Physiology of Acetic Acid Bacteria and Their Role in Vinegar and Fermented Beverages

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ABSTRACT

Acetic acid bacteria (AAB) have, for centuries, been important microorganisms in the production of fermented foods and beverages such as vinegar, kombucha, (water) kefir and lambic beer. Their unique form of metabolism, known as "oxidative" fermentation, mediates the transformation of a variety of substrates into products which are of importance in the food and beverage industry and beyond; the most well-known of which is the oxidation of ethanol into acetic acid. Here, a comprehensive review of the physiology of acetic acid bacteria is presented, with particular emphasis on their importance in the production of vinegar and fermented beverages. In addition, particular reference is addressed towards *Gluconobacter oxydans* due to its biotechnological applications, such as its role in Vitamin C production. The production of vinegar and fermented beverages in which AAB play an important role is discussed, followed by an examination of the literature relating to the health benefits associated with consumption of these products. Acetic acid bacteria hold great promise for future exploitation, both due to increased consumer demand for traditional fermented beverages such as kombucha, and for the development of new types of products. Further studies on the health benefits related to the consumption of these fermented products and guidelines on assessing the safety of AAB for use as microbial food cultures (starter cultures) are, however, necessary in order to take full advantage of this important group of microorganisms.

Introduction

Acetic acid bacteria (AAB), first described as "vinegar bacteria" by Louis Pasteur over 150 years ago, are an important and diverse group of bacteria involved in the production of fermented foods and beverages, especially known for their production of acetic acid (ethanoic acid) in the making of vinegar (Hutkins, 2006; Pasteur, 1864). Acetic acid bacteria are characterized by their ability to oxidize carbohydrates, alcohols and sugar alcohols (polyhydric alcohols or polyols) into their corresponding organic acids, aldehydes or ketones, in a process termed "oxidative fermentation", from which they gain energy (Taban & Saichana, 2017). This unique property also has applications in the production of industrially-relevant compounds and has primarily been exploited in the synthesis of ascorbic acid (Vitamin C) and miglitol (an antidiabetic drug used in the treatment of type II diabetes mellitus) (Shinjoh & Toyama, 2016; Taban & Saichana, 2017). Some genera of AAB are notable for their production of a variety of exopolysaccharides (EPS), the most valuable of which are bacterial cellulose (BC) and acetan. BC offers several advantages over plant-derived cellulose, particularly because it is free of hemicellulose and lignin associated with cellulose (Dağbağlı & Göksungur, 2017). Acetic acid bacteria are associated with, and have been isolated from, carbohydrate-rich and acidic environments such as fruits and flowers, and are involved in the production of a variety of fermented foods and beverages including vinegar, kombucha, lambic beers, kefir and nata de coco (Table 1). They also play an important role in the cocoa fermentation process (Pothakos et al., 2016). Unsurprisingly, AAB have also been associated with the spoilage of foods and alcoholic beverages such as beer, wine, cider and fruit juices (Taban & Saichana, 2017). The purpose of this review is to provide an overview of the physiology and biochemistry of AAB and to provide an understanding of how the unique capabilities of these microorganisms are important in the production of vinegar and other fermented beverages. Other technologically-relevant aspects, such as EPS biosynthesis and their role in the bioconversion of products useful

72 in the food, chemical and pharmaceutical industries, such as ascorbic acid, are also considered.
73 While recent reviews from others such as Ho et al. (2017) and De Roos & De Vuyst (2018a)
74 have addressed specific topics, the aim here was to provide a broad and comprehensive review
75 on the physiology and application of AAB (De Roos & De Vuyst, 2018a; Ho, Lazim, Fazry,
76 Zaki, & Lim, 2017).

Characteristics of the AAB and their identification

Acetic acid bacteria are Gram-negative or Gram-variable, obligate aerobes, and are classified in the family *Acetobacteraceae*. They are non-spore forming, ellipsoidal to rod-shaped cells that can occur singly, in pairs or in short chains (Malimas et al., 2017). The members of the *Acetobacteraceae* family are separated into two groups, the acetous and the acidophilic groups, of which AAB are included in the former (Komagata, Iino, & Yamada, 2014). Eighteen genera are currently reported in the acetous group; the most important in terms of fermented foods are *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, and *Komagataeibacter* (Table 2) (Giudici, De Vero, & Gullo, 2017). In addition, the aforementioned genera contain many species while the remaining genera consist of only one or two (Yamada, 2016). Some genera are motile, having either peritrichous or polar flagella. The majority of species are catalase-positive and oxidase-negative, however, there are exceptions. Growth in the presence of 0.35% acetic acid has been used as a criterion; however, while members of the genera *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, and *Komagataeibacter* grow in the presence of 0.35% acetic acid, not all genera can e.g. *Asaia*. Acetic acid bacteria are typically considered to be mesophilic, with the optimum temperature for growth being around 30^{o}C (Malimas et al., 2017). At higher temperatures, growth reduces significantly, with none usually occurring above 34^{o}C (Saichana, Matsushita, Adachi, Frebort, & Frebortova, 2015). The mesophilic character of AAB, which require strict temperature control, poses a challenge for industrial application. However, thermotolerant strains that can grow at a temperature of up to 42^{o}C have been identified (Saichana et al., 2015). It is noteworthy that the genera constituting the AAB are undergoing continuous revision and taxonomic changes and the reader is directed elsewhere for further information on this topic, such as Malimas et al. (2017). Acetic acid bacteria are notable for their direct oxidation of carbohydrates and sugar alcohols from which they accumulate large amounts of the corresponding oxidation products, gleaning metabolic energy

from the process. This so-called “oxidative fermentation” is a key metabolic characteristic in AAB and is carried out by membrane dehydrogenases and will be considered in greater detail in subsequent sections (Matsushita & Matsutani, 2016).

Acetic acid bacteria have primarily been isolated from sugary, acidic and alcoholic habitats, materials such as flowers or fruits, or from the fermented foods which they inhabit, such as vinegar, beer, cider and cocoa pulp-bean mass. As AAB occur as symbionts of insects, this can be another isolation source (Mamlouk & Gullo, 2013). Typically, species of *Gluconobacter* are associated with carbohydrate-rich environments, whereas species of *Acetobacter* can be found in alcohol-enriched niches (Raspor & Goranovic, 2008).

Nutrient media for the isolation and growth of AAB can vary, mainly in the types of carbon sources which are included, as strains from different environments or niches differ in their nutritional requirements (Table 3). For example, glucose yeast extract carbonate (GYC) medium is commonly used for the isolation and growth of strains originating from carbohydrate-rich environments. Calcium carbonate is added to buffer the acid production, with a zone of clearing being observed around colonies of AAB when grown on agar media to which it has been added. For the isolation and growth of strains originating from ethanol- and acetic acid-rich environments the use of media containing ethanol and acetic acid is recommended (e.g. acetic acid ethanol (AE) medium) (Gullo & Giudici, 2008). When isolating strains from environmental sources or matrices likely to contain other microorganisms, AAB can be selected for by reducing the pH of the growth medium to pH 4.4 and/or by adding antimicrobial agents such as cycloheximide for the inhibition of yeasts or penicillin to inhibit lactic acid bacteria (LAB) (De Vero, Gullo, & Giudici, 2017). Media which contain alternative carbon sources to glucose, such as mannitol or malt extract, are commonly used for isolating AAB e.g. yeast extract peptone mannitol (YPM) medium and malt yeast extract agar (MYA) medium (Mamlouk & Gullo, 2013). Deoxycholate mannitol sorbitol (DMS) agar, which

contains a number of carbon sources has been used for the selective isolation and enumeration of presumptive AAB, for example, from cocoa pulp-bean mass and lambic beer. A modified version (mDMS) containing lactic acid, acetic acid and ethanol is advisable for the isolation of a number of AAB species (Camu et al., 2008; De Roos, Verce, Aerts, Vandamme, & De Vuyst, 2018a; Papalexandratou et al., 2013; Wieme et al., 2014).

The capability to accurately identify microorganisms is indisputable, not only from the perspective of obtaining a basic understanding of the microorganism(s) being applied, (particularly where the properties and desired traits that different species of microorganism possess, or ideally should possess, can vary), but also from a food safety perspective. Many DNA-based methods have been used for the identification of AAB, both as single isolated strains and as members of complex food matrices, such as fermenting wine (Gonzalez, Hierro, Poblet, Mas, & Guillamon, 2005). More recently, as the number of described AAB species has risen taxonomically, in part as a consequence of the use of such molecular techniques, the requirement for higher resolution and better differentiation between species has increased, necessitating the development of, firstly, molecular targets with increasing resolving power, and secondly, non-DNA-based methods that are sensitive and rapid (Andrés-Barrao et al., 2013).

Early molecular methods focused on the 16S rRNA gene and included Restriction Fragment Length Polymorphism Analysis (RFLP) of the gene or PCR amplification and direct sequencing (Andrés-Barrao et al., 2017). RFLP was a fingerprinting method that allowed identification to the species level, but required the restriction pattern of a known species as a comparison for identification. Also, direct sequencing of the gene necessitates that the sequence of a known species has been deposited in a database and can be used similarly for comparison. In addition, due to the sequence of the 16S rRNA gene being highly conserved between some species, differentiation between such species is not always possible. Therefore,

RFLP of the spacer region between the 16S and 23S rRNA genes (internal transcribed spacer, ITS) has been used to provide such differentiation (Gonzalez & Mas, 2011). Intergenic regions are known to have higher variability than functional, protein-coding sequences, thus being able to resolve closely related species (Barry, Colleran, Glennon, Dunican, & Gannon, 1991). However, due to frequent variations and high divergences of intergenic sequences, even among strains of the same species, direct sequencing of the ITS was not useful for identification to species level (Ruiz, Poblet, Mas, & Guillamon, 2000). Nevertheless, the use of a polyphasic approach including the sequencing of both the 16S rRNA gene and the ITS region for use in phylogenetic analysis has enabled differentiation of closely related strains (Gonzalez & Mas, 2011). Alternative loci such as the *adhA* gene, *recA* gene and *tuf* gene can be used for polygenetic studies, showing similar results to phylogenies prepared with 16S rRNA and ITS sequences (Greenberg et al., 2006; Trcek, 2005).

Another method of phylogenetic analysis which has gained prominence is Multilocus Sequence Typing (MLST). This is based on the phylogenetic analysis of concatenated sequences from single-copy, ubiquitous, protein-coding genes, typically house-keeping genes, which evolve faster than rRNA. Construction of phylogenetic trees based on concatenated sequences of the housekeeping genes *dnaK*, *rpoB* and *groEL*, produced similar results to those obtained with the 16S rRNA gene and delineation of closely related species of the *K. liquefaciens* and *K. xylinus* groups. In addition, trees based solely on individual *dna*K, *gro*EL and *rpo*B sequences showed similar topology to that of the tree based on the concatenated sequence of the same genes (Cleenwerck, De Vos, & De Vuyst, 2010). An increasingly applied format of MLST is the use of whole-genome sequences (as opposed to only a few house-keeping genes) in the phylogenetic analysis. Here, all of the genes that are present in all isolated strains or species under investigation (i.e. the core genome) are concatenated and used to build the phylogenetic tree (Matsutani, Hirakawa, Yakushi, & Matsushita, 2011).

For genotyping and identification to the strain level, methods such as Random Amplification of Polymorphic DNA (RAPD) and Amplified Length Fragment Polymorphism (ALFP), and techniques based on amplification of repetitive sequences, such as Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR), Repetitive Extragenic Palindromic-PCR (REP-PCR) and $(GTG)_5$-PCR have been variously applied in studies, all of which are fingerprinting techniques. Both ERIC-PCR and $(GTG)_5$-PCR have been demonstrated to be most suitable for the differentiation of isolates to strain level, in some studies being used to monitor the population dynamics of AAB in traditional wine vinegar production at the strain level (De Vuyst et al., 2008; Papalexandratou & De Vuyst, 2011; Vegas et al., 2010).

Alternatives to DNA-dependant molecular methods are increasingly being explored as accurate, rapid and high throughput means of microbial identification. One such method is Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) which has originally been exploited in the field of clinical microbiology for the rapid identification of human pathogens, but has in recent years developed in the area of food microbiology (Croxatto, Prod'hom, & Greub, 2012; De Roos, et al., 2018a; Spitaels et al., 2014a; Spitaels et al., 2015). MALDI is a soft ionization method used with mass spectrometry for the analysis of large organic biomolecules. Briefly, the sample is bombarded with a high-energy laser beam leading to ionization of the sample in the form of cations. These ions are then accelerated in an electric field to a speed that depends on the mass-to-charge (*m/z*) ratio of each specific particle produced upon sample ionisation. The particles then enter a TOF mass analyser and travel along a field-free flight path towards the detector. The time required for each particle to reach the detector is precisely measured and is dependent on its *m/z* ratio. The *m/z* ratio of each particle is determined and a mass spectrum is generated, representing both *m/z* and signal intensity of the detected ions. The mass spectrum generated from a bacterium corresponds to high-abundance soluble proteins, predominantly ribosomal proteins and other

abundant cytosolic proteins, and is unique to a bacterium because protein composition differs between different bacterial genera and species (Andrés-Barrao et al., 2017; Bourassa & Butler-Wu, 2015). MALDI-TOF is comparable to 16S rRNA gene sequencing in its ability to differentiate to species level, as phylogenetic dendrograms produced by both methods were identical except for certain outlier strains which were positioned away from their expected taxonomic position on the tree (Andrés-Barrao et al., 2013). MALDI-TOF is not however suitable for differentiation at the strain level due to a strong effect of the growth medium used on the proteomic profile of the strains (Wieme et al., 2014). However, efforts to minimise the effect of the growth medium, with the potential to enable inter-strain discrimination, have been applied in some studies; for instance, sub-culturing of isolates under investigation multiple times on the same, defined agar medium prior to MALDI-TOF analysis (De Roos, et al., 2018a; Spitaels, Wieme, & Vandamme 2016).

Physiology and metabolism of AAB

- Aerobic respiration

Similar to many aerobic bacteria, AAB gain the majority of their energy by performing a type of aerobic respiration (Matsushita & Matsutani, 2016). In the general process of aerobic respiration, initially pyruvate is completely oxidised to carbon dioxide (CO_2) in the citric acid cycle. Subsequently, the reduced electron acceptors formed in the citric acid cycle are shuttled to the respiratory chain in the cytoplasmic membrane. Here, oxidation of the reduced electron carriers by components of the respiratory chain (oxidative phosphorylation) results in the formation of water, along with exclusion of protons from the cytoplasm, producing a proton gradient. Equalisation of this proton-motive force via transfer of protons back into the cell through a transmembrane ATPase (F_1F_0-type ATP synthase) leads to the biosynthesis of energy in the form ATP (Madigan, Martinko, Bender, Buckley, & Stahl, 2015).

The basic components of the AAB respiratory machinery consist of two periplasmic dehydrogenases: a membrane-bound proton pumping transhydrogenase, a non-proton translocating NADH: ubiquinone oxidoreductase, and two terminal oxidases of the ubiquinol oxidase-type. Ubiquinone (UQ) acts as the electron shuttle, in its reduced form, ubiquinol (UQH_2), between these respiratory proteins. The function of the transhydrogenase and NADH: ubiquinone oxidoreductase is the regeneration of $NADP^+$ and NAD^+, respectively, with the concomitant exclusion of protons in the case of the transhydrogenase complex. The terminal oxidases accept the electrons from ubiquinone, transferring them to molecular oxygen, the final electron acceptor, forming water (Figure 1). AAB have two terminal ubiquinol oxidases, designated cytochrome *bo*$_3$ ubiquinol oxidase and cytochrome *bd* quinol oxidase. Cytochrome *bo*$_3$ ubiquinol oxidase catalyses 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. In addition, it has been found that, in *G. oxydans*, the cytochrome *bd* quinol oxidase is particularly active at low pH (Hanke et al., 2012). Thus, it is suggested that cytochrome bo_3 oxidase may serve as a major terminal oxidase at the early growth phase, when the culture pH is closer to neutral, and when the pH is decreased as a result of the production of a large amount of oxidized products, cytochrome *bd* quinol oxidase maintains oxidative fermentation under acidic conditions by complementing the function of cytochrome bo_3 oxidase (Miura et al., 2013).

Compared to some microorganisms which obtain energy via respiration (e.g. *Escherichia coli*), the energy yield, and thus biomass yield, of AAB are relatively low (Luttik, Van Spanning, Schipper, Van Dijken, & Pronk, 1997). This can be attributed to the absence of certain key respiratory chain components in these microorganisms. For example, the genome of *Gluconobacter oxydans* 621H lacks genes encoding cytochrome c oxidase (complex IV; despite encoding genes for a cytochrome bc1 complex and for a soluble cytochrome c) and the proton-translocating NADH: ubiquinone oxidoreductase (complex I; *G. oxydans* has a non-proton-translocating NADH: ubiquinone oxidoreductase instead) (Prust et al., 2005). Thus, *G. oxydans* lacks two components which would normally perform proton translocation leading to the generation of a proton-motive force. However, not all AAB are as deficient in their respiratory machinery, for example, *A. pasteurianus* 386B, a strain isolated from a spontaneous cocoa bean fermentation, encodes a complete proton-translocating complex I (Illeghems, De Vuyst, & Weckx, 2013).

Therefore, due to the inadequate coupling of the electron transport with proton translocation, the proton-translocating potential and thus ability for energy transduction in *G. oxydans* is relatively limited. Thus, a low amount of energy is conserved by the microorganism, which

limits its growth rates, with most of the energy being lost as heat (Matsushita, Nagatani, Shinagawa, Adachi, & Ameyama, 1989). In this context, while appearing inefficient, and as will be discussed further below, the presence of membrane-bound (periplasmic) dehydrogenases in AAB enable rapid oxidation of substrates via "oxidative fermentation" at the cell membrane level, without the need for time-consuming intracellular transport; this generates the necessary proton-motive force and allows rapid energy conservation, in addition to generating an unfavourable environment for competing microorganisms through the production of acidic products of oxidation (Zahid, 2017).

- Oxidative fermentation

"Oxidative fermentation" is a process of incomplete oxidation of substrates which are oxidised by primary dehydrogenases of a respiratory chain, with the concomitant release of oxidised products into the surrounding medium. Bacteria capable of performing oxidative fermentation are termed "oxidative bacteria". Acetic acid bacteria are most prominent of such bacteria and commonly oxidise ethanol, carbohydrates and sugar alcohols to the various corresponding products such as organic acids, aldehydes and ketones (Table 4) (Matsushita & Matsutani, 2016). Oxidative fermentation can be considered as an "overflow metabolism", from which lower amounts of energy are conserved compared to if the substrates were completely oxidised to CO_2 and water by aerobic respiration (Deppenmeier & Ehrenreich, 2009). Examples of oxidative fermentation reactions include the conversion of ethanol to acetic acid (carried out by almost all genera of AAB and from where these bacteria get their name), and the conversion of glucose via glucono delta-lactone to gluconic acid/gluconate (GA), amongst others. Oxygen availability is of prime importance and profoundly affects the fermentation rate and productivity (Gullo, Verzelloni, & Canonico, 2014). The high accumulation of acidic products

in their environment give AAB an advantage over competitive microorganisms (Matsutani et al., 2014).

Oxidative fermentation reactions are performed by respiratory chains in AAB that are similar to the respiratory chains discussed above that oxidise reduced electron carriers (i.e. nicotinamide adenine dinucleotide phosphate [NAD(P)]-dependent dehydrogenases) and reduce ubiquinone (Figure 1). That is, in the process, electrons are channelled by ubiquinone to molecular oxygen at terminal oxidases while protons are abstracted to create a proton-motive force. This proton-motive force could be used by ATP synthase, or to perform some other energetic work. However, the dehydrogenase enzymes in the respiratory chains involved in oxidative fermentations are enzymes which specifically oxidise substrates such as ethanol, carbohydrates and sugar alcohols (as opposed to reduced electron carriers such as NADH). These enzymes contain a prosthetic group and are typically either PQQ (pyrroloquinoline quinone)-dependent dehydrogenases (quinoproteins and quinoprotein-cytochrome c complexes) or FAD-dependent dehydrogenases (flavoprotein-cytochrome c complexes), some of which work on the same substrate but produce different oxidation products (Adachi et al., 2003) (Table 4). MCD (methylsuccinyl-CoA dehydrogenase)-dependent dehydrogenases (molybdoprotein-cytochrome c complexes) have also been identified in some genera (Thurner, Vela, Thöny-Meyer, Meile, & Teuber, 1997). The cytochrome subunit of these dehydrogenases is responsible for the transfer of electrons to, and thus reduction of, ubiquinone to ubiquinol (Matsushita, Yakushi, Toyama, Shinagawa, & Adachi, 1996). In addition, these respiratory chains are located on the periplasmic face of the cytoplasmic membrane, while those involved in the oxidation of reduced electron carriers are located in the cytosol of the cell. The cytosolic NAD(P)-dependent dehydrogenases have no role in oxidative fermentation. Indeed, it appears that the cytosolic respiratory chain competes with the oxidative fermentation (periplasmic) respiratory chains regarding electron transfer and energetics and that both forms of respiration

occur in different growth phases (discussed further below) (Matsushita & Matsutani, 2016). Thirty-two membrane-bound dehydrogenases have been identified in the genome of *G. oxydans* 621H with 11 known and 21 unknown substrate specificities (Richhardt, Luchterhand, Bringer, Buchs, & Bott, 2013).

Ethanol oxidation

One of the most well-known and important oxidative fermentation reactions performed by AAB is the production of acetic acid (vinegar) from ethanol. Both *Acetobacter* and *Komagataeibacter* species have a strong ability to produce acetic acid and both genera also show high resistance to high ethanol and acetic acid levels, which are important traits for industrial vinegar production (Taban & Saichana, 2017). Ethanol oxidation is catalysed by two membrane-bound enzymes located on the outer surface of the cytoplasmic membrane (periplasmic side). Ethanol is first oxidized to acetaldehyde by a PQQ-dependent alcohol dehydrogenase (ADH) and acetaldehyde is further oxidized to acetic acid by aldehyde dehydrogenase (ALDH). The prosthetic group of ALDH has been shown to be different between genera, being either PQQ- or MCD-dependent (Gómez-Manzo et al., 2010; Thurner et al., 1997). ADH is stable over a broad pH range of 2.3 to 8.0 and retained more than 90% activity when incubated on ice for 30 minutes. ALDH, while also stable at acidic pHs (optimum pH 4 – 5, but can also function at lower pH values), is more heat stable than ADH, retaining more than 50% activity after 30 min at 60^{o}C (Kanchanarach et al., 2010a). However, ALDH is sensitive to the level of oxygen present; when this is either too low or too high its activity falls, allowing acetaldehyde to accumulate in the medium (Mamlouk & Gullo, 2013; Rubio-Fernandez, Desamparados Salvador, & Fregapane, 2004).

335 The ADH activity in *Acetobacter* species has been found to be more stable under acid
336 conditions than that of *Gluconobacter* species, which may partly explain why acetobacters are
337 more proficient in acetic acid production than gluconobacters and gluconacetobacters
338 (Matsushita, Toyama, & Adachi, 1994). In addition, when *G. oxydans* is grown in media
339 supplemented with a fixed amount of ethanol at the beginning, the levels of acetic acid
340 produced by the strain are lower when glucose is present as a carbon source and GA is therefore
341 produced; that is, acetic acid is still produced by *G. oxydans*, albeit at lower levels if glucose
342 is available for GA production (authors own observations).

343 Inactive forms of ADH have been identified from strains of *Gluconobacter* and
344 *Gluconacetobacter*. Ethanol-oxidation-deficient strains of *Gluconobacter* have a single
345 nucleotide polymorphism that results in a truncated signal peptide and therefore incorrect
346 localisation of the protein in the cytoplasmic membrane. When these gluconobacters are grown
347 in acidic or high aeration conditions, they produce a large amount of ADH protein, but ADH
348 activity remains unchanged, suggesting the presence of an inactive protein. Such inactive ADH
349 displays a tenth of the activity of the active form (Gomez-Manzo et al., 2012; Matsushita,
350 Yakushi, Takaki, Toyama, & Adachi, 1995). Certain cultivation conditions such as low pH
351 and/or high aeration also reduce ADH activity, such as in *Gluconobacter suboxydans*, where
352 low aeration was shown to favour active over inactive ADH formation (Matsushita et al., 1995).

353 ADH displays a wide specificity for short-chain alcohols, except methanol. Glycerol can be a
354 substrate, yielding glyceraldehyde, but only under high concentrations does ADH oxidise
355 glycerol at a significant rate. Aldehydes can also be oxidised by ADH, and in some AAB, at a
356 similar rate to alcohols. This has led to the suggestion that ADH alone (and not in concert with
357 ALDH) can perform the acetic acid fermentation, which has been shown for the strain *Ga.*
358 *diazotrophicus* Pal5 (Gomez-Manzo et al., 2015).

When ethanol has been completely oxidised and depleted, some genera of AAB, namely *Acetobacter*, *Gluconacetobacter* and *Komagataeibacter*, can assimilate acetic acid and oxidise it completely to CO_2 and water using the citric acid cycle and glyoxylate shunt, which is known as acetate "overoxidation" (Sievers & Swings, 2005). There also appears to be an irreversible metabolic change, after which they are unable to oxidize ethanol again; this is evidently unfavourable in vinegar production as it leads to lower acetic acid yields (Raspor & Goranovic, 2008). *Gluconobacter* and some other AAB genera do not overoxidise acetate as they lack key enzymes in the citric acid cycle and glyoxylate shunt (Deppenmeier & Ehrenreich, 2009; Mamlouk & Gullo, 2013). Because of this difference in oxidative potential, gluconobacters are sometimes referred to as "under-oxidisers" and acetobacters (and gluconacetobacters) as "over-oxidisers" (Bartowsky & Henschke, 2008). Overoxidation can be avoided if a small proportion of ethanol is maintained in the medium (Raspor & Goranovic, 2008).

Oxidation of carbohydrates and carbon metabolism in Gluconobacter oxydans

Catalysed by various periplasmic dehydrogenases involved in oxidative fermentation, AAB oxidise a number of carbohydrates to their corresponding carboxylic acids, obtaining metabolic energy in the process through the ultimate generation of a proton-motive force, as described above. As mentioned, while *Acetobacter* and *Komagataeibacter* are efficient at ethanol oxidation, *Gluconobacter* species are particularly proficient in carbohydrate and sugar alcohol oxidation (Matsushita & Matsutani, 2016). The production of GA and associated ketogluconates from glucose by *Gluconobacter oxydans* will be described below to illustrate carbohydrate oxidation in AAB, followed by a description of carbon metabolism in *G. oxydans*. Gluconic acid and its salts has wide application in various industries such as the food, construction, textile and pharmaceutical sectors (discussed further in the section

383 "Biotechnological applications of *Gluconobacter oxydans* relevant to the food industry") and
384 *G. oxydans* represents an alternative to the current use of the fungus *Aspergillus niger* as a
385 source of GA (García-García et al., 2017).

386 Acetic acid bacteria oxidise D-glucose firstly to D-glucono-δ-lactone by PQQ-dependent
387 glucose dehydrogenase (PQQ-GDH) and then to D-gluconate, either spontaneously or via a
388 gluconolactonase located in the cytoplasmic membrane (Raspor & Goranovic, 2008). D-
389 gluconate can be further converted to ketogluconates by other periplasmic dehydrogenases. It
390 can be oxidised further to either 2-keto-D-gluconate (2KGA) by an FAD-containing gluconate
391 dehydrogenase (FAD-GADH) or 5-keto-D-gluconate (5KGA) by PQQ-glycerol
392 dehydrogenase (PQQ-GLDH). In some AAB strains, 2KGA is later converted to 2,5-diketo-
393 gluconate (2,5-diKGA) by FAD-containing 2KGA dehydrogenase (FAD-2KGADH)
394 (Shinagawa, Ano, Yakushi, Adachi, & Matsushita, 2009; Toyama et al., 2007). Conversion of
395 GA to its associated ketogluconates is both strain- and growth condition-dependent. Factors
396 such as glucose concentration, pH and the level of oxygenation effect the yield and ratio of GA
397 to ketogluconates (García-García et al., 2017; Saichana et al., 2015). It is clear that the further
398 oxidation of GA to ketogluconates is undesirable if the production of D-gluconate is to be
399 maximised (Mamlouk & Gullo, 2013); this will be discussed further in the section
400 "Biotechnological applications of *Gluconobacter oxydans* relevant to the food industry".

401 When AAB are grown on alcohols, carbohydrates or sugar alcohols two growth phases
402 (biphasic growth, phase I and II) can be observed (Malimas et al., 2017). In both phases, the
403 majority of energy is obtained from respiratory chains and the generation of a proton-motive
404 force; however, in the second growth phase some of the energy is obtained through assimilation
405 and metabolic catabolism of the oxidised products produced in the first growth phase. In the
406 case of glucose, for example, the early, logarithmic growth phase results from the energy
407 derived from the oxidative fermentation of glucose, which produces gluconate and a small

amount of 2KGA, as described above. In the process, a proton-motive force is generated which drives ATP biosynthesis. When glucose is used up, the cells enter a stationary phase. However, a second growth phase is subsequently observed, which is primarily due to the further oxidation of gluconate to 2KGA. In both phases I and II a small proportion of gluconate is assimilated by the cells and is subsequently catabolised intracellularly in the reactions of primarily the Pentose Phosphate pathway (PPP), but the Entner Doudoroff pathway (EDP) and the citric acid cycle also have a role (Prust et al., 2005). These reactions yield additional energy primarily via the generation of reduced electron acceptors (e.g. NADH) which are subsequently oxidised by the cytosolic respiratory chains (NAD(P)-dependent dehydrogenases), thus generating a proton-motive force. Actually, only a small proportion of the glucose (phase I) and gluconate (phase II) are assimilated and catabolised intracellularly, the majority of the energy being yielded by oxidative fermentation reactions (Bringer & Bott, 2016). In this way, the cytosolic NAD(P)-dependent respiratory chains primarily have a role in the later phase of growth.

Catabolism of GA and associated ketogluconate products is possible only via the PPP and EDP because AAB do not have the enzyme, phosphofructokinase, and thus glycolysis is not active. In addition, while most AAB genera can completely oxidise substrates to CO_2 and H_2O in the citric acid cycle, in *Gluconobacter* this cycle is incomplete as they lack the enzymes succinyl-CoA synthetase and succinate dehydrogenase (Bringer & Bott, 2016).

While the metabolism of intracellular, assimilated glucose and gluconate, primarily via 6-phosphogluconate, may involve the PPP, EDP and citric acid cycle, the majority of energy gained from intracellular metabolism is derived from the oxidative PPP, whereas the EDP is dispensable. Carbon labelling of glucose has demonstrated the carbon flux during cellular metabolism of glucose in *G. oxydans*, showing that the majority of energy is derived from the PPP which operates in a cycle (Hanke et al., 2013). The key enzymes in the cycle are glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase; deletion of 6-

phosphogluconate dehydrogenase was observed to severely limit the growth of *G. oxydans*. In contrast, deletion of an integral enzyme in the EDP (2-keto-3-deoxy-6-phosphogluconate aldolase) had little effect on strain growth (Bringer & Bott, 2016; Hanke et al., 2013).

The initial generation of oxidised products of carbohydrates and sugar alcohols allows AAB to rapidly deplete the availability of carbon sources for competing microorganisms, while also producing an inhibitory environment for such competitors due to the resulting low pH generated by the accumulation of the acidic products; in addition, the potential for these products to be assimilated and used partly as an energy source by AAB provides a further competitive advantage (García-García et al., 2017).

- Tolerance to acidic environments

Acetic acid is an effective antimicrobial compound, yet most species of AAB are able to produce and tolerate high concentrations, from 6 - 10% (v/v) and some up to 15 – 20% (v/v) (Emde, 2006; Schüller, Hertel, & Hammes, 2000). Halstead et al. (2015) found that acetic acid at concentrations from 5% to as low as 0.3% were capable of preventing growth of both planktonic cells and biofilms formed by a range of pathogenic microorganisms that typically affect burns patients, including *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Gram-negative isolates were generally found to be more susceptible than Gram-positive strains (Halstead et al., 2015). This antibacterial efficacy raises the question as to how AAB can tolerate such high acetic acid levels. Different genera of AAB display varying tolerances to acetic acid; species of *Acetobacter* such as *A. aceti* and *A. pasteurianus* tolerate acidities of about 6 – 10%, while *Komagataeibacter* are generally more acid tolerant. Strains of *K. xylinus* and *K. hansenii*, for example, show resistance to 10 - 15% acetic acid, while strains of *K. europaeus* and *K. oboediens*, have been isolated from submerged

vinegar fermentation processes with acidities as high as 15 - 20% (Andrés-Barrao & Barja, 2017; Emde, 2006).

Acetic acid is a particularly effective antimicrobial, because at a relatively high pH (pH 4.7 = pKa of acetic acid) it exists primarily in its undissociated form and can enter the cell (Hirshfield, Terzulli, & O'Byrne, 2003). The properties of undissociated organic acids such as fat-solubility and neutral charge enable them to passively diffuse through the cell membrane of the target microorganism; in the cytoplasm the higher intracellular pH causes the acid to become dissociated, producing primarily hydrogen ions (H^+), but also acetate ions (CH_3COO^-). These ions are toxic to the cell and interfere with cellular processes such as enzyme activity, DNA replication and transcription, and protein expression, therefore effecting the normal growth of the microorganism (Chen, Chen, Giudici, & Chen, 2016; Russell & Diez-Gonzalez, 1997).

AAB employ a number of strategies to resist the detrimental effects of acetic acid, which will be discussed briefly here. Readers are directed to Andrés-Barrao & Barja (2017) for a more in-depth appraisal of the topic. The mechanisms which AAB use to tolerate high concentrations of acetic acid can be broadly classified as follows, 1) adaption of and protection of intracellular proteins to and against acid stress, 2) metabolism of acetic acid which enters the cell (overoxidation), 3) acetic acid efflux from the cell and 4) prevention of acetic acid from entering the cell (Figure 3).

Many intracellular proteins of AAB have adapted to tolerate a low cytoplasmic pH. For example, certain enzymes in particularly acid tolerant AAB (e.g. *A. pasteurianus*) have structural modifications, such as a higher number of inter-subunit hydrogen bonds and an increased number of arginine-containing salt bridges that not only confer stability in the acidic cytoplasm of AAB, but may also contribute to thermotolerance (Settembre, Chittuluru, Mill,

Kappock, & Ealick, 2004). The heat-shock systems, GroESL and DnaJK, are also important in acetic acid resistance. These chaperones and chaperonins are general stress proteins that protect other proteins from denaturation and aggregation caused by heat, but also other environmental stresses such as oxidative, acid, salt, and starvation stresses (Andrés-Barrao & Barja, 2017). Under both [sudden] acid-shock conditions, induced through the addition of 3% acetic acid to a growing culture, and more continuous stress conditions in the presence of high ethanol and increasing acetic acid levels (e.g. conditions which occur during vinegar fermentation), increased protein expression of the chaperone system consisting of GroESL, DnaKJ and GrpE occurs (Andrés-Barrao et al., 2012; Matsushita et al., 1994). In addition, disruption of RpoH, an important RNA polymerase sigma factor in the regulation of the heat-shock response proteins such as GroEL, DnaK, DnaJ, resulted in reduced expression of concomitant genes and decreased resistance to ethanol (5%) and acetic acid (1%) stress conditions in a strain of *A. pasteurianus* (Okamoto-Kainuma et al., 2011).

As described above, when ethanol has been completely oxidised and depleted, some genera of AAB assimilate acetic acid and oxidise it completely to CO_2 and water using the citric acid cycle and glyoxylate shunt (acetate "overoxidation") (Sievers & Swings, 2005). Thus, overoxidation is a mechanism by which the intracellular acetic acid level can be decreased, whilst also gleaning energy from the process through the oxidative reactions of the citric acid cycle. In addition, acetic acid may be assimilated from the environment causing the pH to rise. In both cases, the effect is essentially a detoxification of acetic acid and reduced acid stress on the bacterium. A fully functioning citric acid cycle is necessary for acetic acid overoxidation, which has been found to be the case for the most acid tolerant AAB such as *Acetobacter* and *Komagataeibacter*; other genera such as *Gluconobacter* do not possess a complete citric acid cycle which maybe an important factor in explaining their comparatively lower tolerance to acetic acid (Mamlouk & Gullo, 2013; Prust et al., 2005). In addition, this lower acid tolerance

may be linked to the reduced acetic acid productivity of *Gluconobacter* strains compared to those of *Acetobacter* and *Komagataeibacter*, which are commonly used in industrial vinegar production. In those AAB capable of overoxidation, a number of genes involved in the citric acid cycle and glyoxylate shunt are up-regulated during acetic acid fermentation, primarily at the end of ethanol oxidation. These include *aarA* (encoding for a citrate synthase), *aarC* (encoding for a succinyl CoA:acetate CoA-transferase) and *aconitase* (isomerises citrate) (Sakurai, Arai, Ishii, & Igarashi, 2012). It is noteworthy that during vinegar fermentation, it is desirable to maintain a low level of residual ethanol, to avoid the overoxidation of acetic acid which would lead to productivity losses. Therefore, in this case, acetic acid cannot be detoxified by this mechanism and other strategies must be employed to maintain acetic acid tolerance (Andrés-Barrao & Barja, 2017).

The export of intracellular acetate is another strategy employed by AAB to tolerate acetic acid. Two types of export systems have been identified, an efflux pump driven by a proton-motive force (anti-port of H^+ ions) and an ATP-binding cassette (ABC) transporter (Matsushita, Inoue, Adachi, & Toyama, 2005; Nakano, Fukaya, & Horinouchi, 2006). This ABC transporter has been found in *Acetobacter* and *Komagataeibacter* species and confers resistance to acetic acid, in addition to other short-chain organic acids such as formic acid, propionic acid and lactic acid (Nakano & Fukaya, 2008). Export of acetic acid may play a crucial role in cell survival under the high acidity conditions prevailing during the industrial production of vinegar, because, as mentioned above, under these conditions assimilation of acetate via the citric acid cycle is prevented in order to maintain acetic acid productivity.

Another strategy used by AAB to resist acid stress is the exclusion of acetic acid and its prevention from entering the cell. To this end, certain species, for example those of the genus *Komagataeibacter*, are reported to have a higher content of phosphatidylcholine (PC) in their lipid membranes (Nakano & Ebisuya, 2016). In addition, during acetic acid fermentation, as

the acid content began to rise, the ratio of PC was observed to increase and that of phosphatidylglycerol decreased, suggesting the importance of PC in acetic acid tolerance (Higashide et al., 1996). The cell membranes of *Komagataeibacter* have also been found to possess a high content of hopanoids, especially tetrahydoxybacteriohopane (THBH), which may have a role in acetic acid, but also ethanol, tolerance (Nakano & Ebisuya, 2016). Carbohydrate polymers attached to the outer membrane may also play a role in protecting against acetic acid ingress into the cell. The intracellular content of acetate was found to be significantly higher in a strain of *A. pasteurianus* displaying a non-polysaccharide-producing phenotype, compared to a polysaccharide-producing strain (Kanchanarach et al., 2010b).

Finally, it is noteworthy that there appears to be a link between acetic acid resistance and the yield or productivity during acetic acid fermentation. During investigations into the mechanisms of acetic acid resistance, strains which were modified to overexpress certain genes involved in acetic acid resistance, namely, aconitase, an enzyme in the citric acid cycle (Nakano, Fukaya, & Horinouchi, 2004) or AatA, the putative ABC transporter involved in acetic acid export (Nakano et al., 2006), not only displayed increased resistance to acetic acid but also produced higher acetic acid yields. Thus, the higher acetic acid productivity of *Komagataeibacter* and *Acetobacter* species may partly be a consequence of their natural intrinsic tolerance to acetic acid, when compared to species such as *Gluconobacter*, which in general, display lower acetic acid productivity.

Cellulose and other exopolysaccharides produced by AAB

Cellulose is the most abundant natural polymer on the planet, with high economic value. Plants (e.g. wood and cotton) and ocean phytoplankton and algae are nature's largest source of cellulose (Tonouchi, 2016). Certain bacteria, most notably AAB of the genus *Komagataeibacter* (formerly *Gluconacetobacter*), are capable of producing large amounts of cellulose (Lin et al., 2013). The chemical structure of plant and bacterial cellulose (BC) is identical, that is, composed of β-1,4-linked glucopyranosyl residues; however, the structure of BC is unique as it is composed of ultrafine fibres that form a network of pure cellulose (Tonouchi, 2016). BC can also be known as bacterial nanocellulose (Klemm et al., 2011). BC is a linear, unbranched, water-insoluble homoexopolysaccharide of exceptionally high purity and containing no contaminating substances, in contrast to plant cellulose which contains lignin and hemicellulose (arabinoxylan), which can be tightly bound and complexed with cellulose. Therefore, high amounts of energy are required for the purification of plant cellulose, which is not the case for BC. In addition, BC exhibits high crystallinity, water absorption, tensile strength and mechanical strength (Mohite & Patil, 2014a). These properties make it a favourable alternative to plant cellulose.

Several species of AAB within the genera *Acetobacter*, *Gluconacetobacter*, *Gluconobacter* and *Komagataeibacter* produce cellulose. However, only *Komagataeibacter* produce it at commercial levels, with *K. xylinu*s and *K. hansenii* being most notable (Lin et al., 2013). BC-producing strains have been isolated from a number of environments, including fruits, flowers, fermented foods (e.g. vinegar) and beverages.

The ecological benefit of cellulose production for AAB is not fully clear. It is commonly understood that its production forms a scaffold which floats on the liquid surface, as seen particularly in static cultures, allowing the bacteria access to both nutrients in the medium and

sufficiently high levels of oxygen in the air, required for their oxidative metabolism (Tonouchi, 2016). As described above, EPS production may contribute to acetic acid tolerance. In addition, in natural environments such as rotting fruit, cellulose production may protect the bacteria from predation or from the damaging effects of UV light or dissection through moisture loss. Cellulose production appears to be variable in *G. oxydans*, with some strains capable of producing it having been identified previously (Jia et al., 2004; Valera, Torija, Mas, & Mateo, 2015). In a screening of 77 different AAB strains, representing 35 different species, Valera et al. (2015) found that of the three *G. oxydans* strains examined, two produced cellulose on all the six different media used to investigate its production. Both of these strains were isolates from beer (Valera et al., 2015). However, yields of cellulose from *G. oxydans* are much lower than those of *Acetobacter xylinum* (presently *K. xylinus*) (Jia et al., 2004). This could be due to the fact that in AAB, both cellulose production and glucose oxidation (to GA and ketogluconates) are in competition for the same glucose as substrate. Thus, in *G. oxydans*, which has a particularly high oxidative capacity for carbohydrates, the majority of glucose may be oxidised to GA, resulting in low availability for cellulose production. In contrast, *K. xylinus* may utilise the majority of glucose for cellulose production (De Muynck et al., 2007). Indeed, Keshk and Sameshima (2006) demonstrated that productivity of BC from *Ga. xylinus* (now *K. xylinus*) increased significantly in the presence of lignosulfonate, an antioxidant compound that inhibited the formation of GA. In its presence, one strain of *Ga. xylinus* produced up to 16 g/L BC (Keshk & Sameshima, 2006). A similar effect was also observed in the presence of Vitamin C, which is also an antioxidant compound (Keshk, 2014).

BC is synthesised from a variety of carbohydrates and organic acids by AAB (Serrato et al., 2013). The cellulose biosynthesis pathway can be considered as an off-shoot of the PPP. Glucose is the polymeric unit that cellulose is composed of, therefore, other substrates must be converted to glucose-6-phosphate, a key intermediate, through various intracellular metabolic

599 reactions. Cellulose biosynthesis is comparable to that of heteropolysaccharides in other
600 bacteria, such as LAB, the building block of which are sugar nucleotides (Lynch, Zannini,
601 Coffey, & Arendt, 2018a). In the cellulose biosynthesis pathway, glucose-6-phosphate is
602 converted to glucose-1-phosphate, which is subsequently metabolised to uridine
603 diphosphoglucose (UDP-glucose), the direct precursor of cellulose. Uridine diphosphoglucose
604 biosynthesis is controlled by phosphoglucomutase and UDPG-pyrophosphorylase. Following
605 UDP-glucose formation, glucose polymerisation to produce cellulose is mediated by cellulose
606 synthase, a complex of proteins spanning the periplasmic space between the cytoplasmic and
607 outer membranes. The cellulose synthase complex facilitates the polymerisation of UDP-
608 glucose, translocation of the polymer across the membranes and assembly of the glucan chains
609 extracellularly (Tonouchi, 2016). The protein subunits that constitute the cellulose synthase
610 complex are encoded in an operon (the *bcs* operon), together with accessory proteins that assist
611 cellulose biosynthesis. Following polymerisation of the individual β-1,4-glucan chains, these
612 are brought together in a hierarchical manner to form the higher-order cellulose ribbon. In this
613 way, several glucan chains form a ~1.5 nm wide protofibril, protofibrils are arranged into ~2-
614 4 nm microfibrils; finally, microfibrils are bundled together forming a ~20-100 nm cellulose
615 ribbon (Jozala et al., 2016). In general, a single ribbon or fibre is produced by a bacterial cell,
616 from BC synthase complexes (sometimes termed terminal complexes) that are arranged on one
617 side of the cell only (Kimura, Chen, Saxena, Brown, & Itoh, 2001). This ribbon is ultrafine,
618 being about one thousandth the width of a plant cellulose fibre (Tonouchi, 2016). As mentioned
619 above, carbon sources other than glucose can be used for BC biosynthesis, however, besides
620 mannitol and arabitol, all other sources result in lower BC productivity than on glucose
621 (Khajavi, Esfahani, & Sattari, 2011).

622 Aside from the carbon source, the nitrogen source and level of other micronutrients influence
623 the growth of microorganisms as well as the productivity of BC (Dağbağlı & Göksungur,

2017). A number of agro-industrial waste by-products have been investigated for use in BC production, particularly those high in sucrose, fructose, nitrogen and vitamins (Castro et al., 2011).

Apart from the substrate material affecting yield, the method of production (e.g. static vs. stirred culture) can influence the structure and fibre network arrangement of the BC produced, thus having an effect on its physical and mechanical properties. Under static conditions, bacterial cellulose is produced in a gelatinous form. However, under agitation, shear stress seems to inhibit gel formation, resulting in the production of small aggregates, or in some cases, distinctive spheres of BC (Mohite & Patil, 2014b). In addition, in agitated cultures the fibrous network of BC is more disordered due to the physical effects of agitation (Watanabe, Tabuchi, Morinaga, & Yoshinaga, 1998). A negative effect of agitation is that it can induce mutations in certain strains, resulting in decreased BC production (Krystynowicz et al., 2002). Therefore, both the nature of the substrate material (carbon, nitrogen, micronutrient composition and concentration) and the method of production (static or stirred culture, type of bioreactor) are factors which contribute to the productivity and yield of BC.

Due to physicochemical properties such as structure-forming potential, high purity and bio-compatibility, BC has numerous potential applications, for example in the medical (e.g. as an artificial skin) and food industries (Tonouchi, 2016). Due to the scope of this review, only those applications relevant to the food industry will be described briefly below. Many current applications continue to employ plant-derived cellulose and it is used in food as a thickening, gelling, stabilising or water-binding agent (Shi, Zhang, Phillips, & Yang, 2014). For example, cellulose and its derivatives (known in the food industry as cellulosics) such as carboxymethylcellulose (CMC) and hydroxypropylmethylcellulose (HPMC) are important in the ice-cream and bakery industries, being used as a stabilising agent and a texture-modifying hydrocolloid, respectively (Murray, 2009). In particular, in gluten-free bread making, the use

649 of HPMC can increase moisture retention, increase loaf volume and decrease crumb hardness
650 and the rate of staling (Mir, Shah, Naik, & Zargar, 2016). BC has functionality in areas where
651 plant cellulose is limited. This is particularly attributed to the high purity, crystalline structure
652 and high water-binding capacity of BC. Thus, it is recommended where low use levels, foam
653 stabilisation, and stability over a wide range of pH, temperature and freeze-thaw conditions are
654 desirable. Such products in which it has potential use include pourable and spoonable dressings,
655 whipped toppings and aerated desserts, fermented and frozen dairy products, and sauces (Park
656 & Khan, 2009). As a raw material, BC is a distinct constituent of Nata and kombucha tea. Nata
657 is a BC gel, consumed as a desert in the Philippines, but becoming more popular worldwide. It
658 is produced by fermenting coconut water (Nata de Coco) or pineapple juice (Nata de Piña) as
659 a substrate with cellulose-producing AAB. The cellulose gel produced is cut into pieces and
660 consumed, having the flavour of the original substrate (Shi et al., 2014). Kombucha tea is
661 produced by fermenting sweetened tea with a symbiotic culture of bacteria and yeast (SCOBY)
662 or "tea fungus", of which cellulose-producing AAB constitute important members and produce
663 a characteristic cellulose pellicle on the surface of the medium (De Roos & De Vuyst, 2018a).
664 This pellicle harbours the community of bacteria and yeast which ferment the tea, producing a
665 mildly acidic beverage. Kombucha tea is discussed further below.

666

667 *Acetan*

668 Acetan is formed by cellulose-producing AAB and is composed of a cellulose backbone,
669 substituted on alternate glucose residues with a charged pentasaccharide side chain. This side
670 chain is composed of D-glucose, D-mannose, L-rhamnose and D-glucuronic acid (Dağbağlı &
671 Göksungur, 2017). Acetan biosynthesis is similar to that of heteropolysaccharides in other
672 bacteria, consisting of formation of a lipid-linked oligosaccharide intracellularly followed by

673 polymerisation in the periplasmic space and export and release extracellularly (Tonouchi,
674 2016). Production of acetan appears to affect cellulose biosynthesis as mutant acetan-non-
675 producing strains produce a cellulose that differs in its structure and crystallinity (Watanabe et
676 al., 1998). Acetan has reportedly been widely used in various industries as a viscosifier and
677 emulsifier; however, it is not listed in the current European regulation on food additives
678 (European Commission, 2008; Ishida, Sugano, & Shoda, 2002).

679

680 *Levan*

681 Levan is a homoexopolysaccharide composed of fructose units (i.e. a fructan) linked via β-2,6
682 glycosidic bonds. It is one of two types of fructans, the other being inulin (a β-2,1 linked
683 fructan) (Monsan et al., 2001). Among the AAB, strains from the genera *Gluconobacter*,
684 *Komagataeibacter*, *Kozakia* and *Neoasaia* are known to produce levan (Dağbağlı &
685 Göksungur, 2017; Jakob et al., 2013). Levan is biosynthesised by an extracellular enzyme,
686 known as levansucrase (a fructosyltransferase) secreted by the bacterium and using sucrose as
687 a substrate (Srikanth, Reddy, Siddartha, Ramaiah, & Uppuluri, 2015). Jakob et al. (2012)
688 screened 21 strains of AAB, representing 5 different genera for their ability to produce EPS on
689 sucrose-based media. Of the strains investigated, only those in the genera *Gluconobacter*,
690 *Neoasaia*, and *Kozakia* were shown to produce EPS of the levan type. A yield of 12 g/L was
691 obtained from a strain of *Gluconobacter frateurii* (Jakob, Steger, & Vogel, 2012). Levan, due
692 to its hydrocolloid properties, has potential applications in the food industry, for example, as
693 an emulsifier, stabiliser, texture modifier or fat substitute. EPSs, including levan, have
694 particularly been exploited in the bakery industry, and, through their application with
695 sourdough technology, represent a natural alternative to commercial hydrocolloids for the
696 enhancement of both conventional wheat-containing baked goods and gluten-free products

(Lynch, Coffey, & Arendt, 2018b). To date, EPS-producing strains of LAB have mainly been investigated for such application, but, in recent years AAB have become of increasing interest in this area (Jakob et al., 2012). Application by Jakob et al. (2012) of purified levan (1 - 2% w/w) from AAB to a wheat bread system increased the bread volume, softened the crumb and retarded staling (Jakob et al., 2012). Furthermore, in sucrose-supplemented spelt dough, a strain of *Kozakia baliensis* has been shown to produce up to 49 g/kg (of flour) EPS, followed by 33 g/kg in whole wheat, and 32–36 g/kg in wheat and rye doughs (Hermann, Petermeier, & Vogel, 2015). Ua-Arak et al. (2016) also demonstrated their potential for in-situ application and ability to grow and produce EPS in a gluten-free (buckwheat) sourdough system, with levan productivity of between 16 and 20 g/kg, in molasses-supplemented flour (Ua-Arak, Jakob, & Vogel, 2016). In a later study, the application of this sourdough improved the bread sensory and quality parameters, which included increasing specific volume and increasing crumb softness. However, the positive effects of sourdough application were masked by the impact of the natural acidification during fermentation. Thus, while the ability of AAB to produce a large quantity of high molecular mass levan is attractive and shows potential in bakery applications, a challenge remains due to the necessity for high levels of oxygen for growth during in-situ sourdough fermentation, coupled with the strong acidification (Ua-Arak, Jakob, & Vogel, 2017).

Dextran

Dextran is a homoexopolysaccharide composed of glucose units (i.e. a glucan) linked via α-1,6 glycosidic bonds (at least 50%) and variable amounts of α-1,4, α-1,3 and α-1,2 linkages, with or without branching. Dextrans are principally produced by species of LAB such as *Leuconostoc,* which use sucrose as a substrate (Monsan et al., 2001). The enzyme which

721 mediates the production of dextran in LAB is known as a dextransucrase (Lynch et al. 2018a).
722 Many strains of *G. oxydans* produce dextran using a similar enzyme, known as dextran
723 dextrinase. However, instead of sucrose, dextran dextrinase uses maltodextrins (α-1,4 glucan)
724 as the substrate for dextran formation. Dextran cannot be produced from un-hydrolysed starch
725 however. This has led to the suggestion that dextran dextrinase can act on the non-reducing
726 ends of linear α-1,4 chain structures, but not on structures close to branch points in starch
727 (Naessens, Cerdobbel, Soetaert, & Vandamme, 2005). *G. oxydans* appears to produce both an
728 intracellullar and extracellullular dextran dextrinase, the extracellular form of which is
729 particularly high in the presence of hydrolysed starch and maltodextrins. However, it was
730 previously unclear whether these two forms of the enzyme were in fact the same enzyme, and
731 whether the strain is stimulated to simply secrete the intracellular dextran dextrinase when
732 hydrolysed starch and maltodextrins are present in the environment (Naessens et al., 2005).
733 Recently, both forms of the enzyme were indeed shown to be identical (Sadahiro, Mori, Saburi,
734 Okuyama, & Kimura, 2015). Compared to commercial dextran of a similar molecular mass
735 produced by *Leuconostoc mesenteroides*, dextran produced by *G. oxydans* has a higher degree
736 of branching and displays lower viscosity. Therefore, the latter may be more suitable for food
737 applications not requiring a thickening functionality such as use as a dietary fibre or as a low-
738 calorie bulking agent (Naessens et al., 2005).

Vinegar fermentation

Legal definition of vinegar

The definition of vinegar itself varies from country to country and production is regulated by different laws (Table 5). “Vinegar” primarily is defined as a liquid product produced by the fermentation of carbohydrate sources and must contain a minimum of 3.75 to 5% (w/v) acetic acid. Based on the different laws on vinegar, the unifying parameters for its classification are the acidity, level of residual ethanol and their ratio to one another.

History of vinegar

The history of vinegar production, which dates back more than 10,000 years (Johnston & Gaas, 2006) represents a keystone example of microbial biotransformation. Vinegar, from the French word, *vinaigre*, meaning “sour wine” that in turn came from the Latin, *vinum acetum*, “wine vinegar” has always been considered the “poor relation” among fermented foods: it is not considered a “food”, nor does it have high nutritional value, and it is made through the biotransformation of richer, more nutritive products. Vinegar can be produced using almost anything that is a source of fermentable carbohydrate, including wine, molasses, beer, various fruits, honey or whey (Johnston & Gaas, 2006). Hippocrates of Kos (460-377 BC), father of modern medicine, recommended vinegar as a treatment for a number of diseases including the common cold and cough (Food: A Culinary History, 2000) and for the treatment of sores (Johnston & Gaas, 2006). The great military leader, Hannibal of Carthage (c. 200 BC) reportedly used vinegar to dissolve rocks that blocked his army's path over the Alps. Pliny the Elder (AD 23-79), recounts that the Egyptian Queen, Cleopatra (c. 50 BC) made a bet with the Roman general Mark Anthony that she could host the most expensive banquet costing ten million sesterces. He laughed at her, but during the meal, for the desert, she had a bowl of

763 strong vinegar brought in and dissolved a pearl of inestimable value in it, and then drank the
764 resulting potion (Mazza & Murooka, 2009). In the 8th century, Samurai warriors of Japan used
765 vinegar as a tonic, believing that it gave them power and strength (Ho et al., 2017). The study
766 of its medical properties and its use was widespread in the Middle Ages, becoming a common
767 remedy. During the Great Plague in the fourteenth century in France, vinegar was used to offer
768 protection against the bubonic plague. In England, to prevent the spread of plague during the
769 1660s money was deposited in troughs of vinegar in order to disinfect the coins (Solieri &
770 Giudici, 2009). A chronicle recounts that a concoction made from vinegar, garlic, mint and
771 other herbs, supposedly invented by thieves, allowed them to rob the houses of plague victims
772 without contracting the disease themselves. Fittingly, the mixture was called "Four Thieves
773 Vinegar" and is still manufactured today, mainly in France (Mazza & Murooka, 2009).
774 Through the centuries, from the beginnings of agriculture until today, vinegar has been
775 employed by every culture in some form; as a condiment, as a pickling or preserving agent, as
776 a disinfectant and cleansing agent and as a beverage.

777

778 *Fermentative processes in vinegar production*

779 Following the international definition of vinegar, in this review only vinegars derived from
780 alcoholic and subsequent acetous fermentation processes of agriculturally-produced raw
781 materials are considered. In Table 6 a list of vinegars is presented, but cannot be considered
782 exhaustive because of the countless varieties produced worldwide. The diversity of raw
783 materials used in the production of vinegar is very broad, ranging from by-products and
784 agricultural surpluses to high quality substrates for the most prized vinegars, such as Aceto
785 Balsamico Tradizionale (Italy) and Vinagre de Jerez (Spain). In Mediterranean countries, wine
786 vinegar is unquestionably the most common type of vinegar. In general, fruit vinegars are

787 common in Europe while cereal vinegars are more common in China and Japan (Lu et al.,
788 2017). However, worldwide, most of the vinegar produced is "white" vinegar, made from
789 diluted alcohol (Table 6).

790 Even though the raw materials and end products of vinegar fermentation are diverse, the actual
791 production processes are similar. In general, there are two fermentation steps involved in the
792 vinegar production, which are alcoholic and acetous fermentation, as depicted in Figure 3.
793 During production, ethanol formed by yeast, during fermentation under anaerobic conditions,
794 normally by strains of *Saccharomyces cerevisiae*, is converted to acetic acid (acetification) by
795 AAB, mainly members of the genus *Acetobacter* and *Gluconacetobacter* (now mainly
796 *Komagataeibacter*) under aerobic conditions (Budak, Aykin, Seydim, Greene, & Guzel-
797 Seydim, 2014). However, other microorganisms, such as fungi and LAB, may be involved in
798 certain vinegar types. Besides *S. cerevisiae*, other yeast species ubiquitously found on fruit and
799 vegetables may play a role in vinegars produced from these substrates; the lactose-fermenting
800 yeast, *Kluyveromyces marxianus*, is responsible for whey fermentation (Parrondo, Garcia, &
801 Diaz, 2009). A complex association of yeasts, AAB and LAB is involved in the fermentation
802 of kombucha and cocoa pulp-bean mass. It is probable that numerous AAB species involved
803 in vinegar fermentation have yet to be described because of the difficulties associated with their
804 cultivation on laboratory media (Cleenwerck & De Vos, 2008; Rainieri & Zambonelli, 2009).
805 Depending on the rate of acetic acid formation during vinegar production, the acetous
806 fermentation can be separated into two types of process: The Orléans method (slow) and the
807 submerged and generator methods (quick).
808 The Orléans method, also called "mother of vinegar system" or French method is one of the
809 oldest techniques for producing vinegar, and is an example of a surface fermentation method.
810 Historically, Orléans was a big port on the longest French river, the Loire. Wines arriving there
811 from all regions of France often suffered during transportation, turning sour en route. These

812 wines were sold to the vinegar brewers of Orléans, instead of being sent to Paris, their
813 destination (Bourgeois & Barja, 2009). In the Orléans process, acetification of ethanol into
814 acetic acid is started by "seed vinegar", or "mother of vinegar", an undefined starter culture
815 obtained from the previous vinegar fermentation. This process is called backslopping. This
816 procedure promotes the initial number of desirable microorganisms (yeasts and AAB) over the
817 indigenous population (De Vuyst, 2000). The acetification is performed by a static culture of
818 AAB that grow at the interface between the liquid and air where the oxygen concentration is
819 high (Sengun, 2015). In order to facilitate the oxidation of the ethanol and to leave an air
820 chamber, barrels are filled to approximately two-thirds their capacity and side holes enable
821 circulation. A funnel extends to the base allowing wine to be added at the bottom of the barrel,
822 and preventing disruption of the "mother of vinegar" biofilm of AAB that forms on the liquid
823 surface (Mas, Torija, García-Parrilla, & Troncoso, 2014). During the first week acetous
824 fermentation is activated, after which the liquid is transferred to another vessel. Acetous
825 fermentation is slow, occurring primarily on the surface of the liquid, where there is sufficient
826 oxygen to facilitate the conversion of ethanol to acetic acid. As the substrate ferments, the
827 changed environmental conditions (e.g. reduced pH) favour the most competitive indigenous
828 microbiota, and the more stringent the growth conditions, the greater the selective pressure.
829 Acetous fermentation continues for between 8 and 14 weeks depending on various factors,
830 including the initial composition of the substrate and alcoholic solution, the nature of the
831 microorganisms present, the sufficiency of oxygen supplied and the fermentation temperature
832 (Dabija & Hatnean, 2014). This slow fermentation is difficult to control, with a high risk of
833 spoilage, and is now suitable only for small-scale production. A microbial succession occurs,
834 as in many spontaneous fermentations, in which LAB and yeasts often dominate initially. These
835 consume carbohydrates and produce lactic acid and ethanol, respectively, which inhibit many
836 spoilage microorganisms, extending the shelf life of the resulting product. Acidity reaches a

maximum level after approximately three months. Vinegars produced by this slow, traditional method are considered of high quality due to their organoleptic complexity. The complexity and the resulting product quality are strongly influenced by (i) the substrate raw material and its preparation, (ii) the metabolism of the AAB, which produce additional products of oxidation and some ester compounds besides acetic acid, (iii) the interaction between the vinegar and the wood of the barrels, and (iv) the aging process, which incorporates all the aforementioned characteristics (Mas et al., 2014).

Other surface fermentation methods such as the trickling (or quick vinegar) generator processes (of which the Schutzenbach system or German rapid acetification method is an early example) and the submerged process were developed to reduce the acetification time while maintaining or increasing the quality. The rationale of the trickling generator processes is to increase the surface area for oxygen contact with the fermenting vinegar. Thereby, alcoholic substrates are circulated and trickled through vessels or vats containing an inert, non-compacting material, such as wood shavings or charcoal, on which a film of bacteria (AAB) is present. As the alcoholic substrate trickles downward through the material, contact with AAB and oxygen results in efficient oxidation to acetic acid. Once the substrate reaches the bottom it is re-circulated over the bed to promote increased transformation to acetic acid (Hutkins, 2006). Therefore, the acidity successively increases. The trickling generator system has undergone a number of developments over the years. The Schutzenbach system, originating in 1823, developed the use of the solid packing material as a support for the bacteria and on which the vinegar was spread. Ham, in 1924, further developed this system, including forced aeration through the bed (counter-current to the downward flow of substrate) and the pumping and re-distribution of the substrate over the bed by means of a sparger (Adams, 1998; Bourgeois & Barja, 2009). The Frings generator used wooden chips as the inert packing material. Today, while surface fermentations are still used for the production of low volume, high quality

vinegars such as the Orléans method for the production of Aceto Balsamico di Modena, most of the world's vinegar is produced at large volume in submerged fermentations, described below (Emde, 2014).

Submerged culture systems provide a much faster alternative and involve the suspension of AAB in the acetifying medium and application of stringent aeration to meet the high oxygen demand. The oxidative process occurs at the liquid-air interface of the bubbles, where the AAB convert ethanol into acetic acid, with limited production of other metabolites. The most successful, commonly applied submerged culture system is the Acetator®, built and sold by Heinrich Frings GmbH & Co. (Bonn, Germany). A significant design feature is its self-priming aerator, which provides for highly efficient dispersion of air in the liquid substrate (Adams, 1998).

This submerged method is more straightforward than the traditional method, consisting of application of the raw material and inoculum to the fermenter, the actual fermentation step, and final unloading of the bioprocessed product (Figure 4). A portion of the product is left in the vessel to act as starter culture for the next cycle (Tesfaye, Morales, García-Parrilla, & Troncoso, 2002). This method was introduced for the production of vinegar at the beginning of 20th century and is today employed for the production of most commercial vinegars of high consumption (Tesfaye et al., 2002). A disadvantage of this rapid method is that the high airflow leads to significant stripping of the volatile components from the original substrate, producing a more organoleptically limited product. Despite this, the rapidity of the process (vinegar can be produced in 24-hour cycles) and the high acidity achievable (acetic acid levels of up to 23–25%, compared to 6–13% with traditional systems) are key advantages. Higher acidity makes transportation more cost-effective by reducing water transport.

Role of AAB in other fermented beverages

Kombucha tea

Kombucha tea is traditionally prepared with water, tea, sugar and a kombucha culture ("tea fungus") in open vessels at room temperature for 1–3 weeks. This non-alcoholic, fermented beverage has a sharp acidity and specific flavour (De Roos & De Vuyst, 2018a). It is consumed traditionally in Eastern Europe and Asia. It is noteworthy that in Japan, the term "konbu-cha" can also refer to an unfermented beverage prepared with brown algae (*Laminaria* kelp). Consumption of kombucha was first recorded in 220 BC in Manchuria. More than one thousand years ago "tea fungus" was already consumed in Japan, China and India; then in Russia, Poland and the Baltic States starting in about 1915; in the Balkans, Germany and Eastern Europe around 1925; and in Spain, Italy, France and Switzerland in about 1955 (Kraft, 1959). Similar to other traditional beverages, the popularity of kombucha increased due to its purported beneficial health effects and its ease of preparation. To prepare a basic kombucha ferment, tea leaves are steeped in boiling water, 5 - 15% w/v of sucrose is added, and the mixture is brought to room temperature. A "mother" kombucha pellicle, produced from a previous kombucha fermentation, is placed into the tea along with liquid from a previous ferment (10 - 20% v/v). This pellicle is sometimes referred to as a symbiotic culture of bacteria and yeasts (SCOBY), and is comprised of primarily AAB and ethanol-forming yeasts in a thick cellulose pellicle (Figure 5). The pellicle floats on the liquid surface, at the air interface and grows vertically, increasing biomass with cellulose striations as the fermentation matures. After 1 - 3 weeks incubation at 20 - 30°C, the tea becomes a sweet and sour, naturally carbonated beverage due to microbial activity (Chen & Liu, 2000; Jayabalan, Malbaša, Lončar, Vitas, & Sathishkumar, 2014; Malbaša et al., 2006).

The primary metabolites in a kombucha fermentation are sugars and organic acids. Yeasts convert sucrose to glucose and fructose, and produce ethanol, preferentially from the fructose (Blanc, 1996, Dufresne & Farnworth, 2000). Acetic acid bacteria then convert this ethanol into acetic acid (which gives kombucha its sour flavour) and glucose to GA. The pH of the medium decreases to around 2.6, signifying the maturation of the beverage, ready for consumption (Malbaša et al., 2006). If fermentation is not halted or slowed, the concentrations of acetic acid and GA can increase to levels of greater than 4 g/100 mL, but the beverage is then unsuitable for consumption due to a strong vinegar flavour (Chen & Liu, 2000).

The microbial composition of kombucha varies depending on the source of the inoculum. The basic requirements for a kombucha ferment are at least one cellulose-producing AAB, and at least one yeast that can split sucrose into glucose and fructose. Multiple microorganisms can be present in a kombucha ferment, performing these essential roles along with producing additional secondary metabolites that contribute to the final beverage (Jarrell, Cal, & Bennett, 2000; Mayser, Fromme, Leitzmann, & Gründer, 1995; Roussin, 1996). The most frequently isolated genera of bacteria from kombucha are *Acetobacter, Komagataeibacter, Gluconacetobacter* and *Lactobacillus* (Chen & Liu, 2000; Trovatti, Serafim, Freire, Silvestre, & Neto, 2011). The predominant AAB found in kombucha ferments are *K. xylinus*, *A. pasteurianus*, *A. aceti*, and *G. oxydans* (Liu, Hsu, Lee, & Liao, 1996; Marsh, O'Sullivan, Hill, Ross, & Cotter, 2014). *K. xylinus* – previously known as *Ga. xylinus* (Yamada et al., 2012), and *A xylinum* (Yamada, Hoshino, & Ishikawa, 1997) – are the most abundant (80 - 99%) prokaryotes in kombucha (Chen & Liu, 2000; Marsh et al., 2014) and are responsible for the formation of the cellulosic pellicle (Chen & Liu, 2000; Zhu, Li, Zhou, Lin, & Zhang, 2014). Other AAB species have potential for both cellulose production and nitrogen fixation (Cleenwerck, De Wachter, González, De Vuyst, & De Vos, 2009; Dutta & Gachhui, 2007; Tan, Ren, Cao, Chen, & Tang, 2012).

Water kefir

Water kefir is a fermented, low-alcohol beverage with acidic and fruity flavours (De Roos & De Vuyst, 2018a). It is produced via spontaneous fermentation of a water solution containing approximately 8% (w/v) sucrose, (dried) fruits (e.g., figs), and water kefir grains (these "grains" are essentially a polysaccharide mass encapsulating a complex microbial association of bacteria and yeasts which serve as a starter culture) in a closed container at room temperature for 2–4 days (Figure 6) (Fiorda et al., 2017; Laureys & De Vuyst, 2014). Currently the main market for this beverage is in the USA, Japan, France, and Brazil, where it is consumed for its reported functional properties (Fiorda et al., 2017). The water kefir grain inoculum, and the nature of the substrate (such as the type of fruit used) determines the grain growth, the microbial species diversity, the metabolites formed and their concentrations (Laureys, Aerts, Vandamme, & De Vuyst, 2018; Laureys & De Vuyst, 2017; Laureys, Van Jean, Dumont, & De Vuyst, 2017). In general, the microbiota of water kefir is known to be a stable consortium of different LAB (such as *Lactobacillus, Leuconostoc*), AAB (*Acetobacter, Gluconacetobacter*, and *Gluconobacter*) and yeasts (*Kluyveromyces*, *Brettanomyces*, *Pichia*, and *Saccharomyces*). A strong symbiosis between these three microbial groups is documented (Fiorda et al., 2017). Yeast metabolism promotes the growth of acidophilic bacterial species such as LAB and AAB. Glucose and fructose are made available for LAB growth through the action of yeast invertase on sucrose. Ethanol produced by yeasts may be metabolised to acetic acid by any viable AAB present (Magalhaes, Pereira, Dias, & Schwan, 2010). Growth of AAB particularly takes place under aerobic conditions, leading to increased acetic acid content, which may be unwanted (Gulitz, Stadie, Ehrmann, Ludwig, & Vogel, 2013; Laureys et al., 2017; Marsh, O'Sullivan, Hill, Ross, & Cotter, 2013). Under anaerobic conditions they remain in a viable but non-culturable (VBNC) state, being metabolically dormant, but can start to grow when oxygen

becomes accessible (Laureys et al., 2017). While several species from the genera *Acetobacter*, *Gluconacetobacter*, and *Gluconobacter* have been recovered from water kefir fermentation processes, *Acetobacter* species seem to be best adapted to this ecosystem (Laureys & De Vuyst, 2014; Laureys et al., 2017; Magalhaes et al., 2010; Marsh et al., 2013).

Lambic beer

Lambic sour beers are among the oldest types of refreshing, alcoholic, acidic beers still brewed and which have become increasingly popular worldwide (De Roos & De Vuyst, 2018a, 2018b; Pothakos et al., 2016). In particular, sour beers are now attracting interest in the USA. In the American craft-brewing sector, American coolship ales, for instance, mimic the lambic beer production method (Bokulich, Bamforth, & Mills, 2012). Such beers were once a seasonal product from craft breweries, but today some produce solely sour beers, much like traditional Belgian lambic breweries.

Lambic beer is the result of a spontaneous fermentation process of a barley and unmalted wheat extract (wort) that continues for 1 - 3 years (De Keersmaecker, 1996). The fermentation process is not driven by yeasts or bacteria applied as starter cultures, but by a spontaneous inoculum from the environment. Microbial growth begins during the cooling of the boiled wort which occurs overnight in a shallow open vessel, known as a cooling tun or coolship. These beers are traditionally brewed close to the Senne river valley, near Brussels, Belgium (Spitaels et al., 2014a). The following morning the cooled wort is assumed to have been inoculated with the specific air microbiota of this region and is transferred into wooden casks which are stored at cellar or ambient temperatures, i.e., typically between 15 and 25°C. The wort then ferments and matures in these casks. In addition to inoculation from the environment during the coolship step, microorganisms present on the interior surfaces of the casks also contribute to the fermenting wort, helping to establish a stable microbial community (De Roos, Van der Veken, & De Vuyst, 2018). The end product is a non-carbonated sour beer that mainly serves as a

985 base for gueuze or fruit lambic beers. Several studies have shown a microbial succession of
986 *Enterobacteriaceae* and wild (oxidative) yeasts, including a yeast fermentation phase with
987 *Saccharomyces cerevisiae* and/or *Saccharomyces pastorianus,* an acidification phase with
988 *Pediococcus damnosus* and/or *Lactobacillus brevis,* and a maturation phase with *Dekkera*
989 *(Brettanomyces) bruxellensis* (De Roos & De Vuyst, 2018b). AAB are only occasionally
990 isolated during the lambic beer fermentation and maturation process, probably due to their
991 VBNC state (Spitaels et al., 2014a; Spitaels et al., 2015). However, two new AAB species have
992 been described that seem to be characteristic for lambic beers, namely *Acetobacter lambici*
993 (Spitaels et al., 2014b) and *Gluconobacter cerevisiae* (Spitaels et al., 2014c). It is possible that
994 the AAB, being obligate aerobes, are concentrated at the wort/air interface and, hence, are
995 missed during submerged sampling of the casks. Indeed, this has recently been proven to be
996 the case by De Roos et al. (2018a), who showed that the liquid nearest the interface was
997 characterized by higher AAB counts and higher concentrations of their metabolites (De Roos
998 et al., 2018a).

Biotechnological applications of *Gluconobacter oxydans* relevant to the food industry

Apart from their historical and key role in the production of fermented foods such as vinegar, AAB are also important for the production of useful compounds that find application in the food industry. The formation of GA by *Gluconobacter oxydans*, and their role in the process of Vitamin C synthesis are discussed here.

Gluconic acid production and regulation

The applications of GA in the food industry include use as an acidity regulator (E574–E580), with raising, sequestering and flavour-enhancing properties. Gluconic acid enhances the sensory characteristics of foods by imparting a bitter but refreshing taste. Gluconic acid and its derivative glucono-δ-lactone are also used as food preservatives. Its ketogluconate, 5-KGA, also has important uses, including as a precursor in the production of tartaric acid, xylaric acid, as well as for Vitamin C production (Cañete-Rodríguez et al., 2016). Two methods are commonly employed for the biotechnological production of GA, use of the fungus *Aspergillus niger*, or use of an AAB strain, primarily *G. oxydans*. The biochemistry of the production of GA and associated ketogluconates has been described above. This section will examine the factors influencing GA production in *G. oxydans*. This subject was reviewed recently by García-García et al. (2017) and will be discussed briefly below (García-García et al., 2017). The main factors which affect the production of GA include, in decreasing order of importance, pH, initial concentration of glucose in the medium, concentration of calcium carbonate added to the medium and the dissolved oxygen level.

Using *G. oxydans* 621H it was found that below pH 3.5 - 4 uptake and assimilation of GA into the PPP is almost completely inhibited (Olijve & Kok, 1979). This is likely to be related to the pH optima for the various enzymes involved in these processes – the periplasmic

dehydrogenases involved in oxidative fermentation have pH optima in the acidic range of pH 3 – 6, while those cytoplasmic (NAD(P)-dependent) dehydrogenases have optima in the alkaline range of pH 8 – 11 (García-García et al., 2017). Therefore, the production of GA and associated pH drop may promote its own production and accumulation as the periplasmic dehydrogenases become more active. As outlined above, GA can be further oxidised to ketogluconates such as 2KGA and 5KGA, thus reducing the GA yield. Olijve and Kok (1979) and Weenk et al. (1984) found glucose to be rapidly oxidized virtually quantitatively to GA without formation of any ketogluconates if the pH of the fermentation was uncontrolled (which lead to a rapid pH drop), or if the pH was adjusted to 2.5 at the beginning of the fermentation (Olijve & Kok, 1979; Weenk, Olijve, & Harder, 1984). However, control of the pH at 5.5, or the addition of calcium carbonate to the medium, lead to 2KGA and 5KGA formation once all initial glucose had been utilised (Weenk et al., 1984). The presence of calcium carbonate in the medium promotes ketogluconate production (Beschkov, Velizarov, & Peeva, 1995). It has been postulated that, in the absence of pH control, the formation of ketogluconates may be completely inhibited (Beschkov et al., 1995; Velizarov & Beschkov, 1994).

The initial glucose concentration also strongly influences the production of GA and ketogluconates. Olijve and Kok (1979) found that high glucose concentrations (0.9 – 2.7 g/L) led to rapid GA accumulation, while at lower concentrations the assimilation of glucose and metabolism in the PPP was favoured (Olijve & Kok, 1979). This is understandable from an ecological viewpoint when considering the level of glucose in the environment. If the concentration is low, AAB will preferentially assimilate the glucose for biomass production; in contrast, in the case of high glucose concentrations, the majority of it will be oxidised to gluconate and ketogluconates, thus making it unavailable for competing microorganisms, while also lowering the pH of the environment (García-García et al., 2017). Because GA is produced from the oxidation of glucose and the amount which is formed is directly proportional to the

initial glucose concentration, it is logical that above a certain concentration of glucose, the amount of GA formed will become inhibitory due to the resultant low pH, thus preventing further production. Thus, a glucose concentration above 90 g/L led to a reduced rate and yield of GA compared to a lower glucose concentration. Long lag phases were observed due to the combined effect of high glucose concentration and the low pH due to GA formation (Velizarov & Beschkov, 1994, 1998). High glucose concentrations (~90 g/L) also favoured GA production over ketogluconate formation (Beschkov et al., 1995; García-García et al., 2017).

Compared to the effects of pH and glucose concentration, dissolved oxygen (DO) has a less important role on the production of GA. However, Buse et al. (1992) found that DO control had a significant impact on the formation of 2,5-diKGA in *G. oxydans* ATCC 9937 (formerly *Gluconobacter oxydans* subsp. *melanogenum*). This was related to changes in the activity of the enzyme gluconate dehydrogenase (the first enzyme in the conversion of GA to ketogluconates) at different oxygenation levels. Low oxygen (<30%) delayed the production of this enzyme (Buse, Qazi, & Onken, 1992). Thus, low DO levels appear to inhibit GA conversion to ketogluconates and it is conceivable that relatively low DO levels may have a similar effect in *G. oxydans*.

Vitamin C production and potential for direct formation by G. oxydans

Since 1934 and until the late 1990s, the "Reichstein process" has been used as the main process for the production of Vitamin C, also known as L-ascorbic acid (Asc), of which more than 110,000 tones are produced annually (Bremus, Herrmann, Bringer-Meyer, & Sahm, 2006). This process, although refined and improved over the years, contains a number of chemical steps, and only a single microbially-catalysed step, and is highly energy intensive. Therefore, it has been replaced, particularly in China, by the so-called (Classical) Two-Step Fermentation

Process which is less costly and more environmentally friendly (Yang & Xu, 2016). Figure 7 shows the various routes for Asc production; while different processes have been studied, the Two-Step Fermentation process is today the primary method used for industrial Asc production (Wang et al., 2018), with China supplying 80% of the global demand. Royal DSM remains the sole Western Vitamin C producer (Pappenberger & Hohmann, 2014). Each of the routes to Asc, particularly with reference to the microorganism involved and especially *G. oxydans*, will be discussed further below.

D-glucose, D-sorbitol or D-sorbose can be considered as common starting materials for the process; however, glucose must be chemically converted to sorbitol which is typically via hydrogenation (Yang & Xu, 2016). Central in each process is the production of the precursor, 2-keto-L-gulonic acid (2-KLGA). For a detailed treatment of this topic, readers are also referred to the recent review by Wang et al. (2018) (Wang et al., 2018).

The Reichstein process: This process consists mainly of chemical reactions for Asc synthesis, with only one microbially-catalysed step. This is the oxidation of D-sorbitol to L-sorbose and which is performed by AAB due to their very efficient oxidation of carbohydrates and sugar alcohols. Originally this bioconversion was performed by *Acetobacter aceti* subsp. *xylinum* (now *Komagataeibacter xylinus*); subsequently *Gluconobacter suboxydans* (now *G. oxydans*) was introduced due to its greater capacity for oxidation and has been employed to date, with an almost 100% conversion rate on an industrial scale (Pappenberger & Hohmann, 2014; Yang & Xu, 2016). This is a key reaction, common to each route to Asc production, as can be seen in Figure 7. The key enzyme mediating the conversion of D-sorbitol to L-sorbose is glycerol dehydrogenase (GLDH), a PQQ-dependent membrane protein. This enzyme is also known as D-sorbitol dehydrogenase (SLDH), but it is a major polyol dehydrogenase in *G. oxydans* with broad substrate specificity for other sugar alcohols besides sorbitol, such as D-mannitol, D-arabitol, meso-erythritol, D-adonitol and glycerol (Figure 1) (Matsushita et al., 2003; Sugisawa

& Hoshino, 2002). A second membrane-bound sorbitol oxidising enzyme, which is specific for sorbitol, has been found in *G. oxydans* and other *Gluconobacter* species but is FAD-dependent i.e. FAD-SLDH (Shinjoh & Toyama, 2016). Genome analysis of the industrial strain *G. oxydans* H24 identified three sorbitol oxidising enzymes, two membrane bound and one cytoplasmic, namely PQQ-SLDH (PQQ-GLDH), FAD-SLDH and NADP-SLDH, respectively (Ge et al., 2013). Among these, PQQ-GLDH is believed to play the primary role in converting D-sorbitol to L-sorbose (Matsushita et al., 2003).

Two-Step Fermentation process: This process can be considered as an improvement on the Reichstein process. Two fermentations are performed in this process; the first, the bioconversion of D-sorbitol to L-sorbose is the same as that of the Reichstein process and is performed by *G. oxydans*. The second fermentation involves the conversion of sorbose to the Asc precursor, 2-KLGA. The subsequent transformation of 2-KLGA into Asc is performed through a number of chemical steps, as for the Reichstein process. Therefore, it is the second fermentation that differentiates the Two-Step Fermentation process from the Reichstein process (Yang & Xu, 2016). This fermentation is performed by a two-strain co-culture system, neither of which is actually an AAB. This dual culture consists of the 2-KLGA-producing strain and a "helper" or companion strain which promotes the growth of the 2-KLGA producer. Early studies identified the 2-KLGA producer as *G. oxydans*, but it was later renamed *Ketogulonicigenium vulgare*, a Gram-negative, facultatively anaerobic, chemoheterotrophic soil microorganism (Urbance, Bratina, Stoddard, & Schmidt, 2001). The companion strain, typically a species of *Bacillus*, is considered to stimulate *K. vulgare* growth and 2-KLGA accumulation by releasing particular metabolites (Feng, Zhang, & Zhang, 2000). *B. megaterium* and *B. cereus* were the primary companion strains applied in industrial Asc fermentation, and while many spore-forming strains have been found to be suitable as companion strains, only *K. vulgare* has been used in industrial fermentation so far (Feng et al.,

2000; Jiao, Zhang, Xie, Yuan, & Chen, 2002; Urbance et al., 2001). Two enzymes in *K. vulgare* are key in the oxidation of L-sorbose to 2-KLGA. These are L-sorbose/L-sorbosone dehydrogenase (SSDH) and L-sorbosone dehydrogenase (SNDH). SSDH is a unique PQQ-dependent membrane dehydrogenase, with dual catalytic ability, catalysing not only the conversion of L-sorbose to L-sorbosone but also that of L-sorbosone to 2-KLGA (Asakura & Hoshino, 1999). In addition, the SNDH of *K. vulgare* has been found to catalyse the direct conversion of L-sorbosone to Asc (Miyazaki, Sugisawa, & Hoshino, 2006). Thus, via the action of SSDH and SNDH it is possible for *K. vulgare* to directly produce Asc from L-sorbose and/or L-sorbosone (Sugisawa, Miyazaki, & Hoshino, 2005). Genome sequencing of the industrial strain, *K. vulgare* Y25, found that it contained four genes encoding SSDH and one plasmid-encoded gene for SNDH (Liu et al., 2011). The absence of genes or operons for the biosynthesis of many amino acids, nucleotides and cofactors may explain its dependence on a companion strain. As stated, the primary microorganism in the Two-Step Fermentation process is not an AAB *sensu stricto*; however, homologous enzymes to those employed by *K. vulgare* have been found in *G. oxydans* (Shinjoh & Toyama, 2016), and the potential of this bacterium for the direct production of 2-KLGA and Asc is described next.

Microbial production of 2-KLGA and Asc by *G. oxydans*: There has been interest in the further exploitation of *G. oxydans* in Asc production, beyond its use for sorbitol oxidation. This has been supported by the observation in recent decades of enzymes in certain strains of *G. oxydans* which perform similar bioconversions to those in *K. vulgare*, strengthening the possibility of, in the future, using a single microorganism for direct 2-KLGA or Asc production. Thus, strains of *G. oxydans* have been demonstrated to produce 2-KLGA from both D-sorbitol and L-sorbose, albeit at very low yields with wild-type strains. For example, *G. oxydans* NBRC3292 (formerly *G. oxydans* IFO3292 and *G. melanogenus* ATCC15163) was shown to produce 6.5g/L 2-KLGA from 50 g/L sorbitol over a 150 h fermentation time (Motizuki et al., 1962).

A similar strain, *G. oxydans* NBRC3293 (formerly *G. oxydans* IFO3293 and *G. melanogenus* IFO3293) produced 2.8 g/L 2-KLGA from 25 g/L L-sorbose over 168 h fermentation (Sugisawa et al., 1990). A progenitor strain of IFO3293, designated SPO1, was isolated and produced 13g/L of 2-KLGA from 50 g/L L-sorbose. Subsequent strain improvement studies with strain SPO1 using mutagens such as UV irradiation resulted in the isolation of genetically modified strains producing 50 to 60 g/L 2-KLGA from 100g/L D-sorbitol or 100 g/L L-sorbose over 80 to 100 h. Particular mutant isolates arising from the above strain improvement studies, which have been subsequently extensively studied regarding Asc production by *G. oxydans*, are strains UV10 and N44-1 (Pappenberger & Hohmann, 2014). L-sorbose dehydrogenase (SDH) was identified in the mutant strain UV10. The enzyme was membrane bound and FAD-dependent with a high activity for L-sorbose (L-sorbosone as product). In addition, L-sorbosone itself was also identified as a substrate for SDH, with 2-KLGA as the product. Thus, this SDH in *G. oxydans* UV10 has very similar activity to the SSDH of *K. vulgare* described above. Similarly, a SNDH was identified in *G. oxydans* UV10, this enzyme being found in the cytosolic fraction and being NAD(P)-dependent, with 2-KLGA as the product (Hoshino, Sugisawa, & Fujiwara, 1991; Pappenberger & Hohmann, 2014). A second strain of *G. oxydans*, T-100, was subsequently shown to contain the same dehydrogenases (Saito et al., 1997). Based on genome analysis, homologs of the associated genes are present in many *Gluconobacter* sp. (Gao, Zhou, Liu, Du, & Chen, 2012; Wang et al., 2018). Thus, the formation of 2-KLGA in *G. oxydans* is mediated by either of two enzymes: the membrane bound SDH i.e. from L-sorbose or L-sorbosone, or, the cytosolic SNDH, from L-sorbosone. The more recent observation of small amounts of Asc production by *G. oxydans* NBRC3293 when provided with L-sorbosone has led to the identification of a second type of L-sorbosone dehydrogenase in *G. oxydans*. In contrast to the FAD-dependent cytosolic SNDH of *G. oxydans* UV10, this enzyme was membrane bound and PQQ-dependent and was designated SNDHai (Berry, Lee, Mayer, &

Shinjoh, 2003). A homologous enzyme, designated SNDHak was identified in *K. vulgare* (Miyazaki et al., 2006). Although SNDHai uses L-sorbosone as a substrate, which it converts directly to Asc, it displays much higher activity for the cyclic polyol, myo-inositol; thus, it has been suggested that L-sorbosone oxidation by SNDHai may be a case of fortuitous cross-reactivity with the non-physiological L-sorbosone (Pappenberger & Hohmann, 2014). As shown in Figure 7(d), as little as three periplasmic oxidation steps are required to convert sorbitol to Vitamin C – mediated by GLDH, SDH and SNDHai – all of which are endogenous *G. oxydans* enzymes (Pappenberger & Hohmann, 2016). Despite low yields of Asc, the identification of SNDHai has, nevertheless, increased the possibility that *G. oxydans* may, at some future point, be used as the sole microorganism for the direct production of Vitamin C. With respect to the yield of Asc, there are three challenges which need to be overcome. Firstly, the low affinity of SNDHai for L-sorbosone, as described above. Secondly, the presence of the cytoplasmic SNDH in *G. oxydans* means that a certain proportion of the L-sorbosone will be converted to 2-KLGA and therefore not to Asc. Thirdly, and most critically, is the stability of Asc once it is produced. This is primarily due to the reductive properties of Asc and its propensity to become oxidised, forming L-dehydroascorbic acid. If L-dehydroascorbic acid is not rapidly reduced to again form Asc, it spontaneously and irreversibly degrades with a half-life of minutes. In this way, Asc is oxidised by molecular oxygen, and this reaction is accelerated at above neutral pH and in the presence of trace amounts of transition metal ions. The challenge is that such trace metals are required as a growth factor and, moreover, the strictly oxidative metabolism of AAB means that the presence of molecular oxygen is clearly unavoidable (Pappenberger & Hohmann, 2016). Attempts to overcome such barriers have included the use of resting cells in media in the absence of detrimental substances or with the use of engineered strains. Using the latter approach, a near 90% substrate conversion and yields of 10 g/L Asc were achieved using a genetically engineered strain of *G. oxydans* that was over-

expressing genes for SDH and SNDHai and also had a gene knockout for cytoplasmic SNDH (Pappenberger & Hohmann, 2016). Thus, it appears that at the current state of the science and knowledge, significant yields of Asc using (wild-type) *G. oxydans* alone are not yet possible without the use of engineered strains.

It is noteworthy that *G. oxydans* has also the potential to be used for the biosynthesis of 2-KLGA via a different mechanism to the D-sorbitol pathway, that is, via the biosynthesis of GA and its associated ketogluconates - the 2,5-diKGA pathway (Figure 7(e)). In this system the bioconversion of glucose as substrate into 2-KLGA is performed by cell preparations containing the necessary complement of enzymes such as glucose dehydrogenase, D-gluconate and 2-keto-D-gluconate dehydrogenase, including cytochrome C as a co-factor. A final enzyme, 2,5-diKGA reductase, performs the conversion of 2,5-diKGA into 2-KLGA (Hancock & Viola, 2002). As has been discussed above, specific *G. oxydans* strains have the potential to produce 2,5-diKGA from glucose oxidation via GA. Indeed, resting cells of *G. oxydans* have been used as a source of gluconate dehydrogenase and 2-keto-D-gluconate dehydrogenase for 2-KLGA formation via the 2,5-diKGA pathway (Hancock & Viola, 2002; Ji & Gao, 2001). However, most research into this pathway have used species of *Erwinia* and *Corynebacterium* as the enzyme sources (Wang et al., 2018).

Beneficial effects of AAB-fermented products

Health benefits associated with vinegar consumption

Vinegar is today primarily used as a condiment or seasoning alone, or as a seasoning and preservative agent in salad dressings, mayonnaise, ketchup and similar condiment sauces for its desirable organoleptic properties. However, in historical times it was used medicinally, as described above (Budak et al., 2014). A number of beneficial effects on health have been claimed to be associated with the consumption of vinegar, and while these are numerous, only few are based on clear evidence (Mas, Troncoso, García-Parrilla, & Torija, 2016). Health benefits associated with vinegar include antimicrobial activity, antioxidant activity, modulation of the glycaemic response, positive effects on cardiovascular health, such as cholesterol-lowering and antihypertensive action, positive effects in weight loss, improvement of appetite, reduction of fatigue and anticancer activity (Chen et al., 2016). Organic acids, primarily acetic acid, and polyphenols have been attributed as the main functional compounds in vinegar and are present in all varieties at varying levels (Chen et al., 2016). Other bioactive compounds, their presence and concentration which can vary depending on the type of vinegar and substrate material, may also contribute to the functionality e.g. tryptophol as an anticancer compound in Japanese black soybean vinegar (Inagaki et al., 2007). Certain factors influence the chemical composition of vinegar and thus its functional properties, including the raw material, the production process (acetification method) and the amount of time spent aging in wood (Guerreiro, de Oliveira, Ferreira, & Catharino, 2014).

The antibacterial effects of vinegar have mainly been investigated in the context of in-vitro application to food products, such as fresh fruits and vegetables, for the inhibition of pathogenic bacteria. The antibacterial mechanism of vinegar is primarily due to its acetic acid content. When the bactericidal effects of a number of organic acids, including lactic acid, acetic acid,

citric acid, and malic acid on *Escherichia coli* O157:H7 were investigated, acetic acid was found to be most effective, followed by lactic acid, citric acid, and malic acid. Other pathogens inhibited by acetic acid included *Salmonella enterica* subsp. *enterica*, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, *Aeromonas hydrophila*, and *Bacillus cereus* (Entani, Asai, Tsujihata, Tsukamoto, & Ohta, 1998). Chang and Fang (2007) observed a 3-log reduction in numbers of *E. coli* O157:H7 when rice vinegar containing 5% acetic acid was applied to lettuce for 5 min at 25°C. Use of lower acetic acid levels led to less of a reduction within the same time (Chang & Fang, 2007). The antimicrobial activity of organic acids is influenced by the target bacterial strain(s), temperature, pH, acid concentration, and ionic strength (Budak et al., 2014). The properties of undissociated organic acids such as fat-solubility and neutral charge enable them to diffuse through the cell membrane of the target microorganism and enter the cell; in the cytoplasm, the higher intracellular pH causes the acid to become dissociated, producing hydrogen ions. Hydrogen ion production thus reduces the intracellular pH and interferes with cellular processes, such as enzyme activity, DNA replication and transcription, and protein expression, therefore effecting the normal growth of the microorganism (Chen et al., 2016). Acetic acid is a particularly effective antimicrobial because at a relatively high pH (pH 4.7 = pKa of acetic acid) it exists primarily in its undissociated form and can enter the cell. Other organic acids, for example lactic acid, are not as effective; lactic acid cannot easily enter cells at a pH>3.8 (its pKa) as it exists primarily in its dissociated form.

A high oxidant and low antioxidant level in the human body is associated with the development of chronic, inflammatory diseases, such as cancer and cardiovascular disease (Srdic-Rajic & Konic Ristic, 2016). Intake of dietary antioxidants inhibit the formation of peroxides and their absorption in the gastrointestinal tract (Verzelloni, Tagliazucchi, & Conte, 2007). Vinegar exhibits antioxidant capacity which has been associated with the presence of polyphenols and

derived phenolic compounds, such as, gallic acid, caffeic acid, *p*-coumaric acid and ferulic acid amongst many others (Garcia-Parrilla, Torija, Mas, Cerezo, & Troncoso, 2017). In addition, carotenoids, phytosterols and vitamins, such as Vitamins C and E, and melanoidins also contribute to the antioxidant capacity (Ho et al., 2017; Tagliazucchi, Verzelloni, & Conte, 2010). As stated above, the processes used in vinegar production can influence its chemical composition, and it is noteworthy that, in the case of the phenolic composition, contact with wood can influence the phenolic content, due to polyphenol release via alcoholysis of wood lignin (Tesfaye et al., 2002).

It has been demonstrated in-vitro that the antioxidant capacity of Traditional Balsamic Vinegar (TBV) was equal to that of a 0.2% Vitamin C solution (Chen et al., 2016). Tagliazucchi et al. (2008) showed, in-vitro, that TBV had antioxidant activity equal to or higher than that of red wine, with 45% of the antioxidant activity due to the total polyphenolic fraction, primarily tannins, and 45% due to melanoidins and other lower molecular mass Maillard reaction products (Tagliazucchi, Verzelloni, & Conte, 2008). TBV melanoidins were responsible for preventing the pro-oxidant and cytotoxic effects of heme during simulated gastric digestion of meat (Verzelloni, Tagliazucchi, & Conte, 2010). Kurosu, a Japanese vinegar produced from unpolished rice, has been reported to have a high antioxidant activity. An ethyl acetate extract of kurosu inhibited myeloperoxidase activity, hydrogen peroxide generation and lipid peroxidation in mouse skin cells, and had the highest antioxidant activity compared to ethyl acetate extracts of grain vinegar, apple vinegar and wine vinegar (Nishidai et al., 2000).

Vinegar has been described to have an antiglycaemic effect and to improve blood glucose control and insulin resistance. Indeed, before the advent of pharmacological hypoglycaemic agents, vinegar "teas" were consumed by diabetics to help manage their condition (Johnston & Gaas, 2006). Many types of vinegars including apple cider vinegar, ginsam vinegar (an Asian vinegar produced from *Panax ginseng*), and tomato vinegar are capable of reducing

postprandial blood glucose and alleviating insulin resistance as well as promoting insulin production. This antiglycaemic effect of vinegar was first reported by Ebihara and Nakajima (1988). The authors found that co-administration of 2% acetic acid with a high glycaemic load meal consisting of 10% corn starch, significantly reduced the blood glucose response in rats (Ebihara & Nakajima, 1988). In human subjects, the consumption of sucrose accompanied by vinegar decreased the area under the insulin response curve by 20% (Johnston & Gaas, 2006). Administration of 20 mL white vinegar (equivalent to 5% acetic acid) as a salad dressing with a mixed meal consisting of white bread (50 g carbohydrate) and lettuce reduced the glycaemic response by over 30% in healthy individuals. Neutralisation of the acetic acid with sodium bicarbonate destroyed this antiglycaemic effect (Brighenti et al., 1995). The substitution of pickled cucumber for fresh cucumber in a meal consisting of bread, butter and yogurt reduced the glycaemic index (GI) by over 30% in healthy subjects (Östman, Liljeberg Elmståhl, & Björck, 2001). Similar results were observed by Johnston et al. (2010), who demonstrated that the postprandial glucose response was reduced by 23% in healthy individuals fed a meal of a bagel and juice containing 10 g apple cider vinegar (5% acidity), but not by the same meal containing neutralised vinegar. It was concluded that the neutralised salt of acetic acid (acetate) does not appear to possess antiglycaemic properties. In addition, ingestion of vinegar five hours before the meal reduced the antiglycaemic effect compared to consumption of vinegar with the meal (Johnston, Steplewska, Long, Harris, & Ryals, 2010).

Vinegar has also been shown to modulate the glucose response and insulin sensitivity in diabetic individuals. In patients with type 2 diabetes, dietary consumption of acetic acid significantly reduced the level of glycated haemoglobin (0.16%) during a 12-week experiment (Johnston, White, & Kent, 2009). Furthermore, in healthy individuals at risk of developing type II diabetes mellitus, ingestion of 0.75 g acetic acid as a vinegar drink twice daily at mealtime, for 12 weeks, reduced fasting blood glucose levels, and to a greater extent than diabetic

pharmaceutical medications (Johnston, Quagliano, & White, 2013). This is in agreement with an earlier study by Johnston et al. (2004) which showed that ingestion of a vinegar drink (20 g vinegar, 40 g water, 1 tablespoon saccharine) by individuals with insulin resistance (pre-diabetic) had a marked reduction in postprandial glycaemia (64%) and improved insulin sensitivity (34%) (Johnston, Kim, & Buller, 2004).

It has been reported that the reduction of postprandial glucose affected by vinegar in high GI meals is not observed for low GI meals (Liatis et al., 2010). However, vinegar ingestion with both high- and even low-GI meals does improve insulin sensitivity, independent of blood glucose level, as glucose uptake was enhanced after both meal types when vinegar was also ingested (Mitrou et al., 2015). Indeed, even the improvement by vinegar consumption of insulin sensitivity alone is noteworthy, particularly in insulin-resistant (pre-diabetic) subjects, as trials have demonstrated that slowing the progression to diabetes in high-risk individuals and improving their insulin sensitivity may increase the probability that such individuals may revert to a normal, glucose-tolerant state over time (Johnston & Gaas, 2006).

Postprandial blood glucose levels are primarily determined by 1) the rate that glucose enters the blood and 2) the rate at which it is consumed in-vivo. The rate of gastric emptying, digestion, and absorption in the small intestine determine the rate that glucose enters the blood (Chen et al., 2016). It is not yet fully understood how vinegar modulates glucose metabolism. Acetic acid in vinegar may supress carbohydrate absorption in the gut, more specifically through reducing disaccharidase activity and decreasing the digestion of disaccharides and oligosaccharides. Thus, monosaccharide absorption in the gut is reduced, lowering the blood glucose level (Johnston et al., 2013). Ogawa et al. (2000) demonstrated that acetic acid significantly inhibited the disaccharidase (e.g. sucrase, lactase) activity of Caco-2 cells, an effect not seen with other organic acids such as lactic acid or citric acid (Ogawa et al., 2000). In addition, acetic acid may regulate glucose metabolism by promoting uptake of glucose by

the liver and skeletal muscle and its conversion to glycogen stores (Hlebowicz, Darwiche, Bjorgell, & Almer, 2007). In this case, the mechanism by which glucose metabolism is regulated is believed to be through activation of the adenosine monophosphate-activated protein kinase (AMPK) pathway. Acetic acid is a building block for the biosynthesis of acetyl-coenzyme A (acetyl-CoA). During acetyl-CoA biosynthesis, adenosine triphosphate (ATP) is consumed and adenosine monophosphate (AMP) is produced, increasing the AMP/ATP ratio and leading to activation of the AMPK pathway. This causes a reduction in blood glucose levels and concomitant glycogen biosynthesis (Sakakibara, Yamauchi, Oshima, Tsukamoto, & Kadowaki, 2006). Another mechanism by which vinegar may reduce the postprandial blood glucose level is via effecting delayed gastric emptying. Hlebowicz et al. (2007) demonstrated that consumption of 30 mL of apple vinegar decreased the postprandial gastric emptying rate by 10% (Hlebowicz et al., 2007). The stabilisation of the postprandial blood glucose level through the mechanism described above may also increase postprandial satiety, thus reducing dietary intake and, as such, a further increase in blood glucose (Chen et al., 2016).

There is evidence that vinegar consumption can also affect lipid metabolism and, by association, promote weight loss. Studies, in particular in animal models, have highlighted that vinegar consumption can increase the concentration of high-density lipoprotein (HDL) cholesterol while reducing triglycerides, total cholesterol, and low-density lipoprotein (LDL) cholesterol (Fushimi et al., 2006). Similar effects on lipid levels were observed in human subjects displaying hyperlipidaemia and in obese individuals following consumption of apple cider vinegar (Beheshti et al., 2012; Kondo, Kishi, Fushimi, Ugajin, & Kaga, 2009a). Obese individuals, following long term apple vinegar consumption, also displayed significantly reduced body weight and body mass index (Kondo et al., 2009a). Regulation of lipid metabolism by vinegar intake is understood to be due to acetic acid, and, in a similar mechanism to glycaemic control, via activation of the AMPK pathway. Activation of this

pathway decreases the biosynthesis of lipids, specifically through inhibition of genes related to fatty acid biosynthesis, and also increases their breakdown and excretion. It is suggested that lipid oxidation, due to acetic acid for example, stimulates expression of certain oxidase enzymes, resulting in lipolysis (Samad, Azlan, & Ismail, 2016). The effect of acetic acid on postprandial satiety, as mentioned above, may also have a role in simply decreasing food, and thus fat, intake (Chen et al., 2016). Acetic acid, and thus vinegar, may have a role in reducing hypertension (blood pressure) through inhibiting angiotensin-converting enzyme (ACE) which reduces plasma levels of the strong vasoconstrictive, angiotensin II (Samad et al., 2016).

Few studies on the anticancer properties of vinegar or acetic acid are available. The effect of kurosu, a Japanese rice vinegar on the proliferation of a number of human cancer cell lines, including colon adenocarcinoma, lung carcinoma, breast adenocarcinoma, bladder carcinoma, and prostate carcinoma cells has been studied. It was reported to up-regulate the expression of enzymes involved in DNA repair and cell apoptosis in cells, and to inhibit the growth of all tested cell lines in a dose-dependent manner (Nanda et al., 2004). Kurosu was also shown by Baba et al. (2013) to inhibit the proliferation of human squamous cell carcinoma cells via programmed necrosis (Baba, Higashi, & Kanekura, 2013). The antioxidative nature of certain types of vinegar is also postulated to have a role in anticancer activity. Kibizu, a Japanese vinegar made from sugar cane, inhibited the growth of human leukaemia cells due to its high radical-scavenging capacity (Mimura et al., 2004).

While there is significant evidence linking vinegar consumption to the modulation of blood glucose levels, scientific studies linking vinegar intake to other health benefits such as control of lipid levels and anticancer effects remain equivocal (Johnston & Gaas, 2006). As shown in Table 7, the majority of studies have been performed in animal models; therefore, more human studies, including large-scale and long-term clinical trials are required before any definitive health claims can be made (Karabiyikli & Sengun, 2017).

Health benefits associated with kombucha

As reported by Dufresne and Farnworth (2000), a myriad of health benefits have been attributed to the consumption of kombucha, from protecting against diabetes to counteracting ageing and improving eyesight, mostly based on drinkers testimonials, and few of which have been investigated scientifically (Dufresne & Farnworth, 2000). Those health benefits that have been studied have primarily been performed in animal models, with very little data related to studies on humans (Jayabalan et al., 2014). Many of the claimed beneficial effects of kombucha have been correlated with its antioxidant activity. This has been mainly attributed to the tea substrate and the presence of tea polyphenols, Vitamin C and D-saccharic acid-1,4-lactone (DSL). Kombucha tea has a higher polyphenol content than un-fermented tea which is hypothesised to be due to structural modification of tea polyphenols by enzymes produced during fermentation by bacteria and yeast (Jayabalan et al., 2014). The polyphenol content of kombucha, and thus its antioxidant capacity can be dependent on a number of factors, such as, the fermentation time and conditions, variety of tea substrate, and the kombucha culture microbiota, which itself can vary depending of the substrate and fermentation conditions, and which, in turn determines the nature of the metabolites produced and present in the final beverage. In kombucha prepared with different types of tea, reducing power, hydroxyl radical scavenging ability, and anti-lipid peroxidation were decreased, while total phenolic compounds and scavenging activity against DPPH and the superoxide radical increased with a prolonged fermentation time (Jayabalan, Subathradevi, Marimuthu, Sathishkumar, & Swaminathan, 2008). Preparation of kombucha with different starter cultures of mixed AAB and a single yeast species on green tea had lower antioxidant capacity compared to a native (complex culture) kombucha (Malbaša, Lončar, Vitas, & Čanadanović-Brunet, 2011). In a recent study, the polyphenol content of kombucha was shown to be dependent on fermentation temperature, with a higher level at low (20^{o}C)

compared to high (30°C) temperatures. This was linked to the varying species dominance at the different temperatures (De Filippis, Troise, Vitaglione, & Ercolini, 2018).

Investigations in cell lines animal models found that kombucha can protect against hepatotoxicity caused by several toxicants such as paracetamol, carbon-tetrachloride, aflatoxin B1 and acetaminophen (Jayabalan, Baskaran, Marimuthu, Swaminathan, & Yun, 2010; Pauline et al., 2001; Wang et al., 2014). Bellassoued et al. (2015) demonstrated that the high thiobarbituric acid reactive substances (TBARS) concentration was significantly reduced in the liver and kidney of rats fed with cholesterol-rich diets after the treatment with fermented tea (Bellassoued et al., 2015). The anti-toxigenic effect of kombucha has been attributed to its antioxidant activity and the hepatoprotective effects against acetaminophen were primarily attributed to the presence of DSL (Wang et al., 2014). Early investigations of the potential health effects and detoxifying capacity of kombucha had primarily attributed the effects to its acidic composition; in particular, the ability of glucuronic acid to bind toxic components (glucuronidation) and to increase their excretion from the body was cited. However, the possibility that what was actually being measured in those early studies as glucuronic acid was in fact 2KGA, has been debated (Dufresne & Farnworth, 2000). Indeed, the concentration of glucuronic acid in kombucha may be as much as ten thousand times lower than that of GA (Lončar, Petrovič, Malbača, & Verac, 2000).

Given that acetic acid is a primary metabolite of the microbial consortia in kombucha, it could be hypothesised that those health benefits attributed to the presence of acetic acid, as observed in vinegar, may also contribute to the potential health effects of kombucha, notwithstanding the difference in acetic acid concentration between both fermented products. Srihari et al. (2013a) observed that the daily administration for 45 days, of a kombucha extract, reduced glycated haemoglobin and increased the plasma insulin level, thus demonstrating an antiglycaemic effect. However, the authors attributed the effect mainly to the polyphenolic

component of kombucha and non-specifically to the presence of organic acids and B-complex vitamins (Srihari, Karthikesan, Ashokkumar, & Satyanarayana, 2013a).

Kombucha has for many decades been claimed by drinkers to have anticancer effects, even in a population study conducted in 1951 in Russia by the "Central Oncological Research Unit" and the Russian Academy of Sciences in Moscow. The antiproliferative activity of kombucha produced from black tea has been demonstrated against a number of cancer cell lines, such as HeLa (cervix epithelial carcinoma) and HT-29 (colon adenocarcinoma) cells (Cetojevic-Simin, Bogdanovic, Cvetkovic, & Velicanski, 2008). In addition, an ethyl acetate extract of black tea kombucha caused cytotoxic effects on 786-O (human renal carcinoma) and U2OS (human osteosarcoma) cells and significantly reduced cell invasion and motility of these cells in addition to A549 (human lung carcinoma) cells (Jayabalan et al., 2011). Srihari et al. (2013b) demonstrated that a lyophilized extract of kombucha significantly reduced the survival of prostate cancer cells via down regulation of angiogenesis stimulators (Srihari, Arunkumar, Arunakaran, & Satyanarayana, 2013b). Again, the presence of polyphenols and their phenolic degradation products, as a consequence of fermentation, have been generally attributed as the anticancer bioactive compounds in kombucha.

Similar to those studies on the health effects of vinegar, many of those investigating similar benefits associated with the consumption of kombucha tea have relied on in vitro and animal model investigations. Therefore, clinical trials and further in vivo evaluations are necessary in order to confirm the claimed health benefits of kombucha tea. In particular, it is questionable, and data is necessary to support such claims, as to whether the efficacious concentrations for the positive effects observed through in-vitro studies would be achievable in-vivo in humans.

Regulatory aspects on the use of AAB as food cultures

The application of microbial food cultures in the production of fermented foods, used in both traditional backslopping practices and in recent decades as defined starter cultures, has an important role in ensuring the quality and safety of these products, in addition to imparting desirable flavour, aroma and textural properties (Bourdichon et al., 2012a). Today, with the large and every expanding variety of fermented foods and beverages that exists, and the possibility of applying new strains and species in foods, where they previously may not have been used, ensuring consumer safety is of prime importance. Following a brief description of the regulations pertinent to microbial food cultures in the European Union (EU) and the United States of America (USA), aspects related to the application of AAB will be considered.

Microbial Food Cultures and Regulation in the European Union

In the EU, the European Food Safety Authority (EFSA) operates the Qualified Presumption of Safety (QPS) approach (European Food Safety Authority, 2007). Hereby, a taxonomic unit (usually species) that is notified to the EFSA is pre-assessed for its safety based on aspects such as the associated body of knowledge, a history of apparent safe use in food, scientific literature, clinical aspects and industrial application. In this approach, familiarity is a critical aspect to support evidence of a microbial presumption of safety (Russo, Spano, & Capozzi, 2017). Familiarity, as a concept, is "taken to include practical experience of use of the organism(s) including its history of use for particular purposes and any body of literature on the biology of the taxonomic unit" (European Food Safety Authority, 2005), which could be translated to the body of knowledge supporting evidence for the historical use or consumption of such microorganisms in fermented foods, with no apparent adverse effects. For those microorganisms which have not traditionally been significantly consumed within the EU (prior

to May 1997), or, are not generally associated with foods, the concept of a "novel food" has been devised and is regulated separately (European Commission, 2015).

If a species is deemed safe by the EFSA Panel on Biological Hazards it is placed on the QPS list which is published by EFSA (Ricci et al., 2017a). However, individual strains must still satisfy certain criteria, or qualifications, before being applied in food (and beverages) or feed. As an example, a generic qualification for all bacterial taxonomic units on the QPS list is that strains should not harbour any acquired antimicrobial resistance genes to clinically relevant antimicrobials (Ricci et al., 2017a). In addition, as an alternative to exclusion from the list, certain bacterial species may have specific qualifications placed on them, which give them QPS status, but only when used for a defined application (Leuschner et al., 2010). Thus, for those species that are on the QPS list, they may be permitted for use in food or feed once the strain(s) being applied satisfy the attached qualifications. All microorganisms not on the QPS list remain subject to a full safety assessment (European Food Safety Authority, 2007). However, it is not entirely clear, at least from documents and literature from EFSA, what constitutes a full safety assessment. To this end, Pariza et al. (2015) proposed a decision tree that could be used for the safety evaluation of both non-QPS and QPS strains (Pariza, Gillies, Kraak-Ripple, Leyer, & Smith, 2015) (Figure 8). This will be discussed further below with relevance to AAB. In addition, microbial cultures assessed under the novel foods regulation (and not on the QPS list) must undergo a full safety assessment (Laulund, Wind, Derkx, & Zuliani, 2017).

The absence of a species from the QPS list does not necessarily imply a risk associated with its use. Individual strains may be safe, but the body of knowledge may not be sufficient to exclude any potential risk. In addition, the EFSA may not have been requested to date to evaluate the taxonomic unit in question (Bourdichon et al., 2012a). It is also possible that, for some microorganisms on the QPS list, EFSA may have been requested to evaluate a taxonomic unit for use in a defined application, and the scope of the evaluation may have been narrow, to

exclude only the potential risks associated with the application for which the microorganism was notified. Such microorganisms are included on the list, with a qualification of a specific use (e.g. QPS applies only when used for vitamin production).

There remains some ambiguity and uncertainty around the QPS approach and regulation, as outlined by Laulund et al. (2017) (Laulund et al., 2017). It appears that microbial food cultures with a history of safe use in food are considered as traditional food ingredients and are legally permitted for use in food in the EU without pre-market authorisation. On the other hand, those with no history of use in foods would be considered as novel food, and require full safety assessment, as mentioned above. This seems to suggest that it would be possible to place a product on the market that uses microbial cultures that have not undergone any safety assessment, if the manufacture considers that such cultures have a historical safe use in food. It also raises two questions. Firstly, what exactly defines microbial food cultures? Given the use of the wording "history of safe" and "traditional food ingredients", does this suggest complex, multi-strain cultures? What about single strain starter cultures? Secondly, how is a history of safe use defined and how can it be proven? To address these questions and the gaps in the regulation, the European Food and Feed Cultures Association (EFFCA) have proposed a definition of food cultures, which outlines what constitutes a microbial food culture. Their 2015 definition defines microbial food cultures as "safe live bacteria, yeasts or moulds used in food production, and they are in themselves a characteristic food ingredient. FC [food culture] preparations are formulations, consisting of concentrates (>10^8 CFU per g or mL) of one or more live and active microbial species and/or strains, including unavoidable media components carried over from the fermentation and components, which are necessary for their survival, storage and to facilitate their application in the food production process, and are in some cases standardised to a low count with carriers" (European Food and Feed Cultures Association, 2018). In addition, to address the question of cultures with a history of safe use in food, EFFCA

have, in association with the International Dairy Federation (IDF), compiled an "Inventory of Microorganisms with a documented history of use in food", the first inventory of FC with a documented significant use in food production before 1997 (Laulund et al., 2017). The first IDF/EFFCA Inventory, published in 2002, and primarily addressing cultures used in the dairy fermentation industry, was updated in 2012 with an expanded scope to include microbial cultures used in a wider range of food products (including meat, vegetable, cereals, beverages and vinegar) (Bourdichon et al., 2012b). One of the main criteria for inclusion in the inventory is a documented presence in fermented foods, and not just an incidental isolate (Bourdichon et al., 2012c).

Therefore, it appears that a food producer could conceivably place a product on the market that uses a single microbial culture or a number of cultures, which have an associated history of safe use in food, proven due to their inclusion in the IDF/EFFCA Inventory, and therefore without the need for pre-authorisation or safety assessment of the employed strains. However, it is likely that food producers would preferably opt to assess the safety of any strains employed as much as possible, primarily to ensure the safety of consumers, but secondly, to minimise liability, especially if the species is not on the QPS list.

Microbial Food Cultures and Regulation in the United States of America

In the USA, the Food and Drug Administration (FDA) operates the Generally Recognised as Safe (GRAS) system. This system is applied to a wide range of ingredients and is not specific to microbial FCs in the way that the EU QPS system is. Substances (including microbial cultures) added to food can be considered either as additives or GRAS substances. If they are considered as additives, then a pre-market authorisation by the FDA is required. However, if their use pre-dates 1958, or they have GRAS status, then no pre-market approval is required

(Russo et al., 2017). Achieving GRAS status is, similar to the QPS system, built on evidence of safety; in the case of GRAS, this must be guaranteed, with reasonable certainty, through examination of the body of knowledge (typically scientific publications) and the assessment and consensus of a panel of experts as to the safety of the substance or FC under its conditions for use (Bourdichon et al., 2012a). In contrast to QPS, the GRAS designation encompasses the substance / microbial culture and its specific application or usage (e.g. in a particular type of product), rather than applying solely to the microorganism itself; thus, while for QPS the evaluation is at the level of the taxonomic unit (species), and applies to that unit independent of application, GRAS designation can be at the species or strain level because the specific application (including formulation, dosages etc.) is considered (Ricci et al., 2017b).

From a legal standpoint, with a GRAS determination the onus and liability are placed on the food company, as a GRAS designation is issued based on evidence evaluated by the food manufacturer and the panel of experts engaged by that company. This is in contrast to the food additive designation, where the onus is placed to a greater degree on the FDA, where a full safety assessment by the authority is required. However, by law, neither a GRAS determination nor the notification to the FDA of the new use of a microbial culture is mandatory for a food company (Russo et al., 2017). Nevertheless, performance of a safety evaluation and the attainment of GRAS status would serve to reduce the liability on the food manufacturer.

Safety aspects of AAB, with emphasis on G. oxydans

Unlike LAB which are today primarily applied as single, defined starter cultures, the application of AAB is still via undefined, mixed or complex cultures, where the mode of transfer of the microorganisms to the next fermentation is through the traditional process of "backslopping". This is, for example, the case for both traditional and industrial methods of

acetous vinegar fermentation. This has been described to be, firstly, due to the nutritionally fastidious nature of AAB, which are difficult to preserve as a dried starter; and, secondly, vinegar is generally viewed as an inexpensive commodity and its production has therefore not warranted the development or use of expensive starter cultures (Solieri & Giudici, 2009). This is also the case for other fermented foods in which AAB play a dominant role; thus, as their use has been seen as traditional and viewed as safe through a long history of use, this group of microorganisms have not been considered to a large extent with regard to the regulations surrounding microbial FCs. However, with the increasing consumer interest in fermented food products it is foreseeable that the industry will require the future development of defined AAB starter cultures, which will necessitate the safety evaluation of such strains.

The only AAB (and one of only two Gram-negative bacteria) listed on the QPS list is *Gluconobacter oxydans* (EFSA Panel on Biological Hazards, 2018). *G. oxydans* was assessed by the EFSA Panel on Biological Hazards for the first time in 2013 and recommended for the QPS list. The species was subject to a qualification, however; QPS only applies when the species is used for vitamin production (EFSA Panel on Biological Hazards, 2013). It is not stated in the Scientific Opinion exactly why the qualification was put in place; the panel noted the general non-pathogenic nature of the genus and species to humans and animals (De Muynck et al., 2007), and a review of over 5,000 references raised no human or animal safety concerns. The possible pathogenic effect of [unidentified or unstated] *Gluconobacter* species was mentioned by two studies, however, these cases involved individuals with compromised immune systems (Alauzet et al., 2010; Bassetti et al., 2013). While the panel noted the reported rare occurrence of infections, as well as colonization with AAB in patients with underlying chronic diseases and/or indwelling devices, and the potential of some *Gluconobacter* species (although not *G. oxydans*) to be opportunistic pathogens (Alauzet et al., 2010), no article reported safety concerns related to consumption of foods and feed. The potential for

monobactam antibiotic production by strains of *G. oxydans*, as reported in one study in 1982 was noted (Wells et al., 1982), with a decision to follow this aspect in future QPS reviews. Nevertheless, the Panel placed *G. oxydans* on the QPS list with a qualification that it applies only when the species is used for vitamin production. While it was not stated why the panel placed this qualification on the taxonomic unit, it was noted that the qualification was "relevant for the intended use for which the species was notified". Whether the Panel placed this qualification on the taxonomic unit because of unanswered questions around the safety of the species, or, considered the safety of the species only in the context of the notified (narrow) application is not known (EFSA Panel on Biological Hazards, 2013). In the intervening time period to the present (August 2018), no new safety concerns were raised by EFSA and *G. oxydans* has been maintained on the QPS list (EFSA Panel on Biological Hazards, 2018). In the USA, to date, no products using *Gluconobacter* species have been submitted to the FDA for GRAS designation (Food and Drug Administration, 2018).

The 2012 IDF/EFFCA Inventory lists 3 genera of AAB, encompassing 18 species. Species from the genera *Acetobacter*, *Gluconacetobacter* (some of which would now be *Komagataeibacter*) and *Gluconobacter* are included for their roles in vinegar, vegetable, coffee and cocoa fermentations (Bourdichon et al., 2012b). Thus, the historic and safe use of these species, with relative certainty, is unquestionable. With regard to any future safety assessment of these species, the following paragraphs will outline some important considerations, using the decision tree outlined in Figure 8 to guide the discussion, and with emphasis on *G. oxydans*.

Strain characterisation and genome sequencing: It is generally accepted today that bacterial isolates are identifiable to species level using molecular approaches such as 16S rRNA gene sequencing (Weisburg, Barns, Pelletier, & Lane, 1991). In addition, whole genome sequencing of strains is now commonly performed and can prove indispensable for the safety assessment, providing additional insights into the genetic basis of strain safety. Different bioinformatics

tools and databases can be used to screen for the presence of virulence factors and for antibiotic resistance genes e.g. microbial virulence factors can be screened for using the MvirDb database (Zhou et al., 2006), while the Comprehensive Antibiotic Resistance Database (McArthur et al., 2013) and ResFinder (Zankari et al., 2012) can be used to search for antibiotic resistance determinants (Laulund et al., 2017). Currently, in the National Center for Biotechnology Information (NCBI) database there are approximately 170 sequenced genomes of AAB strains of the genera *Acetobacter*, *Gluconacetobacter*, *Gluconobacter* and *Komagataeibacter*, with 14 *G. oxydans* genomes, 5 of which are complete (NCBI, 2018).

Screening for undesirable attributes and metabolites: With regard to the AAB strain producing virulence factors or toxins, a whole genome sequence can be used to screen for genetic elements encoding such traits, as described above. Besides those studies detailed in the 2013 QPS update, which highlighted the rare occurrence of AAB as potential opportunistic pathogens, such cases related to individuals with underlying chronic diseases and/or indwelling devices (EFSA Panel on Biological Hazards, 2013). In the case of *G. oxydans*, no further studies in the intervening time period raised any new concerns (EFSA Panel on Biological Hazards, 2018). In addition, their long history of safe use and the presence of many AAB species on the 2012 IDF/EFFCA Inventory is testament, within the limits of reasonable certainty, to their safety when consumed by healthy individuals. Unlike the LAB, some species of which are biogenic amine producers, AAB have not been found to produce these toxigenic compounds (Landete, Ferrer, & Pardo, 2007). However, in-vitro tests can be performed on strains to identify biogenic amine producers; in addition, the genome sequence of strains can be screened for putative responsible amino acid decarboxylase genes (or their homologues). The question of antibiotic resistance is fundamental in assessing the safety of a strain. Chiefly, the strain must be free of functional and transferable antibiotic resistance genes. Proven intrinsic (natural) resistance is generally acceptable; the resistance determinant must not be transferable – this is to prevent the

horizontal or lateral transfer of (acquired) resistance to antimicrobials of human and veterinary importance from FC microorganisms to (potentially pathogenic) commensal microorganisms in the gut (Pariza et al., 2015). Unlike LAB, for which guidelines and procedures have been developed by the regulatory authorities to inform the testing and interpretation of antibiotic susceptibilities of this microbial group (EFSA FEEDAP, 2012; International Organization for Standardization, 2010), no such distinct guidelines are in place for AAB. In this case, it is recommended that methods described by the Clinical and Laboratory Standard Institute be used. Again, the genome sequence can also be useful to search for genes conferring antibiotic resistance, with particular focus on genes associated with mobile genetic elements (plasmids, conjugative transposons) that would be potentially transferrable (Laulund et al., 2017). In the 2013 QPS update when *G. oxydans* was admitted to the QPS list, the Panel reported that no evidence of resistance to antibiotics was found in any of the papers screened. According to only one publication, strains of some *Gluconobacter* species, although not *G. oxydans*, may be multi-resistant to some antimicrobial agents. In addition, such strains were isolated from hospitalised patients and the nature of the resistance (intrinsic or acquired) was not investigated (Alauzet et al., 2010). When performing antibiotic susceptibility testing of strains, it is important to differentiate between intrinsic and acquired antibiotic resistance and have knowledge of the potential for intrinsic resistance in the strains being assessed; for example, bacteria differ in their susceptibility to penicillin G; Gram-positive bacteria are generally sensitive, while most Gram-negative bacteria are naturally resistant because this compound cannot penetrate the outer cell membrane (Madigan et al., 2015). Gram-negative bacteria are also resistant to glycopeptide antibiotics (such as vancomycin) for the same reason. Although arguable, for food products where the microbial culture has been killed or inactivated (through an intense pasteurisation, for example), it appears, at least in the past, that the presence of acquired microbial resistance was not considered to be an issue and was acceptable (Russo et

al., 2017). However, if these dead cells are damaged or lysed in some way, either because of food processing steps or passage through the gastrointestinal tract, it should be considered whether mobile genetic elements carrying antibiotic resistance genes could still be released into the gut environment and taken up, for example, via transformation by other microorganisms present (European Food Safety Authority, 2008).

The ability of microbial food cultures to produce antimicrobial agents is also appreciable because such agents could select for resistance in the host bacterial population (Bourdichon et al., 2012a). As mentioned above, the potential for monobactam antibiotic production by strains of *G. oxydans* was reported in a 1982 study (EFSA Panel on Biological Hazards, 2013; Wells et al., 1982). The Panel stated the decision to follow this aspect in future QPS reviews; however, up to and including the latest QPS update, no further information has emerged.

In general, there is only a very small body of knowledge and few studies related to such safety aspects of AAB - antimicrobial susceptibility and potential pathogenicity. More research is required, research which will also inform regulatory authorities and enable the development of guidelines which should 1) guide academia and industry on the safety testing of AAB strains and 2) allow streamlining of the pre-market approval process for the use of such strains as starter cultures in both traditional and novel food products.

Genetic modification: If a strain has been isolated from a natural environment or system (e.g. a fermented food product produced by traditional methods), it is unlikely to have been genetically modified. If the strain has been genetically modified, regulatory approval and an assessment of the safety of the expressed product is required in many countries (Pariza, 2007).

Strain origin: Consideration of the origin of the microbial FC focuses on its isolation from a food with a demonstrated history of safe consumption and its significant role in the production and characteristics of that food. As outlined in previous sections in this review, AAB, in

particular members of the genera *Acetobacter*, *Gluconobacter*, *Gluconacetobacter* and *Komagataeibacter*, are key members in fermented food products such as vinegar and kombucha (Table 1), which are often sources for the isolation of new strains. Such fermented foods have a long history of safe consumption, particularly in Eastern Europe, Russia and Asia. Therefore, the long history of safe consumption of members of the AAB is self-evident. Nonetheless, non-food products such as flowers can also be sources of new AAB strains; the acceptability of the use of such strains, not strictly satisfying the guidelines of having been isolated from food, could be an important topic for discussion.

The safety of a strain can only be assessed based on the existing body of scientific knowledge. In addition, this knowledge should be assessed by an authoritative group and/or a group of qualified scientific experts (Pariza et al., 2015). While *G. oxydans* is on the QPS list, with qualifications, no other AAB has yet been placed on the list. However, as outlined above, 8 species, including *G. oxydans*, are included in the 2012 IDF/EFFCA Inventory (Bourdichon et al., 2012b). Thus, the IDF/EFFCA Panel would be considered as an authoritative group of scientific experts, and their inclusion of these AAB in the Inventory should be considered as an affirmation, with reasonable certainty, of their safety. Nevertheless, such expert evaluations should not be taken alone to constitute the absolute safety of a species and it is important to consider, particularly when dealing with individual strains, the safety of a culture with respect to the results of in-vitro tests and an analysis of the genome. In addition, it is noteworthy that the term safe or safety, in this context, means that that there is a reasonable certainty in the minds of competent scientists that a substance or microorganism is not harmful under the intended conditions of use. However, as observed by Lauland et al. (2017), it is impossible in the present state of scientific knowledge to establish with complete certainty the absolute harmlessness of the use of any microorganism (Laulund et al., 2017). It is also important to

consider recent scientific findings that may have emerged since the last peer review or scientific evaluation, to ensure that the safety of a species is still valid.

Exposure levels: The level of exposure of consumers to any new microbial FC could be dependent on a number of factors, such as the proposed applications of the strain, the number of different food products in which the strain will be applied, the amount of the strain (inoculum and final cell counts) within the product and the (post-fermentation) product processing conditions. A key consideration is whether groups (e.g. age groups, groups with particular medical conditions) which have not traditionally consumed or been exposed to the strain, will be thus through its use as a starter culture or probiotic (Laulund et al., 2017). With respect to AAB, the fact that certain populations may not have significantly consumed products produced by this group of microorganisms (i.e. acetous fermented food and beverages) to a large extent in the past should be considered e.g. Western European counties where kombucha has not traditionally been consumed. Nevertheless, if the consumption of such products has been considered, historically, to be safe in one population (e.g. in Asia), it is unlikely to be harmful to another, albeit the gastrointestinal microbiota might be diet dependent.

Any processing performed on the final food or beverage product may also effect the level of exposure to a microbial FC. For example, pasteurisation processes, depending on the conditions chosen, may significantly reduce the number of viable cells remaining. In addition, there is the option to completely inactivate the strain through an intense heat treatment. This may be advisable if there is a contraindication to having live cells in the final product. An example of this is the approval by EFSA in 2015 of the use of *Bacteroides xylanisolvens* DSM 23964 as a novel food for use in fermented milk products, under the Novel Food Regulation No 258/97 (EFSA Panel on Dietetic Products, 2015; European Commission, 1997). The usage of the strain was restricted to the fermentation of pasteurized milk products and only heat-treated (75^{o}C for 1 hour) and therefore inactivated cells of *B. xylanisolvens* were allowed in the final product. At

the same time, the EFSA Panel on Biological Hazards carried out a QPS assessment of the species; however, while no safety concerns could be identified the panel found that the published studies about *B. xylanisolvens* were too few and not sufficient to definitively exclude safety issues. Therefore, the microorganism was not included on the QPS list. The fact that the strain under investigation had no history of use in the food industry, and no strain in the genus *Bacteroides* has a proven history of use in food production was also considered. In addition, a gene encoding β-lactam antibiotic resistance was found in the genome. However, no mobile genetic elements were found, and the Panel considered the transfer of genes therefore unlikely due to this fact, in addition to the heat inactivation of the cells (Brodmann et al., 2017; EFSA Panel on Biological Hazards, 2015). Even though *B. xylanisolvens* was not placed on the QPS list, the positive outcome in terms of designation as novel food opens the possibility of using other species or strains of bacteria as microbial FC, even if the body of knowledge is insufficient and/or safety concerns cannot be fully excluded; however, with the qualification that no viable cells remain in the product. This is noteworthy if the strain has been applied for reasons beyond its fermentative capacity – for example, as viability is an essential condition for probiotic activity, inactivated cells should not be promoted for their potentially probiotic properties, and statements such as "contains live and active cultures" should not be made if the microbial FC has been inactivated. Nevertheless, non-viable cells may still elicit immunogenic effects and may have health benefits, hence, the term "paraprobiotic" has been coined to describe such cultures (Taverniti & Guglielmetti, 2011).

As an aside, one commercial beverage product which is produced via a *Gluconobacter oxydans* fermentation of malt-base (wort, or similar sugar source) is Bionade® [patent: DE4012000A1]. Following the fermentation, the *G. oxydans* cells are stated to be removed via filtration (www.bionade.de/en/production-process).

In conclusion, in light of their long history of safe consumption, it appears unlikely that the application of certain species of AAB (e.g. *Acetobacter*, *Gluconobacter*) would pose a safety risk. However, in the interest of consumer safety it would be prudent that any strains of AAB, applied as microbial FC in the future, would be thoroughly evaluated for their safety using a QPS approach or following a decision tree similar to that shown in Figure 8. However, relatively few studies have been conducted with regard to the safety of this microbial group, which means that the body of scientific knowledge in the area is lacking, especially when compared to the LAB (for example, prevalence and breadth of resistance to antimicrobials, production of toxins etc.). In addition, distinct guidelines from the responsible regulatory authorities (e.g. EFSA) are required, that deal with this important microbial group; however, it is clear and understood that such guidelines can only be built on a pre-existing body of scientific knowledge.

Conclusion

AAB are most commonly known for their role in vinegar production, yet, this diverse group of bacteria play an important part in the production of other fermented products such as kombucha and water kefir. Their highly efficient oxidative metabolism is unique and can be harnessed for the production of compounds that find application not only in the food and beverage area e.g. GA, ascorbic acid, cellulose. The diversity of uses for this group of bacteria is particularly highlighted by *Gluconobacter oxydans*. However, an understanding of their characteristics and metabolism is critical to maximising the potential of these bacteria, especially with respect to their strict oxidative requirements. Therefore, such an understanding of their metabolism can be applied to enable process optimisations for decreased process times or increased product yields, for example. In addition, further research may likely uncover other additional metabolic transformations performed by these bacteria which may have as yet unknown important applications.

With rising consumer interest in fermented foods and beverages, linked to purported health benefits (both scientifically proven and anecdotal) and an increasing strive by consumers towards a more health-conscious lifestyle, the AAB, like the LAB, are well poised for future exploitation – both in the re-imagining of traditional foods or beverages, such as kombucha, and in the development of new types of products. However, further and more extensive studies and trials are needed with regard to the health benefits related to the consumption of AAB-fermented products. In addition, guidance needs to be put forward by the relevant regulatory authorities, regarding the safety aspects of the application of this important group of microorganisms. However, it is clear that more fundamental studies on the safety aspects of this microbial group are required, upon which guidance from regulatory authorities can be based.

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Author Contributions

Kieran M. Lynch and Emanuele Zannini wrote the manuscript with critical input and corrections by Elke K. Arendt. Kieran M. Lynch did the final editing. Stuart Wilkinson and Luk Daenen critically evaluated the manuscript and provided valuable input and suggestions.

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Table 1: Acetic acid bacteria commonly isolated from vinegar and fermented beverages

Food product	**AAB species found associated with the fermentation**	**References**
Vinegar (Traditional Balsamic Vinegar)	*Acetobacter pasteurianus, A. aceti, Komagataeibacter xylinus, K. europaeus, K. hansenii*	(De Vero et al., 2006), (Gullo, Caggia, De Vero, & Giudici, 2006),
Kombucha	*K. xylinus, A. pasteurianus, K. hansenii, A. aceti, Ga. saccharivorans, other Acetobacter, Gluconobacter, and Gluconacetobacter spp.*	(Jayabalan et al., 2014), (Tan et al., 2012), (Liu et al., 1996), (Wang et al., 2014), (Marsh et al., 2014)
Water kefir	*A. lovaniensis, A. fabarum, A. cerevisiae, A. aceti, A. ghanensis, A. lovaniensis/fabarum, A. sicerae*	(Magalhaes et al., 2010), (Miguel, Cardoso, Magalhães, & Schwan, 2011), (Gulitz et al., 2013) (Stadie, Gulitz, Ehrmann, & Vogel, 2013), (Laureys & De Vuyst, 2014)
Lambic beer	*A. lambici, G. cerevisiae*	(De Roos & De Vuyst, 2018a, 2018b; Spitaels et al., 2014b; Spitaels et al., 2014c)

2808 Table 2: Key characteristics of the four genera of AAB commonly associated with food

Characteristic	*Acetobacter*	*Gluconobacter*	*Gluconacetobacter*	*Komagataeibacter*
Type strain	*A. aceti* NBRC 14818^T	*G. oxydans* NBRC 14819^T	*Ga. liquefaciens* NBRC 12388^T	*K. xylinus* JCM 7644^T
Production of acetic acid from ethanol	+	+	+	+
Flagellation	Per	Pol	Per	N
Oxidation of (to CO_2 and H_2O)				
Acetate	+	-	+	+
Lactate	+	-	+	+
Growth on				
30% glucose (w/v)	-	-[b]	-	nd
1% glucose (w/v)	+	+	+	+
Glutamate agar	-	-	+	+
Mannitol agar	vw	+	+	+
Raffinose	-	-	-	nd
Utilisation of methanol	-	-	-	nd
Growth in the presence of				
0.35% acetic acid (w/v)	+	+	+	+
1% KNO_3 (w/v)	-	-	-	nd
Water soluble brown pigment production	-	-[b]	+	-
Production of dihydroxyacetone from glycerol	+	+	+	+
Production of levan-like polysaccharide	-	-[b]	-	-[b]
Assimilation of ammoniac nitrogen on				
Glucose	-	+	+	nd
Mannitol	-	+	+	+
Ethanol	w	-	-	nd
Production of				
2-ketogluconate	+	+	+	+
5-ketogluconate	+	+	+	+
2,5-diketogluconate	-	-[b]	+	-
Acid production from				
Mannitol	-	+	-	-
Sorbitol	-	+	-	-
Dulcitol	-	w	-	nd
Glycerol	-	+	-	nd
Raffinose	-	-	-	nd
Ethanol	+	+	+	+

Per: peritrichous; Pol: polar; N: none; +, positive; -, negative; nd, not determined; b: some strains in the genus are positive; w, weakly positive; vw, very weakly positive. Modified from Yamada, 2016.

Table 3: Media commonly used for the growth of AAB and their composition

Ingredient (%)	GYC	AE	YPM	MYA	DMS	mDMS
Glucose	10	0.5	-	-	0.1	0.1
Yeast extract	1	0.3	0.5	0.5	0.3	0.3
Peptone	-	0.4	0.3	-	1.0	1.0
Calcium carbonate	2	-	-	-	-	
Ethanol	-	3	-	6	-	0.5
Glacial acetic acid	-	3	-	-	-	0.3
Lactic acid	-	-	-	-	-	0.6
Mannitol	-	-	2.5	-	0.1	0.1
Sorbitol	-	-	-	-	0.1	0.1
Malt extract	-	-	-	1.5	-	-
Calcium lactate	-	-	-	-	1.5	-
Potassium phosphate	-	-	-	-	0.1	0.1
Sodium deoxycholate	-	-	-	-	0.01	0.01
Magnesium sulfate	-	-	-	-	0.002	0.002
Bromocresol	-	-	-	-	0.003	0.003
Bacteriological agar	1.5	0.9	1.2	1.5	1.0	1.8

GYC, glucose-yeast extract-calcium carbonate; AE, acetic acid-ethanol; YPM, yeast extract-peptone-mannitol; MYA, malt extract-yeast extract-acetic acid; DMS, deoxycholate-mannitol-sorbitol; mDMS, modified deoxycholate-mannitol-sorbitol.

2847

2848 Table 4: Primary membrane-bound dehydrogenases of AAB performing oxidative
2849 fermentations.

Enzyme	Abbreviation	Prosthetic group	Substrate	Product	Importance	References
Alcohol dehydrogenase	ADH	PQQ	Ethanol	Acetaldehyde	Vinegar production	(Yakushi & Matsushita, 2010)
Aldehyde dehydrogenase	ALDH	MCD	Acetaldehyde	Acetic acid	Vinegar production	(Thurner et al., 1997)
Glucose dehydrogenase	GDH	PQQ	D-glucose	δ-glucono-lactone	Gluconate and keto-gluconate(s) production	(Ameyama, Shinagawa, Matsushita, & Adachi, 1981b)
Gluconate dehydrogenase	GADH	FAD	D-gluconate	2-keto-D-gluconate	Gluconate and keto-gluconate(s) production	(Shinagawa, Matsushita, Adachi, & Ameyama, 1984)
2-keto-D-gluconate dehydrogenase	GADH	FAD	2-Keto-D-gluconate	2,5-diketo-D-gluconate	Gluconate and keto-gluconate(s) production	(Toyama et al., 2007)
Glycerol dehydrogenase	GLDH	PQQ	(Polyols) D-gluconate Glycerol D-mannitol D-sorbitol D-arabitol Ribitol Meso-erythritol	(ketones) 5-keto-D-gluconate Dihydroxyacetone D-fructose L-sorbose D-xylulose L-ribulose Erythrulose	Polyol oxidation	(Matsushita et al., 2003)
Sorbitol dehydrogenase	SLDH	FAD	D-sorbitol	L-sorbose	Vitamin C production	(Shinagawa, Matsushita, Adachi, & Ameyama, 1982)
L-sorbose dehydrogenase	SDH	FAD	L-sorbose	L-sorbosone	Potential Vitamin C production	(Sugisawa, Hoshino, Nomura, & Fujiwara, 1991)
L-sorbosone dehydrogenase*	SNDHai	PQQ	L-sorbosone	L-ascorbic acid	Potential Vitamin C production	(Berry et al., 2003; Pappenberger & Hohmann, 2014)
Fructose dehydrogenase	FDH	FAD	D-fructose	5-keto-D-fructose	Potential low-calorie sweetener	(Ameyama, Shinagawa, Matsushita, & Adachi, 1981a)
Quinate dehydrogenase	QDH	PQQ	Quinate	3-dehydroquinate	Precursor for protocatechuic acid production	(Vangnai et al., 2004)

					(antioxidant and anti-inflammatory compound)	
Myo-Inositol dehydrogenase	IDH	PQQ	Myo-inositol	2-keto-myo-inositol	No clear industrial importance	(Hoelscher, Weinert-Sepalage, & Goerisch, 2007)
Glycerol dehydrogenase	GLDH	PQQ	(N-hydroxyethyl)-1-amino-1-deoxy-d-sorbitol	(N-hydroxyethyl)-6-amino-6-deoxy-l-sorbose	Production of the antidiabetic drugs, 1-deoxynojirimycin and miglitol	(Schedel, 2000)

*Note: Another enzyme of the same name, SNDH-FAD, is located in the cytoplasm and converts L-sorbosone to 2-keto-L-gulonic acid.

2856 Table 5. Definition of vinegar around the world.

Regulation	Definition	Reference
Malaysian Food Act 1983 and Food Regulation 1985,	Liquid product prepared from the alcoholic fermentation and subsequent acetous fermentation of any suitable food. The final product shall not contain less than 4 percent weight per volume (w/v) of acetic acid and shall not contain any mineral acid. The vinegar may also contain permitted preservatives, caramel as a colouring substance and spices as permitted flavouring substances	(Malaysian Food Regulations, 1985)
Codex Alimentarius Commission	Liquid suitable for human consumption and produced exclusively from appropriate products containing starch or sugars or starch and sugars by double fermentation processes, alcoholic and acetous. Vinegar shall not contain more than 0.5% alcohol, and stabiliser is not permitted for use in fermented vinegars according to European law. The vinegar itself shall not contain less than 50 g per litre (w/v) of acetic acid	(Codex Alimentarius Commission, 1987)
U.S. Food and Drug Administration (FDA)	There are no standards of identity for vinegar established under the Federal Food, Drug and Cosmetic Act. Nevertheless, the FDA considers that an acceptable guideline for vinegars that they must contain in excess of 4 g of acetic acid per 100 mL. Vinegar is made by the alcoholic and subsequent acetous fermentation of fruit juice	(United States of America Food and Drug Administration, 1977)
Commission Regulation (EU) 2016/263	1) Liquid produced by double fermentation, i.e., alcoholic and acetic from agricultural origin such as fruit, cereal, grains, wine, cider or malt. Plants or part of plants, including fruit, spices, salt or sugar may be added for flavouring. 2) Diluted acetic acid (diluted with water to 4-30 % by volume).	(European Commission, 2016)
Food Standards Australia New Zealand 2.10.1	Sour liquid prepared by the acetous fermentation with or without alcoholic fermentation of any suitable foodstuff and includes blends and mixtures of vinegar. This vinegar must contain not less than 40 g/kg of acetic acid.	(Food Standards Australia New Zealand Act, 1991)
Food Safety and Standards Authority of India	Products obtained by the alcoholic and acetic acid fermentation of any suitable medium such as fruit, malt, or molasses, with or without the addition of caramel and spices. They shall not be fortified with acetic acid. The acidity, calculated as the acetic acid content, shall not be less than 3.75% (m/v), the total solids (m/v) shall not less than 1.5%, and the total ash content shall not be less than 0.18%	(Food Safety and Standards Authority of India, 2012)

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The Ministry of Food and Drug Safety - Korea (MFDS)	1) Brewed vinegar that is produced by fermenting grains, fruits or alcoholic drinks or by mixing and ripening them with a grain-saccharified solution or fruit juice 2) Synthetic vinegar that is manufactured by diluting glacial acetic acid or acetic acid with drinking water. The total acid content is quantified as the acetic acid content, which is in the range of 4.0 to 29.0% (w/v), and tar colour should not be detected	(Ministry of Food and Drugs Safety, 2014)
Chinese National Standard code of condiments (2004) Edible vinegar. No.14834, N5239	Products obtained from both fermentation or artificial process (acetic acid blended with other ingredients, such as flavours)	(Chinese National Standard, 2004)

2867 Table 6. Overview of vinegars from around the world: raw materials, intermediate product, vinegar name and geographical distribution.

Category	Raw material	Edible part	Intermediate	Main carbon sources	Vinegar name	Geographical distribution[d]
Vegetable[a]	Bamboo sap	Bamboo sap	Fermented bamboo sap	Sucrose	Bamboo vinegar[b]	Japan, Korea
	Palm sap	Sap (xylem fluid)	Palm wine (toddy, tari, tuack, tuba)	Sucrose	Palm vinegar, toddy vinegar	Southeast Asia, Africa
	Tea and Sugar		Kombucha	Sucrose	Kombucha vinegar	Russia, Asia (China, Japan, Indonesia)
	Onion	Bulbs	Onion alcohol	Fructose, glucose, sucrose	Onion vinegar	East and Southeast Asia
	Tomato		-		Tomato vinegar	Japan, East Asia
	Sugarcane	Stalks	Fermented sugar cane juice	Sucrose	Cane vinegar	France, USA
			Basi		Sukang iloko	Philippines
					Kibizu	Japan
Grains	Malt	Seeds (caryopsis)	Beer	Maltose	Malt vinegar	Northern Europa, USA
	Rice	Seeds (caryopsis)	Koji/moromi	Starch	Komesu, kurosu (Japanese)	East and Southeast Asia
					Heicu (Chinese)	
	Barley	Seeds (caryopsis)	Beer	Starch	Vinegar	Germany, Austria, Netherlands
	Millet	Seeds	Koji/moromi	Starch	Black vinegar	China, East Asia
	Wheat	Seeds (caryopsis)	Koji/moromi	Starch	Black vinegar	China, East Asia
	Sorghum	Seeds (caryopsis	Koji/moromi	Starch	Black vinegar	China, East Asia
Fruit	Apple	Fruit (pome)	Cider	Fructose, glucose, sucrose	Cider vinegar	USA, Canada
	Grape	Fruits (berry)	Raisin	Fructose, glucose	Raisin (grape) vinegar	Turkey and Middle East
			Red or white wine		Wine vinegar	Widespread
			Sherry wine		Sherry (jerez) vinegar	Spain
			Cooked must		Balsamic vinegar	Italy
	Coconut	Coconut water	Fermented coconut water	Glucose, fructose	Coconut water vinegar	Philippines, Sri Lanka
	Date	Fruits (drupe)	Fermented date juice	Sucrose	Date vinegar	Middle East
	Mango	Fruits	Fermented mango juice		Mango vinegar	East and Southeast Asia
	Red date	Fruits (drupe)	Fermented jujube juice	Sucrose	Jujibe vinegar	China
	Raspberry	Fruits (berry)	Fermented raspberry juice	Fructose, glucose	Raspberry vinegar	East and Southeast Asia
	Black currant	Fruits (berry)	Fermented black currant juice	Fructose, glucose	Blackcurrant vinegar	East and Southeast Asia
	Blackberry	Fruits (berry)	Fermented blackberry juice	Fructose, glucose	Blackberry vinegar	East and Southeast Asia
	Mulberry	Fruits (berry)	Fermented mulberry juice	Fructose, glucose	Mulberry vinegar	East and Southeast Asia
	Plum	Fruits (drupe)	Umeboshi[c] fermented plum juice	Sucrose, fructose, glucose	Ume-su	Japan
	Cranberry	Fruits (drupe)	Fermented cranberry juice	Fructose, glucose	Cranberry vinegar	East and Southeast Asia
	Kaki	Fruit (pome)	Fermented persimmon juice	Fructose, glucose, sucrose	Persimmon vinegar	South Korea
					Kakisu	Japan
Animal	Whey	Whey	Fermented whey	Lactose	Whey vinegar	Europe
	Honey	Honey	Diluted honey win, tej	Fructose, glucose	Honey vinegar	Europe, America, Africa

2868 [a]Vegetable is not a botanical term and is used to refer to an edible plant part; some botanical fruits, such as tomatoes, are also generally considered to be vegetables. [b] Obtained by bamboo sap fermentation (González
2869 & De Vuyst, 2009).
2870 [c] Umeboshi are pickled *ume* fruits. *Ume* is a species of fruit-bearing tree of the genus *Prunus*, which is often called a plum but is actually more closely related to the apricot. [d] Listed in order, from the largest to the
2871 smallest amount.
2872 Reprinted with permission from (Solieri & Giudici, 2009).

2873 Table 7. Studies examining the potential health benefits associated with vinegar consumption.

Vinegar type	Function	Country	Subject(s)	Results	Reference
Spirit vinegar	Antibacterial	Japan	Food-borne pathogenic bacteria	The growth of all strains evaluated was inhibited with a 0.1% concentration of acetic acid in the vinegar.	(Entani et al., 1998)
Rice vinegar	Antibacterial	China	*E. coli* O157:H7	Treatment of inoculated lettuce (10^7 CFU/g bacteria) with vinegar (5% acetic acid) for 5 min would reduce 3 logs population at 25 °C.	(Chang & Fang, 2007)
Grape vinegar	Antibacterial	Turkey	Salmonella typhimurium	Treatment of carrot samples with vinegar (4.03% acetic acid) for different exposure times (0, 15, 30 and 60 min) caused significant reductions ranging between 1.57 and 3.58 log CFU/g.	(Sengun & Karapinar, 2004)
Acetic acid solution	Anti-infection	Kuwait	96 patients with chronic suppurative otitis media	The patients received ear irrigation with 2% acetic acid solution three times per week (3 weeks, followed for up to 3 years). 55 patients had resolution of their original otorrhen, whereas 19 patients developed healed ear drum perforation. 14 patients (15%) showed recurrence and 8 of them had no response to the treatment.	(Aminifarshidmehr, 1996)
Fermented vinegar	Anti-infection	Korea	15 patients with chronic granular myringitis	The patients were treated with irrigation of the external canal with dilute vinegar solution (pH = 2.43) twice to four times per day. All patients had resolution of their original otorrhoea within three weeks.	(Jung, Cho, Yoo, Lim, & Chae, 2002)
Shanxi aged vinegar	Antioxidative	China	Hyperlipidemic mouse	Fed with a diet with 1% freeze-dried powder of Shanxi aged vinegar for 35 d resulted in a significant increase in antioxidant activity in mice.	(Liu & Yang, 2015)
Zhenjiang aromatic vinegar	Antioxidative	China	Ageing accelerating mice	Instilled with 1.2 g/kg/d vinegar for 35 d resulted in a significant increase in antioxidant activity in mice.	(Xu, Tao, & Ao, 2005)

Kurosu	Antioxidative	Japan	Mice	The ethyl acetate extract of Kurosu significantly suppressed the 12-*O*- tetradecanoylphorbol-13-acetate induced myeloperoxidase activity and H_2O_2 generation in mouse.	(Nishidai et al., 2000)
Traditional balsamic vinegar	Antioxidative	Italy	In vitro	Reducing capacity: 218.85 ± 6.86 mg Vc/100 mL Antiradical activity: 298.10 ± 6.25 mg Vc/100 mL	(Tagliazucchi et al., 2008)
Traditional balsamic vinegar	Antioxidative	Italy	In vitro	Reducing capacity: 27.12 ± 11.1 μM Tes/mL Antiradical activity: 33.52 ± 19.3 μM Tes/mL	(Bertelli et al., 2015)
Traditional balsamic vinegar	Antioxidative	Italy	Simulated gastric	The vinegar melanoidins (4.5 mg/mL) significantly inhibited the lipid peroxidation during simulated gastric digestion of meat.	(Verzelloni et al., 2010)
Balsamic vinegar	Antioxidative	Japan	Macrophage:THP-1	Balsamic vinegar (0.01%) significantly inhibited the low density lipoprotein (LDL) oxidation and lipid accumulation in macrophages	(Iizuka et al., 2010)
Red wine vinegar	Antioxidative	Italy	In vitro	Reducing capacity: 48.18 ± 2.00 mg Vc/100 mL Antiradical activity: 85.40 ± 1.73 mg Vc/100 mL	(Verzelloni et al., 2007)
White vinegar	Blood glucose control	Sweden	12 healthy volunteers	Supplementation of a meal with vinegar (18 g) reduced postprandial responses of blood glucose and insulin and increased the subjective rating of satiety.	(E. Östman, Granfeldt, Persson, & Björck, 2005)
Bitter buckwheat vinegar	Blood glucose control	China	Diabetic rats	Oral intake of vinegar (2 mL/kg/d) for 4 weeks reduced about 17% blood glucose in rats.	(Ma, Xia, & Jia, 2010)
Rice vinegar	Blood glucose control	China	Diabetic rats	Oral intake of vinegar (2 mL/kg/d) for 30 d improved fasting hyperglycemia and body weight loss through attenuating insulin deficiency, pancreatic beta-cell deficit, and hepatic glycogen depletion in rats.	(Gu et al., 2012)
Apple vinegar	Blood glucose control	America	11 patients with type 2 diabetes	Vinegar ingestion (30 mL) at bedtime moderates waking glucose concentrations in adults with well-controlled type 2 diabetes mellitus.	(White & Johnston, 2007)

Apple vinegar	Blood glucose control	America	27 patients with type 2 diabetes	Oral intake of vinegar (1.4g/d) for 12 weeks significantly reduced haemoglobin A1c values in individuals with type 2 diabetes mellitus.	(Johnston et al., 2009)
Apple vinegar	Blood glucose control	America	8 healthy volunteers	Supplementation of a meal with vinegar (10 g) reduced about 23% postprandial responses of blood glucose.	(Johnston et al., 2010)
Apple vinegar	Blood glucose control	Japan	7 patients with polycystic ovary syndrome	Oral intake of vinegar (15 g/d) for 90 - 110 d improved insulin sensitivity in individuals with polycystic ovary syndrome.	(Wu et al., 2013)
Vinegar	Blood glucose control	Greece	10 patients with type 1 diabetes	Supplementation of a meal with vinegar (30 mL) reduced about 20% postprandial responses of blood glucose.	(Mitrou et al., 2010)
Acetic acid solution	Regulation of lipid metabolism	Japan	Human umbilical vein endothelial cell	Vinegar intake enhances flow-mediated vasodilatation via upregulation of endothelial nitric oxide synthase activity.	(Sakakibara et al., 2010)
Shanxi aged vinegar	Regulation of lipid metabolism	China	Hyperlipidemic mice	Fed with a diet with 1% freeze-dried powder of vinegar for 35 d resulted in a significant reduction of triglyceride, total cholesterol and LDL in mouse.	(Liu & Yang, 2015)
Sorghum vinegar	Regulation of lipid metabolism	China	Rats	Fed with a diet with extract of vinegar (100 mg/kg) protected the rats against thrombotic death induced by collagen and epinephrine.	(Fan et al., 2009)
Grape vinegar	Regulation of lipid metabolism	Iran	Rabbits with high cholesterol diet	Oral intake of 10 mL vinegar significantly reduced LDL-cholesterol, oxidized-LDL malondialdehyde and total cholesterol in rabbits after 3 hours.	(Setorki, Asgary, Eidi, & Khazaei, 2010)
Grape vinegar	Regulation of lipid metabolism	Egypt	Diabetic rats	Fed with a diet with 15% vinegar for 6 weeks significantly reduced LDL-cholesterol and total cholesterol in rats.	(Soltan & Shehata, 2012)

Apple vinegar	Regulation of lipid metabolism	Iran	19 patients with hyperlipidemia	Oral intake of vinegar (30 mL/d, twice) for 8 weeks significantly reduced triglyceride, total cholesterol and LDL in individuals with hyperlipidemia.	(Beheshti et al., 2012)
Persimmon vinegar	Regulation of lipid metabolism	Korea	Mouse with high lipid diet	Oral intake of vinegar (2 mL/kg/d) for 16 weeks significantly reduced triglyceride and total cholesterol in mouse.	(Moon & Cha, 2008)
Acetic acid solution	Weight loss	Japan	Obese mice	Fed with 0.3 or 1.5% acetic acid solution for 6 weeks significantly inhibited the accumulation of body fat and hepatic lipids without changing food consumption or skeletal muscle weight.	(Kondo, Kishi, Fushimi, & Kaga, 2009b)
Corn vinegar	Weight loss	China	Obese mice	Oral intake of vinegar (0.3 mL/d) for 30 d significantly reduced body weight, fat coefficient, triglyceride and total cholesterol in mouse.	(Li et al., 2009)
Purple sweet potato vinegar	Weight loss	China	Obese mice	Oral intake of vinegar (10 mL/ kg/d) for 30 d significantly reduced body weight, fat coefficient, LDL, triglyceride and total cholesterol in mouse.	(Liu et al., 2015)
Apple vinegar	Weight loss	Japan	150 obese Japanese	Oral intake of vinegar (15 mL/d) for 12 weeks significantly reduced body weight, body fat mass and serum triglyceride levels in subjects.	(Kondo et al., 2009a)
Apple vinegar	Weight loss	Mexico	Rats with high-caloric diets	Oral intake of vinegar (0.8 mL/ kg/d) for 4 weeks significantly reduced body weight, fat coefficient, LDL, triglyceride and total cholesterol in rats.	(De Dios Lozano, Juárez-Flores, Pinos-Rodríguez, Aguirre-Rivera, & Álvarez-Fuentes, 2012)
Mulberry vinegar	Weight loss	China	Obese mice	Oral intake of vinegar (0.1 mL/d) for 30 d significantly reduced body weight, fat coefficient, triglyceride and total cholesterol in mouse.	(Wei et al., 2005)
Hawthorn Vinegar	Weight loss	Turkey	37 Obese patients with cardiovascular disease	Oral intake of vinegar (40 mL/d) for 4 weeks significantly reduced body weight, body fat mass and serum triglyceride levels in subjects.	(Kadas, Akdemir Evrendilek, & Heper, 2014)

Shanxi aged vinegar	Anti-carcinogenic	China	Cancer cells (A549, Hep-G2, MDA-MDB-231, HeLa)	Ethyl acetate extract of vinegar (0.01%) significantly inhibited the proliferation of cancer cells *in vitro*.	(Chen & Gullo, 2015)
Kurosu	Anti-carcinogenic	Japan	Rats with colon cancer	Fed with water containing 0.05% ethyl acetate extract of Kurosu for 35 weeks significantly inhibited azoxymethane-induced colon carcinogenesis in rats.	(Shimoji et al., 2004)
Kurosu	Anti-carcinogenic	Japan	Cancer cell (Caco-2, A549, MCF-7, 5637, LNCaP)	Ethyl acetate extract of vinegar (0.025%) significantly inhibited the proliferation of cancer cells *in vitro*.	(Nanda et al., 2004)
Black soybeans vinegar	Anti-carcinogenic	Japan	Leukaemia U937 cells	Ethyl acetate extract of vinegar (10 mg/mL) significantly inhibited the proliferation of cancer cells *in vitro*.	(Inagaki et al., 2007)
Post-distillation slurry vinegar	Anti-carcinogenic	Japan	Mice with Sarcoma 180 and Colon 38 tumour cells	Fed with a diet with 0.5% vinegar for 72 d significantly decreased the sizes of tumours and prolonged life spans of mouse.	(Seki, Morimura, Shigematsu, Maeda, & Kida, 2004)
Sugarcane vinegar	Anti-carcinogenic	Japan	Leukaemia cells: HL-60, THP-1, Molt-4, U-937, K-562	Fraction eluted by 40% methanol from vinegar significantly inhibited the proliferation of leukaemia cells *in vitro*.	(Mimura et al., 2004)
Zhenjiang Aromatic Vinegar	Anti-fatigue	China	Mouse	Fed with a diet with vinegar (300 mg/kg/d) for 28 d significantly improved anti-fatigue abilities of mouse.	(Lu & Zhou, 2002)
Mulberry vinegar	Anti-fatigue	China	Mouse	Fed with a diet with vinegar (0.2 mL/d) for 20 d significantly improved anti-fatigue abilities of mouse.	(Zhang, Li, & Du, 2007)
Grain vinegar	Prevention of osteoporosis	Japan	Ovariectomized rats	Fed with a diet with 0.4% vinegar for 32 d significantly increased intestinal absorption of calcium in rats.	(Kishi et al., 1999)

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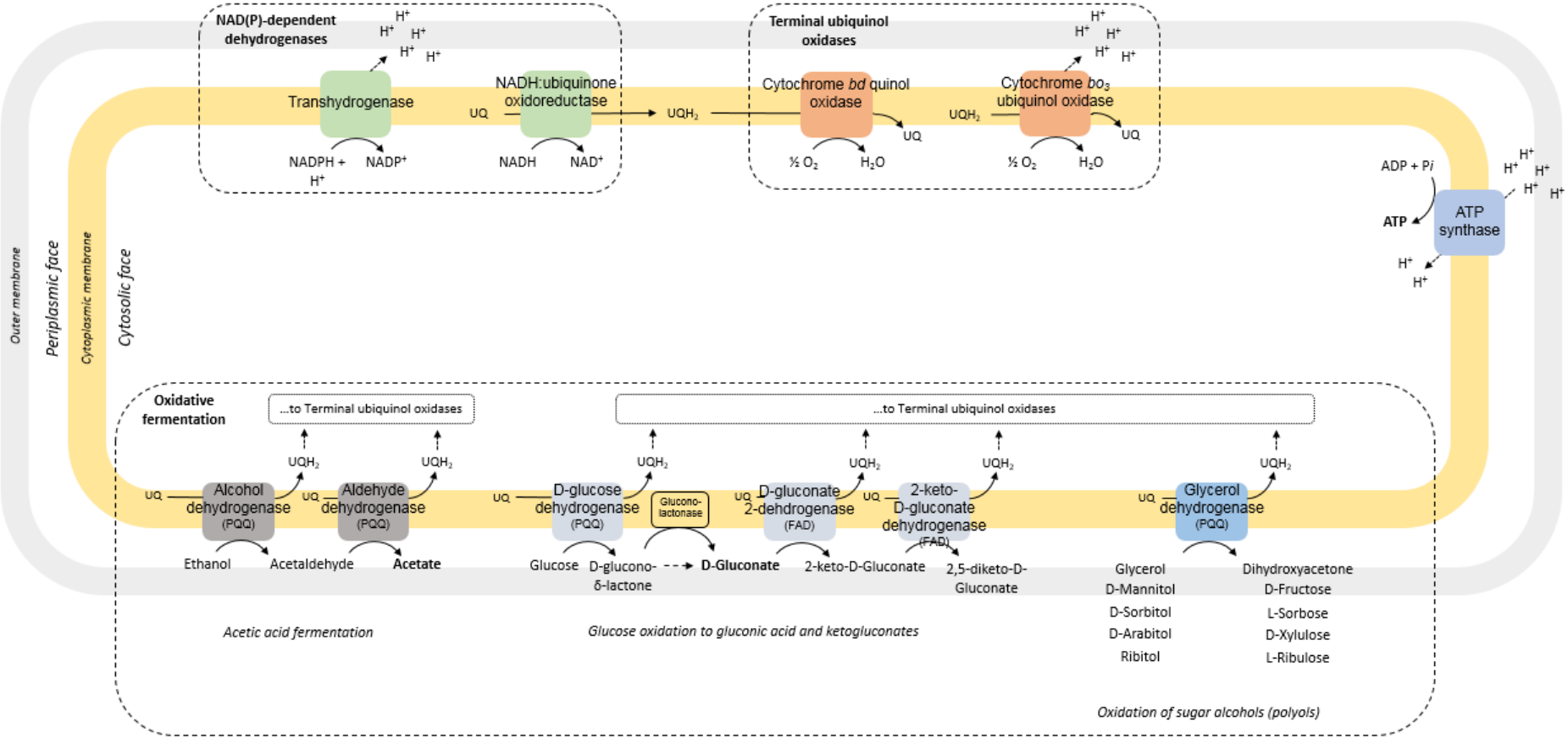

Figure 1. Respiratory and oxidative fermentation chains and associated dehydrogenases in Acetic acid bacteria.

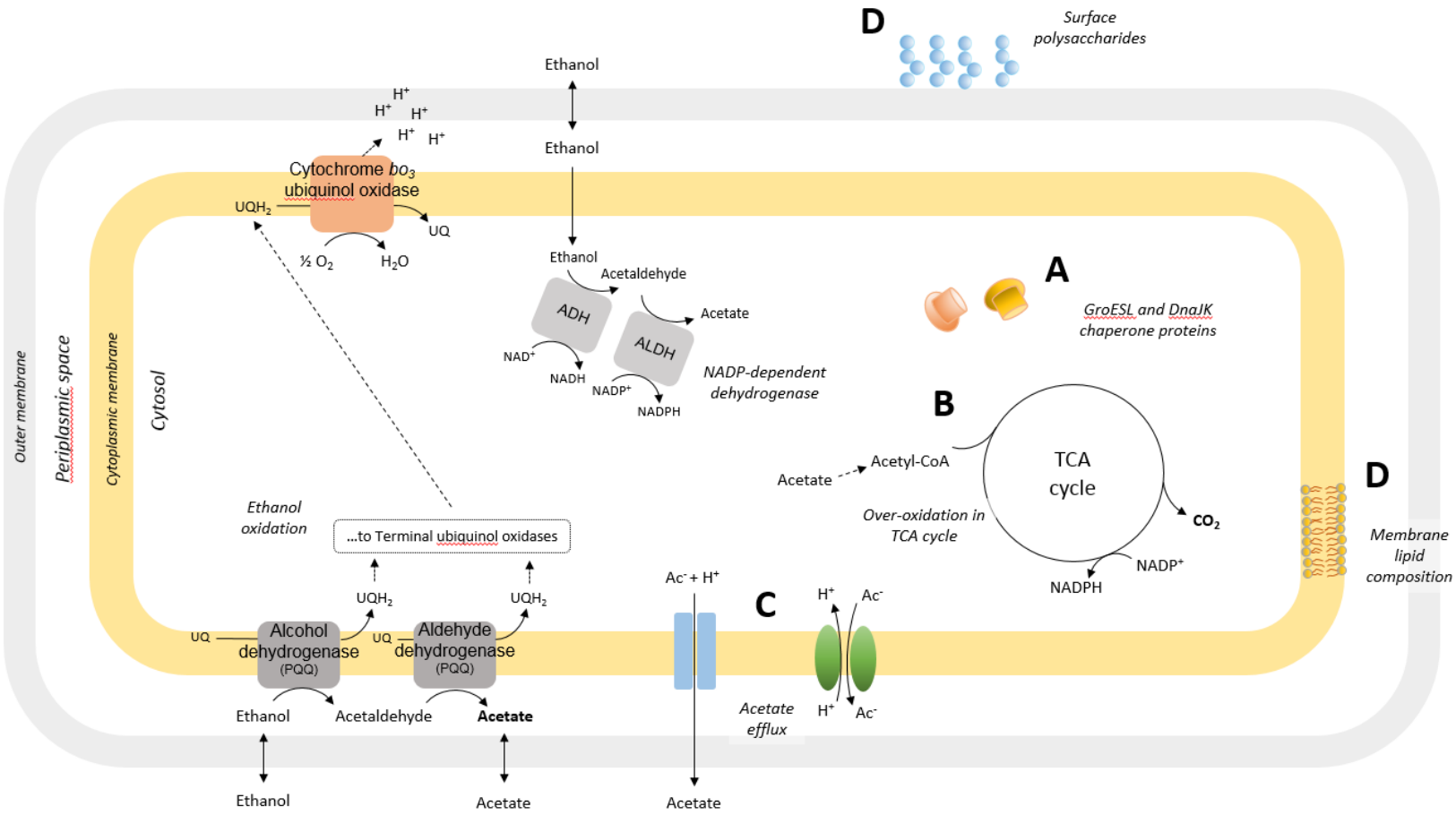

Figure 2. Strategies of acetic acid resistance employed by Acetic acid bacteria. a) adaption of and protection of intracellular proteins to and against acid stress, b) metabolism (overoxidation) of intracellular acetic acid, c) efflux of acetic acid from the cell and d) prevention of acetic acid from entering the cell.

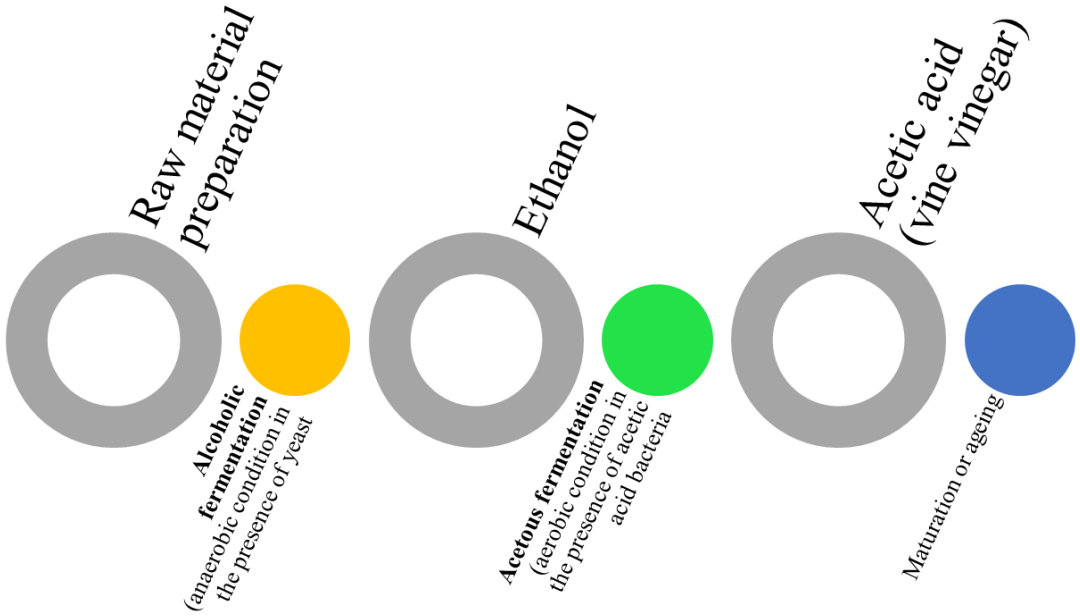

Figure 3. The process of wine vinegar-making from grape juice.

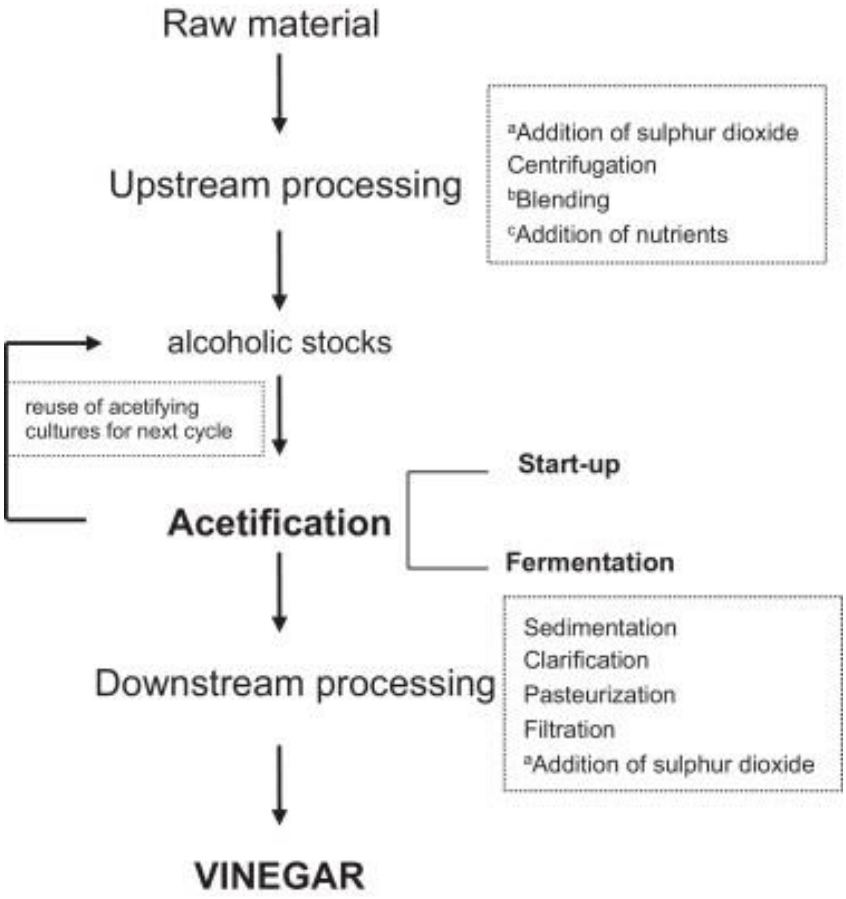

Figure 4. Schematic representation of vinegar production in submerged system. [a]At concentrations specified by legislation; [b] Blending with high acidity vinegar, to block undesired alcoholic fermentation; c nutrients containing carbon and nitrogen sources, vitamins and minerals are supplemented especially to produce high acidity vinegar (>12% of acetic acid) from alcoholic stocks containing no carbon sources except for ethanol. Reprinted with permission from Gullo et al., 2014.

Figure 5. Kombucha cellulose pellicle (SCOBY).

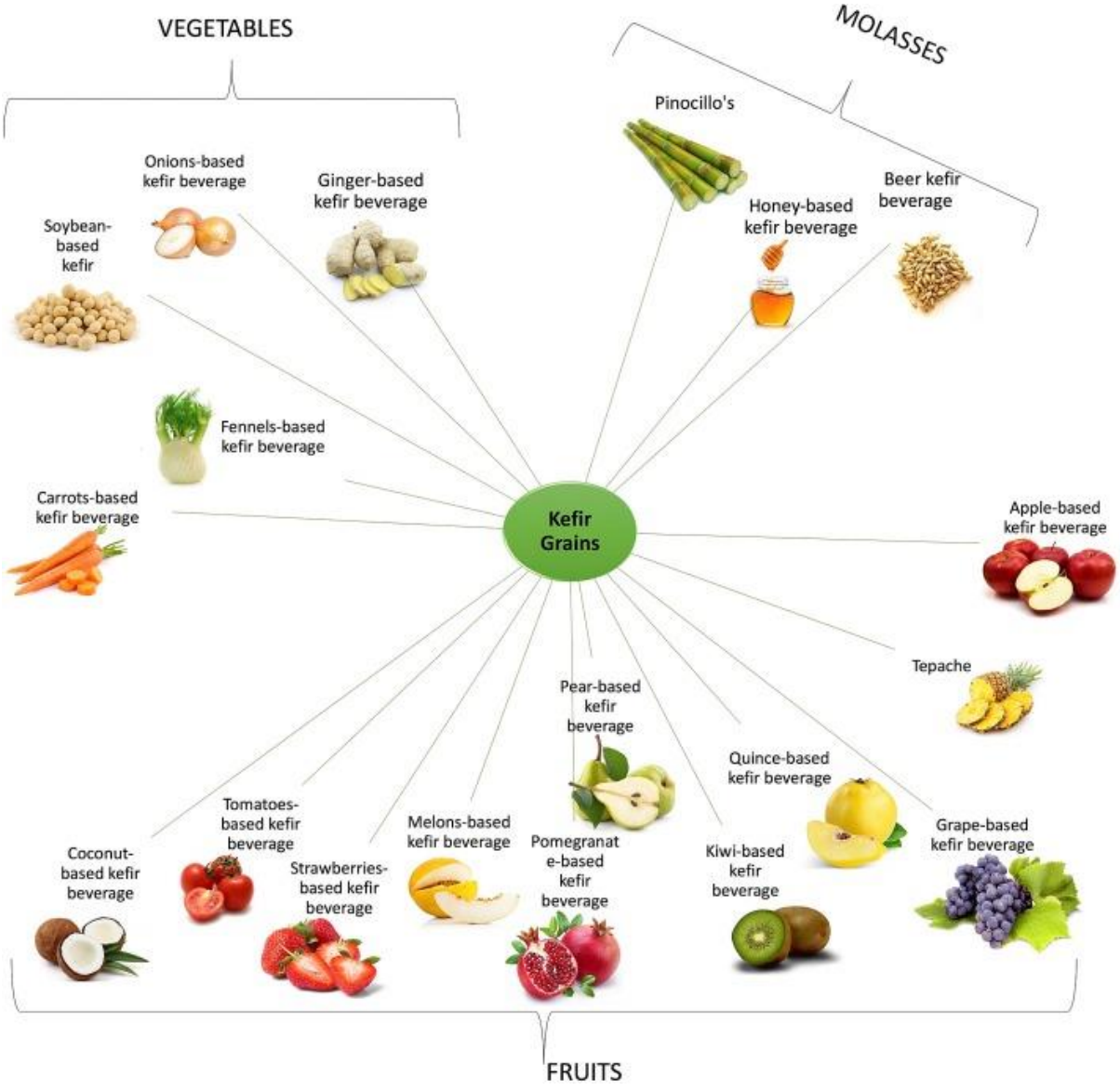

Figure 6. Different non-dairy products from sugary kefir fermentation. Reprinted with permission from Fiorda et al., 2017.

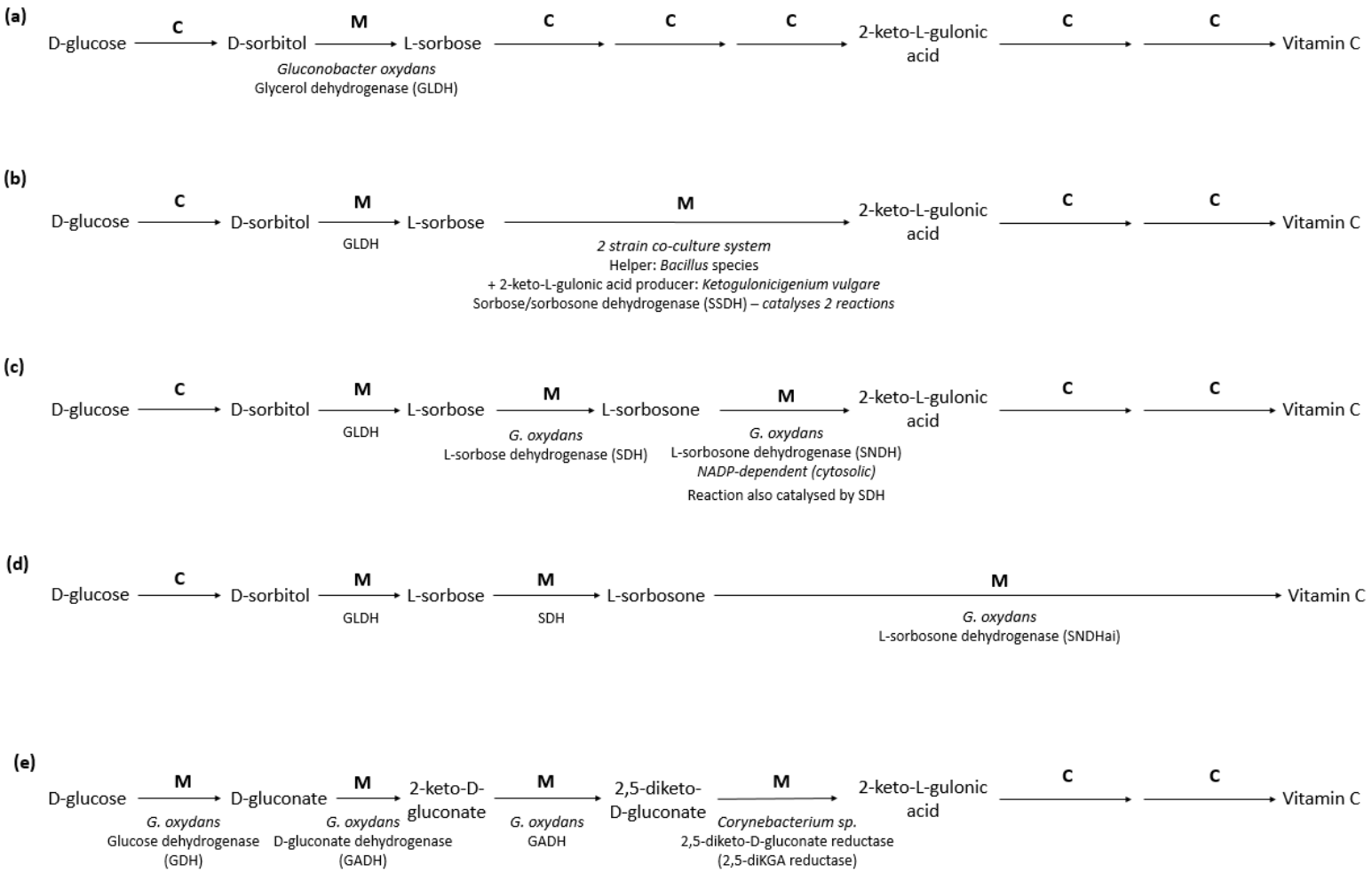

Figure 7. Processes used in, or having the potential for, Vitamin C production. (a) The Reichstein Process, microbial production of L-sorbose from D-sorbitol; (b) The Two-Step Fermentation Process, microbial production of 2-KLGA from L-sorbose via a 2-step fermentation; (c) Microbial production of 2-KLGA by *G. oxydans*; (d) direct microbial production of Vitamin C by *G. oxydans*; (e) microbial production of 2-KLGA via the 2,5-diKGA pathway. C: chemical reaction, M: microbial bioconversion.

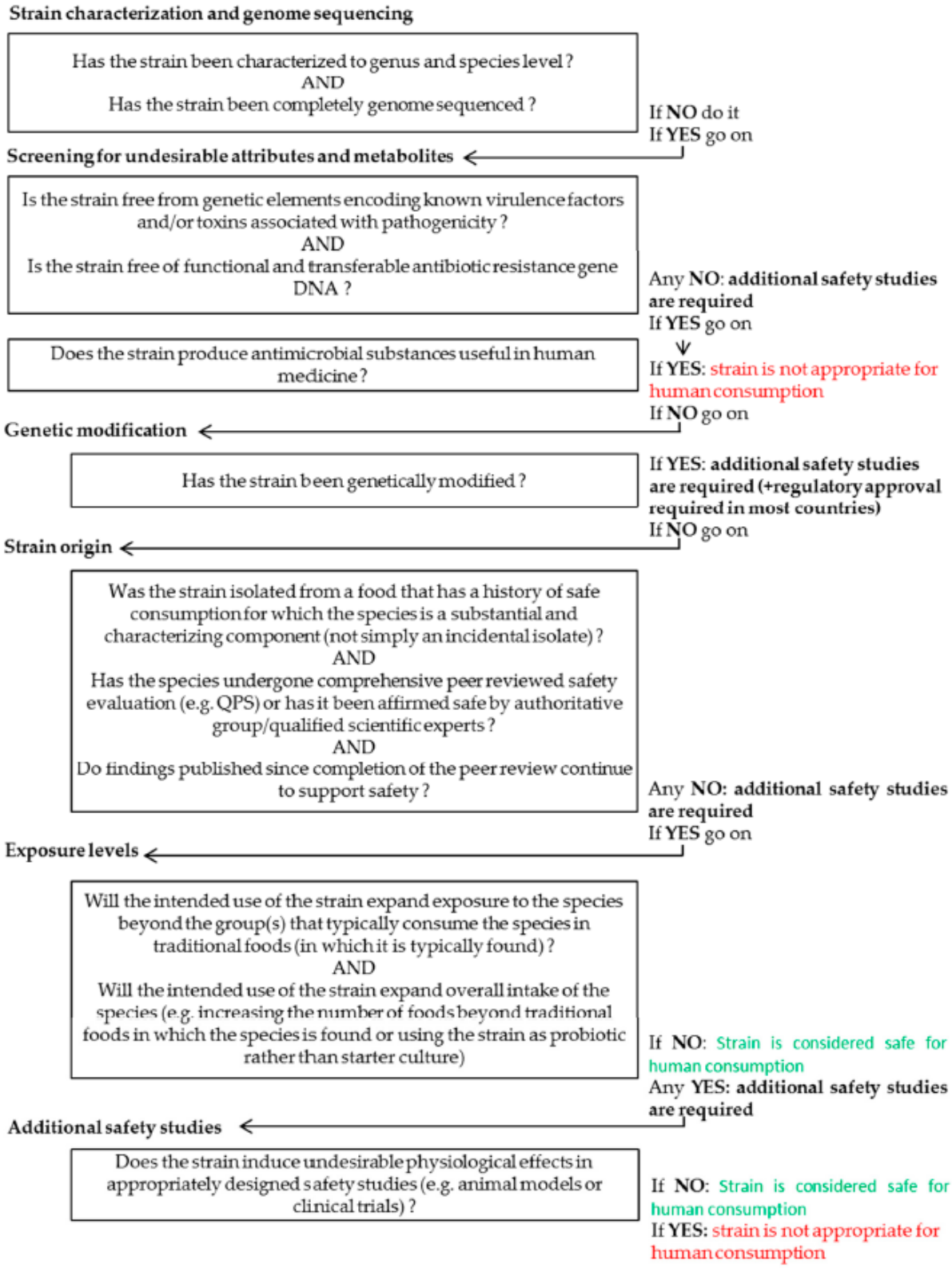

Figure 8. Decision tree for the safety assessment of microbial strains to be used in food applications. Reprinted from Laulund et al., 2017.

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