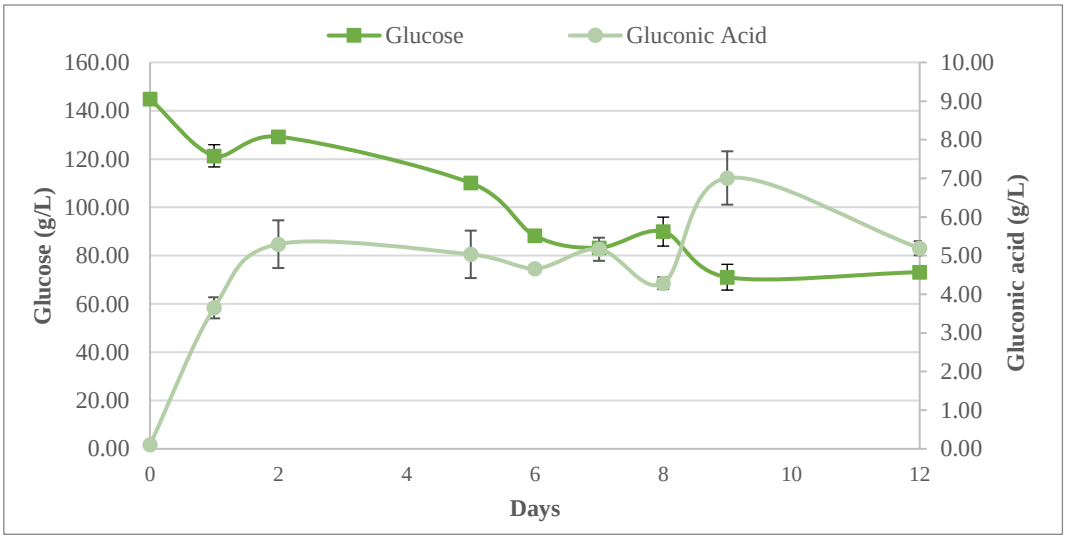


Figure 3.3 Fermentation trend of blackcurrant juice 25° Brix: (a) pH and titratable acidity (b) glucose consumption and GA production with relative standard deviations.

pH and titratable acidity values of *blackberry juice* at 25°C follow a regular trend, with a final increase in titratable acidity and a final decrease in pH. As can be seen in **Figure 3.4a**, the decrease in pH values corresponds to an increase in acidity and on day 12 of fermentation the minimum pH value and maximum acidity content is reached. After 5 days of fermentation, a substantial decrease in pH is noted and an equally substantial increase in titratable acidity. The bacterial strain after 5 days of fermentation started to produce a considerable quantity of GA, as confirmed by the **Figure 3.4b**, which highlights a considerable increase in GA produced after 5 days of fermentation, causing an increase in acidity in the medium, with a consequent lowering of the pH. The results regarding glucose consumption and GA production of 25° Brix blackberry juice are shown in **Figure 3.4b**. The quantity of glucose decreases constantly and it is assumed that in the first days of fermentation the bacterial strain uses glucose to obtain primary energy for its survival and subsequently, on the fifth day of fermentation, an important production of GA is found [105]. The amount of glucose decreases following a constant trend and the amount of GA increases, until reaching, after 9 days of fermentation, 62.7209 g/L in the medium, the maximum peak. After 9 days of fermentation, the bacterial strain continues to consume glucose, but a decrease in the GA content is observed. This trend is attributable to the oxidation of the GA into ketogluconic acids, in fact it has been found that a small amount of 2-keto-D-gluconate (ketogluconic acid) may be formed during the oxidation of glucose to GA [52]. Furthermore, may be that the large amount of accumulated GA in the medium has created an acidic environment, resulting in a decrease in pH, which led to slower growth and a gradual decrease in cell viability and a repression of GA production [106].

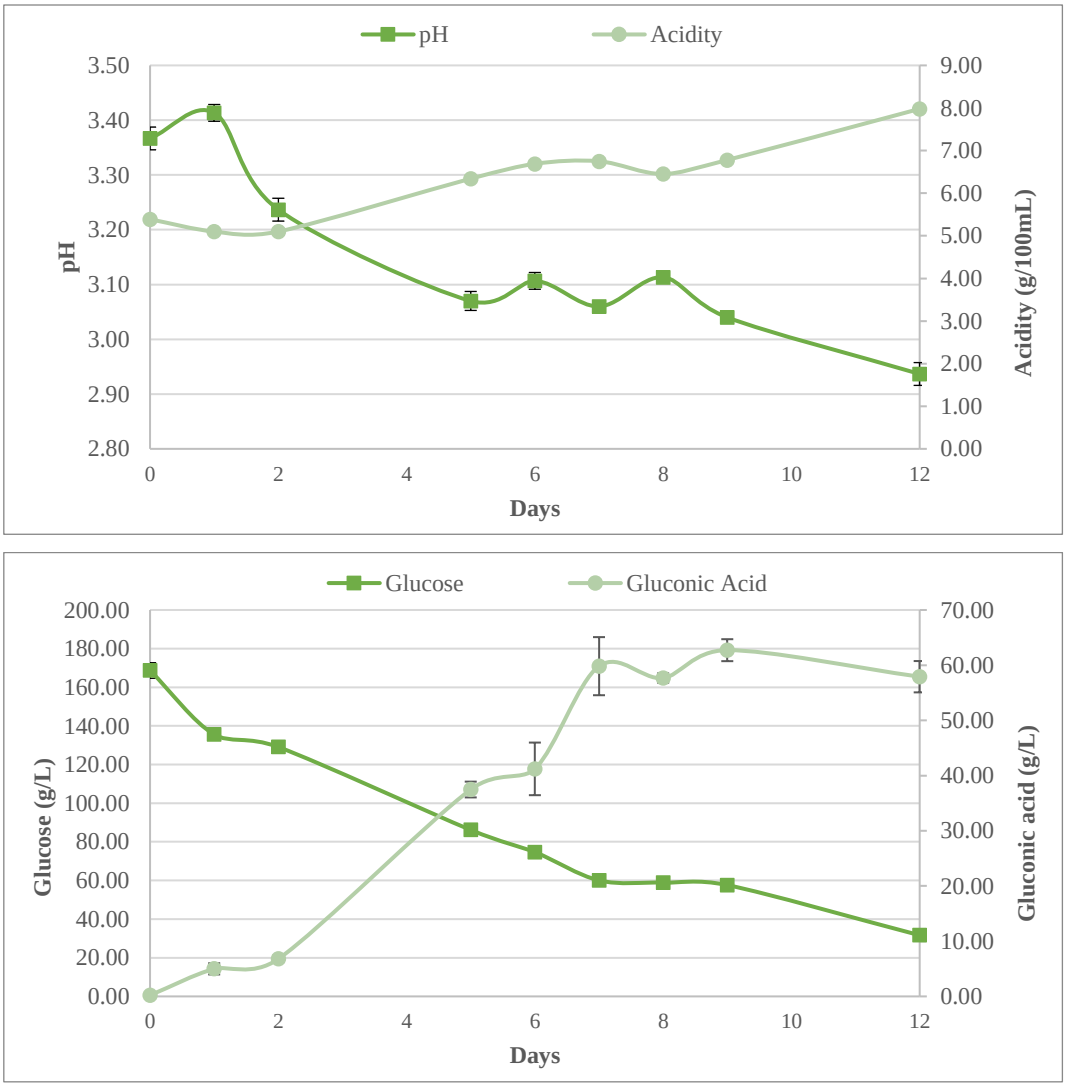


Figure 3.4 Fermentation trend of blackberry juice 25° Brix: (A) pH and titratable acidity (B) glucose consumption and GA production with relative standard deviations.

The measured parameter values of the 25°C Brix *raspberry juice* are shown in **Figures 3.5a-b**. A correlation can be found between pH and titratable acidity. This correlation is highlighted on the fifth day of fermentation, in which a clear decrease in pH can be noted, and an equally clear increase in the acidity value. The final values obtained from the fermented beverage after 12 days of fermentation are a high final acidity and a acidic pH, an indication that gluconic fermentation has taken place. A constant and regular decrease in glucose and an exponential production of GA up to the sixth day of fermentation, then maintain constant values and reach the maximum value after 12 days of fermentation. The trend of the production of GA is interesting, which has a notable increase in the first days of the fermentation process, attributable to the initial conditions of the matrix, and a stationary phase, from day 7 to day 12, in which a slight increase is recorded the amount of GA. This behaviour could be related to a slowdown in cell viability due to the acidic environment created after the production of GA [106].

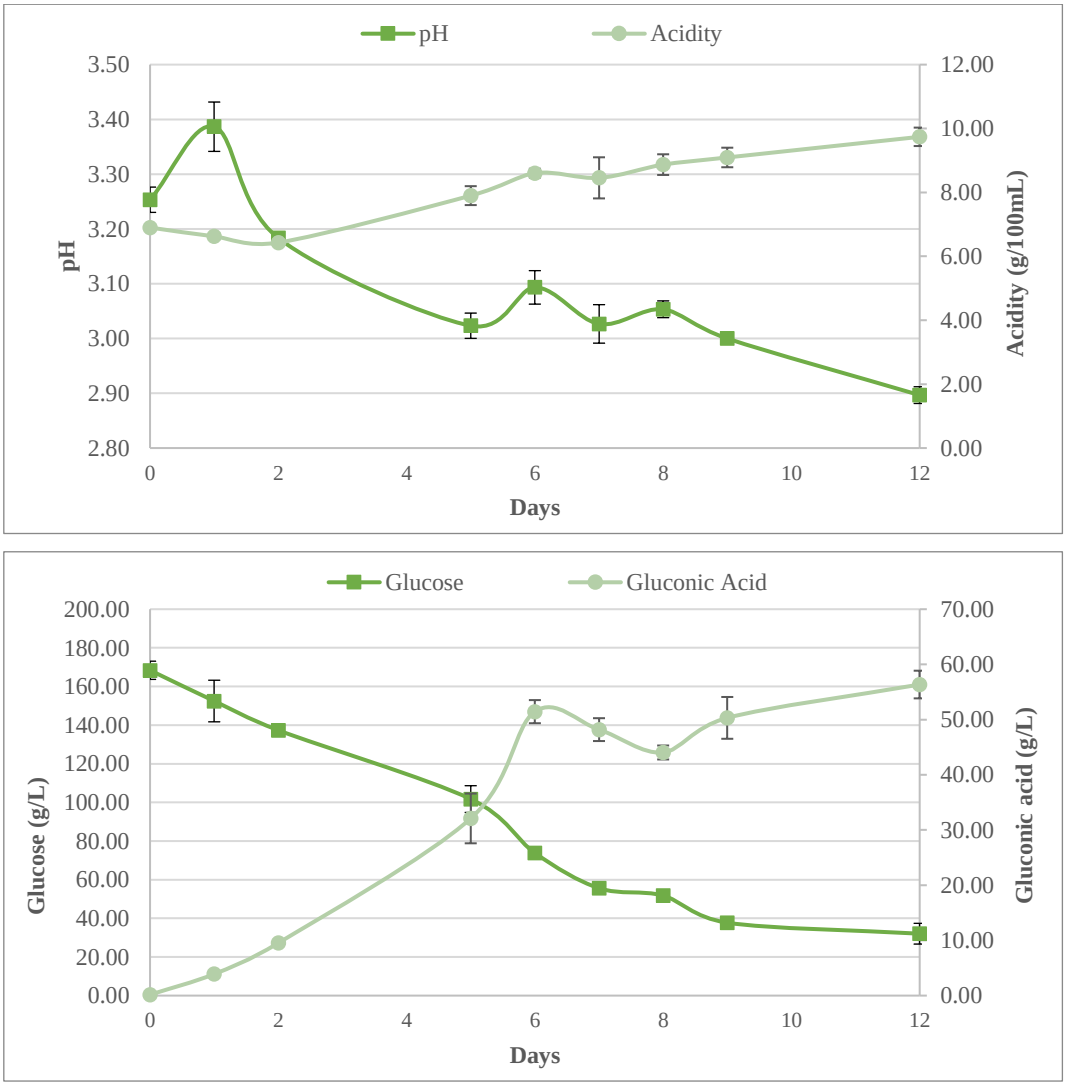


Figure 3.5 Fermentation trand of raspberry juice 25° Brix: (A) pH and titratable acidity (B) glucose consumption and GA production with relative standard deviations.

3.3.3.2 Comparison of juices at 25° Brix during fermentation

As previously mentioned, the 3 juices at 25° Brix have different chemical-physical characteristics and for this reason the fermented drinks obtained have different values (**Table 3.4**).

	Days	pH	Acidity (g/100mL)	Glucose (g/L)	Gluconic acid (g/L)
Blackberry	**0**	$3.37^{a} \pm 0.02$	$5.39^{c} \pm 0.22$	$168.686^{a} \pm 4.014$	$0.208^{c} \pm 0.0006$
	1	$3.41^{b} \pm 0.02$	$5.10^{c} \pm 0.15$	$135.978^{b} \pm 0.489$	$4.994^{c} \pm 1.0167$
	2	$3.24^{c} \pm 0.02$	$5.10^{c} \pm 0.31$	$129.088^{c} \pm 0.915$	$6.785^{c} \pm 0.5780$
	5	$3.07^{de} \pm 0.02$	$6.34^{b} \pm 0.08$	$86.162^{d} \pm 1.917$	$37.474^{b} \pm 1.430$
	6	$3.11^{d} \pm 0.02$	$6.69^{b} \pm 0.07$	$74.657^{e} \pm 3.150$	$41.217^{b} \pm 4.766$
	7	$3.06^{e} \pm 0.02$	$6.75^{b} \pm 0.16$	$60.042^{f} \pm 03605$	$59.822^{a} \pm 5.264$
	8	$3.11^{d} \pm 0.02$	$6.45^{b} \pm 0.22$	$58.866^{f} \pm 0.934$	$58.109^{a} \pm 0.559$
	9	$3.04^{e} \pm 0.02$	$6.78^{b} \pm 0.21$	$57.205^{f} \pm 1.374$	$62.721^{a} \pm 1.990$
	12	$2.94^{f} \pm 0.02$	$7.98^{a} \pm 0.11$	$31.647^{g} \pm 0.174$	$57.913^{a} \pm 2.838$
Raspberry	**0**	$3.25^{b} \pm 0.02$	$6.90^{d} \pm 0.16$	$168.387^{a} \pm 4.710$	$0.161^{f} \pm 0.051$
	1	$3.39^{a} \pm 0.02$	$6.63^{d} \pm 0.10$	$152.471^{b} \pm 10.772$	$3.913^{ef} \pm 0.298$
	2	$3.18^{b} \pm 0.02$	$6.43^{d} \pm 0.02$	$137.620^{c} \pm 0.854$	$9.448^{e} \pm 0.279$
	5	$3.02^{cd} \pm 0.02$	$7.90^{c} \pm 0.30$	$101.724^{d} \pm 6.092$	$32.098^{d} \pm 4.506$
	6	$3.09^{c} \pm 0.02$	$8.60^{bc} \pm 0.14$	$74.051^{e} \pm 1.967$	$51.428^{ab} \pm 2.963$
	7	$3.03^{cd} \pm 0.02$	$8.46^{bc} \pm 0.64$	$55.591^{f} \pm 1.598$	$47.831^{bc} \pm 2.952$
	8	$3.05^{cd} \pm 0.02$	$8.87^{b} \pm 0.32$	$51.783^{fg} \pm 1.951$	$44.045^{c} \pm 1.262$
	9	$3.00^{d} \pm 0.02$	$9.09^{ab} \pm 0.31$	$37.166^{gh} \pm 2.830$	$50.910^{abc} \pm 5.150$
	12	$2.90^{e} \pm 0.02$	$9.74^{a} \pm 0.29$	$32.053^{h} \pm 5.359$	$56.354^{a} \pm 2.504$
Blackcurrant	**0**	$3.09^{a} \pm 0.02$	$9.84^{a} \pm 0.39$	$144.237^{a} \pm 1.445$	$0.106^{d} \pm 0.019$
	1	$3.01^{a} \pm 0.02$	$9.30^{a} \pm 0.12$	$121.331^{b} \pm 4.607$	$3.650^{c} \pm 0.273$
	2	$2.77^{c} \pm 0.02$	$9.61^{a} \pm 0.21$	$129.922^{b} \pm 2.203$	$5.297^{b} \pm 0.617$
	5	$2.76^{c} \pm 0.02$	$9.52^{a} \pm 0.10$	$110.079^{c} \pm 1.592$	$5.033^{b} \pm 0614$
	6	$2.87^{b} \pm 0.02$	$9.53^{a} \pm 0.09$	$88.881^{d} \pm 1.410$	$4.664b^{c} \pm 0.079$
	7	$2.82^{bc} \pm 0.02$	$9.37^{a} \pm 0.17$	$83.291^{d} \pm 1.922$	$5.165^{b} \pm 0.299$
	8	$2.88^{b} \pm 0.02$	$9.58^{a} \pm 0.21$	$89.960^{d} \pm 5.993$	$4.286^{bc} \pm 0.154$
	9	$2.84^{bc} \pm 0.02$	$9.30^{a} \pm 0.06$	$71.084^{e} \pm 5.331$	$7.010^{a} \pm 0.691$
	12	$2.78^{c} \pm 0.02$	$9.61^{a} \pm 0.12$	$73.592^{e} \pm 3.442$	$5.192^{b} \pm 0.183$

Table 3.4 Values of pH, titratable acidity, quantity of glucose and GA of the three initial juices and of the fermented beverages at the different fermentation times at 25° Brix. Values are reported as average of three replicates ± standard deviation. Significant differences are shown by different letters ($p \leq 0.05$).

As can be seen in **Figure 3.6**, it is possible to note different initial pH values, in fact currant juice has a lower pH than blackberry and raspberry juices, which have higher values. The trend is similar for all three matrices, in fact there is a general decrease in pH, with the only difference that after 12 days of fermentation currant juice reaches lower values than blackberry and raspberry juice, which reach very similar values.

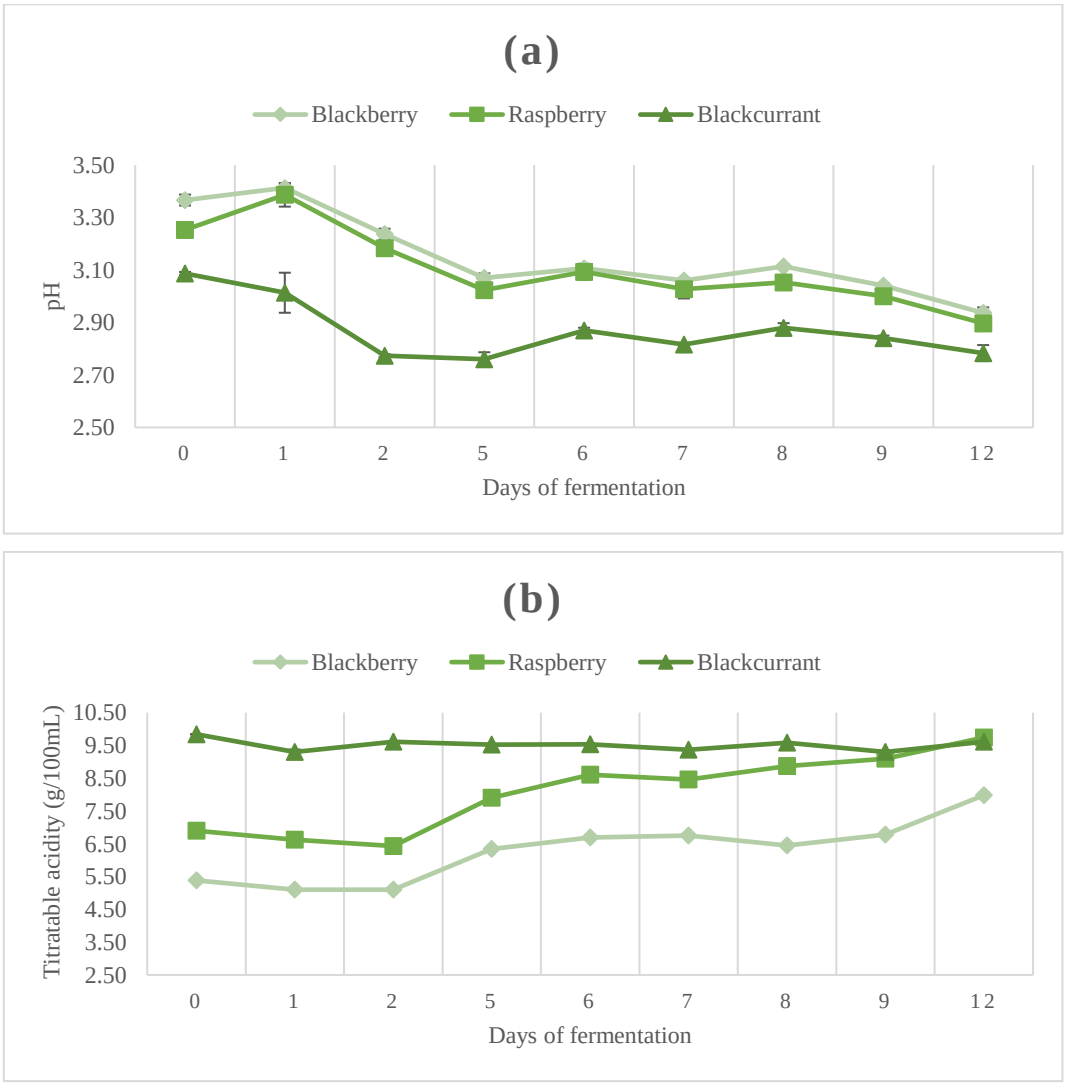


Figure 3.6 Comparison of (a) pH and (b) titratable acidity trend of juices at 25° Brix with relative standard deviations.

The titratable acidity in the initial juices has different values and, as noted in **Table 3.4**, the currant juice has a much higher titratable acidity than the blackberry and raspberry juice. During fermentation, the titratable acidity of currants remains constant, with a slight decrease at the end of fermentation. While for blackberry and raspberry juice a similar general trend is highlighted, with a final increase in titratable acidity, the final acidity of the raspberry fermented drink is higher than blackberry, and reaches a higher value than the blackcurrant fermented drink (**Figure 3.4b**). Due to

the increase in acidity, there is a slowdown in bacterial activity in raspberry juice, which is found in the repression of the production of GA.

The main factor that quantifies the fermentation trend are represented by the consumption of glucose and the production of GA. The three juices initially have similar amounts of glucose, and as reported in **Figure 3.7a**, blackberry and raspberry have slightly higher contents than currant juice. The 3 juices follow a very similar trend, in which it is observed that as the days of fermentation increase, the glucose content decreases. The main difference is based on speed of glucose consumption by *G. oxydans* 621 H, in fact **Figure 3.7a** underlines that in raspberry and blackberry juices glucose consumption is much faster than in currant juice.

Comparing **Figure 3.7a** with **Figure 3.7b,** which shows the content of GA produced by the bacterial strain in the 3 juices, it can be observed that the currant juice, was found unsuitable for being subjected to a gluconic fermentation, in fact the production of GA is low, and in 12 days of fermentation, the quantity remains stationary. Probably, because of unsuitable conditions for the adaptation of the strain, the glucose consumed is mainly used for the survival of the bacterial strain. The blackberry and raspberry juices at 25° Brix were instead more suitable for gluconic fermentation, since, as shown in **Figure 3.7b**, the production of gluconic is high. It is noted that up to day 5, in raspberry and blackberry juices, the trend of GA production is minimal, this may be related to the time necessary for the microorganism to adapt to the medium, or to the high glucose content initial, which inhibits the conversion of glucose to GA. Previous studies have shown that an initial glucose content higher than 90 g/L can slow down the oxidation of glucose into GA, causing an increase in the stationary phase in species belonging to the genus *Gluconobacter* [107]. The stationary phase lasts until day 5, where, comparing **Figure 3.7**, the consumption of glucose is highlighted up to the achievement of 86.1617 g/L in blackberry juice and 101.7240 g/L in raspberry juice, and the consequent increase in the production of GA. The blackberry juice reaches the maximum peak quantity of GA, 62.7209 g/L, after 9 days of fermentation, then decrease slightly, probably due to the oxidation of GA and the production of ketogluconic acids. Raspberry juice, on the other hand, reaches the maximum quantity of GA, 56.3540 g/L, after 12 days of fermentation.

Considering the parameters analysed, among the juices at 25° Brix, blackberry and raspberry juice are certainly the most suitable for undergoing gluconic fermentation as they are characterized by a less stressful environment for the adaptation of the bacterial strain and a greater quantity of GA product, compared to blackcurrant juice.

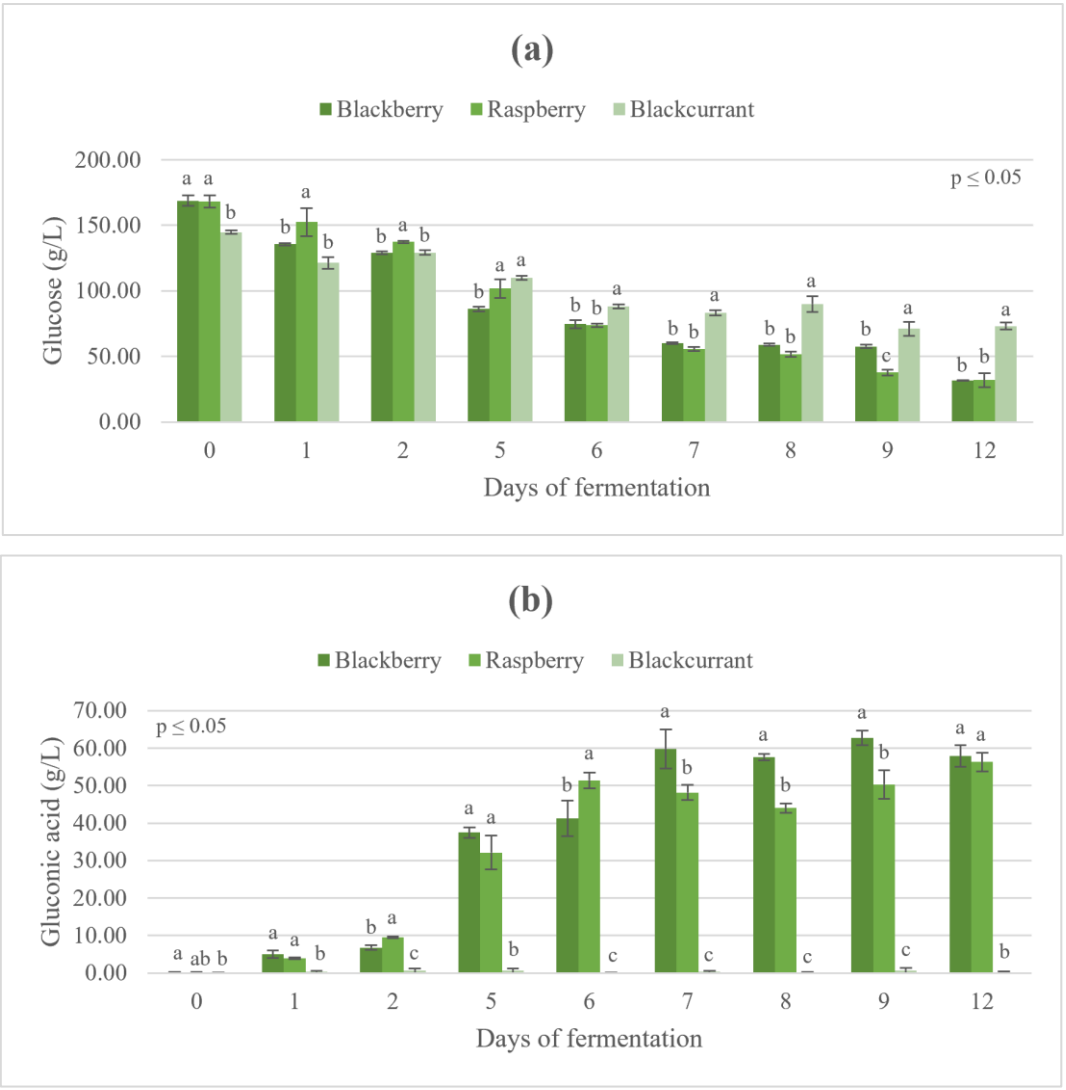


Figure 3.7 Comparison of the amount of (a) glucose consumed and (b) GA produced in the three 25 ° Brix juices. Bar plots indicate the average glucose consumption (a) and gluconic acid production (b) by three replicates ± standard deviation. Significant difference at same days of fermentation is shown by different letters ($p \leq 0.05$).

3.3.3.3 Chemical-physical characterization of the initial juices at 10° Brix

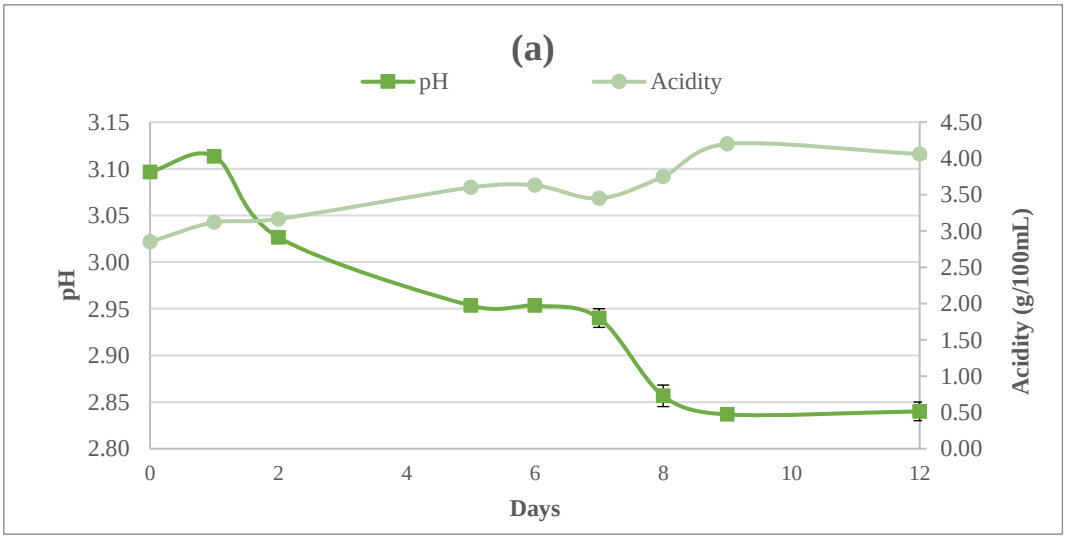
Table 3.5 shows the characteristics of the three juices at 10° Brix, which allow us to highlight similarities and differences. The initial pH value was found to be similar for all matrices, as was the initial amount of GA which was practically nil, on the contrary the titratable acidity and the initial amount of glucose were found to be different. As regards titratable acidity, the highest value is represented by blackcurrant juice, which differs from the other two juices, even if the general acidity of the three juices is in any case not a very high value, therefore the environment should not be too stressful for the adaptation of the bacterial strain, also considering the pH values. The amount of initial glucose is different, as raspberry juice has a smaller amount than blackcurrant and blackberry juice.

	pH	Titratable acidity (g/100mL)	Glucose (g/L)	Gluconic acid (g/L)
Blackberry	$3.46^{a} \pm 0.01$	$1.95^{b} \pm 0.05$	$98.610^{a} \pm 0.489$	$0.478^{a} \pm 0.002$
Raspberry	$3.30^{b} \pm 0.01$	$1.63^{c} \pm 0.11$	$36.215^{b} \pm 1.998$	$0.040^{b} \pm 0.012$
Blackcurrant	$3.09^{c} \pm 0.01$	$2.85^{a} \pm 0.04$	$87.201^{a} \pm 0.678$	$0.118^{ab} \pm 0.006$

Table 3.5 pH, titratable acidity, glucose and GA values of the three initial juices at 10° Brix. Values are reported as average of three replicates ± standard deviation. Significant differences among initial matrixes are shown by different letters ($p \leq 0.05$).

3.3.3.4 Evaluation of parameters of juices at 10° Brix during fermentation

The analysed characteristics of *blackcurrant juice* at 10° Brix are observed in **Figures 3.8. Figure 3.8a** highlights that, for almost all the fermentative days, there is a decrease in pH followed by a corresponding increase in titratable acidity. The pH decreases slightly after 12 days of fermentation, while the acidity shows a more evident increase, indicating that the production of GA has made the medium more acidic. **Figure 3.8** shows the trend of glucose and GA, and shows a net consumption of glucose after 2 days of fermentation, followed by a constant decrease until day 12, where the minimum value is reached. The quantity of GA grows in a linear and constant, until it reaches a stationary phase after 8 days of fermentation, due to the low glucose content remaining in the medium.



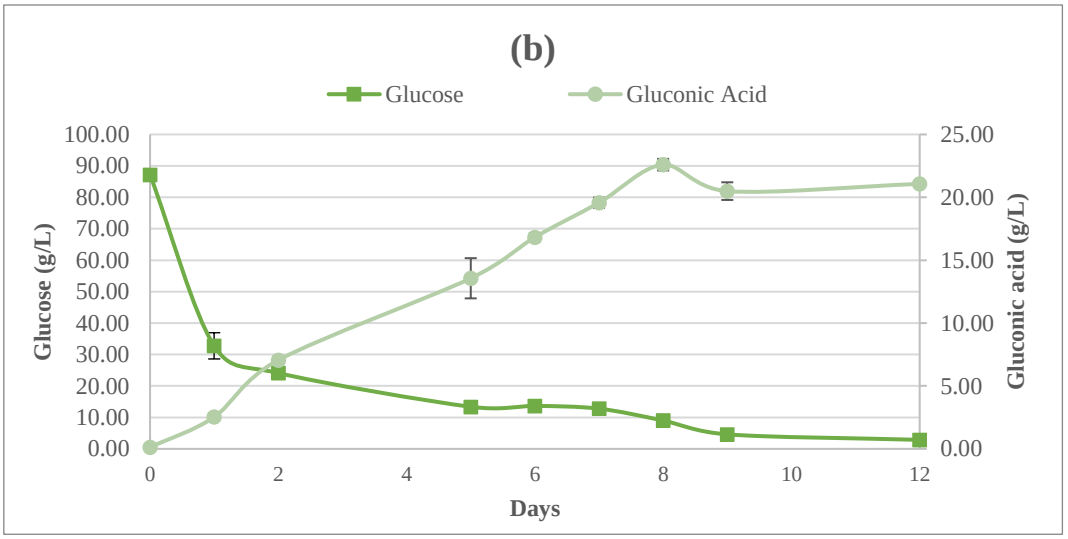


Figure 3.8 Fermentation trend of blackcurrant juice 10° Brix: (a) pH and titratable acidity (b) glucose consumption and GA production with relative standard deviations.

The pH and titratable acidity values of 10 ° Brix *blackberry juice* follow a regular trend, since the entire fermentation process, the decrease in pH coincides with an increase in titratable acidity. At the end of the fermentation process, the pH decreased and the titratable acidity increased, indicating that gluconic fermentation has taken place. **Figure 3.9** shows the evolution of glucose consumption and GA production, and underlines the amount of glucose clearly decreases after only one day of fermentation, being almost totally consumed after only 6 days of fermentation. As far as the production of GA is concerned, a clear increase can be seen after 2 fermentation days and then a slight increase up to the fifth fermentation day. From the sixth day onwards, the trend is not linear, characterized by non-constant quantitative growth and decrease. This trend indicates that the initial pH and titratable acidity values are favourable to the adaptation of the bacterial strain. An exponential consumption of glucose suggested that the fermentation process suitable for this matrix can stop after 5 days of fermentation. At the fifth day, was observed an almost total depletion of available glucose and an amount of GA produced that is close to the maximum value.

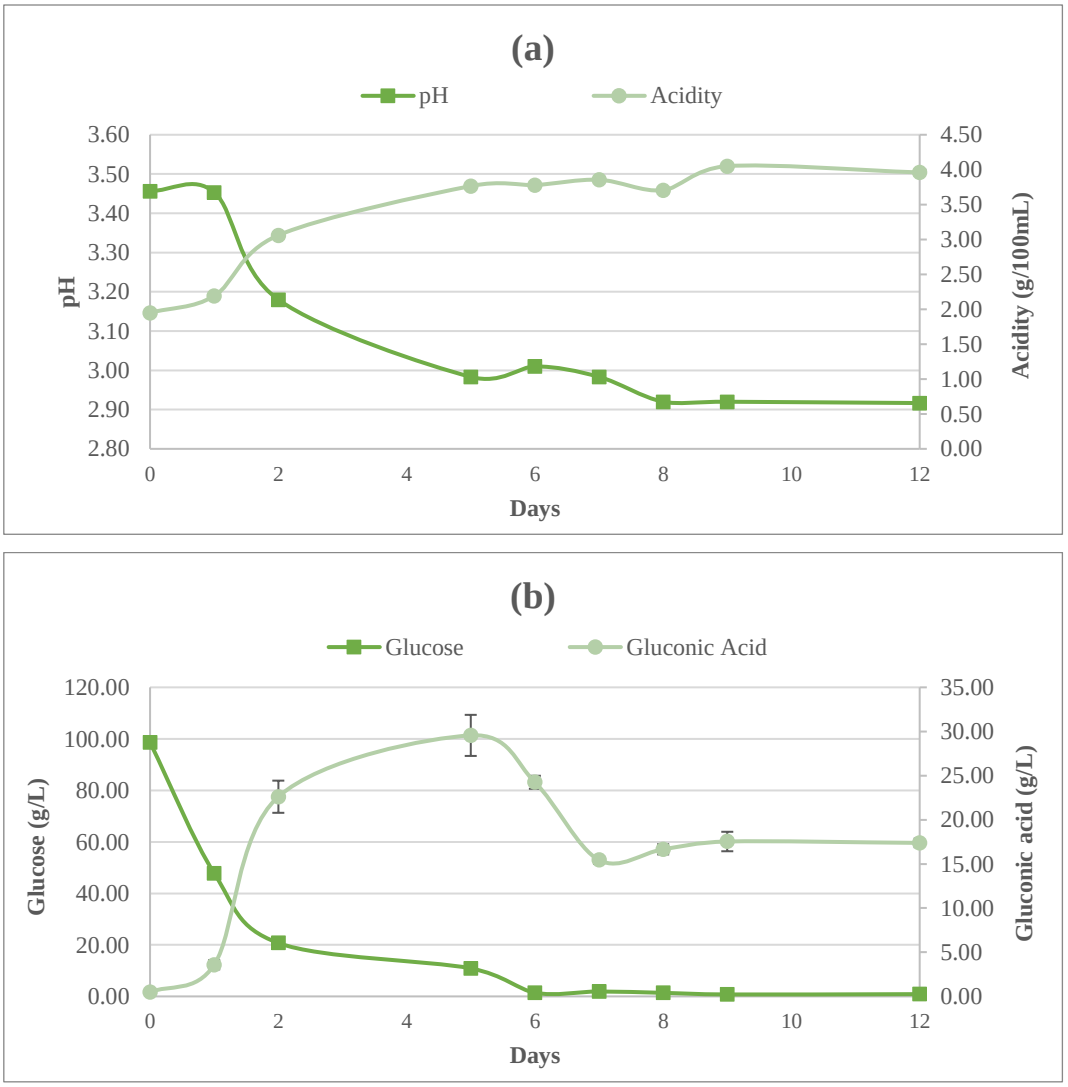


Figure 3.9 Fermentation trend of blackberry juice 10°Brix: (A) pH and titratable acidity (B) glucose consumption and GA production with relative standard deviations.

The values analysed for *raspberry juice* at 10° Brix show that the initial matrix is an environment where the strain can easily adapt without having too many difficulties. As seen in **Figure 3.10**, the initial pH and titratable acidity values represent suitable conditions for the growth of the bacterial strain since the pH is not too acidic and the titratable acidity has a minimum value. The trend of pH and acidity is closely related, in fact when the pH decreases, the acidity increases for the entire fermentation process, ending with a final decrease in pH and an increase in acidity. Despite these ideal characteristics, the low glucose content did not favour GA production as shown in **Figure 3.10**. In fact, it is noted that the glucose present in the food matrix is almost totally consumed after only two days of fermentation and the production of GA remains constant for the entire process.

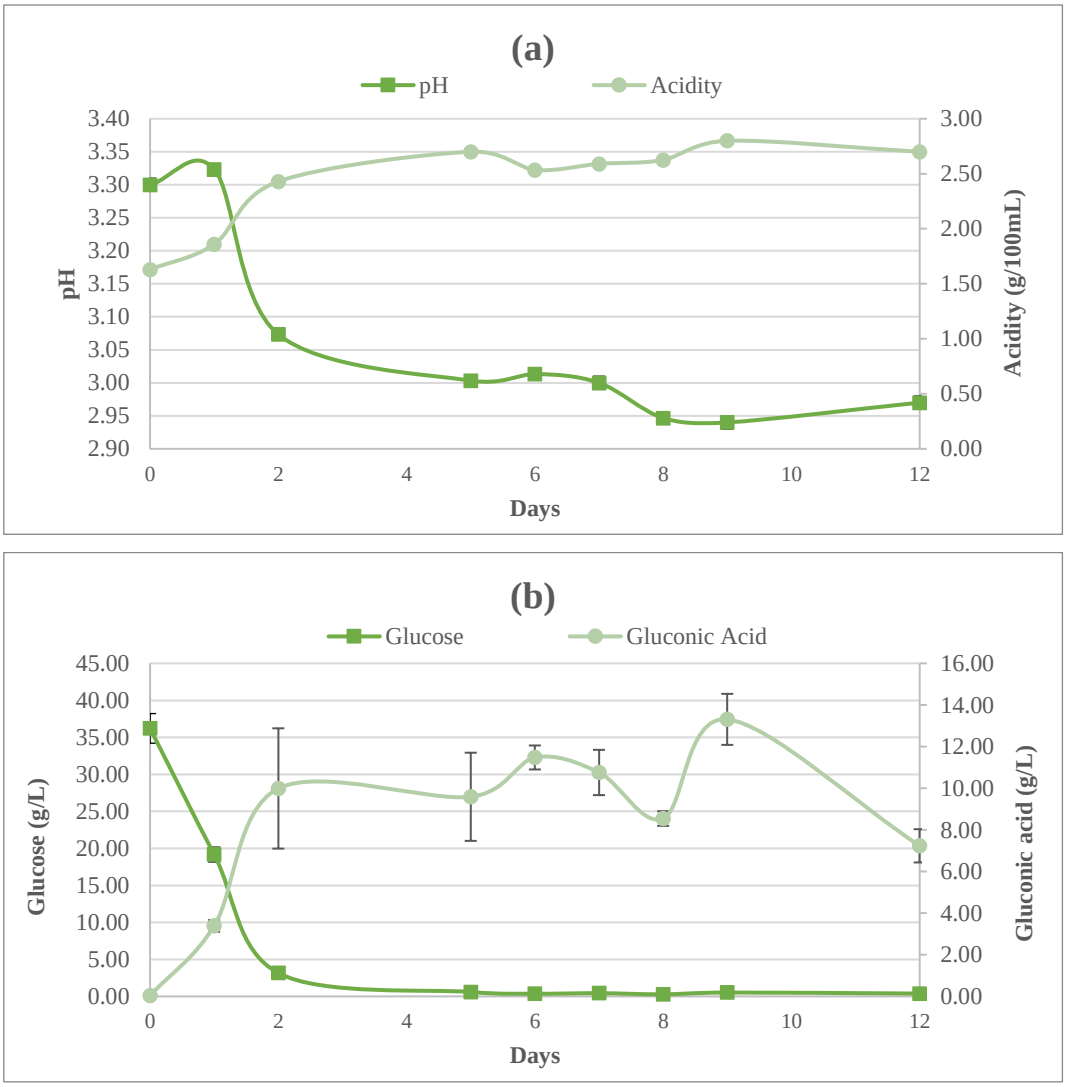


Figure 3.10 Fermentation trend of raspberry juice 10°Brix: (a) pH and titratable acidity (b) glucose consumption and GA production with relative standard deviations.

3.3.3.5 Comparison of juices at 10° Brix during fermentation

	Days	pH	Acidity (g/100mL)	Glucose (g/L)	Gluconic acid (g/L)
Blackberry	**0**	$3.46^{a} \pm 0.02$	$1.95^{d} \pm 0.05$	$98.610^{a} \pm 0.489$	$0.478^{d} \pm 0.002$
	1	$3.45^{a} \pm 0.02$	$2.19^{d} \pm 0.02$	$47.818^{b} \pm 1.636$	$3.581^{d} \pm 0.520$
	2	$3.18^{b} \pm 0.02$	$3.06^{b} \pm 0.10$	$20.737^{c} \pm 1.872$	$22.927^{b} \pm 2.460$
	5	$2.98^{d} \pm 0.02$	$3.77^{b} \pm 0.08$	$10.887^{d} \pm 0.501$	$29.331^{a} \pm 3.242$
	6	$3.01^{c} \pm 0.02$	$3.78^{b} \pm 0.06$	$1.419^{e} \pm 0.050$	$24.074^{b} \pm 0.950$
	7	$2.98^{d} \pm 0.02$	$3.86^{ab} \pm 0.13$	$1.903^{e} \pm 0.147$	$15.338^{c} \pm 0.559$
	8	$2.92^{e} \pm 0.02$	$3.71^{b} \pm 0.11$	$1.370^{e} \pm 0.260$	$16.879^{c} \pm 0.615$
	9	$2.92^{e} \pm 0.02$	$4.05^{a} \pm 0.04$	$0.751^{e} \pm 0.064$	$17.325^{c} \pm 1.470$
	12	$2.91^{e} \pm 0.02$	$3.96^{ab} \pm 0.12$	$0.879^{e} \pm 0.104$	$17.543^{c} \pm 0.571$
Raspberry	**0**	$3.30^{a} \pm 0.02$	$1.63^{c} \pm 0.11$	$36.215^{a} \pm 1.998$	$0.038^{e} \pm 0.012$
	1	$3.32^{a} \pm 0.02$	$1.86^{c} \pm 0.09$	$19.168^{b} \pm 0.998$	$3.389^{de} \pm 0.284$
	2	$3.07^{b} \pm 0.02$	$2.43^{b} \pm 0.05$	$3.160^{c} \pm 0605$	$9.989^{abc} \pm 4.081$
	5	$3.00^{c} \pm 0.02$	$2.70^{a} \pm 0.05$	$0.589^{d} \pm 0.146$	$9.589^{abc} \pm 2.907$
	6	$3.01^{c} \pm 0.02$	$2.54^{ab} \pm 0.07$	$0.362^{d} \pm 0.011$	$11.478^{ab} \pm 0.783$
	7	$3.00^{c} \pm 0.02$	$2.59^{ab} \pm 0.02$	$0.431^{d} \pm 0.077$	$10.757^{abc} \pm 1.509$
	8	$2.95^{d} \pm 0.02$	$2.63^{ab} \pm 0.14$	$0.281^{d} \pm 0.017$	$8.545^{bc} \pm 0.168$
	9	$2.94^{d} \pm 0.02$	$2.80^{a} \pm 0.12$	$0.521^{d} \pm 0.067$	$13.308^{a} \pm 1.733$
	12	$2.97^{d} \pm 0.02$	$2.70^{a} \pm 0.09$	$0.380^{d} \pm 0.024$	$7.234^{cd} \pm 1.062$
Blackcurrant	**0**	$3.10^{a} \pm 0.02$	$2.85^{f} \pm 0.04$	$87.201^{a} \pm 0.678$	$0.119^{g} \pm 0.006$
	1	$3.11^{a} \pm 0.02$	$3.12^{ef} \pm 0.15$	$32.755^{b} \pm 4.171$	$2.533^{f} \pm 0.262$
	2	$3.03^{b} \pm 0.02$	$3.17^{de} \pm 0.16$	$24.123^{c} \pm 0.832$	$7.056^{e} \pm 0.168$
	5	$2.95^{c} \pm 0.02$	$3.60^{bc} \pm 0.03$	$13.379^{de} \pm 0.679$	$13.564^{d} \pm 2.180$
	6	$2.95^{c} \pm 0.02$	$3.63^{bc} \pm 0.10$	$13.667^{d} \pm 0.245$	$16.829^{c} \pm 0.224$
	7	$2.94^{c} \pm 0.02$	$3.45^{cd} \pm 0.06$	$12.779^{de} \pm 0.765$	$19.573^{b} \pm 0.006$
	8	$2.86^{d} \pm 0.02$	$3.75^{b} \pm 0.08$	$9.019^{ef} \pm 1.548$	$22.599^{a} \pm 0.056$
	9	$2.84^{d} \pm 0.02$	$4.20^{a} \pm 0.11$	$4.602^{fg} \pm 0.147$	$20.494^{b} \pm 0.894$
	12	$2.84^{d} \pm 0.02$	$4.06^{a} \pm 0.09$	$2.860^{g} \pm 0.759$	$21.071^{ab} \pm 0.279$

Table 3.6 Values of pH, titratable acidity, quantity of glucose and GA of the three initial juices and of the fermented beverages at the different fermentation times at 10° Brix with relative standard deviations. Values are reported as average of three replicates ± standard deviation. Significant differences along fermentation time are shown by different letters ($p \leq 0.05$).

The chemical-physical analyses carried out on 10° Brix juices have highlighted the different peculiarities of the food matrices and have made it possible to evaluate the suitability or otherwise of the juices and to outline a threshold for an optimal process. Having a general picture of all the parameters, various considerations can be made. **Figure 3.11** shows a generally similar pH trend for

all the juices, where a decrease is found at the end of the fermentation process, where they probably reach similar values. The titratable acidity has low values for juices, probably due to the greater dilution of the initial concentrate and therefore a lower concentration of the acids that determine the titratable acidity. As can be seen from **Figure 3.11**, the acidity increases constantly for all 3 juices, the difference is the absolute value of the parameter, in fact the acidity of the raspberry differs from the currant and blackberry juice, reaching a lower value.

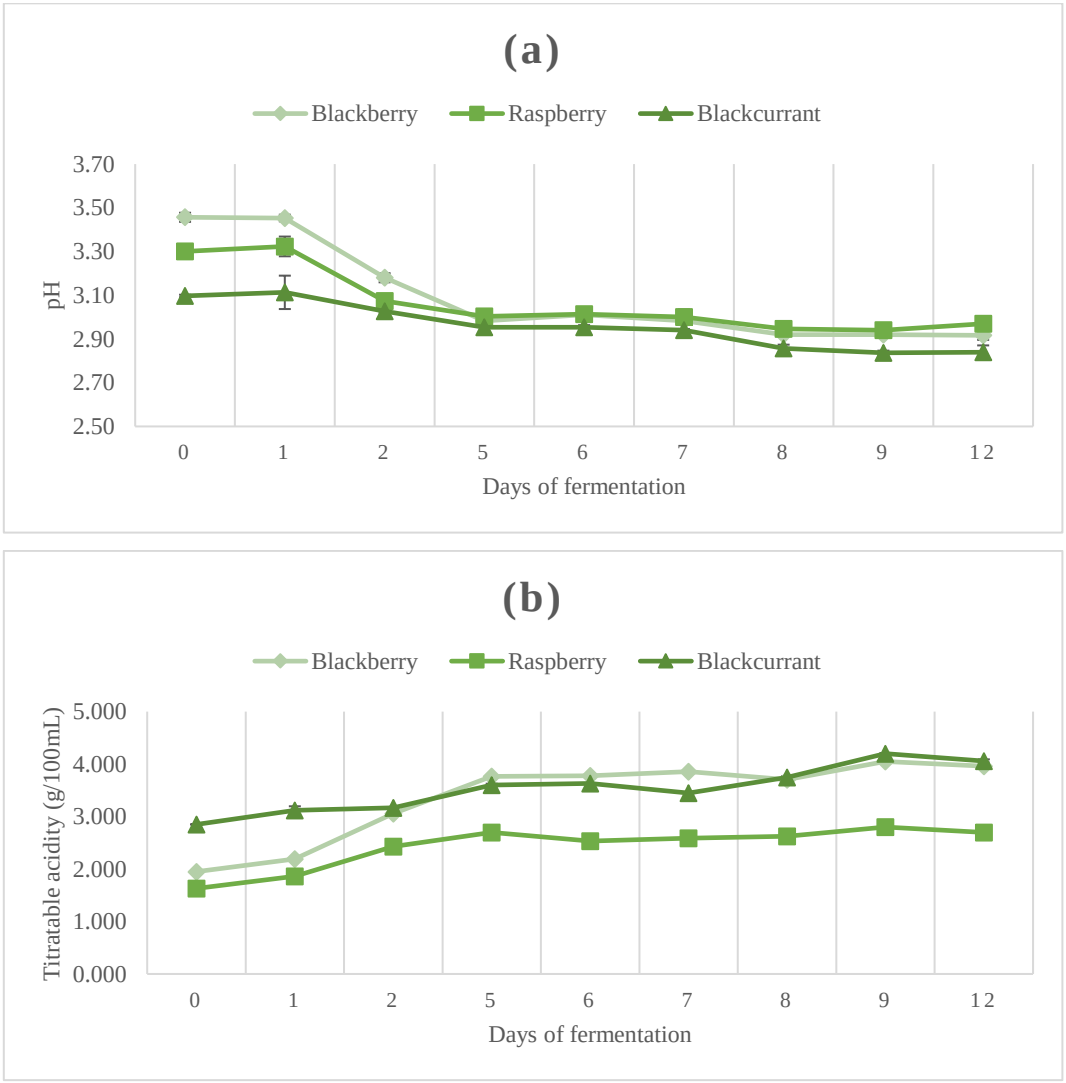


Figure 3.11 Comparison of (a) pH and (b) titratable acidity trend of juices at 10° Brix with relative standard deviations.

Comparing **Figures 3.12a-b** it can be seen that the initial available glucose is high for blackberry and blackcurrant, while for raspberry the value is very low and it follows that the production of GA is higher in blackberry and blackcurrant juice.

It is found that glucose is almost totally consumed after a few days of fermentation, and consequently oxidation into GA occurs in the first half of the fermentation process, when the glucose content is

higher. After the sixth day of fermentation, a constant settling is generally noticed for all the juices, due to the absence of glucose and therefore due to the lack of the substrate necessary for the production of GA. The rapid glucose consumption may be due to the favourable initial conditions of *G. oxydans* 621H growth juices, such as low titratable acidity, and an ideal initial glucose content.

By comparing all the analyzed parameters of the juices at 10° Brix, it can be seen that the raspberry juice was found to be unsuitable for this fermentation due to the low initial amount of glucose available, while the blackberry and currant juices were more suitable for undergoing a gluconic fermentation, considering that the amount of available glucose was higher. Quantitatively, blackberry juice is the medium in which the strain produced the most GA.

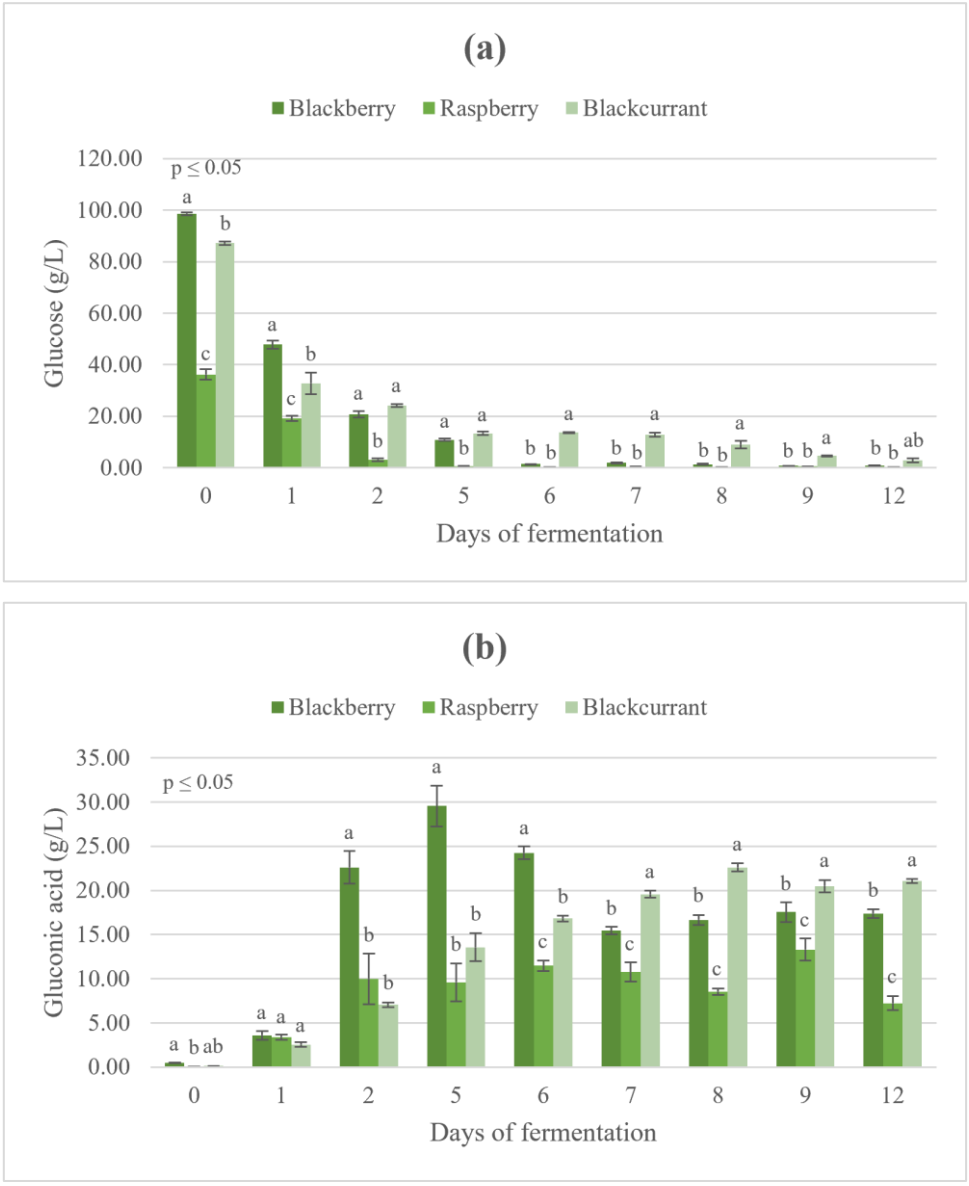


Figure 3.12 Comparison of the amount of (a) glucose consumed and (b) GA produced in the three 10 ° Brix. Bar plots indicate the average glucose consumption (a) and gluconic acid production (b) by three replicates ± standard deviation. Significant difference at same days of fermentation is shown by different letters ($p \leq 0.05$).

3.3.4 Comparison of chemical and physical parameters of juices at 10 and 25° Brix

The starting matrices, having a different soluble solid content, are characterized by different peculiarities and bacterial strain behaviour based on the medium and it was possible to identify differences between the same juices with different Brix degrees.

3.3.4.1 Comparison of pH and titratable acidity of juices at 10 and 25° Brix

Comparing the data obtained on *blackcurrant juice*, differences are in all the parameters evaluated. As can be seen from **Figure 3.13a**, the starting pH is the same for both 10 and 25° Brix juices and the trend is similar. Indeed, pH values of all the samples drop slightly and reach similar values after 12 days of fermentation. It should be emphasized that the decrease in pH at 10° Brix is more gradual than at 25° Brix, where there is a more drastic initial drop, followed by an irregular trend.
Figure 3.13b indicates the variation of titratable acidity occurred in 10° Brix and 25° Brix juices during fermentation. It can be seen that the trend is very constant in the two juices, as the variation after 12 days of fermentation is not so evident, but the two juices differ due to the different titratable acidity value, since for juice at 25° Brix the acidity is much higher.

Blackberry juice, as previously described, has very interesting parameters and by comparing the two juices with different contents of soluble solutes, the different characteristics are highlighted.
The pH turned out to be practically the same, characterized by a constant and similar decrease, in fact from **Figure 3.13a** there is a decrease for both after 12 days of fermentation. The comparison of the titratable acidity, shown in **Figure 3.13b**, indicates a similar trend, where a higher value is reached after 12 days, with the difference that the acidity of the 25° Brix juice is higher than the 10° Brix juice.

The *raspberry juices* present, as given by the analyses, different peculiarities. The pH of both has a very similar decreasing trend, as shown in **Figure 3.13a**, where after two days a drop in value is noted for both, to then have a more or less constant trend. The titratable acidity follows a slight increase for 10°Brix juice, while for 25°Brix juice it is more significant and differs since it has a higher initial and final value **Figure 3.13b**.

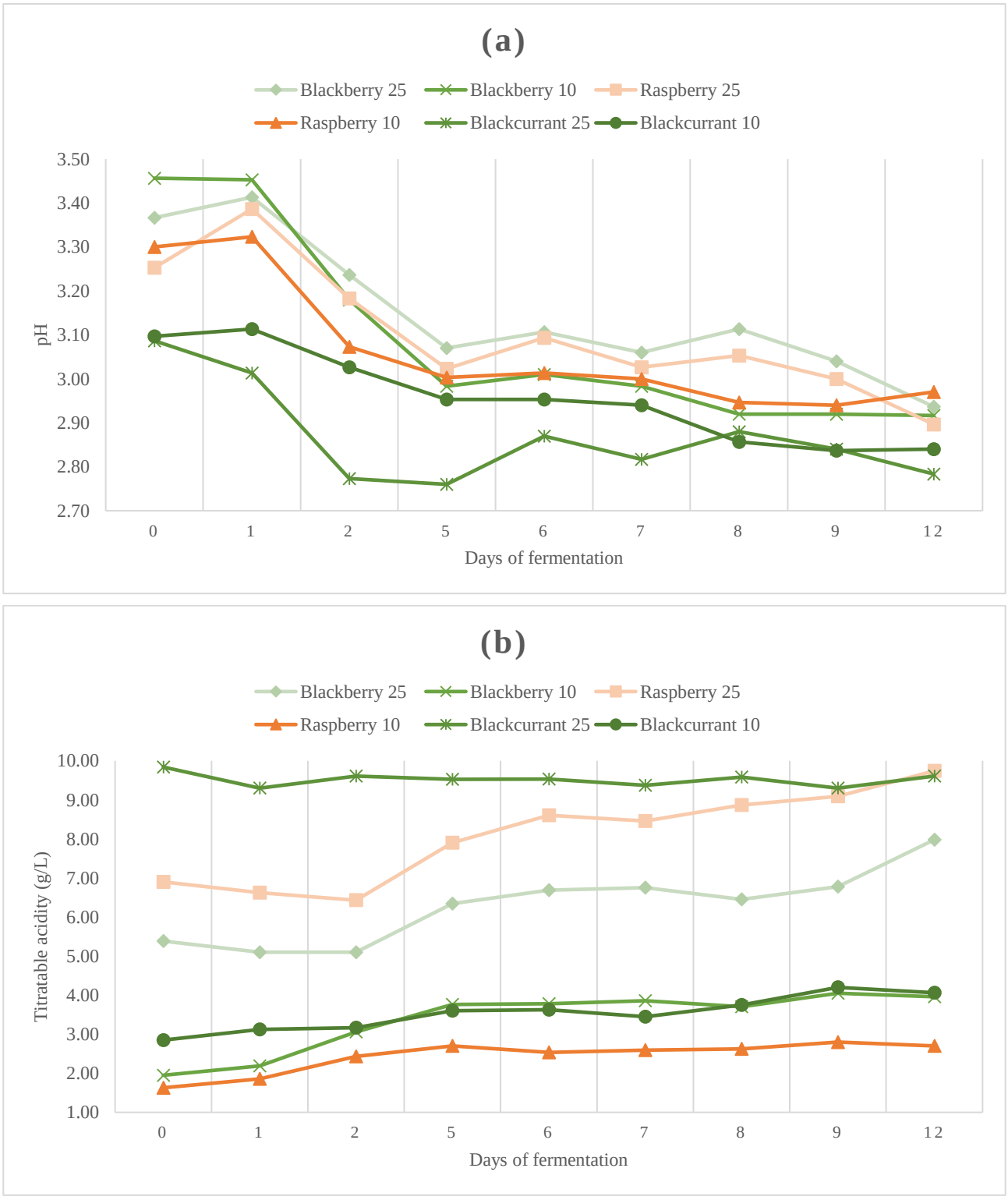


Figure 3.13 (a) pH and (b) titratable acidity of juices at 10° and 25° Brix. Relative standard deviation are expressed in Table 3.4-3.6.

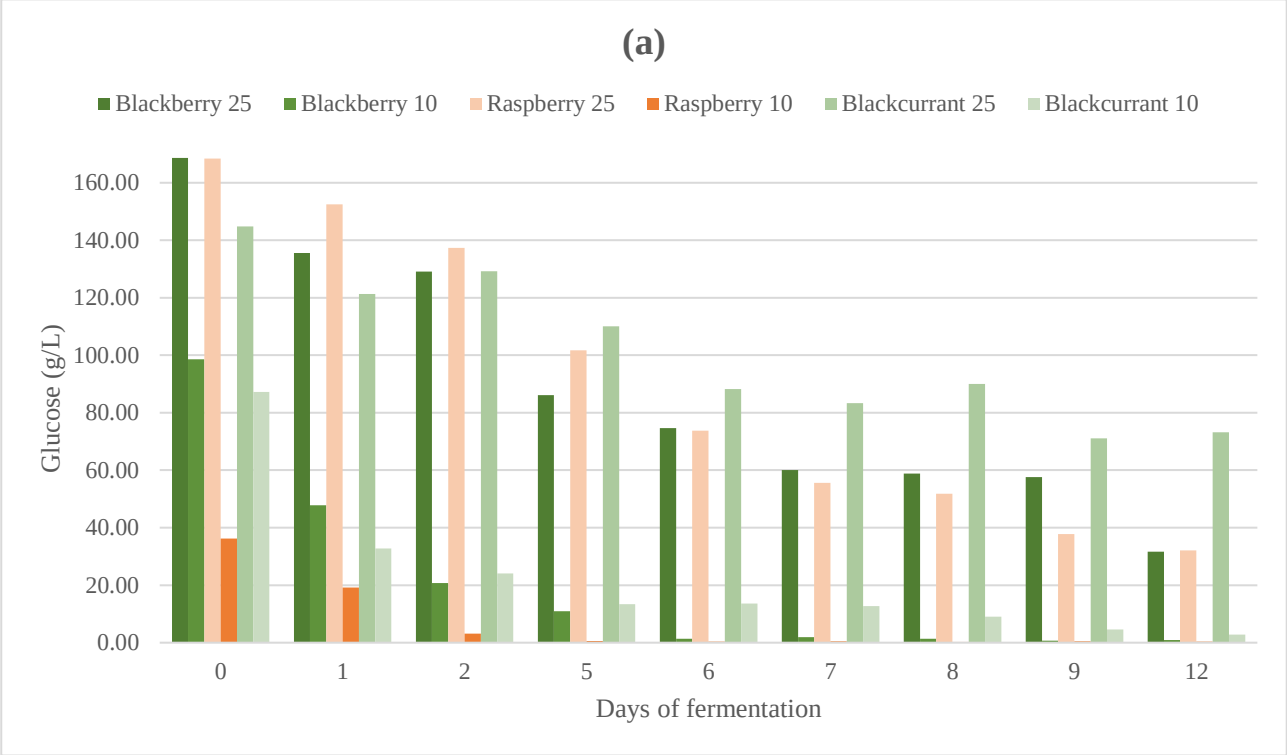
3.3.4.2 Comparison of glucose consumption and GA production of juices at 10 and 25° Brix

The initial glucose content is higher in *blackcurrant juice* at 25°Brix, but it can be seen from **Figure 3.14a**, that glucose in 10°Brix blackcurrants is almost totally consumed after 6 days of fermentation. In fact, the production of gluconic is evident after 6 days (**Figure 3.14b**), then no increase is observed. On the other hand, in 25° Brix juice the trend is not regular, and the consumption of glucose does not have a well-defined trend. This behaviour is mirrored in the production of GA, which is practically zero in juice at 25° Brix. The reason for the different behaviour can be traced back to the different conditions to which the bacterial strain is subjected. In juice at 25° Brix the titratable acidity is clearly higher, and this is a very stressful factor for the production of GA, for which bacterial growth is strongly limited and slowed down, with consequent inhibition of GA production. The most suitable juices is the 10° Brix.

The glucose consumption, as shown in **Figure 3.14a**, shows that the juice at 25° Brix has a higher initial quantity of glucose, which is consumed gradually and is distributed over all the days of fermentation, while in *blackberry juice* at 10 ° Brix, the consumption of glucose occurs essentially in the first 6 days of fermentation. This trend is probably due to the different acidity values of the two matrices, since the strain probably takes more days to adapt to the juice at 25° Brix, considered the higher acidity. This can also be seen in the production of GA, in fact **Figure 3.14b** shows that as regards the 10°Brix juices, the production of GA reached the maxim concentration after 5 days of fermentation. Therefore, no increases in GA were observed in any of the 10° Brix juices after 5 days. This could be related to the low residual glucose present in the juices.. In the juice at 25° Brix, on the other hand, there is an increase in GA which reaches its maximum after 9 days of fermentation, and then decreases until the end of fermentation at 12 days. It is important to underline that in the first 2 days of fermentation, the strain took longer in adapting to the environment using glucose to survive and remain in the latent phase. Finally, however, the yield of GA is clearly higher for juice at 25 ° Brix. Comparing the various parameters, we come to the conclusion that both juices can be used as a matrix to start of a gluconic fermentation. Finally, The 10° Brix juice is suitable for a short fermentation, ranging from 2 to 5 days, depending on the target GA yield and glucose content of the final fermented beverage, while blackberry juice at 25° Brix is best suited for approximately 9 days of fermentation.

The difference in glucose is clear, and it is what has most influenced the production of gluconic acid in *raspberry juices*. As can be seen in **Figure 3.14a** that the available glucose is very high in the juice

at 25° Brix, while in the juice at 10° Brix it is very low. The consumption of glucose in the juice at 25° Brix follows a regular trend, where there is a continuous decrease of the substrate, while for the 10° juice, considering the low initial content, the glucose is consumed after 2 days. This is also found in the production of GA, as seen in **Figure 3.14b**, where after two days in the 10° Brix juice approximately 10 g/L is reached. This value which remains constant for the entire fermentation process. While in the 25° Brix juice, the significant increase is visible after 6 days of fermentation and the peak is reached after 12 days of fermentation. Having compared all the parameters, it can be deduced that the 10° Brix raspberry juice is not suitable for undergoing gluconic fermentation, given the low glucose content, while the 25° Brix raspberry juice was found to be suitable for undergoing this type of fermentation.



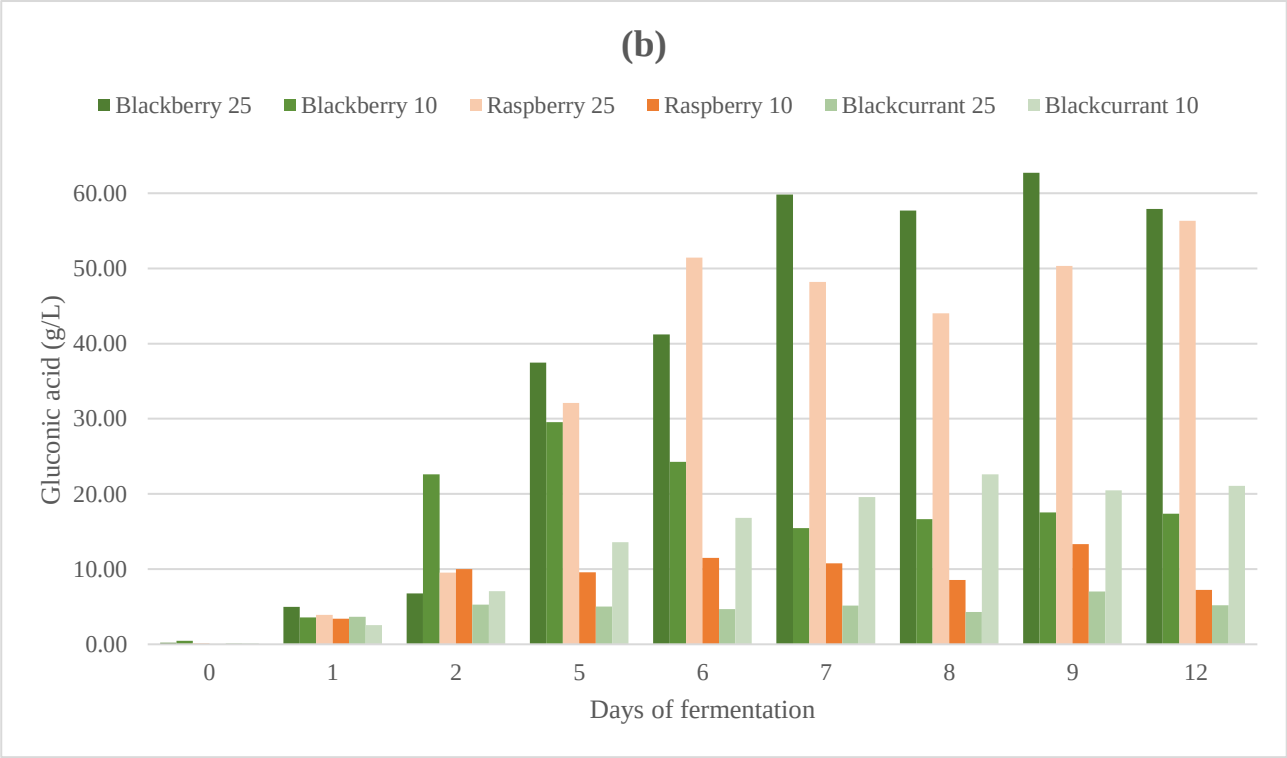


Figure 3.14 (a) glucose consumption and (b) GA production of juices at 10° and 25° Brix. Relative standard deviation are expressed in Table 3.4-3.6.

3.3.5 Evaluation of total phenolic compounds and antioxidant activity in vinegar samples produced from juices and fruits

Based on the result of previews section, blackcurrant fruit was no longer taken under consideration because of its non-optimal performance for the production of GA. On the other hand, blackberry, raspberry were evaluated for phytochemicals and antioxidants activity. Both juices and puree were considered, as for date in **Section 2.3.4**.

Juices and purees were pasteurized at 80°C for 20 minutes and subsequently inoculated with oenological strain *S. cerevisiae* UMCC 855. Finally, alcoholic products were fermented for 6 days with a strain of *K. europaeus* ZJ 555 and two strains of *A. pasteurianus* AB 0220 and DL 13.

The concentration of total phenolic compounds, in general, show a slight increase following the fermentation process (**Table 3.7**).

Products	Start concentration	*K. europaeus* ZJ 555 After fermentation	*A. pasteurianus* AB 0220 After fermentation	*A. pasteurianus* DL 13 After fermentation
Blackberry juice	4087.651^{a} ± 305.000	4155.088^{a} ± 315.440	3882.424^{a} ± 323.020	4258.131^{a} ± 370.191
Blackberry puree	2444.505^{a} ± 158.100	2389.000^{a} ± 177.000	2566.391^{a} ± 144.734	2517.595^{a} ± 182.242
Raspberry juice	3257.415^{a} ± 258.000	3311.200^{a} ± 264.202	3187.222^{a} ± 274.082	3324.242^{a} ± 124.235
Raspberry puree	1365.000^{a} ± 75.770	1358.000^{a} ± 95.040	1119.440^{b} ± 101.118	1155.660^{ab} ± 71.111

Table 3.7 Total phenolic compound concentration expressed in mg / L of gallic acid equivalent (GAE). Values are reported as average of three replicates ± standard deviation. Significant differences are shown by different letters ($p \leq 0.05$).

In particular, as previously seen, destruction of cellular wall improve total phenolic compounds release in fermented fruit juices.

Regarding antioxidant activity expressed as an equivalent amount of vitamin C per 100 mL of sample, the analyses, carried out on the raw materials and the fermented products obtained from them, show tendency to a slight decrease in vitamin C. The data in **Table 3.8** are preliminary and believed interesting to set up further fermentation tests by varying the fermentation conditions in order to acquire information on the influence of the kinetics / fermentation regime on the vitamin C content.

Products	Start concentration	*K. europaeus* ZJ 555 After fermentation	*A. pasteurianus* AB 0220 After fermentation	*A. pasteurianus* DL 13 After fermentation
Blackberry juice	74.70^{a} ± 1.89	58.05^{b} ± 5.02	61.44^{b} ± 5.66	55.83^{b} ± 4.89
Raspberry juice	64.82^{a} ± 4.06	61.33^{a} ± 5.00	55.05^{b} ± 2.36	52.61^{b} ± 2.05

Table 3.8 Antioxidant activity expressed as an equivalent amount of vitamin C per 100 mL of sample (mg vit C/100 mL). Significant differences are shown by different letters ($p \leq 0.05$).

3.4 Conclusions

This part of the research focused on analyzing gluconic fermentation on blackcurrant, raspberry and blackberry juices at 25° and 10° Brix to compare them and evaluate their differences, trying to understand which could be the most suitable matrix and the optimal process. Data highlighted different behaviors of 621H strain belonging to *G. oxydans* species, in the different juices, based on the conditions of the medium, which strongly affected the progress of the fermentation. This made it possible to outline the optimal fermentation processes and evaluate the most promising raw materials for undergoing gluconic fermentation.

The acidity of the environment has strongly influenced the speed and quantity of GA produced, for example in currant juice 25°Brix, which is characterized by a strong initial acidity, the strain is not managed to produce GA in detectable quantities, compared to raspberry juice and blackberry juice of the same amount of soluble solids. The parameter considered, also, strongly affected the rapidity of glucose consumption and GA production, as can be seen in juices at 10° Brix where the lower titratable acidity in the medium allowed the strain to consume glucose very quickly and produce GA in the first days of fermentation. Besides, the initial quantity of glucose in juices greatly influenced the progress of the fermentation. Data shows that greater was the initial quantity, greater was the production of GA during fermentation, in fact in blackberry and raspberry juice at 25° Brix juices, which have an initial glucose content of around 170 g/L, which is higher than all the other matrices, show a clearly higher production of GA than other juices. On the other hand, the speed with which GA is produced could be slowed down by the high initial glucose content. Indeed, in blackberry and raspberry juice at 25°Brix, in which the strain produces after 5 days of fermentation, while in currant and blackberry juices at 10° Brix which contain respectively 87.2010 and 98.6100 g/L, therefore a smaller quantity, there is a more immediate production of GA even if in smaller quantities. However, it should be taken into consideration that 10° Brix juices have lower titratable acidity values than 25° Brix juices, and this has probably influenced the speed of production of GA. The influence of the initial glucose content on the speed of production of gluconic acid is however an aspect to be investigated in order to evaluate its veracity through other studies.

Analyzing the values of the parameters obtained, the 10° Brix raspberry juice and the 25° Brix blackcurrant juice were found to be less suitable for undergoing gluconic fermentation, due to a low initial glucose content and the conditions very acidic of the medium. In the 10° Brix blackberry and blackcurrant juices fermentation produced “intermediate” quantities of GA, respectively 29.3313 g/L

and 22.3345 g/L, while for the 25° Brix raspberry and blackberry juice it should be highlighted the largest amount of GA produced, respectively 56.3540 g/L and 62.7209 g/L.
The ideal conditions for the production of fermented beverages starting from blackcurrant, blackberry and raspberry juice are an initial pH range of the medium with values of 3.10-3.50, as found in blackcurrant and blackberry juice 10° Brix , and raspberry and blackberry juice 25° Brix, where the production of GA took place, even if in different quantities. As previously discussed, acidity was found to be a parameter that strongly influenced the progress and timing of the fermentation. It is interesting to note that in blackcurrant and blackberry juices and 10 ° Brix the initial titratable acidity values are respectively 2.85 g/L and 1.95 g/L and are lower than in blackberry and raspberry juice 25 ° Brix which have values of 5.39 g/L and 6.90 g/L respectively. The strong initial acidity leads the bacteria to consume large quantities of glucose in order to be able to adapt to the food matrix, this translates into a lower yield for the production of GA.

Data obtained, that blackberry and raspberry juice at 25° Brix are certainly the most interesting matrices, as the GA produced is around 60 g/L for both. The conditions of the medium were therefore found to be suitable in terms of GA yield. The initial glucose quantity is almost identical (about 170 g/L), and the GA peak is recorded after 9 days for the blackberry and after 12 days for the raspberry.

In conclusion, among all the matrices tested, blackberry juice proved to be the best for undergoing GA fermentation, considering all the chemical-physical parameters evaluated by the following study. For a future production of fermented beverages starting from these matrices it is important to evaluate the starting parameters so that the conditions are ideal for the strain in question.

General conclusion and perspectives

This PhD project developed in the collaboration between the Laboratory of Food Microbiology/UMCC (Unimore Microbial Culture Collection) of Department of Life Science, University of Modena and Reggio Emilia, and Ponti SpA, was aimed at evaluating the fermentation capacity of fruits and qualitative characteristics of the products obtained thought selective fermentations. In this regard, the purpose of this experimental thesis was to enhance fruits as a raw material for the production of vinegars and non-alcoholic beverages with the use of specific bacterial cultures and monitoring the pH parameters, glucose consumption, GA production, EtOH utilization and AcOH production. The fruits considered were date, blackcurrant, blackberry and raspberry.

The best AAB for industrial production of vinegars and non-alcoholic beverages were *G. oxydans* 621H that produced 61.667 g/L of GA, *K. europaeus* ZJ 555 which produced 58.605 g/L of ACOH and *A. pasteurianus* DL 13 with a production of AcOH of 59.190 g/L. Moreover, results of prototypal trials provide evidence of the feasibility of developing controlled fermentation of dates juices for obtaining beverages at different AcOH content, both in static and submerged systems.

Data highlighted important parameters for fermentation processes. First of all, acidity of environment strongly influenced the speed and quantity of GA produced. The strong initial acidity leads the bacteria to consume large quantities of glucose in order to be able to adapt to the food matrix, this translates into a lower yield for the production of GA. Then, starter glucose concentration positively affect bacterial growth. Higher was the initial amount and greater was production of GA. On the other hand, smaller quantity give an immediate production of GA, even if in minor quantity. Finally, dates, blackberry, and raspberry, that produced around 60 g/L of GA, are the best raw material for production of vinegar and non-alcoholic beverages. Moreover, fermentation slight increase phenolic compounds content, knowing to show a protective effect against many chronic diseases.

In conclusion, considering the rich content in essential nutrients, carbohydrates, fatty acids, proteins, vitamins and mineral salts, the valorisation of date, blackcurrant, blackberry and raspberry fruits could start from the production of gluconic beverages, non-alcoholic beverages and vinegars using increasingly innovative technologies. These outputs offer the chance to valorise juices by selective AcOH fermentations, contributing to development of sustainable bioprocesses.

For a future production of fermented beverages starting from these juices is important to evaluate starting parameters, such as ideal conditions for the starter strain and different fermentation conditions

to identify the best combinations of process parameters that keep the initial concentration of phenolic compounds unchanged.

Appendix A

Characterization of wine vinegar samples from industrial bioreactors

This part of the research activity includes a first insight into the microbial composition of submerged fermentation at Ponti S.p.a. Samples with fermentation deviations detected by the company were provided to the Laboratory of Food Microbiology / UMCC, where isolation and strains characterization was performed. Together with samples, chemical-physical raw data produced by the company where provided (**Table A.1).**

Code	Product description	Titratable acidity %	pH	Ethanol %	d 20/20	SO2 mg/l	Extracts g/l
21AP01769	Wine vinegar - Tank n. 11	9.91	2.79	0.13	1.020	19	16.2
21AP01775	Wine vinegar - Tank n. 14	8.08	2.68	1.6	1.016	30	17.7
21AP01777	Wine vinegar - Tank n. 16	8.29	2.78	2.5	1.016	27	18.8
21AP01776	Wine vinegar - Tank n. 19	6.16	2.91	1.9	1.013	20	17.0
21AP01778	Wine vinegar - Tank n. 20	8.11	2.88	2.9	1.017	32	24.2
21AP01779	End wine vinegar fermentation	0.30	3.59	0.4	1.005	14	-
21AP02122	End wine vinegar fermentation	-	-	-	-	-	-

Table A.1 Samples of vinegar received from Ponti SpA and characteristics.

The isolation strategy was based on the detection of a halo around the colonies that occurs following the bacteriale growth in a medium containing calcium carbonate (GYC). The isolation was carried out from the samples and from serial dilutions of the same (dilution 10 and 100), and after purification, cultures were transferred to liquid GYC broth and incubated for 3 days at 28 ° C. The strains were then stored at -80 ° C in the presence of glycerol as a cryoprotective.

To obtain fresh cultures, single cultures were grown in tubes containing GY broth. Basic phenotypic characterization was done by Gram stain and catalase enzyme assay [108].

Molecular characterization of bacterial strains was performed by DNA extraction and molecular identification by sequencing of the 16S rRNA gene. DNA extraction was carried out using the DNeasy blood tissue kit (Qiagen) and, if the DNA yield was insufficient, a new extraction was performed by sodium dodecylsulfate (SDS) proteinase-cethyltrimethyl ammonium bromide (CTAB) treatment as previously reported [109]. gDNA concentration and quality were evaluated by NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, USA) and by gel electrophoreses (1% gel agarose in 1X TBE buffer), a 100 bp plus DNA ladder (Invitrogen, Carlsbad, CA, USA) was used. PCR reactions were performed using primers 27F (AGRGTTYGATYMTGGCTCAG) and 1490R (TACGGYTACCTTGTTACGACTT). Amplified products were purified using the kit DNA Clean & Concentrator™-5 (ZymoResearch) and automated sequenced (Sanger sequencing technique, BIOFAB).

The sequencing data found were processed (trimming ends, removing bases with low quality and assembling sequences) using CodoCode aligner software to obtained consensus sequences. These were sequences identified by alignment with Blastn and the 16S database of the National Center of Biotechnology Information (NCBI). From the multi-alignment, a neighbor-joining phylogenetic tree was constructed using the Tamura-Nei model and a bootstrap value of 1000 [110]. The phylogenetic tree was visualized and annotated using Interactive Tree Of Life software (iTOL) [111].

The industrial methods for producing wine vinegar are different and allow to obtain high concentrations of acidity in the shortest time possible. The main parameters to be evaluated in the industrial production of vinegar depends mainly on the metabolic needs of the AAB. Since AAB are obligate aerobic bacteria, industrial production systems are equipped with efficient ventilation systems to guarantee a high and constant oxygen supply. The samples analysed in this work come from a submerged fermentation in a semi-continuous system. This type of fermentation system is one of the first to have been developed and allows the production of wine vinegar with high acidity (11-12% g/L). The system basically consists of two bioreactors (tanks): in tank 1 the substrate (wine with an alcohol content between 10.5 and 11% g/L) is fermented until an optimal balance between the concentration of AcOH and EtOH is reached. In this system, the optimum equilibrium is represented by an AcOH concentration of 8% g/L and an EtOH concentration of 3% g/L. Once this balance has been reached, a part of the fermented AcOH is discharged into tank 2, where the acetic fermentation will end (EtOH < 0.5%) when the pre-established concentration of AcOH is reached, in this case a concentration between 10-11% [10].

The samples obtained in this study were obtained from production system tanks at different stages of fermentation. In fact, sample 21AP01769 represents a finished fermentation, samples 21AP01775-76-77-78 were taken from active fermentation tanks and 21AP01779-2122 was end fermentation waste. For sample 21AP02122 it was not possible to determine chemical-physical parameters with standard methods, as it is in waste tank it is not possible to determine parameters such as AcOH, EtOH, SO2, D20/20 with standard tests.

This study carried out a characterization of industrially produced wine vinegar, using culture-dependent methods. Through the use of specific media for the recognition of bacteria capable of carrying out the oxidative fermentation of EtOH (GYC and GEY) it was possible to obtain bacterial isolates which appear on the plate as well-defined and spaced colonies.

Under culture conditions in liquid medium, different isolates showed quite distinct phenotypic characteristics, highlighting a certain degree of diversity. Extracellular matrix production has been observed for some isolates, mainly represented by exopolysaccharides and localized in the liquid-air interface (bacterial biofilm). Bacterial biofilms produced by the isolates object of this study presented different characteristics: some showed a well-defined matrix, others had a high filamentous component and finally others were characterized by a high degree of dispersion. Four of the isolated strains showed biofilm production (P1B22, P1B76, P2B79 and P3B79), the other three (P2B22, P1B79 and P1B78) were free. From a phenotypic point of view, Gram staining was performed highlighting the presence of Gram+ (P2B22, P1B78 and P1B79) and Gram– (P1B22, P1B76, P2B79 and P3B79) bacteria, and the catalase test yielded all isolates as catalase positive except strains P2B22 and P1B79.

Table A.2 shows a summary of the results. The study made it possible to isolate strains belonging to the genera *Acetobacter* and *Komagataeibacter*, whose presence is consistent with the origin of the samples, being organisms producing AcOH and associated with acetic fermentation phenomena. In addition, strains belonging to microbial groups other than AAB have been found: *Lactobacillus paracasei*, *Lactobacillus nagelii* (lactic acid bacteria) and *Lysinibacillus fusiformis*. The presence of lactic bacteria, although not associated with the phenomena of acetic oxidation, is likely in fermented products and widely documented in the literature. The isolation of a strain of *Lysinibacillus fusiformis* can be traced back to occasional contamination.

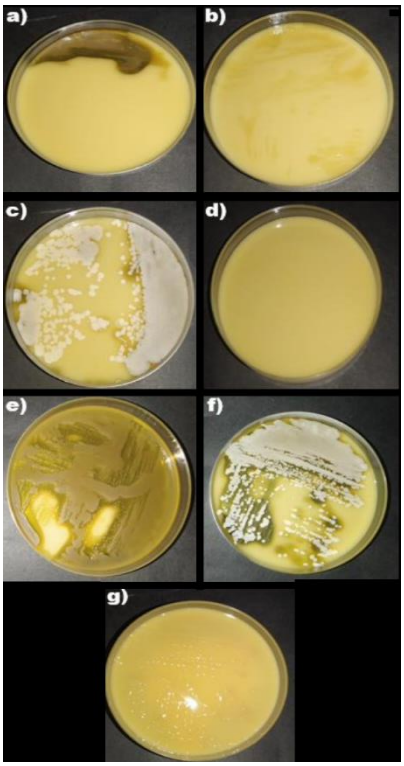


Figure A.1 Isolation colonies on GYC. P1B22 strain (a), P2B22 strain (b), P1B79 strain (c), P1B76 strain (c), control plate (d), P2B79 strain (e), P3B79 strain (f), P1B78 strain (g).

In this work, the samples provided were analysed, but a more in-depth analysis would require isolation from the same samples at different frequencies in order to be able to correlate the fermentation parameters to the microbial species. Furthermore, isolation on culture media can, on the one hand, highlight the presence of bacteria that are not functional to the acetification process, since these are samples that come from processes conducted not in sterile conditions, there may be a microflora that is easy to cultivation. As regards sample 21AP01778, with fermentation parameters indicating fermentation activity (AcOH and EtOH), no AAB were isolated. This phenomenon might seem contradictory but as demonstrated in the scientific literature, AAB from industrial vinegar processes are difficult to isolate. In fact, the physiological characteristics of AAB and the documented inability to grow under laboratory conditions, despite being viable in the original sample, is documented [27].

Code sample	Description	Acetic acid g/100mL	pH	Ethanol g/100mL	D 20/20	SO2 mg/L	Extract g/L	Strain	Species	Biofilm presence	Biofilm aspect	Gram reaction	Catalase reaction
21AP02122	End wine vinegar fermentation	0	--	--	--	--	--	**P1B22**	***Komagataeibacter saccharivorans***	+	Biofilm superficial and compact	-	+
21AP02122	End wine vinegar fermentation	0	--	--	--	--	--	**P2B22**	***Lactobacillus paracasei***	-	-	+	-
21AP01779	End wine vinegar fermentation	0.3	3.59	0.4	1.005	14	--	**P1B79**	***Lactobacillus nagelii***	-	-	+	-
21AP01779	End wine vinegar fermentation	0.3	3.59	0.4	1.005	14	--	**P2B79**	***Acetobacter* tropicalis**	+	Biofilm in fragments	-	+
21AP01779	End wine vinegar fermentation	0.3	3.59	0.4	1.005	14	--	**P3B79**	***Acetobacter okinawensis***	+	Biofilm unic and floating	-	+
21AP01776	Wine vinegar - Tank n. 19	6.16	2.91	1.9	1.013	20	17	**P1B76**	***Komagataeibacter rhaeticus***	+	Biofilm superficial and compact	-	+
21AP01778	Wine vinegar - Tank n. 20	8.11	2.88	2.9	1.017	32	24	**P1B78**	***Lysinibacillus fusiformis***	-	-	+	+

Table A.2 Samples provided, physico-chemical characteristics and strains characterization.

Through the use of target sequences, represented by 16S rRNA, the isolates examined in this study have been characterized (**Table A.3**). Results obtained show a typical taxonomic profile of wine vinegar. The phylogenetic tree (**Figure A.2**) is characterized by the presence of two large clades, in one we find the genera *Komagataeibacter* and *Acetobacter*, belonging to the *Acetobacteraceae* family; the other clade includes the recently reclassified genus *Lactobacillus* taxonomically in 25 different genera, and the genus *Lysinibacillus*, an unusual genus for the wine vinegar described microbial communities.

Isolated	Species	Similitude %	Accession number	Strain
P1B22	*K. saccharivorans*	100%	NR_108135.1	+
P2B22	*Lact. paracasei*	99.03%	NR_025880.1	-
P1B79	*Liq. nagelii*	100%	NR_112754.1	+
P1B76	*K. rhaeticus*	97.80%	NR_113396.1	+
P2B79	*A. tropicalis*	100%	NR_113846.1	+
P3B79	*A.okinawensis*	99.93%	NR_113546.1	+
P1B78	*Lys. fusiformis*	99.93%	NR_042072.1	+

Table A.3 Identification of taxonomic species in industrial wine vinegar.

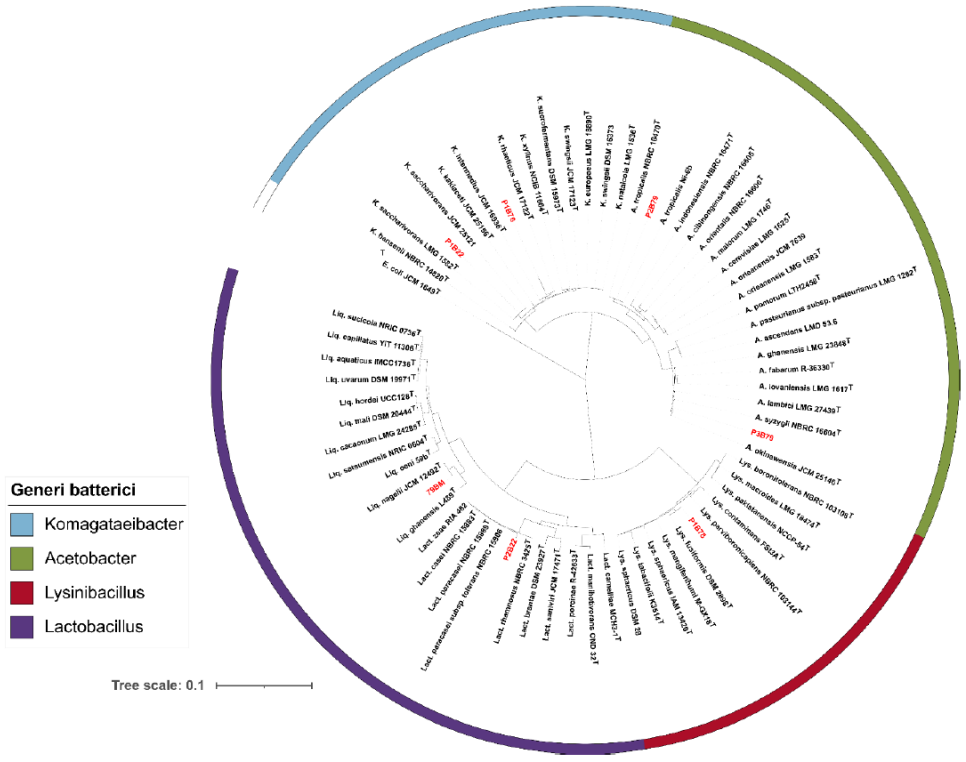


Figure A.2 Phylogenetic tree of known samples and isolated strains

As regards the isolates examined, two (P1B22 and P1B76) were classified in the genus *Komagataeibacter*, in detail assigned to the species *K. saccharivorans* (type strain JCM 25121^T) and to the species *K. rhaeticus* (strain JCM 17122), with a similarity value of 100% for the P1B22 strain (*K. saccharivorans*) and 97.8% for the P1B76 strain (*K. rhaeticus*). Two strains, P2B79 and P3B79, have been included in the subclade of the genus *Acetobacter* with a sequence similarity percentage of 100% with the strain *A. tropicalis* Ni-6b T and 99.93% *A. okinawensis* JCM 25146^T. The strain P1B78 was classified as belonging to the genus *Lysinibacillus*, with a similarity of 99.93% with the type strain *L. fusiformis* DSM 2898^T. The remaining two strains, P1B79 and P2B22, were included in the *Lactobacillus* subclade group. In particular, the P1B79 strain has been assigned to the *Liq. nagelii* JCM 12492^T with a 100% sequence similarity rate, while the P2B22 strain has a 99.03% similarity rate to the *Lact. paracasei* subsp. *tolerans*.

The results obtained from this study show a trend in terms of microbial diversity, similar to that described in the literature. Most of the isolates belong to the *Acetobacteraceae* family (57%) while 28% of the isolates were classified in the *Lactobacillaceae* family. *K. saccharivorans* and *K. rhaeticus*. These are AAB with a particular aptitude to grow in acid environments [112]. No strains belonging to the species *K. europaeus* and *K. xylinus* have been identified in this work. *K. europeus* is the species most associated with the production of industrial vinegars and for which the problem of cultivating the strains has been documented [27]. It is a species whose strains produce high amount of AcOH and resist high concentrations (about 18%) of the same. Although various ad hoc culture media have been formulated over the years, the use of culture dependent methods are not always suitable for the recovery of strains, whereas the presence of *K. europaeus* during production of industrial vinegar has been confirmed by culture independent methods.

Both isolated strains of the genus *Acetobacter* have similar behaviors with regards to their proliferation in vinegar. *A. okinawensis* has been shown to be the dominant species in vinegars, in particular in fruit vinegars (apple), instead *A. tropicalis* [113] has been isolated in Austrian vinegar. The presence of *Lact. paracasei* in fermented beverages is confirmed by the literature, are present several studies where it has been identified within the Chinese sour rice soup [114], a soup based of tomato juice and rice in which the main fermentation product is lactic acid produced by these bacteria. This bacterium is not absent in vinegar, however it is quite rare, generally among lactic acid bacteria the most common is *Lactiplantibacillus plantarum* [115] although *L. paracasei* has also been encountered. Both *Lys. fusiformis* than in *Lys. sphaericus* have been found in caxiri [116], a fermented beverage produced by the indigenous Juruná Brazilians in which bacterial and yeast communities coexist. During fermentation some lactic acid bacteria degrade the root wall of the cassava plant for soften it. However, no studies demonstrating the occurrence in vinegar have been produced.

This preliminary study on the microflora occurring during production of vinegar in submerged system, highlighted the problematic of recovering and cultivating AAB strains from high acidic environments. A metagenomic study on the vinegar microflora from selected fermenting vinegar has been set up and analysis of data is ongoing.

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