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Acetic Acid Bacteria in the Food Industry: Systematics, Characteristics and Applications

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SUMMARY

The group of Gram-negative bacteria capable of oxidising ethanol to acetic acid is called acetic acid bacteria (AAB). They are widespread in nature and play an important role in the production of food and beverages, such as vinegar and kombucha. The ability to oxidise ethanol to acetic acid also allows the unwanted growth of AAB in other fermented beverages, such as wine, cider, beer and functional and soft beverages, causing an undesirable sour taste. These bacteria are also used in the production of other metabolic products, for example, gluconic acid, L-sorbose and bacterial cellulose, with potential applications in the food and biomedical industries. The classification of AAB into distinct genera has undergone several modifications over the last years, based on morphological, physiological and genetic characteristics. Therefore, this review focuses on the history of taxonomy, biochemical aspects and methods of isolation, identification and quantification of AAB, mainly related to those with important biotechnological applications.

Key words: acetic acid bacteria, taxonomy, vinegar, bacterial cellulose, biotechnological products

INTRODUCTION

Acetic acid bacteria (AAB) belong to the family Acetobacteraceae, which includes several genera and species. Currently, they are classified into nineteen genera, including *Acetobacter*, *Acidomonas*, *Ameyamaea*, *Asaia*, *Bombella*, *Commensalibacter*, *Endobacter*, *Gluconacetobacter*, *Gluconobacter*, *Granulibacter*, *Komagataeibacter*, *Kozakia*, *Neoasaia*, *Neokomagataea*, *Nguyenibacter*, *Saccharibacter*, *Swaminathania*, *Swingsia* and *Tanticharoenia* (*1*). The main species responsible for the production of vinegar belong to the genera *Acetobacter*, *Gluconacetobacter, Gluconobacter* and *Komagataeibacter* because of their high capacity to oxidise ethanol to acetic acid and high resistance to acetic acid released into the fermentative medium (*2,3*).

The species most frequently reported in vinegar production comprise *Acetobacter aceti*, *Acetobacter cerevisiae*, *Acetobacter malorum*, *Acetobacter oeni*, *Acetobacter pasteurianus*, *Acetobacter pomorum*, *Gluconacetobacter entanii*, *Gluconacetobacter liquefaciens*, *Gluconobacter oxydans*, *Komagataeibacter europaeus*, *Komagataeibacter hansenii*, *Komagataeibacter intermedius*, *Komagataeibacter medellinensis, Komagataeibacter oboediens* and *Komagataeibacter xylinus* (*4–6*).

The synthesis of other metabolites, for example, L-sorbose from D-sorbitol, as well as dihydroxyacetone from glycerol, has also been described for some species of AAB (*7–10*). Another important feature of AAB is their ability to produce extracellular polymers, for example bacterial cellulose, which is mainly synthesised by species of the *Gluconacetobacter* and *Komagataeibacter* genera. This polymer is highly versatile with unique properties (*e.g.* high water-holding capacity, ultrafine network structure, biocompatibility, high crystallinity) that support a range of commercial applications, for instance, as a wound dressing, functional food additive, and in tablet preparation (*11*).

TAXONOMY

The first attempt at classifying AAB was made by Hansen in 1894 (*12*). However, Beijerinck was the first to establish the genus name *Acetobacter* in 1898 (*13*). In 1925, Visser't Hooft was the first scientist to consider the biochemical characteristics in the classification of AAB (*14*). In 1934 and 1935, Asai (*15,16*) classified them into two main genera: *Acetobacter* and *Gluconobacter*. Frateur (*17*), in 1950, proposed a scheme for the classification of *Acetobacter* that was based on five biochemical criteria: (*i*) the presence of catalase, (*ii*) the oxidation and overoxidation of ethanol to acetic acid, and to carbon dioxide and water, respectively, (*iii*) oxidation of lactate to carbonate, (*iv*) oxidation of glycerol to dihydroxyacetone and (*v*) acid production from D-glucose. In the eighth edition of Bergey's Manual of Determinative Bacteriology (*18*), the classification of AAB was defined as *Acetobacter* and *Gluconobacter*. The genus *Acetobacter* was classified based on the presence/absence of peritrichous flagella and the ability to oxidise acetate and lactate. This genus contained three species (*A. aceti*, *A. pasteurianus* and *A. peroxydans*) and nine subspecies. The genus *Gluconobacter* was classified based on the presence/absence of polar flagella, inability to oxidise acetate and lactate, and the capability to oxidise D-glucose to gluconate, then further oxidise gluconate to 2-ketogluconate and 5-ketogluconate. This genus contains one single species (*G. oxydans*) with four subspecies (*19-21*). Furthermore, all the *Gluconobacter* species examined by Yamada *et al.* (*22,23*) had the coenzyme Q10 (ubiquinone) system. However, those of *Acetobacter* species had the Q9 or 10 (observed in *A. xylinus* strains) system (*24*).

In 1984, a new subgenus of the Q10-equipped acetate-oxidising AAB, namely *Acetobacter liquefaciens* and *Acetobacter xylinum*, was found (*24*). In 1997, the new genus *Gluconacetobacter* was proposed by Yamada *et al.* (*25,26*), based on partial 16S ribosomal RNA (rRNA) sequences and chemotaxonomic comparisons of the ubiquinone systems. As a result, species containing Q10, previously classified as *Acetobacter* (*A. diazotrophicus*, *A. europaeus A. hansenii*, *A. liquefaciens* and *A. xylinus*) were renamed *Gluconacetobacter* (*19*).

Over the last years, new species have been described in the genus *Acetobacter* and *Gluconobacter.* Subsequently, classification adjustments based on physiological characteristics were suggested, and the species belonging to genus *Acetobacter* were phylogenetically divided into two groups. The first group corresponded to the *A. aceti* group, which included *A. aceti*, *A. cerevisiae, A. cibinongensis*, *A. estunensis*, *A. indonesiensis*, *A. malorum, A. nitrogenifigens*, *A. oeni*, *A. orientalis*, *A. orleanensis* and *A. tropicalis*. The second group corresponded to the *A. pasteurianus*, which included *A. lovaniensis*, *A. pasteurianus*, *A. peroxydans*, *A. pomorum* and *A. syzygii*. The *A. aceti* group was phenotypically distinguished from *A. pasteurianus* group by the production of 2-ketogluconate (except for *A. oeni*) and 5-ketogluconate, and production of dihydroxyacetone from glycerol, which was detected in three species (*A. aceti, A. pomorum* and *A. nitrogenifigens*) (*27*). The species of the genus *Gluconobacter* were also phylogenetically divided into two groups: *G. oxydans* group, which includes *G. oxydans* and *G. albidus*, and the *G. cerinus* group, which includes *G. cerinus*, *G. frateurii* and *G. thailandicus*. These two groups were differentiated phenotypically and genetically from each other by growth characteristics on media containing D-arabitol without nicotinic acid addition, as well as by DNA base composition, *i.e.* G+C content (*27*).

In the last decade, the genus *Gluconacetobacter* was proposed to be subdivided into two groups with different morphological, physiological and ecological characteristics. These groups were the *G. liquefaciens* group (including *G. azotocaptans*, *G. diazotrophicus*, *G. liquefaciens* and *G. sacchari*) and the *G. xylinus* group (including *G. entanii, G. europaeus, G. hansenii, G. intermedius, G. nataicola, G. oboediens, G. rhaeticus, G. saccharivorans*, *G. swingisii* and *G. xylinus*) (*27*). Afterwards, according to the genetic analyses and phenotypic characteristics, Yamada *et al.* (*28,29*) proposed the new genus *Komagataeibacter* comprising the species belonging to the *G. xylinus* group. The two genera were differentiated from each other by the production of a water-soluble brown pigment and cell motility. *Gluconacetobacter* species generally produce the water-soluble brown pigment and are motile, whereas the *Komagataeibacter* species do not produce the pigment and are non-motile. In addition, the species of the former genus were associated with plants and isolated mostly from fruits, flowers, coffee and sugarcane. Conversely, the species of the latter genus were isolated chiefly from fermented foods, such as vinegar, kombucha, nata de coco and fruit juice (*28,30*).

CHARACTERISTICS

AAB are strictly aerobic microorganisms, Gram-negative or Gram-variable, catalase-positive and oxidase-negative, ellipsoidal to rod-shaped cells that can occur singly, in pairs or chains. They are also mesophilic microorganisms, and their optimum growth temperature is between 25 and 30 °C. The optimum pH for their growth is 5.0–6.5, but they can also grow at lower pH values (*31,32*).

The species of AAB are well known to have a high capability to oxidise alcohols, aldehydes, sugars or sugar alcohols in the presence of oxygen. As a result of these oxidative activities, the corresponding oxidation products such as carboxylic acids, accumulate in the culture medium. These oxidative reactions are catalysed by primary dehydrogenases, located on the outer surface of the cytoplasmic membrane (*33*).

Many other bacterial species are also able to oxidise ethanol under aerobic conditions, but they are unable to do this under high acidic conditions. AAB strains oxidise ethanol to acetic acid by two sequential catalytic reactions. First, ethanol is oxidised to acetaldehyde, which is catalysed by membrane-bound pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenase (ADH). Then, the generated acetaldehyde is immediately oxidised to acetate by membrane-bound aldehyde dehydrogenase (ALDH), located near ADH (*33–36*). During alcohol oxidation, no aldehyde liberation is observed,

indicating that ADH and ALDH form a multienzyme complex in the bacterial membrane and function sequentially to produce acetic acid from ethanol (*33*). The produced acetic acid is released into the growth medium, where it accumulates to a maximum 5–10 % in *Acetobacter* species and 10–20 % in *Komagataeibacter* species (*37,38*). Some genera can further oxidise the produced acetic acid to CO_2 and H_2O, resulting in so-called acetate oxidation (overoxidation). This ability is useful for distinction from the genus *Gluconobacter*, which does not have the same capability. This condition depends on the composition of the medium, especially when ethanol is used by the bacteria (*39,40*).

During acetification, AAB species occur depending on the concentration of acetic acid. In the first stage of acidification, at low acetic acid concentration, there is a predominance of the *Acetobacter* genus. Subsequently, when the mass per volume ratio of acetic acid exceeds 5 %, the population of *Komagataeibacter* species dominates. Therefore, *Komagataeibacter* species are the main strains involved in submerged acetic acid fermentation to produce vinegar (*38,41*). The *K. europaeus*, *K. intermedius* and *K. oboediens* are typical representatives during spontaneous vinegar production with acidity above 6 %, and *K. europaeus* is described as one of the AAB most frequently found and isolated from submerged vinegar fermentors. This behaviour results from an enhanced resistance of these microorganisms to the highest concentration of acetic acid and their greater adaptation to extreme acidity (*42,43*). In contrast, species of the genus *Acetobacter* are mainly responsible for the traditional surface production of vinegar, in which the final acetic acid content does not exceed 8 %, considered the acetic acid threshold for these bacteria (*38*). Besides the fermentation methods and acetic acid concentration, the species of AAB found in the fermentation medium are also significantly affected by the raw materials used in vinegar production (*44*).

The genera of AAB show similarity in the abundance of the ADH enzyme. However, the ADH of *Gluconobacter* species is less stable under acidic conditions than of other genera, such as *Acetobacter* (*45,46*). This fact, associated with the greater resistance of the cells to acetic acid, may explain the higher productivity of the *Acetobacter* compared to *Gluconobacter* species. Furthermore, the genera of AAB show a difference in oxidation capacity of ethanol, sugar and sugar alcohol. For example, production of gluconic acid from D-glucose and ketogenic activity from glycerol is weak to negligible in *Acetobacter* species but strong in *Gluconobacter* (*46*). Namely, species of the genus *Gluconobacter* have potent catalytic activity in the oxidation of ethanol, D-glucose, gluconic acid, glycerol and sorbitol. Conversely, species of the genera *Acetobacter, Gluconacetobacter* and *Komagataeibacter* possess a powerful system to oxidise ethanol, but only a slight oxidative activity on the sugars. The main biochemical and differential characteristics of the genera of AAB associated with vinegar production are presented in **Table 1** (*28,31,47,48*).

Table 1. Differential characteristics of the genera *Acetobacter*, *Gluconacetobacter*, *Gluconobacter* and *Komagataeibacter*

Characteristic	*Acetobacter*	*Gluconobacter*	*Gluconacetobacter*	*Komagataeibacter*
Motility and flagellation	peritrichous or non-motile	polar or non-motile	peritrichous or non-motile	no
Oxidation of ethanol to acetic acid	+	+	+	+
Oxidation of acetic acid to CO_2 and H_2O	+	–	+	+
Oxidation of lactate to CO_2 and H_2O	+	–	+	+
Growth on 0.35 % acetic-acid-containing medium	+	+	+	+
Growth in the presence of 30 % D-glucose	–	+ or -	+ or –	n.d.
Production of cellulose	–	–	+ or –	+ or –
Ketogenesis (dihydroxyacetone) from glycerol	+ or –	+	+ or –	+ or –
Acid production from:				
Glycerol	+ or –	+	+	n.d.
D-Mannitol	+ or –	+	+ or –	–
Raffinose	–	–	–	n.d.
Production of water-soluble brown pigment	–	variable	variable	–
Production from D-glucose of:				
2-keto-D-gluconic acid	+ or –	+	+ or –	+ or –
5-keto-D-gluconic acid	+ or –	+ or –	+ or –	+ or –
2,5-keto-D-gluconic acid	+ or –	+ or –	+ or –	–
Ubiquinone type	Q9	Q10	Q10	Q10

Data shown are combined from various sources (*28,31,47,48*). +=90 % or more of the strains positive, –=90 % or more of the strains negative; n.d.=not determined

ISOLATION AND PHENOTYPIC IDENTIFICATION

AAB are described as nutritionally demanding microorganisms and difficult to isolate and cultivate on artificial media, especially from fermented beverages. This challenge has been attributed to the phenomenon of the viable but nonculturable (VBNC) state, which causes the inability to cultivate and enumerate the AAB population on growth media, mainly strains isolated from environments with high levels of acetic acid (*31,47,49*). Despite the abundant number of growth media proposed for the isolation and cultivation of AAB strains (**Table 2**; *48,50–61*), not all media support their growth and they can be selective of one strain or another (*31,47*).

The isolation and purification of AAB strains from industrial vinegar must is performed through the use of a liquid or solid medium that provides their nutritional needs. The sources of carbon are mainly D-glucose and D-mannitol, and in some instances, ethanol and acetic acid are added at various concentrations. Nitrogen sources, such as peptone and yeast extract, and minerals, such as KH_2PO_4, Na_2HPO_4 and $MgSO_4$, are also often added for recovery of the microorganisms (*21*). The cultivation on the double-layer agar plate by adding 0.5 % agar and coating with a 1 % agar layer, under high humidity, is the most efficient technique because it provides a wet environment for the formation of acidifying bacterial colonies (*56*). Among selective inhibitors of Gram-positive microbiota, including crystal violet, brilliant green and sodium deoxycholate, it was found that brilliant green is the least inhibitory to the AAB. Sodium deoxycholate reduced the growth of all tested *Acetobacter* species, and violet crystal completely inhibited the growth of the studied *A. aceti* subspecies (*53*).

The traditional methods for classification of AAB species, after isolation, are based on cellular morphology, flagellation and some physiological and biochemical properties. Examples of these attributes are the production of a water-soluble brown pigment, production of cellulose, ability to oxidise sugars and ethanol to acid and ability to oxidise lactate and acetic acid to CO_2 and H_2O, using differentiation medium based on the biochemical characteristics of the AAB genera (*21*).

The genera that can oxidise lactate to CO_2 and H_2O, such as *Acetobacter*, *Gluconacetobacter* and *Komagataeibacter*, may be rapidly distinguished from the genus that cannot oxidise lactate, such as *Gluconobacter*, by inoculation of the strains into dextrose sorbitol mannitol (DSM) agar (*53*). This selective medium contains calcium lactate as the main source of carbon and smaller amounts of other sources, and it is based on the preferential oxidation of the carbon source. When *Acetobacter* grows on DSM agar, the medium changes from yellow to purple, as a result of lactate utilisation, causing a pH increase, which is detected by the bromocresol purple indicator. *Gluconobacter*, being unable to oxidise lactate, preferentially oxidises the minor carbohydrate constituents, producing acid and maintaining the yellow appearance of the medium (*21,53*).

The oxidation of ethanol to acetic acid and overoxidation to CO_2 and H_2O can be detected by several methods. For example, Carr agar (*52*) contains ethanol as a carbon source and

Table 2. Main media for culture, recovery, growth and genus differentiation of acetic acid bacteria

Medium	γ/(g/L) or *φ/(mL/L)	Reference
AE (acetic acid-ethanol)	Glucose 5, yeast extract 3, peptone 4, acetic acid 30*, ethanol 30*, agar 9	(*50*)
BME (basal medium plus ethanol)	Yeast extract 0.5, vitamin-free casamino acids 3, ethanol 3*, agar 15	(*51*)
Carr	Yeast extract 30, ethanol 20*, bromocresol green 0.022, agar 20	(*52*)
Medium for chalk-ethanol test	Glucose 0.5, yeast extract 5, peptone 3, calcium carbonate 15, ethanol 15*, agar 12	(*48*)
DSM (dextrose-sorbitol-mannitol)	Glucose 1, sorbitol 1, mannitol 2, yeast extract 3.3, proteose-peptone 10, calcium lactate 15, KH_2PO_4 1, $MnSO_4 \cdot H_2O$ 0.02, cycloheximide 0.004, bromocresol purple 0.03, brilliant green 0.0295, agar 15	(*53*)
GY (glucose-yeast extract)	Glucose 50, yeast extract 10, agar 15	(*54*)
GYAE (glucose-yeast extract-acetic acid-ethanol)	Glucose 50, yeast extract 10, acetic acid 10*, ethanol 20*, agar 15	(*54*)
GYC (glucose-yeast extract-$CaCO_3$)	Glucose 100, yeast extract 10, calcium carbonate 20, agar 15	(*50*)
GYEC (glucose-yeast extract-ethanol-$CaCO_3$)	Glucose 10, yeast extract 10, calcium carbonate 20, ethanol 30*, agar 10	(*55*)
GYP (glucose-yeast extract-peptone)	Glucose 30, yeast extract 5, peptone 2, agar 15	(*56*)
HS (Hestrin-Schramm)	Glucose 20, yeast extract 5, peptone 5, Na_2HPO_4 2.7, citric acid 1.15	(*57*)
MYA (malt extract-yeast extract-agar)	Malt extract 15, yeast extract 5, ethanol 60*, agar 15	(*58*)
MYP (mannitol-yeast extract-peptone)	Mannitol 25, yeast extract 5, peptone 3, agar 12	(*48*)
RAE (reinforced AE)	Glucose 40, yeast extract 10, peptone 10, $Na_2HPO_4 \cdot 2H_2O$ 3.38, citric acid 1.5, acetic acid 10*, ethanol 20*, agar 10	(*59*)
SYP (sorbitol-yeast extract-peptone)	Sorbitol 50, yeast extract 5, peptone 3, agar 12	(*48*)
YG (yeast extract-glucose)	Glucose 20, yeast extract 5, $(NH_4)_2HPO_4$ 0.26, $MgSO_4 \cdot 7H_2O$ 0.05	(*60*)
YGM (yeast extract-glucose-mannitol)	Glucose 20, mannitol 20, yeast extract 10, acetic acid 5*, ethanol 20*	(*60*)
YPE (yeast extract-peptone-ethanol)	Yeast extract 10, peptone 5, ethanol 20*, agar 15	(*61*)

bromocresol green as a pH indicator. The oxidation of ethanol generates acid, and thus, the medium turns from green to yellow. The strains that can overoxidise ethanol show the same colour change. However, as acetic acid is oxidised to CO_2 and H_2O, the green appearance returns after an extended incubation period (*21*). In another solid medium, the presence of acids is typically revealed by the formation of a clear zone, due to the dissolution of the $CaCO_3$ that exists in the medium. Subsequently, further oxidation of the acetic acid gradually leads to precipitation of $CaCO_3$ and the initial white milky appearance of the medium (*48*). This principle is also used as biochemical evidence of the production of gluconic acid from D-glucose, where the gluconic acid that is formed dissolves the $CaCO_3$ present in the solid medium (*21*).

The production of cellulose by the genera *Komagataeibacter* and *Gluconacetobacter* can be detected by formation of a pellicle on the surface of a liquid medium after growth under static conditions or by the appearance of spheres or irregular masses in the agitated or shaken culture medium (*62*). Notably, the phenotypic/biochemical characteristics of the genera *Acetobacter*, *Gluconacetobacter*, *Gluconobacter* and *Komagataeibacter* can also be found in other genera, for instance, *Frateuria* and *Acidomonas* (*21,48*). Classification based on the phenotypic characteristics leads to other inaccuracies. For example, spontaneous mutation can lead to deficiencies in various physiological properties. Spontaneous mutants of *A. aceti* deficient in ethanol oxidation (*63*) and cellulose-negative mutants of *K. xylinus* with an extreme deficiency in cellulose-forming ability (*64,65*) are known examples. The mutations are related to genetic instability of these strains (*66*). For more accurate identification of AAB genera and species, the molecular methods are currently indicated as the most reliable techniques.

MOLECULAR IDENTIFICATION

AAB are very difficult to correctly identify at species levels based only on biochemical and physiological characteristics. For their proper identification, molecular analysis of the strains in comparison with reference species is recommended. In recent years, a variety of methods based on molecular techniques of DNA extraction and identification by polymerase chain reaction (PCR) have been used for identification of the genera, species and strains of AAB. The main methods reported by authors were: plasmid profiling, PCR amplification and sequencing of a specific region on the 16S rRNA gene, random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR), restriction fragment length polymorphism (RFLP) of the PCR-amplified 16S rRNA gene and 16S-23S rRNA intergenic spacer region, amplified fragment length polymorphism (AFLP), denaturing gradient gel electrophoresis (DGGE) of the PCR-amplified partial 16S rRNA gene, repetitive extragenic palindromic PCR (REP-PCR), enterobacterial repetitive intergenic consensus sequence-PCR (ERIC-PCR), DNA-DNA hybridisation and amplified ribosomal DNA restriction analysis (ARDRA) (*1,67,68*). These methods differ in analysis time, instrumentation and levels of discrimination capacities (*1*). For example, it has been reported that DGGE-PCR technique allowed the distinction of genera (*69*), while ERIC-PCR, PCR of the 16S rRNA gene and plasmid profiling analysis permitted species identification (*70–73*). Moreover, RFLP-PCR of the 16S ribosomal DNA (rDNA) and 16S-23S rDNA enabled a faster identification of the AAB species than lengthy methods, such as DNA-DNA hybridisation (*67*).

Another technique, used successfully in the profiling of proteins from intact bacteria, for the distinction of different genera, species and strains of AAB is matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). The resulting mass spectrum can be regarded as a bacterial protein fingerprint. It contains up to 30 peaks that correspond to soluble proteins of high abundance, which are unique for each bacterium. MALDI-TOF MS is described as a rapid and reliable method for identification of AAB involved in the industrial production of vinegar that allows microorganisms to be distinguished at the species or even subspecies level (*1,2*).

METHODS FOR AAB ENUMERATION

Several authors have reported the difficulty in determination of the population of AAB strains. The adversity is attributed to the VBNC state of several strains that causes significant differences between enumeration by both plating and microscopy. Consequently, plate counting may not be the best method of choice for enumerating viable AAB cells (*4,49*). This approach is further complicated by the arrangement of AAB strains that can occur in pairs, chains or aggregates, which probably represent a single colony when plating in a solid growth medium (*49*). Furthermore, some AAB species grow to form a continuous biofilm of exopolysaccharides (such as dextrans, levans and cellulose from D-glucose metabolism) on the surface of the solid growth medium, which impedes colony forming and subsequent counting (*21,49*). This problem was observed by Spinosa (*21*) when trying to enumerate the total population of AAB from industrial vinegar fermentors. The number of viable cells was obtained by direct counting under an optical microscope, using the Neubauer chamber and a vital dye (0.2 % trypan blue) for differentiation of cell viability.

Alternatively, the enumeration of non-cultivable AAB can be performed by real-time polymerase chain reaction (RT-PCR), using specific primers designed from the 16S rRNA gene. This technique is described as a fast, sensitive and accurate tool for quantifying bacteria and proved to be adequate for enumeration of AAB strains in commercial samples of wines and vinegars, even in samples artificially contaminated with other microorganisms, such as yeasts (*5,74*). Epifluorescence staining techniques have also been developed for the enumeration of total, viable and non-viable AAB cells involved in vinegar production and they were described as reliable, rapid and easy methods for this purpose (*47,75,76*).

PRODUCTS OF AAB METABOLISM

Vinegar

Vinegar is an aqueous solution of acetic acid and other constituents and is known and consumed worldwide as a food condiment and preservative (*55,77*). The use of vinegar dates back more than 10 000 years. Vinegar was known by ancient civilisations and used in folk medicine in wound treatment, as well as a hand-washing agent, to prevent infection. Nowadays, it is commonly used in the preparation of pickles, salad dressings and other food products. Vinegar has also become recognised for its functional properties, such as antibacterial activity, blood pressure reduction, antioxidant activity, reduction in the effects of diabetes and prevention of cardiovascular disease (*78,79*).

This product is the result of a two-stage fermentation. The first step is an anaerobic fermentation (alcoholic fermentation of sugars into ethanol by yeasts) and the second step is an aerobic fermentation (oxidation of ethanol into acetic acid by AAB). The raw materials, consisting of starch or complex carbohydrates, also need saccharification before alcoholic fermentation to release fermentable sugars (*6,80*). The high consumption of vinegar necessitated the elaboration of technological processes for obtaining the product. Currently, there are three key vinegar production methods, namely, slow surface culture fermentation (Orleans or traditional process), generator process (German process) and submerged process (*6,40,81*).

Kombucha

Kombucha is a traditional beverage obtained by fermenting sugary tea with a symbiotic culture of acidophilic yeasts and AAB embedded in a microbial cellulose layer known as tea fungus. The yeasts convert the sugar to organic acids, CO_2 and ethanol. The produced ethanol is later oxidised by AAB to acetic acid (*82,83*). AAB also use D-glucose to synthesise bacterial cellulose and gluconic acid. The main AAB strains found in kombucha are *A. aceti*, *A. pasteurianus G. oxydans* and *K. xylinus*. Many types of yeast have also been identified in kombucha samples, including species of the genera *Brettanomyces*, *Candida*, *Kloeckera*, *Mycoderma*, *Mycotorula*, *Saccharomyces*, *Schizosaccharomyces*, *Torulaspora*, *Pichia* and *Zygosaccharomyces* (*84*). Some beneficial properties, for example improving general health, increasing longevity and as a treatment of gastrointestinal disorders, have been claimed for kombucha. These properties are attributed to the acidic composition and the presence of phenolic antioxidants in this product (*84*).

Gluconic acid

Gluconic acid occurs naturally in fruits, plants and other foods, such as wine, vinegar and honey. It improves the sensory properties of food products, by imparting a bitter but refreshing taste and can also be used as an additive and preservative by the food industry. Gluconic acid can be obtained using chemical and biotechnological methods. However, the latter is the main method used on an industrial scale. A wide variety of bacteria is capable of oxidising D-glucose to gluconic acid. Various AAB genera and strains from other genera, such as *Pseudomonas* and *Zymomonas*, show this ability and can be used in the fermentative process for the biosynthesis (*85*).

G. oxydans, used for the industrial production of gluconic acid, contains two glucose dehydrogenases (GDHs), catalysing the direct oxidation of D-glucose to gluconic acid. In addition to a membrane-bound, PQQ-dependent GDH, a soluble nicotinamide adenine dinucleotide phosphate ($NADP^+$)-dependent, cytoplasmic GDH is also present. From experiments, it was evidenced that the production of gluconic acid primarily results from the direct oxidation of glucose in the periplasmic space and that the activity of membrane-bound GDH was 30-fold higher than that of cytosolic $NADP^+$-dependent GDH (*86,87*).

Due to its role in the aromatic profile of foods, gluconic acid has been proposed to be a quality parameter of food products. Consequently, it is preferable to use AAB strains that simultaneously produce gluconic and acetic acids during fermentation, when the sensory quality of the final product is expected (*88*).

Gluconic acid is also used in the pharmaceutical industry as gluconates of divalent metals, such as Ca^{2+}, Mg^{2+} and Fe^{2+}, which function as mineral supplements to treat hypocalcaemia, hypomagnesaemia and anaemia, respectively. Finally, products of oxidative metabolism of gluconic acid can be obtained through regioselective oxidation by the dehydrogenases of some *Gluconobacter* strains. For example, 5-ketogluconate, a raw material applicable for the production of tartaric acid, and 2-ketogluconate are both produced from gluconic acid by *G. oxydans* strains (*35,85*).

Sorbose and ascorbic acid

AAB, particularly strains of the genus *Gluconobacter* that possess an enormous oxidative capacity, can be used for oxidative conversion of D-sorbitol to L-sorbose, an important intermediate in the industrial production of L-ascorbic acid (vitamin C) (*8,10*). Two different membrane-bound enzymes are central in L-sorbose production from D-sorbitol by *Gluconobacter* strains. One is the PQQ-dependent glycerol dehydrogenase that oxidises many sugar alcohols, for example, glycerol to dihydroxyacetone, D-gluconate to 5-ketogluconate, D-mannitol to D-fructose, and D-arabitol to D-xylulose. The other sorbitol-oxidising enzyme is a flavin adenine dinucleotide-dependent sorbitol dehydrogenase that catalyses the regioselective oxidation of D-sorbitol (*8,35,89*). L-Ascorbic acid has an important role in human and animal nutrition and can be used as an antioxidant in the food industry (*89*). It is mainly synthesised *via* the seven-step Reichstein–Grüssner process, using D-glucose as a starting material. This process involves six chemical steps and one fermentation step, which is the oxidation of D-sorbitol to L-sorbose, catalysed by *G. oxydans* dehydrogenase (*90*).

Bacterial cellulose

Cellulose is a linear homopolymer of β-(1→4)-D-glucose units, alternately rotated 180° (*91,92*). It is synthesised by many different organisms, including plants, algae and some bacteria. Microbiological production of cellulose has attracted interest in recent years, due to the unusual properties and characteristics of bacterial cellulose. Unlike cellulose from plants, which is typically mixed with lignin, hemicelluloses and pectin, bacterial cellulose is extremely pure. Moreover, as mentioned in the introduction, bacterial cellulose displays many unique physicochemical and mechanical properties, such as high crystallinity, a high degree of polymerisation, high water-absorbing and -holding capacities, high tensile strength, high elasticity and excellent biocompatibility and biodegradability (*93,94*).

Due to the need for pure and crystalline cellulose, bacterial cellulose represents a promising alternative to plant-derived cellulose and presents specific applications in various industries. Among its numerous uses, bacterial cellulose is used as a gelling, stabilising and thickening agent in foods, or for skin repair in wound healing and burn treatments, as well as heart valve prostheses and artificial blood vessels in biomedical and pharmaceutical applications (*94–98*). Many species of bacteria excrete bacterial cellulose. However, *K. xylinus* is the most commonly used strain in the biosynthesis because of its capability to produce a relatively high level of bacterial cellulose from a wide range of carbon and nitrogen sources (*99*) and due to its industrial biosynthesis applicability (*81*).

It was suggested that in a liquid medium, bacterial cellulose helps aerobic bacteria to obtain a limited supply of oxygen by floating the cells near the surface. Additionally, bacterial cellulose protects the organism cells from damage by UV light and assists in moisture retention to prevent drying of the natural substrates on which the bacteria are growing (*95,100*). The pathway to produce cellulose from D-glucose by *K. xylinus* consists of four enzymatic steps. The enzymes involved in cellulose biosynthesis are glucose kinase, phosphoglucomutase, uridine diphosphate glucose pyrophosphorylase and the membrane-bound cellulose synthase (*95,101*).

Other exopolysaccharides

Although cellulose is the most common exopolysaccharide produced by AAB, they are also able to produce other important polysaccharides, such as levans. Levan is a branched homopolymer of D-fructofuranosyl residues containing β-(2→6) linkages in the core chain and β-(2→1) linkages at the branching points. It is produced extracellularly from sucrose-based substrates by a variety of bacteria, including *Gluconacetobacter*, *Gluconobacter*, *Komagataeibacter*, *Kozakia* and *Neoasaia* (*102,103*). Levan exhibits important biomedical and functional food properties due to characteristics like biodegradability, biocompatibility and the ability to form nanoparticles, as well as films (*103*). Several studies suggest beneficial effects of levan on the intestinal microbial community in the gut of farmed animals. Other uses of levan include films for packaging and medical applications for healing wounds and burned tissue (*104*). In the food industry, levan is used as an emulsifying agent, colouring and flavouring vehicle and as a fat substitute. Moreover, levan exhibits excellent antioxidant and anti-inflammatory potential (*102,103*). Other microbial exopolysaccharides produced by AAB, include dextran, acetan or xylinan, mannan and gluconacetan (*102*).

NEGATIVE ASPECTS OF AAB

Despite the importance of AAB in the food industry and biotechnological processes, negative aspects are also reported. For example, AAB can act as contaminating and spoiling agents during production, fermentation or maturation of alcoholic drinks, like wine, cider and beer, as well as in fruit-flavoured water and soft beverages (*47,102,105,106*), causing an undesirable sour odour and taste in these products. Another issue can occur in the vinegar industry, when a large volume of cellulose accumulates in the fermentors, mainly during the German process, requiring constant cleaning by the operator. In organic vinegar, which does not use preservative agents, opening the bottle can encourage the growth of cellulose-producing aerobic bacteria that are not completely removed in the filtration process, causing the formation of pellicles either on the surface or in the product. Even in conventional vinegar, which contains preservative agents, this phenomenon can occur (albeit less frequently) if the AAB are not well removed in the filtration, before bottling. The formation of cellulose pellicles in bottled vinegar may generate many complaints by consumers due to the unpleasant appearance of the product. Regarding human pathogens associated with AAB, to date, only two species that can cause opportunistic human infections have been reported, namely *Asaia bogorensis* and *Granulibacter bethesdensis* (*102*).

COMPARATIVE ANALYSIS OF AAB GENOMES

Recent advances in molecular techniques have allowed the complete genome sequencing of AAB strains. Consequently, several complete AAB genomes have been investigated in the last years, which has provided an important source of information about the phenotypic and genotypic characteristics of these strains (*102*). It was suggested that AAB species exhibit high genetic instability (*42,66*). The degree of acetic acid tolerance varies among AAB strains. Species traditionally used in the production of vinegar tolerate higher concentrations of acetic acid than other AAB (*102*). In *A. pasteurianus*, it was proposed that the ethanol and acetic acid tolerance could be partly attributed to the intrinsic properties of the amino acid sequences of the proteins PQQ-ADH and ALDH. Therefore, high concentrations of ethanol would not cause mutations in these proteins, and high conservation of the two enzymes could contribute to the stable industrial performance of this strain (*107*). Also, high levels of PQQ-ADH contribute not only to the enhanced production of acetic acid

but also to increased tolerance to the extreme acid environment (*102*).

G. oxydans 621H has an extraordinary potential to oxidise a variety of carbohydrates, alcohols and other organic compounds because it possesses a wide range of membrane-bound dehydrogenases that supply electrons for the respiratory chain. At least 75 genes in the genome of *G. oxydans* 621H were identified as potential oxidoreductases. Three have previously been characterised as membrane-bound quinoprotein dehydrogenases, but many dehydrogenases remain poorly described and of unknown substrate specificity. The substantial oxidative potential of this organism is, therefore, still not fully explored (*102*).

Genomes of the genus *Komagataeibacter* are yet to be sequenced completely (*102*). However, draft genome sequencing of strains isolated from kombucha demonstrated that the same environment could provide strains with increased/decreased cellulose production ability, namely *K. rhaeticus* and *K. intermedius*, respectively (*108,109*). The selection of strains with high cellulose productivity is promising for the industrial production of this biopolymer, given that the low yield mainly under agitated conditions is a limiting factor for the bioproduction. Comparative genomic analysis of *K. xylinus* NBRC 3288, a strain that does not produce cellulose, with those of the cellulose-producing strains clarified the biological significance of the *bcsB* gene in cellulose production by *Komagataeibacter* species. In this strain, a nonsense mutation caused splitting of *bcsB* into GLX_25070 and GLX_25080, affecting the cellulose synthesis capacity. This single mutation suggests that the *bcsB* gene is indispensable for cellulose production by *Komagataeibacter* species (*102,110*).

CONCLUSIONS

Over the last decades, new species and genera of acetic acid bacteria (AAB) have been suggested. Thereby, their classification and taxonomy have been the object of several modifications and updates, based on molecular, physiological and biochemical characteristics. These bacteria play an important role in the biotechnological and food industries because of their excellent ability to oxidise ethanol, sugar and sugar alcohols, and in the biosynthesis of pure and crystalline cellulose, a biopolymer with important industrial applicability. AAB are also used in the production of vinegar and kombucha beverages, which have antioxidant properties and beneficial effects on human health. However, many factors still affect the recovery, isolation and enumeration of AAB strains from fermented foods, necessitating the study and implementation of new technologies for this purpose.

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