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

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22 **Short title:** Microbiology of Acetic acid bacteria

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25 **Keywords:** Acetic acid bacteria, oxidative fermentation, exopolysaccharides, vinegar,
26 Vitamin C.

29 **ABSTRACT**

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

47 **Introduction**

48 Acetic acid bacteria (AAB), first described as “vinegar bacteria” by Louis Pasteur over 150
49 years ago, are an important and diverse group of bacteria involved in the production of
50 fermented foods and beverages, especially known for their production of acetic acid (ethanoic
51 acid) in the making of vinegar (Hutkins, 2006; Pasteur, 1864). Acetic acid bacteria are
52 characterized by their ability to oxidize carbohydrates, alcohols and sugar alcohols (polyhydric
53 alcohols or polyols) into their corresponding organic acids, aldehydes or ketones, in a process
54 termed “oxidative fermentation”, from which they gain energy (Taban & Saichana, 2017). This
55 unique property also has applications in the production of industrially-relevant compounds and
56 has primarily been exploited in the synthesis of ascorbic acid (Vitamin C) and miglitol (an
57 antidiabetic drug used in the treatment of type II diabetes mellitus) (Shinjoh & Toyama, 2016;
58 Taban & Saichana, 2017). Some genera of AAB are notable for their production of a variety
59 of exopolysaccharides (EPS), the most valuable of which are bacterial cellulose (BC) and
60 acetan. BC offers several advantages over plant-derived cellulose, particularly because it is free
61 of hemicellulose and lignin associated with cellulose (Dağbağlı & Göksungur, 2017). Acetic
62 acid bacteria are associated with, and have been isolated from, carbohydrate-rich and acidic
63 environments such as fruits and flowers, and are involved in the production of a variety of
64 fermented foods and beverages including vinegar, kombucha, lambic beers, kefir and nata de
65 coco (Table 1). They also play an important role in the cocoa fermentation process (Pothakos
66 et al., 2016). Unsurprisingly, AAB have also been associated with the spoilage of foods and
67 alcoholic beverages such as beer, wine, cider and fruit juices (Taban & Saichana, 2017). The
68 purpose of this review is to provide an overview of the physiology and biochemistry of AAB
69 and to provide an understanding of how the unique capabilities of these microorganisms are
70 important in the production of vinegar and other fermented beverages. Other technologically-
71 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).

77 **Characteristics of the AAB and their identification**

78 Acetic acid bacteria are Gram-negative or Gram-variable, obligate aerobes, and are classified
79 in the family *Acetobacteraceae*. They are non-spore forming, ellipsoidal to rod-shaped cells
80 that can occur singly, in pairs or in short chains (Malimas et al., 2017). The members of the
81 *Acetobacteraceae* family are separated into two groups, the acetous and the acidophilic groups,
82 of which AAB are included in the former (Komagata, Iino, & Yamada, 2014). Eighteen genera
83 are currently reported in the acetous group; the most important in terms of fermented foods are
84 *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, and *Komagataeibacter* (Table 2) (Giudici,
85 De Vero, & Gullo, 2017). In addition, the aforementioned genera contain many species while
86 the remaining genera consist of only one or two (Yamada, 2016). Some genera are motile,
87 having either peritrichous or polar flagella. The majority of species are catalase-positive and
88 oxidase-negative, however, there are exceptions. Growth in the presence of 0.35% acetic acid
89 has been used as a criterion; however, while members of the genera *Acetobacter*,
90 *Gluconobacter*, *Gluconacetobacter*, and *Komagataeibacter* grow in the presence of 0.35%
91 acetic acid, not all genera can e.g. *Asaia*. Acetic acid bacteria are typically considered to be
92 mesophilic, with the optimum temperature for growth being around 30°C (Malimas et al.,
93 2017). At higher temperatures, growth reduces significantly, with none usually occurring above
94 34°C (Saichana, Matsushita, Adachi, Frebort, & Frebortova, 2015). The mesophilic character
95 of AAB, which require strict temperature control, poses a challenge for industrial application.
96 However, thermotolerant strains that can grow at a temperature of up to 42°C have been
97 identified (Saichana et al., 2015). It is noteworthy that the genera constituting the AAB are
98 undergoing continuous revision and taxonomic changes and the reader is directed elsewhere
99 for further information on this topic, such as Malimas et al. (2017). Acetic acid bacteria are
100 notable for their direct oxidation of carbohydrates and sugar alcohols from which they
101 accumulate large amounts of the corresponding oxidation products, gleaning metabolic energy

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

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

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

127 contains a number of carbon sources has been used for the selective isolation and enumeration
128 of presumptive AAB, for example, from cocoa pulp-bean mass and lambic beer. A modified
129 version (mDMS) containing lactic acid, acetic acid and ethanol is advisable for the isolation of
130 a number of AAB species (Camu et al., 2008; De Roos, Verce, Aerts, Vandamme, & De Vuyst,
131 2018a; Papalexandratou et al., 2013; Wieme et al., 2014).

132 The capability to accurately identify microorganisms is indisputable, not only from the
133 perspective of obtaining a basic understanding of the microorganism(s) being applied,
134 (particularly where the properties and desired traits that different species of microorganism
135 possess, or ideally should possess, can vary), but also from a food safety perspective. Many
136 DNA-based methods have been used for the identification of AAB, both as single isolated
137 strains and as members of complex food matrices, such as fermenting wine (Gonzalez, Hierro,
138 Poblet, Mas, & Guillamon, 2005). More recently, as the number of described AAB species has
139 risen taxonomically, in part as a consequence of the use of such molecular techniques, the
140 requirement for higher resolution and better differentiation between species has increased,
141 necessitating the development of, firstly, molecular targets with increasing resolving power,
142 and secondly, non-DNA-based methods that are sensitive and rapid (Andrés-Barrao et al.,
143 2013).

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

152 RFLP of the spacer region between the 16S and 23S rRNA genes (internal transcribed spacer,
153 ITS) has been used to provide such differentiation (Gonzalez & Mas, 2011). Intergenic regions
154 are known to have higher variability than functional, protein-coding sequences, thus being able
155 to resolve closely related species (Barry, Colleran, Glennon, Dunican, & Gannon, 1991).
156 However, due to frequent variations and high divergences of intergenic sequences, even among
157 strains of the same species, direct sequencing of the ITS was not useful for identification to
158 species level (Ruiz, Poblet, Mas, & Guillamon, 2000). Nevertheless, the use of a polyphasic
159 approach including the sequencing of both the 16S rRNA gene and the ITS region for use in
160 phylogenetic analysis has enabled differentiation of closely related strains (Gonzalez & Mas,
161 2011). Alternative loci such as the *adhA* gene, *recA* gene and *tuf* gene can be used for
162 polygenetic studies, showing similar results to phylogenies prepared with 16S rRNA and ITS
163 sequences (Greenberg et al., 2006; Treck, 2005).

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

177 For genotyping and identification to the strain level, methods such as Random Amplification
178 of Polymorphic DNA (RAPD) and Amplified Length Fragment Polymorphism (ALFP), and
179 techniques based on amplification of repetitive sequences, such as Enterobacterial Repetitive
180 Intergenic Consensus-PCR (ERIC-PCR), Repetitive Extragenic Palindromic-PCR (REP-PCR)
181 and (GTG)₅-PCR have been variously applied in studies, all of which are fingerprinting
182 techniques. Both ERIC-PCR and (GTG)₅-PCR have been demonstrated to be most suitable for
183 the differentiation of isolates to strain level, in some studies being used to monitor the
184 population dynamics of AAB in traditional wine vinegar production at the strain level (De
185 Vuyst et al., 2008; Papalexandratou & De Vuyst, 2011; Vegas et al., 2010).

186 Alternatives to DNA-dependant molecular methods are increasingly being explored as
187 accurate, rapid and high throughput means of microbial identification. One such method is
188 Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-
189 TOF MS) which has originally been exploited in the field of clinical microbiology for the rapid
190 identification of human pathogens, but has in recent years developed in the area of food
191 microbiology (Croxatto, Prod'hom, & Greub, 2012; De Roos, et al., 2018a; Spitaels et al.,
192 2014a; Spitaels et al., 2015). MALDI is a soft ionization method used with mass spectrometry
193 for the analysis of large organic biomolecules. Briefly, the sample is bombarded with a high-
194 energy laser beam leading to ionization of the sample in the form of cations. These ions are
195 then accelerated in an electric field to a speed that depends on the mass-to-charge (m/z) ratio
196 of each specific particle produced upon sample ionisation. The particles then enter a TOF mass
197 analyser and travel along a field-free flight path towards the detector. The time required for
198 each particle to reach the detector is precisely measured and is dependent on its m/z ratio. The
199 m/z ratio of each particle is determined and a mass spectrum is generated, representing both
200 m/z and signal intensity of the detected ions. The mass spectrum generated from a bacterium
201 corresponds to high-abundance soluble proteins, predominantly ribosomal proteins and other

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

214 **Physiology and metabolism of AAB**

- 215 • Aerobic respiration

216 Similar to many aerobic bacteria, AAB gain the majority of their energy by performing a type
217 of aerobic respiration (Matsushita & Matsutani, 2016). In the general process of aerobic
218 respiration, initially pyruvate is completely oxidised to carbon dioxide (CO₂) in the citric acid
219 cycle. Subsequently, the reduced electron acceptors formed in the citric acid cycle are shuttled
220 to the respiratory chain in the cytoplasmic membrane. Here, oxidation of the reduced electron
221 carriers by components of the respiratory chain (oxidative phosphorylation) results in the
222 formation of water, along with exclusion of protons from the cytoplasm, producing a proton
223 gradient. Equalisation of this proton-motive force via transfer of protons back into the cell
224 through a transmembrane ATPase (F₁F₀-type ATP synthase) leads to the biosynthesis of energy
225 in the form ATP (Madigan, Martinko, Bender, Buckley, & Stahl, 2015).

226 The basic components of the AAB respiratory machinery consist of two periplasmic
227 dehydrogenases: a membrane-bound proton pumping transhydrogenase, a non-proton
228 translocating NADH: ubiquinone oxidoreductase, and two terminal oxidases of the ubiquinol
229 oxidase-type. Ubiquinone (UQ) acts as the electron shuttle, in its reduced form, ubiquinol
230 (UQH₂), between these respiratory proteins. The function of the transhydrogenase and NADH:
231 ubiquinone oxidoreductase is the regeneration of NADP⁺ and NAD⁺, respectively, with the
232 concomitant exclusion of protons in the case of the transhydrogenase complex. The terminal
233 oxidases accept the electrons from ubiquinone, transferring them to molecular oxygen, the final
234 electron acceptor, forming water (Figure 1). AAB have two terminal ubiquinol oxidases,
235 designated cytochrome *bo*₃ ubiquinol oxidase and cytochrome *bd* quinol oxidase. Cytochrome
236 *bo*₃ ubiquinol oxidase catalyses a reaction which contributes to the generation of a proton-
237 motive force while cytochrome *bd* quinol oxidase does not. An important function of the latter

238 terminal oxidase is believed to be the re-oxidation of ubiquinol to ubiquinone, thus rapidly
239 regenerating ubiquinone that can contribute to further reactions in the respiratory chain or in
240 the reactions of oxidative fermentation. In addition, it has been found that, in *G. oxydans*, the
241 cytochrome *bd* quinol oxidase is particularly active at low pH (Hanke et al., 2012). Thus, it is
242 suggested that cytochrome *bo*₃ oxidase may serve as a major terminal oxidase at the early
243 growth phase, when the culture pH is closer to neutral, and when the pH is decreased as a result
244 of the production of a large amount of oxidized products, cytochrome *bd* quinol oxidase
245 maintains oxidative fermentation under acidic conditions by complementing the function of
246 cytochrome *bo*₃ oxidase (Miura et al., 2013).

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

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

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

271

272 • Oxidative fermentation

273 “Oxidative fermentation” is a process of incomplete oxidation of substrates which are oxidised
274 by primary dehydrogenases of a respiratory chain, with the concomitant release of oxidised
275 products into the surrounding medium. Bacteria capable of performing oxidative fermentation
276 are termed “oxidative bacteria”. Acetic acid bacteria are most prominent of such bacteria and
277 commonly oxidise ethanol, carbohydrates and sugar alcohols to the various corresponding
278 products such as organic acids, aldehydes and ketones (Table 4) (Matsushita & Matsutani,
279 2016). Oxidative fermentation can be considered as an “overflow metabolism”, from which
280 lower amounts of energy are conserved compared to if the substrates were completely oxidised
281 to CO₂ and water by aerobic respiration (Deppenmeier & Ehrenreich, 2009). Examples of
282 oxidative fermentation reactions include the conversion of ethanol to acetic acid (carried out
283 by almost all genera of AAB and from where these bacteria get their name), and the conversion
284 of glucose via glucono delta-lactone to gluconic acid/gluconate (GA), amongst others. Oxygen
285 availability is of prime importance and profoundly affects the fermentation rate and
286 productivity (Gullo, Verzelloni, & Canonico, 2014). The high accumulation of acidic products

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

289 Oxidative fermentation reactions are performed by respiratory chains in AAB that are similar
290 to the respiratory chains discussed above that oxidise reduced electron carriers (i.e.
291 nicotinamide adenine dinucleotide phosphate [NAD(P)]-dependent dehydrogenases) and
292 reduce ubiquinone (Figure 1). That is, in the process, electrons are channelled by ubiquinone
293 to molecular oxygen at terminal oxidases while protons are abstracted to create a proton-motive
294 force. This proton-motive force could be used by ATP synthase, or to perform some other
295 energetic work. However, the dehydrogenase enzymes in the respiratory chains involved in
296 oxidative fermentations are enzymes which specifically oxidise substrates such as ethanol,
297 carbohydrates and sugar alcohols (as opposed to reduced electron carriers such as NADH).
298 These enzymes contain a prosthetic group and are typically either PQQ (pyrroloquinoline
299 quinone)-dependent dehydrogenases (quinoproteins and quinoprotein-cytochrome c
300 complexes) or FAD-dependent dehydrogenases (flavoprotein-cytochrome c complexes), some
301 of which work on the same substrate but produce different oxidation products (Adachi et al.,
302 2003) (Table 4). MCD (methylsuccinyl-CoA dehydrogenase)-dependent dehydrogenases
303 (molybdoprotein-cytochrome c complexes) have also been identified in some genera (Turner,
304 Vela, Thöny-Meyer, Meile, & Teuber, 1997). The cytochrome subunit of these dehydrogenases
305 is responsible for the transfer of electrons to, and thus reduction of, ubiquinone to ubiquinol
306 (Matsushita, Yakushi, Toyama, Shinagawa, & Adachi, 1996). In addition, these respiratory
307 chains are located on the periplasmic face of the cytoplasmic membrane, while those involved
308 in the oxidation of reduced electron carriers are located in the cytosol of the cell. The cytosolic
309 NAD(P)-dependent dehydrogenases have no role in oxidative fermentation. Indeed, it appears
310 that the cytosolic respiratory chain competes with the oxidative fermentation (periplasmic)
311 respiratory chains regarding electron transfer and energetics and that both forms of respiration

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

316

317 *Ethanol oxidation*

318 One of the most well-known and important oxidative fermentation reactions performed by
319 AAB is the production of acetic acid (vinegar) from ethanol. Both *Acetobacter* and
320 *Komagataeibacter* species have a strong ability to produce acetic acid and both genera also
321 show high resistance to high ethanol and acetic acid levels, which are important traits for
322 industrial vinegar production (Taban & Saichana, 2017). Ethanol oxidation is catalysed by two
323 membrane-bound enzymes located on the outer surface of the cytoplasmic membrane
324 (periplasmic side). Ethanol is first oxidized to acetaldehyde by a PQQ-dependent alcohol
325 dehydrogenase (ADH) and acetaldehyde is further oxidized to acetic acid by aldehyde
326 dehydrogenase (ALDH). The prosthetic group of ALDH has been shown to be different
327 between genera, being either PQQ- or MCD-dependent (Gómez-Manzo et al., 2010; Thurner
328 et al., 1997). ADH is stable over a broad pH range of 2.3 to 8.0 and retained more than 90%
329 activity when incubated on ice for 30 minutes. ALDH, while also stable at acidic pHs (optimum
330 pH 4 – 5, but can also function at lower pH values), is more heat stable than ADH, retaining
331 more than 50% activity after 30 min at 60°C (Kanchanarach et al., 2010a). However, ALDH is
332 sensitive to the level of oxygen present; when this is either too low or too high its activity falls,
333 allowing acetaldehyde to accumulate in the medium (Mamlouk & Gullo, 2013; Rubio-
334 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).

359 When ethanol has been completely oxidised and depleted, some genera of AAB, namely
360 *Acetobacter*, *Gluconacetobacter* and *Komagataeibacter*, can assimilate acetic acid and oxidise
361 it completely to CO₂ and water using the citric acid cycle and glyoxylate shunt, which is known
362 as acetate “overoxidation” (Sievers & Swings, 2005). There also appears to be an irreversible
363 metabolic change, after which they are unable to oxidize ethanol again; this is evidently
364 unfavourable in vinegar production as it leads to lower acetic acid yields (Raspor & Goranovic,
365 2008). *Gluconobacter* and some other AAB genera do not overoxidise acetate as they lack key
366 enzymes in the citric acid cycle and glyoxylate shunt (Deppenmeier & Ehrenreich, 2009;
367 Mamlouk & Gullo, 2013). Because of this difference in oxidative potential, gluconobacters are
368 sometimes referred to as “under-oxidisers” and acetobacters (and gluconacetobacters) as “over-
369 oxidisers” (Bartowsky & Henschke, 2008). Overoxidation can be avoided if a small proportion
370 of ethanol is maintained in the medium (Raspor & Goranovic, 2008).

371

372 *Oxidation of carbohydrates and carbon metabolism in Gluconobacter oxydans*

373 Catalysed by various periplasmic dehydrogenases involved in oxidative fermentation, AAB
374 oxidise a number of carbohydrates to their corresponding carboxylic acids, obtaining metabolic
375 energy in the process through the ultimate generation of a proton-motive force, as described
376 above. As mentioned, while *Acetobacter* and *Komagataeibacter* are efficient at ethanol
377 oxidation, *Gluconobacter* species are particularly proficient in carbohydrate and sugar alcohol
378 oxidation (Matsushita & Matsutani, 2016). The production of GA and associated
379 ketogluconates from glucose by *Gluconobacter oxydans* will be described below to illustrate
380 carbohydrate oxidation in AAB, followed by a description of carbon metabolism in *G. oxydans*.
381 Gluconic acid and its salts has wide application in various industries such as the food,
382 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

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

421 Catabolism of GA and associated ketogluconate products is possible only via the PPP and EDP
422 because AAB do not have the enzyme, phosphofructokinase, and thus glycolysis is not active.
423 In addition, while most AAB genera can completely oxidise substrates to CO₂ and H₂O in the
424 citric acid cycle, in *Gluconobacter* this cycle is incomplete as they lack the enzymes succinyl-
425 CoA synthetase and succinate dehydrogenase (Bringer & Bott, 2016).

426 While the metabolism of intracellular, assimilated glucose and gluconate, primarily via 6-
427 phosphogluconate, may involve the PPP, EDP and citric acid cycle, the majority of energy
428 gained from intracellular metabolism is derived from the oxidative PPP, whereas the EDP is
429 dispensable. Carbon labelling of glucose has demonstrated the carbon flux during cellular
430 metabolism of glucose in *G. oxydans*, showing that the majority of energy is derived from the
431 PPP which operates in a cycle (Hanke et al., 2013). The key enzymes in the cycle are glucose-
432 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase; deletion of 6-

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

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

442

- 443 • Tolerance to acidic environments

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

457 vinegar fermentation processes with acidities as high as 15 - 20% (Andrés-Barrao & Barja,
458 2017; Emde, 2006).

459 Acetic acid is a particularly effective antimicrobial, because at a relatively high pH (pH 4.7 =
460 pKa of acetic acid) it exists primarily in its undissociated form and can enter the cell
461 (Hirshfield, Terzulli, & O'Byrne, 2003). The properties of undissociated organic acids such as
462 fat-solubility and neutral charge enable them to passively diffuse through the cell membrane
463 of the target microorganism; in the cytoplasm the higher intracellular pH causes the acid to
464 become dissociated, producing primarily hydrogen ions (H⁺), but also acetate ions (CH₃COO⁻
465). These ions are toxic to the cell and interfere with cellular processes such as enzyme activity,
466 DNA replication and transcription, and protein expression, therefore effecting the normal
467 growth of the microorganism (Chen, Chen, Giudici, & Chen, 2016; Russell & Diez-Gonzalez,
468 1997).

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

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

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

494 As described above, when ethanol has been completely oxidised and depleted, some genera of
495 AAB assimilate acetic acid and oxidise it completely to CO₂ and water using the citric acid
496 cycle and glyoxylate shunt (acetate “overoxidation”) (Sievers & Swings, 2005). Thus,
497 overoxidation is a mechanism by which the intracellular acetic acid level can be decreased,
498 whilst also gleaning energy from the process through the oxidative reactions of the citric acid
499 cycle. In addition, acetic acid may be assimilated from the environment causing the pH to rise.
500 In both cases, the effect is essentially a detoxification of acetic acid and reduced acid stress on
501 the bacterium. A fully functioning citric acid cycle is necessary for acetic acid overoxidation,
502 which has been found to be the case for the most acid tolerant AAB such as *Acetobacter* and
503 *Komagataeibacter*; other genera such as *Gluconobacter* do not possess a complete citric acid
504 cycle which maybe an important factor in explaining their comparatively lower tolerance to
505 acetic acid (Mamlouk & Gullo, 2013; Prust et al., 2005). In addition, this lower acid tolerance

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

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

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

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

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

550 **Cellulose and other exopolysaccharides produced by AAB**

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

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

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

574 sufficiently high levels of oxygen in the air, required for their oxidative metabolism (Tonouchi,
575 2016). As described above, EPS production may contribute to acetic acid tolerance. In addition,
576 in natural environments such as rotting fruit, cellulose production may protect the bacteria from
577 predation or from the damaging effects of UV light or desiccation through moisture loss.
578 Cellulose production appears to be variable in *G. oxydans*, with some strains capable of
579 producing it having been identified previously (Jia et al., 2004; Valera, Torija, Mas, & Mateo,
580 2015). In a screening of 77 different AAB strains, representing 35 different species, Valera et
581 al. (2015) found that of the three *G. oxydans* strains examined, two produced cellulose on all
582 the six different media used to investigate its production. Both of these strains were isolates
583 from beer (Valera et al., 2015). However, yields of cellulose from *G. oxydans* are much lower
584 than those of *Acetobacter xylinum* (presently *K. xylinus*) (Jia et al., 2004). This could be due to
585 the fact that in AAB, both cellulose production and glucose oxidation (to GA and
586 ketogluconates) are in competition for the same glucose as substrate. Thus, in *G. oxydans*,
587 which has a particularly high oxidative capacity for carbohydrates, the majority of glucose may
588 be oxidised to GA, resulting in low availability for cellulose production. In contrast, *K. xylinus*
589 may utilise the majority of glucose for cellulose production (De Muynck et al., 2007). Indeed,
590 Keshk and Sameshima (2006) demonstrated that productivity of BC from *Ga. xylinus* (now *K.*
591 *xylinus*) increased significantly in the presence of liginosulfonate, an antioxidant compound that
592 inhibited the formation of GA. In its presence, one strain of *Ga. xylinus* produced up to 16 g/L
593 BC (Keshk & Sameshima, 2006). A similar effect was also observed in the presence of Vitamin
594 C, which is also an antioxidant compound (Keshk, 2014).

595 BC is synthesised from a variety of carbohydrates and organic acids by AAB (Serrato et al.,
596 2013). The cellulose biosynthesis pathway can be considered as an off-shoot of the PPP.
597 Glucose is the polymeric unit that cellulose is composed of, therefore, other substrates must be
598 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 arabinol, 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 & Gökşungur,

624 2017). A number of agro-industrial waste by-products have been investigated for use in BC
625 production, particularly those high in sucrose, fructose, nitrogen and vitamins (Castro et al.,
626 2011).

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

639 Due to physicochemical properties such as structure-forming potential, high purity and bio-
640 compatibility, BC has numerous potential applications, for example in the medical (e.g. as an
641 artificial skin) and food industries (Tonouchi, 2016). Due to the scope of this review, only those
642 applications relevant to the food industry will be described briefly below. Many current
643 applications continue to employ plant-derived cellulose and it is used in food as a thickening,
644 gelling, stabilising or water-binding agent (Shi, Zhang, Phillips, & Yang, 2014). For example,
645 cellulose and its derivatives (known in the food industry as cellulose derivatives) such as
646 carboxymethylcellulose (CMC) and hydroxypropylmethylcellulose (HPMC) are important in
647 the ice-cream and bakery industries, being used as a stabilising agent and a texture-modifying
648 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

697 (Lynch, Coffey, & Arendt, 2018b). To date, EPS-producing strains of LAB have mainly been
698 investigated for such application, but, in recent years AAB have become of increasing interest
699 in this area (Jakob et al., 2012). Application by Jakob et al. (2012) of purified levan (1 - 2%
700 w/w) from AAB to a wheat bread system increased the bread volume, softened the crumb and
701 retarded staling (Jakob et al., 2012). Furthermore, in sucrose-supplemented spelt dough, a strain
702 of *Kozakia baliensis* has been shown to produce up to 49 g/kg (of flour) EPS, followed by 33
703 g/kg in whole wheat, and 32–36 g/kg in wheat and rye doughs (Hermann, Petermeier, & Vogel,
704 2015). Ua-Arak et al. (2016) also demonstrated their potential for in-situ application and ability
705 to grow and produce EPS in a gluten-free (buckwheat) sourdough system, with levan
706 productivity of between 16 and 20 g/kg, in molasses-supplemented flour (Ua-Arak, Jakob, &
707 Vogel, 2016). In a later study, the application of this sourdough improved the bread sensory
708 and quality parameters, which included increasing specific volume and increasing crumb
709 softness. However, the positive effects of sourdough application were masked by the impact of
710 the natural acidification during fermentation. Thus, while the ability of AAB to produce a large
711 quantity of high molecular mass levan is attractive and shows potential in bakery applications,
712 a challenge remains due to the necessity for high levels of oxygen for growth during in-situ
713 sourdough fermentation, coupled with the strong acidification (Ua-Arak, Jakob, & Vogel,
714 2017).

715

716 *Dextran*

717 Dextran is a homoexopolysaccharide composed of glucose units (i.e. a glucan) linked via α -
718 1,6 glycosidic bonds (at least 50%) and variable amounts of α -1,4, α -1,3 and α -1,2 linkages,
719 with or without branching. Dextrans are principally produced by species of LAB such as
720 *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 intracellular and extracellular 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).

739 **Vinegar fermentation**

740 *Legal definition of vinegar*

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

746

747 *History of vinegar*

748 The history of vinegar production, which dates back more than 10,000 years (Johnston & Gaas,
749 2006) represents a keystone example of microbial biotransformation. Vinegar, from the French
750 word, *vinaigre*, meaning “sour wine” that in turn came from the Latin, *vinum acetum*, “wine
751 vinegar” has always been considered the “poor relation” among fermented foods: it is not
752 considered a “food”, nor does it have high nutritional value, and it is made through the
753 biotransformation of richer, more nutritive products. Vinegar can be produced using almost
754 anything that is a source of fermentable carbohydrate, including wine, molasses, beer, various
755 fruits, honey or whey (Johnston & Gaas, 2006). Hippocrates of Kos (460-377 BC), father of
756 modern medicine, recommended vinegar as a treatment for a number of diseases including the
757 common cold and cough (Food: A Culinary History, 2000) and for the treatment of sores
758 (Johnston & Gaas, 2006). The great military leader, Hannibal of Carthage (c. 200 BC)
759 reportedly used vinegar to dissolve rocks that blocked his army's path over the Alps. Pliny the
760 Elder (AD 23-79), recounts that the Egyptian Queen, Cleopatra (c. 50 BC) made a bet with the
761 Roman general Mark Anthony that she could host the most expensive banquet costing ten
762 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

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

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

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

865 Submerged culture systems provide a much faster alternative and involve the suspension of
866 AAB in the acetifying medium and application of stringent aeration to meet the high oxygen
867 demand. The oxidative process occurs at the liquid-air interface of the bubbles, where the AAB
868 convert ethanol into acetic acid, with limited production of other metabolites. The most
869 successful, commonly applied submerged culture system is the Acetator®, built and sold by
870 Heinrich Frings GmbH & Co. (Bonn, Germany). A significant design feature is its self-priming
871 aerator, which provides for highly efficient dispersion of air in the liquid substrate (Adams,
872 1998).

873 This submerged method is more straightforward than the traditional method, consisting of
874 application of the raw material and inoculum to the fermenter, the actual fermentation step, and
875 final unloading of the bioprocessed product (Figure 4). A portion of the product is left in the
876 vessel to act as starter culture for the next cycle (Tesfaye, Morales, García-Parrilla, &
877 Troncoso, 2002). This method was introduced for the production of vinegar at the beginning
878 of 20th century and is today employed for the production of most commercial vinegars of high
879 consumption (Tesfaye et al., 2002). A disadvantage of this rapid method is that the high airflow
880 leads to significant stripping of the volatile components from the original substrate, producing
881 a more organoleptically limited product. Despite this, the rapidity of the process (vinegar can
882 be produced in 24-hour cycles) and the high acidity achievable (acetic acid levels of up to 23–
883 25%, compared to 6–13% with traditional systems) are key advantages. Higher acidity makes
884 transportation more cost-effective by reducing water transport.

885 **Role of AAB in other fermented beverages**

886 *Kombucha tea*

887 Kombucha tea is traditionally prepared with water, tea, sugar and a kombucha culture (“tea
888 fungus”) in open vessels at room temperature for 1–3 weeks. This non-alcoholic, fermented
889 beverage has a sharp acidity and specific flavour (De Roos & De Vuyst, 2018a). It is consumed
890 traditionally in Eastern Europe and Asia. It is noteworthy that in Japan, the term “konbu-cha”
891 can also refer to an unfermented beverage prepared with brown algae (*Laminaria* kelp).
892 Consumption of kombucha was first recorded in 220 BC in Manchuria. More than one thousand
893 years ago “tea fungus” was already consumed in Japan, China and India; then in Russia, Poland
894 and the Baltic States starting in about 1915; in the Balkans, Germany and Eastern Europe
895 around 1925; and in Spain, Italy, France and Switzerland in about 1955 (Kraft, 1959). Similar
896 to other traditional beverages, the popularity of kombucha increased due to its purported
897 beneficial health effects and its ease of preparation. To prepare a basic kombucha ferment, tea
898 leaves are steeped in boiling water, 5 - 15% w/v of sucrose is added, and the mixture is brought
899 to room temperature. A “mother” kombucha pellicle, produced from a previous kombucha
900 fermentation, is placed into the tea along with liquid from a previous ferment (10 - 20% v/v).
901 This pellicle is sometimes referred to as a symbiotic culture of bacteria and yeasts (SCOBY),
902 and is comprised of primarily AAB and ethanol-forming yeasts in a thick cellulose pellicle
903 (Figure 5). The pellicle floats on the liquid surface, at the air interface and grows vertically,
904 increasing biomass with cellulose striations as the fermentation matures. After 1 - 3 weeks
905 incubation at 20 - 30°C, the tea becomes a sweet and sour, naturally carbonated beverage due
906 to microbial activity (Chen & Liu, 2000; Jayabalan, Malbaša, Lončar, Vitas, & Sathishkumar,
907 2014; Malbaša et al., 2006).

908

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

917 The microbial composition of kombucha varies depending on the source of the inoculum. The
918 basic requirements for a kombucha ferment are at least one cellulose-producing AAB, and at
919 least one yeast that can split sucrose into glucose and fructose. Multiple microorganisms can
920 be present in a kombucha ferment, performing these essential roles along with producing
921 additional secondary metabolites that contribute to the final beverage (Jarrell, Cal, & Bennett,
922 2000; Mayser, Fromme, Leitzmann, & Gründer, 1995; Roussin, 1996). The most frequently
923 isolated genera of bacteria from kombucha are *Acetobacter*, *Komagataeibacter*,
924 *Gluconacetobacter* and *Lactobacillus* (Chen & Liu, 2000; Trovatti, Serafim, Freire, Silvestre,
925 & Neto, 2011). The predominant AAB found in kombucha ferments are *K. xylinus*, *A.*
926 *pasteurianus*, *A. aceti*, and *G. oxydans* (Liu, Hsu, Lee, & Liao, 1996; Marsh, O'Sullivan, Hill,
927 Ross, & Cotter, 2014). *K. xylinus* – previously known as *Ga. xylinus* (Yamada et al., 2012),
928 and *A xylinum* (Yamada, Hoshino, & Ishikawa, 1997) – are the most abundant (80 - 99%)
929 prokaryotes in kombucha (Chen & Liu, 2000; Marsh et al., 2014) and are responsible for the
930 formation of the cellulosic pellicle (Chen & Liu, 2000; Zhu, Li, Zhou, Lin, & Zhang, 2014).
931 Other AAB species have potential for both cellulose production and nitrogen fixation
932 (Cleenwerck, De Wachter, González, De Vuyst, & De Vos, 2009; Dutta & Gachhui, 2007; Tan,
933 Ren, Cao, Chen, & Tang, 2012).

934

935 *Water kefir*

936 Water kefir is a fermented, low-alcohol beverage with acidic and fruity flavours (De Roos &
937 De Vuyst, 2018a). It is produced via spontaneous fermentation of a water solution containing
938 approximately 8% (w/v) sucrose, (dried) fruits (e.g., figs), and water kefir grains (these
939 “grains” are essentially a polysaccharide mass encapsulating a complex microbial association
940 of bacteria and yeasts which serve as a starter culture) in a closed container at room temperature
941 for 2–4 days (Figure 6) (Fiorda et al., 2017; Laureys & De Vuyst, 2014). Currently the main
942 market for this beverage is in the USA, Japan, France, and Brazil, where it is consumed for its
943 reported functional properties (Fiorda et al., 2017). The water kefir grain inoculum, and the
944 nature of the substrate (such as the type of fruit used) determines the grain growth, the microbial
945 species diversity, the metabolites formed and their concentrations (Laureys, Aerts, Vandamme,
946 & De Vuyst, 2018; Laureys & De Vuyst, 2017; Laureys, Van Jean, Dumont, & De Vuyst,
947 2017). In general, the microbiota of water kefir is known to be a stable consortium of different
948 LAB (such as *Lactobacillus*, *Leuconostoc*), AAB (*Acetobacter*, *Gluconacetobacter*, and
949 *Gluconobacter*) and yeasts (*Kluyveromyces*, *Brettanomyces*, *Pichia*, and *Saccharomyces*). A
950 strong symbiosis between these three microbial groups is documented (Fiorda et al., 2017).
951 Yeast metabolism promotes the growth of acidophilic bacterial species such as LAB and AAB.
952 Glucose and fructose are made available for LAB growth through the action of yeast invertase
953 on sucrose. Ethanol produced by yeasts may be metabolised to acetic acid by any viable AAB
954 present (Magalhaes, Pereira, Dias, & Schwan, 2010). Growth of AAB particularly takes place
955 under aerobic conditions, leading to increased acetic acid content, which may be unwanted
956 (Gulitz, Stadie, Ehrmann, Ludwig, & Vogel, 2013; Laureys et al., 2017; Marsh, O'Sullivan,
957 Hill, Ross, & Cotter, 2013). Under anaerobic conditions they remain in a viable but non-
958 culturable (VBNC) state, being metabolically dormant, but can start to grow when oxygen

959 becomes accessible (Laureys et al., 2017). While several species from the genera *Acetobacter*,
960 *Gluconacetobacter*, and *Gluconobacter* have been recovered from water kefir fermentation
961 processes, *Acetobacter* species seem to be best adapted to this ecosystem (Laureys & De Vuyst,
962 2014; Laureys et al., 2017; Magalhaes et al., 2010; Marsh et al., 2013).

963

964 *Lambic beer*

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

972 Lambic beer is the result of a spontaneous fermentation process of a barley and unmalted wheat
973 extract (wort) that continues for 1 - 3 years (De Keersmaecker, 1996). The fermentation process
974 is not driven by yeasts or bacteria applied as starter cultures, but by a spontaneous inoculum
975 from the environment. Microbial growth begins during the cooling of the boiled wort which
976 occurs overnight in a shallow open vessel, known as a cooling tun or coolship. These beers are
977 traditionally brewed close to the Senne river valley, near Brussels, Belgium (Spitaels et al.,
978 2014a). The following morning the cooled wort is assumed to have been inoculated with the
979 specific air microbiota of this region and is transferred into wooden casks which are stored at
980 cellar or ambient temperatures, i.e., typically between 15 and 25°C. The wort then ferments
981 and matures in these casks. In addition to inoculation from the environment during the coolship
982 step, microorganisms present on the interior surfaces of the casks also contribute to the
983 fermenting wort, helping to establish a stable microbial community (De Roos, Van der Veken,
984 & 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).

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

1000 Apart from their historical and key role in the production of fermented foods such as vinegar,
1001 AAB are also important for the production of useful compounds that find application in the
1002 food industry. The formation of GA by *Gluconobacter oxydans*, and their role in the process
1003 of Vitamin C synthesis are discussed here.

1004

1005 *Gluconic acid production and regulation*

1006 The applications of GA in the food industry include use as an acidity regulator (E574–E580),
1007 with raising, sequestering and flavour-enhancing properties. Gluconic acid enhances the
1008 sensory characteristics of foods by imparting a bitter but refreshing taste. Gluconic acid and
1009 its derivative glucono- δ -lactone are also used as food preservatives. Its ketogluconate, 5-KGA,
1010 also has important uses, including as a precursor in the production of tartaric acid, xylaric acid,
1011 as well as for Vitamin C production (Cañete-Rodríguez et al., 2016). Two methods are
1012 commonly employed for the biotechnological production of GA, use of the fungus *Aspergillus*
1013 *niger*, or use of an AAB strain, primarily *G. oxydans*. The biochemistry of the production of
1014 GA and associated ketogluconates has been described above. This section will examine the
1015 factors influencing GA production in *G. oxydans*. This subject was reviewed recently by
1016 García-García et al. (2017) and will be discussed briefly below (García-García et al., 2017).
1017 The main factors which affect the production of GA include, in decreasing order of importance,
1018 pH, initial concentration of glucose in the medium, concentration of calcium carbonate added
1019 to the medium and the dissolved oxygen level.

1020 Using *G. oxydans* 621H it was found that below pH 3.5 - 4 uptake and assimilation of GA into
1021 the PPP is almost completely inhibited (Olijve & Kok, 1979). This is likely to be related to the
1022 pH optima for the various enzymes involved in these processes – the periplasmic

1023 dehydrogenases involved in oxidative fermentation have pH optima in the acidic range of pH
1024 3 – 6, while those cytoplasmic (NAD(P)-dependent) dehydrogenases have optima in the
1025 alkaline range of pH 8 – 11 (García-García et al., 2017). Therefore, the production of GA and
1026 associated pH drop may promote its own production and accumulation as the periplasmic
1027 dehydrogenases become more active. As outlined above, GA can be further oxidised to
1028 ketogluconates such as 2KGA and 5KGA, thus reducing the GA yield. Olijve and Kok (1979)
1029 and Weenk et al. (1984) found glucose to be rapidly oxidized virtually quantitatively to GA
1030 without formation of any ketogluconates if the pH of the fermentation was uncontrolled (which
1031 lead to a rapid pH drop), or if the pH was adjusted to 2.5 at the beginning of the fermentation
1032 (Olijve & Kok, 1979; Weenk, Olijve, & Harder, 1984). However, control of the pH at 5.5, or
1033 the addition of calcium carbonate to the medium, lead to 2KGA and 5KGA formation once all
1034 initial glucose had been utilised (Weenk et al., 1984). The presence of calcium carbonate in the
1035 medium promotes ketogluconate production (Beschkov, Velizarov, & Peeva, 1995). It has been
1036 postulated that, in the absence of pH control, the formation of ketogluconates may be
1037 completely inhibited (Beschkov et al., 1995; Velizarov & Beschkov, 1994).

1038 The initial glucose concentration also strongly influences the production of GA and
1039 ketogluconates. Olijve and Kok (1979) found that high glucose concentrations (0.9 – 2.7 g/L)
1040 led to rapid GA accumulation, while at lower concentrations the assimilation of glucose and
1041 metabolism in the PPP was favoured (Olijve & Kok, 1979). This is understandable from an
1042 ecological viewpoint when considering the level of glucose in the environment. If the
1043 concentration is low, AAB will preferentially assimilate the glucose for biomass production;
1044 in contrast, in the case of high glucose concentrations, the majority of it will be oxidised to
1045 gluconate and ketogluconates, thus making it unavailable for competing microorganisms, while
1046 also lowering the pH of the environment (García-García et al., 2017). Because GA is produced
1047 from the oxidation of glucose and the amount which is formed is directly proportional to the

1048 initial glucose concentration, it is logical that above a certain concentration of glucose, the
1049 amount of GA formed will become inhibitory due to the resultant low pH, thus preventing
1050 further production. Thus, a glucose concentration above 90 g/L led to a reduced rate and yield
1051 of GA compared to a lower glucose concentration. Long lag phases were observed due to the
1052 combined effect of high glucose concentration and the low pH due to GA formation (Velizarov
1053 & Beschkov, 1994, 1998). High glucose concentrations (~90 g/L) also favoured GA production
1054 over ketogluconate formation (Beschkov et al., 1995; García-García et al., 2017).

1055 Compared to the effects of pH and glucose concentration, dissolved oxygen (DO) has a less
1056 important role on the production of GA. However, Buse et al. (1992) found that DO control
1057 had a significant impact on the formation of 2,5-diKGA in *G. oxydans* ATCC 9937 (formerly
1058 *Gluconobacter oxydans* subsp. *melanogenum*). This was related to changes in the activity of
1059 the enzyme gluconate dehydrogenase (the first enzyme in the conversion of GA to
1060 ketogluconates) at different oxygenation levels. Low oxygen (<30%) delayed the production
1061 of this enzyme (Buse, Qazi, & Onken, 1992). Thus, low DO levels appear to inhibit GA
1062 conversion to ketogluconates and it is conceivable that relatively low DO levels may have a
1063 similar effect in *G. oxydans*.

1064

1065 *Vitamin C production and potential for direct formation by G. oxydans*

1066 Since 1934 and until the late 1990s, the “Reichstein process” has been used as the main process
1067 for the production of Vitamin C, also known as L-ascorbic acid (Asc), of which more than
1068 110,000 tones are produced annually (Bremus, Herrmann, Bringer-Meyer, & Sahm, 2006).
1069 This process, although refined and improved over the years, contains a number of chemical
1070 steps, and only a single microbially-catalysed step, and is highly energy intensive. Therefore,
1071 it has been replaced, particularly in China, by the so-called (Classical) Two-Step Fermentation

1072 Process which is less costly and more environmentally friendly (Yang & Xu, 2016). Figure 7
1073 shows the various routes for Asc production; while different processes have been studied, the
1074 Two-Step Fermentation process is today the primary method used for industrial Asc production
1075 (Wang et al., 2018), with China supplying 80% of the global demand. Royal DSM remains the
1076 sole Western Vitamin C producer (Pappenberger & Hohmann, 2014). Each of the routes to
1077 Asc, particularly with reference to the microorganism involved and especially *G. oxydans*, will
1078 be discussed further below.

1079 D-glucose, D-sorbitol or D-sorbose can be considered as common starting materials for the
1080 process; however, glucose must be chemically converted to sorbitol which is typically via
1081 hydrogenation (Yang & Xu, 2016). Central in each process is the production of the precursor,
1082 2-keto-L-gulonic acid (2-KLGA). For a detailed treatment of this topic, readers are also
1083 referred to the recent review by Wang et al. (2018) (Wang et al., 2018).

1084 The Reichstein process: This process consists mainly of chemical reactions for Asc synthesis,
1085 with only one microbially-catalysed step. This is the oxidation of D-sorbitol to L-sorbose and
1086 which is performed by AAB due to their very efficient oxidation of carbohydrates and sugar
1087 alcohols. Originally this bioconversion was performed by *Acetobacter aceti* subsp. *xylinum*
1088 (now *Komagataeibacter xylinus*); subsequently *Gluconobacter suboxydans* (now *G. oxydans*)
1089 was introduced due to its greater capacity for oxidation and has been employed to date, with
1090 an almost 100% conversion rate on an industrial scale (Pappenberger & Hohmann, 2014; Yang
1091 & Xu, 2016). This is a key reaction, common to each route to Asc production, as can be seen
1092 in Figure 7. The key enzyme mediating the conversion of D-sorbitol to L-sorbose is glycerol
1093 dehydrogenase (GLDH), a PQQ-dependent membrane protein. This enzyme is also known as
1094 D-sorbitol dehydrogenase (SLDH), but it is a major polyol dehydrogenase in *G. oxydans* with
1095 broad substrate specificity for other sugar alcohols besides sorbitol, such as D-mannitol, D-
1096 arabitol, meso-erythritol, D-adonitol and glycerol (Figure 1) (Matsushita et al., 2003; Sugisawa

1097 & Hoshino, 2002). A second membrane-bound sorbitol oxidising enzyme, which is specific for
1098 sorbitol, has been found in *G. oxydans* and other *Gluconobacter* species but is FAD-dependent
1099 i.e. FAD-SLDH (Shinjoh & Toyama, 2016). Genome analysis of the industrial strain *G.*
1100 *oxydans* H24 identified three sorbitol oxidising enzymes, two membrane bound and one
1101 cytoplasmic, namely PQQ-SLDH (PQQ-GLDH), FAD-SLDH and NADP-SLDH, respectively
1102 (Ge et al., 2013). Among these, PQQ-GLDH is believed to play the primary role in converting
1103 D-sorbitol to L-sorbose (Matsushita et al., 2003).

1104 Two-Step Fermentation process: This process can be considered as an improvement on the
1105 Reichstein process. Two fermentations are performed in this process; the first, the
1106 bioconversion of D-sorbitol to L-sorbose is the same as that of the Reichstein process and is
1107 performed by *G. oxydans*. The second fermentation involves the conversion of sorbose to the
1108 Asc precursor, 2-KLGA. The subsequent transformation of 2-KLGA into Asc is performed
1109 through a number of chemical steps, as for the Reichstein process. Therefore, it is the second
1110 fermentation that differentiates the Two-Step Fermentation process from the Reichstein
1111 process (Yang & Xu, 2016). This fermentation is performed by a two-strain co-culture system,
1112 neither of which is actually an AAB. This dual culture consists of the 2-KLGA-producing strain
1113 and a “helper” or companion strain which promotes the growth of the 2-KLGA producer. Early
1114 studies identified the 2-KLGA producer as *G. oxydans*, but it was later renamed
1115 *Ketogulonicigenium vulgare*, a Gram-negative, facultatively anaerobic, chemoheterotrophic
1116 soil microorganism (Urbance, Bratina, Stoddard, & Schmidt, 2001). The companion strain,
1117 typically a species of *Bacillus*, is considered to stimulate *K. vulgare* growth and 2-KLGA
1118 accumulation by releasing particular metabolites (Feng, Zhang, & Zhang, 2000). *B.*
1119 *megaterium* and *B. cereus* were the primary companion strains applied in industrial Asc
1120 fermentation, and while many spore-forming strains have been found to be suitable as
1121 companion strains, only *K. vulgare* has been used in industrial fermentation so far (Feng et al.,

1122 2000; Jiao, Zhang, Xie, Yuan, & Chen, 2002; Urbance et al., 2001). Two enzymes in *K. vulgare*
1123 are key in the oxidation of L-sorbose to 2-KLGA. These are L-sorbose/L-sorbose
1124 dehydrogenase (SSDH) and L-sorbose dehydrogenase (SNDH). SSDH is a unique PQQ-
1125 dependent membrane dehydrogenase, with dual catalytic ability, catalysing not only the
1126 conversion of L-sorbose to L-sorbose but also that of L-sorbose to 2-KLGA (Asakura &
1127 Hoshino, 1999). In addition, the SNDH of *K. vulgare* has been found to catalyse the direct
1128 conversion of L-sorbose to Asc (Miyazaki, Sugisawa, & Hoshino, 2006). Thus, via the action
1129 of SSDH and SNDH it is possible for *K. vulgare* to directly produce Asc from L-sorbose and/or
1130 L-sorbose (Sugisawa, Miyazaki, & Hoshino, 2005). Genome sequencing of the industrial
1131 strain, *K. vulgare* Y25, found that it contained four genes encoding SSDH and one plasmid-
1132 encoded gene for SNDH (Liu et al., 2011). The absence of genes or operons for the biosynthesis
1133 of many amino acids, nucleotides and cofactors may explain its dependence on a companion
1134 strain. As stated, the primary microorganism in the Two-Step Fermentation process is not an
1135 AAB *sensu stricto*; however, homologous enzymes to those employed by *K. vulgare* have been
1136 found in *G. oxydans* (Shinjoh & Toyama, 2016), and the potential of this bacterium for the
1137 direct production of 2-KLGA and Asc is described next.

1138 Microbial production of 2-KLGA and Asc by *G. oxydans*: There has been interest in the further
1139 exploitation of *G. oxydans* in Asc production, beyond its use for sorbitol oxidation. This has
1140 been supported by the observation in recent decades of enzymes in certain strains of *G. oxydans*
1141 which perform similar bioconversions to those in *K. vulgare*, strengthening the possibility of,
1142 in the future, using a single microorganism for direct 2-KLGA or Asc production. Thus, strains
1143 of *G. oxydans* have been demonstrated to produce 2-KLGA from both D-sorbitol and L-
1144 sorbose, albeit at very low yields with wild-type strains. For example, *G. oxydans* NBRC3292
1145 (formerly *G. oxydans* IFO3292 and *G. melanogenus* ATCC15163) was shown to produce
1146 6.5g/L 2-KLGA from 50 g/L sorbitol over a 150 h fermentation time (Motizuki et al., 1962).

1147 A similar strain, *G. oxydans* NBRC3293 (formerly *G. oxydans* IFO3293 and *G. melanogenus*
1148 IFO3293) produced 2.8 g/L 2-KLGA from 25 g/L L-sorbose over 168 h fermentation
1149 (Sugisawa et al., 1990). A progenitor strain of IFO3293, designated SPO1, was isolated and
1150 produced 13g/L of 2-KLGA from 50 g/L L-sorbose. Subsequent strain improvement studies
1151 with strain SPO1 using mutagens such as UV irradiation resulted in the isolation of genetically
1152 modified strains producing 50 to 60 g/L 2-KLGA from 100g/L D-sorbitol or 100 g/L L-sorbose
1153 over 80 to 100 h. Particular mutant isolates arising from the above strain improvement studies,
1154 which have been subsequently extensively studied regarding Asc production by *G. oxydans*,
1155 are strains UV10 and N44-1 (Pappenberger & Hohmann, 2014). L-sorbose dehydrogenase
1156 (SDH) was identified in the mutant strain UV10. The enzyme was membrane bound and FAD-
1157 dependent with a high activity for L-sorbose (L-sorbose as product). In addition, L-
1158 sorbose itself was also identified as a substrate for SDH, with 2-KLGA as the product. Thus,
1159 this SDH in *G. oxydans* UV10 has very similar activity to the SSDH of *K. vulgare* described
1160 above. Similarly, a SNDH was identified in *G. oxydans* UV10, this enzyme being found in the
1161 cytosolic fraction and being NAD(P)-dependent, with 2-KLGA as the product (Hoshino,
1162 Sugisawa, & Fujiwara, 1991; Pappenberger & Hohmann, 2014). A second strain of *G. oxydans*,
1163 T-100, was subsequently shown to contain the same dehydrogenases (Saito et al., 1997). Based
1164 on genome analysis, homologs of the associated genes are present in many *Gluconobacter* sp.
1165 (Gao, Zhou, Liu, Du, & Chen, 2012; Wang et al., 2018). Thus, the formation of 2-KLGA in *G.*
1166 *oxydans* is mediated by either of two enzymes: the membrane bound SDH i.e. from L-sorbose
1167 or L-sorbose, or, the cytosolic SNDH, from L-sorbose. The more recent observation of
1168 small amounts of Asc production by *G. oxydans* NBRC3293 when provided with L-sorbose
1169 has led to the identification of a second type of L-sorbose dehydrogenase in *G. oxydans*. In
1170 contrast to the FAD-dependent cytosolic SNDH of *G. oxydans* UV10, this enzyme was
1171 membrane bound and PQQ-dependent and was designated SNDH_{ai} (Berry, Lee, Mayer, &

1172 Shinjoh, 2003). A homologous enzyme, designated SNDHak was identified in *K. vulgare*
1173 (Miyazaki et al., 2006). Although SNDHai uses L-sorbose as a substrate, which it converts
1174 directly to Asc, it displays much higher activity for the cyclic polyol, myo-inositol; thus, it has
1175 been suggested that L-sorbose oxidation by SNDHai may be a case of fortuitous cross-
1176 reactivity with the non-physiological L-sorbose (Pappenberger & Hohmann, 2014). As
1177 shown in Figure 7(d), as little as three periplasmic oxidation steps are required to convert
1178 sorbitol to Vitamin C – mediated by GLDH, SDH and SNDHai – all of which are endogenous
1179 *G. oxydans* enzymes (Pappenberger & Hohmann, 2016). Despite low yields of Asc, the
1180 identification of SNDHai has, nevertheless, increased the possibility that *G. oxydans* may, at
1181 some future point, be used as the sole microorganism for the direct production of Vitamin C.
1182 With respect to the yield of Asc, there are three challenges which need to be overcome. Firstly,
1183 the low affinity of SNDHai for L-sorbose, as described above. Secondly, the presence of the
1184 cytoplasmic SNDH in *G. oxydans* means that a certain proportion of the L-sorbose will be
1185 converted to 2-KLGA and therefore not to Asc. Thirdly, and most critically, is the stability of
1186 Asc once it is produced. This is primarily due to the reductive properties of Asc and its
1187 propensity to become oxidised, forming L-dehydroascorbic acid. If L-dehydroascorbic acid is
1188 not rapidly reduced to again form Asc, it spontaneously and irreversibly degrades with a half-
1189 life of minutes. In this way, Asc is oxidised by molecular oxygen, and this reaction is
1190 accelerated at above neutral pH and in the presence of trace amounts of transition metal ions.
1191 The challenge is that such trace metals are required as a growth factor and, moreover, the
1192 strictly oxidative metabolism of AAB means that the presence of molecular oxygen is clearly
1193 unavoidable (Pappenberger & Hohmann, 2016). Attempts to overcome such barriers have
1194 included the use of resting cells in media in the absence of detrimental substances or with the
1195 use of engineered strains. Using the latter approach, a near 90% substrate conversion and yields
1196 of 10 g/L Asc were achieved using a genetically engineered strain of *G. oxydans* that was over-

1197 expressing genes for SDH and SNDH_{ai} and also had a gene knockout for cytoplasmic SNDH
1198 (Pappenberger & Hohmann, 2016). Thus, it appears that at the current state of the science and
1199 knowledge, significant yields of Asc using (wild-type) *G. oxydans* alone are not yet possible
1200 without the use of engineered strains.

1201 It is noteworthy that *G. oxydans* has also the potential to be used for the biosynthesis of 2-
1202 KLGA via a different mechanism to the D-sorbitol pathway, that is, via the biosynthesis of GA
1203 and its associated ketogluconates - the 2,5-diKGA pathway (Figure 7(e)). In this system the
1204 bioconversion of glucose as substrate into 2-KLGA is performed by cell preparations
1205 containing the necessary complement of enzymes such as glucose dehydrogenase, D-gluconate
1206 and 2-keto-D-gluconate dehydrogenase, including cytochrome C as a co-factor. A final
1207 enzyme, 2,5-diKGA reductase, performs the conversion of 2,5-diKGA into 2-KLGA (Hancock
1208 & Viola, 2002). As has been discussed above, specific *G. oxydans* strains have the potential to
1209 produce 2,5-diKGA from glucose oxidation via GA. Indeed, resting cells of *G. oxydans* have
1210 been used as a source of gluconate dehydrogenase and 2-keto-D-gluconate dehydrogenase for
1211 2-KLGA formation via the 2,5-diKGA pathway (Hancock & Viola, 2002; Ji & Gao, 2001).
1212 However, most research into this pathway have used species of *Erwinia* and *Corynebacterium*
1213 as the enzyme sources (Wang et al., 2018).

1214 **Beneficial effects of AAB-fermented products**

1215 *Health benefits associated with vinegar consumption*

1216 Vinegar is today primarily used as a condiment or seasoning alone, or as a seasoning and
1217 preservative agent in salad dressings, mayonnaise, ketchup and similar condiment sauces for
1218 its desirable organoleptic properties. However, in historical times it was used medicinally, as
1219 described above (Budak et al., 2014). A number of beneficial effects on health have been
1220 claimed to be associated with the consumption of vinegar, and while these are numerous, only
1221 few are based on clear evidence (Mas, Troncoso, García-Parrilla, & Torija, 2016). Health
1222 benefits associated with vinegar include antimicrobial activity, antioxidant activity, modulation
1223 of the glycaemic response, positive effects on cardiovascular health, such as cholesterol-
1224 lowering and antihypertensive action, positive effects in weight loss, improvement of appetite,
1225 reduction of fatigue and anticancer activity (Chen et al., 2016). Organic acids, primarily acetic
1226 acid, and polyphenols have been attributed as the main functional compounds in vinegar and
1227 are present in all varieties at varying levels (Chen et al., 2016). Other bioactive compounds,
1228 their presence and concentration which can vary depending on the type of vinegar and substrate
1229 material, may also contribute to the functionality e.g. tryptophol as an anticancer compound in
1230 Japanese black soybean vinegar (Inagaki et al., 2007). Certain factors influence the chemical
1231 composition of vinegar and thus its functional properties, including the raw material, the
1232 production process (acetification method) and the amount of time spent aging in wood
1233 (Guerreiro, de Oliveira, Ferreira, & Catharino, 2014).

1234 The antibacterial effects of vinegar have mainly been investigated in the context of in-vitro
1235 application to food products, such as fresh fruits and vegetables, for the inhibition of pathogenic
1236 bacteria. The antibacterial mechanism of vinegar is primarily due to its acetic acid content.
1237 When the bactericidal effects of a number of organic acids, including lactic acid, acetic acid,

1238 citric acid, and malic acid on *Escherichia coli* O157:H7 were investigated, acetic acid was
1239 found to be most effective, followed by lactic acid, citric acid, and malic acid. Other pathogens
1240 inhibited by acetic acid included *Salmonella enterica* subsp. *enterica*, *Vibrio*
1241 *parahaemolyticus*, *Staphylococcus aureus*, *Aeromonas hydrophila*, and *Bacillus cereus*
1242 (Entani, Asai, Tsujihata, Tsukamoto, & Ohta, 1998). Chang and Fang (2007) observed a 3-log
1243 reduction in numbers of *E. coli* O157:H7 when rice vinegar containing 5% acetic acid was
1244 applied to lettuce for 5 min at 25°C. Use of lower acetic acid levels led to less of a reduction
1245 within the same time (Chang & Fang, 2007). The antimicrobial activity of organic acids is
1246 influenced by the target bacterial strain(s), temperature, pH, acid concentration, and ionic
1247 strength (Budak et al., 2014). The properties of undissociated organic acids such as fat-
1248 solubility and neutral charge enable them to diffuse through the cell membrane of the target
1249 microorganism and enter the cell; in the cytoplasm, the higher intracellular pH causes the acid
1250 to become dissociated, producing hydrogen ions. Hydrogen ion production thus reduces the
1251 intracellular pH and interferes with cellular processes, such as enzyme activity, DNA
1252 replication and transcription, and protein expression, therefore effecting the normal growth of
1253 the microorganism (Chen et al., 2016). Acetic acid is a particularly effective antimicrobial
1254 because at a relatively high pH (pH 4.7 = pKa of acetic acid) it exists primarily in its
1255 undissociated form and can enter the cell. Other organic acids, for example lactic acid, are not
1256 as effective; lactic acid cannot easily enter cells at a pH>3.8 (its pKa) as it exists primarily in
1257 its dissociated form.

1258 A high oxidant and low antioxidant level in the human body is associated with the development
1259 of chronic, inflammatory diseases, such as cancer and cardiovascular disease (Srdic-Rajic &
1260 Konic Ristic, 2016). Intake of dietary antioxidants inhibit the formation of peroxides and their
1261 absorption in the gastrointestinal tract (Verzelloni, Tagliazucchi, & Conte, 2007). Vinegar
1262 exhibits antioxidant capacity which has been associated with the presence of polyphenols and

1263 derived phenolic compounds, such as, gallic acid, caffeic acid, *p*-coumaric acid and ferulic acid
1264 amongst many others (Garcia-Parrilla, Torija, Mas, Cerezo, & Troncoso, 2017). In addition,
1265 carotenoids, phytosterols and vitamins, such as Vitamins C and E, and melanoidins also
1266 contribute to the antioxidant capacity (Ho et al., 2017; Tagliazucchi, Verzelloni, & Conte,
1267 2010). As stated above, the processes used in vinegar production can influence its chemical
1268 composition, and it is noteworthy that, in the case of the phenolic composition, contact with
1269 wood can influence the phenolic content, due to polyphenol release via alcoholysis of wood
1270 lignin (Tesfaye et al., 2002).

1271 It has been demonstrated in-vitro that the antioxidant capacity of Traditional Balsamic Vinegar
1272 (TBV) was equal to that of a 0.2% Vitamin C solution (Chen et al., 2016). Tagliazucchi et al.
1273 (2008) showed, in-vitro, that TBV had antioxidant activity equal to or higher than that of red
1274 wine, with 45% of the antioxidant activity due to the total polyphenolic fraction, primarily
1275 tannins, and 45% due to melanoidins and other lower molecular mass Maillard reaction
1276 products (Tagliazucchi, Verzelloni, & Conte, 2008). TBV melanoidins were responsible for
1277 preventing the pro-oxidant and cytotoxic effects of heme during simulated gastric digestion of
1278 meat (Verzelloni, Tagliazucchi, & Conte, 2010). Kurosu, a Japanese vinegar produced from
1279 unpolished rice, has been reported to have a high antioxidant activity. An ethyl acetate extract
1280 of kurosu inhibited myeloperoxidase activity, hydrogen peroxide generation and lipid
1281 peroxidation in mouse skin cells, and had the highest antioxidant activity compared to ethyl
1282 acetate extracts of grain vinegar, apple vinegar and wine vinegar (Nishidai et al., 2000).

1283 Vinegar has been described to have an antiglycaemic effect and to improve blood glucose
1284 control and insulin resistance. Indeed, before the advent of pharmacological hypoglycaemic
1285 agents, vinegar “teas” were consumed by diabetics to help manage their condition (Johnston &
1286 Gaas, 2006). Many types of vinegars including apple cider vinegar, ginsam vinegar (an Asian
1287 vinegar produced from *Panax ginseng*), and tomato vinegar are capable of reducing

1288 postprandial blood glucose and alleviating insulin resistance as well as promoting insulin
1289 production. This antiglycaemic effect of vinegar was first reported by Ebihara and Nakajima
1290 (1988). The authors found that co-administration of 2% acetic acid with a high glycaemic load
1291 meal consisting of 10% corn starch, significantly reduced the blood glucose response in rats
1292 (Ebihara & Nakajima, 1988). In human subjects, the consumption of sucrose accompanied by
1293 vinegar decreased the area under the insulin response curve by 20% (Johnston & Gaas, 2006).
1294 Administration of 20 mL white vinegar (equivalent to 5% acetic acid) as a salad dressing with
1295 a mixed meal consisting of white bread (50 g carbohydrate) and lettuce reduced the glycaemic
1296 response by over 30% in healthy individuals. Neutralisation of the acetic acid with sodium
1297 bicarbonate destroyed this antiglycaemic effect (Brighenti et al., 1995). The substitution of
1298 pickled cucumber for fresh cucumber in a meal consisting of bread, butter and yogurt reduced
1299 the glycaemic index (GI) by over 30% in healthy subjects (Östman, Liljeberg Elmståhl, &
1300 Björck, 2001). Similar results were observed by Johnston et al. (2010), who demonstrated that
1301 the postprandial glucose response was reduced by 23% in healthy individuals fed a meal of a
1302 bagel and juice containing 10 g apple cider vinegar (5% acidity), but not by the same meal
1303 containing neutralised vinegar. It was concluded that the neutralised salt of acetic acid (acetate)
1304 does not appear to possess antiglycaemic properties. In addition, ingestion of vinegar five hours
1305 before the meal reduced the antiglycaemic effect compared to consumption of vinegar with the
1306 meal (Johnston, Steplewska, Long, Harris, & Ryals, 2010).

1307 Vinegar has also been shown to modulate the glucose response and insulin sensitivity in
1308 diabetic individuals. In patients with type 2 diabetes, dietary consumption of acetic acid
1309 significantly reduced the level of glycated haemoglobin (0.16%) during a 12-week experiment
1310 (Johnston, White, & Kent, 2009). Furthermore, in healthy individuals at risk of developing type
1311 II diabetes mellitus, ingestion of 0.75 g acetic acid as a vinegar drink twice daily at mealtime,
1312 for 12 weeks, reduced fasting blood glucose levels, and to a greater extent than diabetic

1313 pharmaceutical medications (Johnston, Quagliano, & White, 2013). This is in agreement with
1314 an earlier study by Johnston et al. (2004) which showed that ingestion of a vinegar drink (20 g
1315 vinegar, 40 g water, 1 tablespoon saccharine) by individuals with insulin resistance (pre-
1316 diabetic) had a marked reduction in postprandial glycaemia (64%) and improved insulin
1317 sensitivity (34%) (Johnston, Kim, & Buller, 2004).

1318 It has been reported that the reduction of postprandial glucose affected by vinegar in high GI
1319 meals is not observed for low GI meals (Liatis et al., 2010). However, vinegar ingestion with
1320 both high- and even low-GI meals does improve insulin sensitivity, independent of blood
1321 glucose level, as glucose uptake was enhanced after both meal types when vinegar was also
1322 ingested (Mitrou et al., 2015). Indeed, even the improvement by vinegar consumption of insulin
1323 sensitivity alone is noteworthy, particularly in insulin-resistant (pre-diabetic) subjects, as trials
1324 have demonstrated that slowing the progression to diabetes in high-risk individuals and
1325 improving their insulin sensitivity may increase the probability that such individuals may revert
1326 to a normal, glucose-tolerant state over time (Johnston & Gaas, 2006).

1327 Postprandial blood glucose levels are primarily determined by 1) the rate that glucose enters
1328 the blood and 2) the rate at which it is consumed in-vivo. The rate of gastric emptying,
1329 digestion, and absorption in the small intestine determine the rate that glucose enters the blood
1330 (Chen et al., 2016). It is not yet fully understood how vinegar modulates glucose metabolism.
1331 Acetic acid in vinegar may suppress carbohydrate absorption in the gut, more specifically
1332 through reducing disaccharidase activity and decreasing the digestion of disaccharides and
1333 oligosaccharides. Thus, monosaccharide absorption in the gut is reduced, lowering the blood
1334 glucose level (Johnston et al., 2013). Ogawa et al. (2000) demonstrated that acetic acid
1335 significantly inhibited the disaccharidase (e.g. sucrase, lactase) activity of Caco-2 cells, an
1336 effect not seen with other organic acids such as lactic acid or citric acid (Ogawa et al., 2000).
1337 In addition, acetic acid may regulate glucose metabolism by promoting uptake of glucose by

1338 the liver and skeletal muscle and its conversion to glycogen stores (Hlebowicz, Darwiche,
1339 Bjorgell, & Almer, 2007). In this case, the mechanism by which glucose metabolism is
1340 regulated is believed to be through activation of the adenosine monophosphate-activated
1341 protein kinase (AMPK) pathway. Acetic acid is a building block for the biosynthesis of acetyl-
1342 coenzyme A (acetyl-CoA). During acetyl-CoA biosynthesis, adenosine triphosphate (ATP) is
1343 consumed and adenosine monophosphate (AMP) is produced, increasing the AMP/ATP ratio
1344 and leading to activation of the AMPK pathway. This causes a reduction in blood glucose levels
1345 and concomitant glycogen biosynthesis (Sakakibara, Yamauchi, Oshima, Tsukamoto, &
1346 Kadowaki, 2006). Another mechanism by which vinegar may reduce the postprandial blood
1347 glucose level is via effecting delayed gastric emptying. Hlebowicz et al. (2007) demonstrated
1348 that consumption of 30 mL of apple vinegar decreased the postprandial gastric emptying rate
1349 by 10% (Hlebowicz et al., 2007). The stabilisation of the postprandial blood glucose level
1350 through the mechanism described above may also increase postprandial satiety, thus reducing
1351 dietary intake and, as such, a further increase in blood glucose (Chen et al., 2016).

1352 There is evidence that vinegar consumption can also affect lipid metabolism and, by
1353 association, promote weight loss. Studies, in particular in animal models, have highlighted that
1354 vinegar consumption can increase the concentration of high-density lipoprotein (HDL)
1355 cholesterol while reducing triglycerides, total cholesterol, and low-density lipoprotein (LDL)
1356 cholesterol (Fushimi et al., 2006). Similar effects on lipid levels were observed in human
1357 subjects displaying hyperlipidaemia and in obese individuals following consumption of apple
1358 cider vinegar (Beheshti et al., 2012; Kondo, Kishi, Fushimi, Ugajin, & Kaga, 2009a). Obese
1359 individuals, following long term apple vinegar consumption, also displayed significantly
1360 reduced body weight and body mass index (Kondo et al., 2009a). Regulation of lipid
1361 metabolism by vinegar intake is understood to be due to acetic acid, and, in a similar
1362 mechanism to glycaemic control, via activation of the AMPK pathway. Activation of this

1363 pathway decreases the biosynthesis of lipids, specifically through inhibition of genes related to
1364 fatty acid biosynthesis, and also increases their breakdown and excretion. It is suggested that
1365 lipid oxidation, due to acetic acid for example, stimulates expression of certain oxidase
1366 enzymes, resulting in lipolysis (Samad, Azlan, & Ismail, 2016). The effect of acetic acid on
1367 postprandial satiety, as mentioned above, may also have a role in simply decreasing food, and
1368 thus fat, intake (Chen et al., 2016). Acetic acid, and thus vinegar, may have a role in reducing
1369 hypertension (blood pressure) through inhibiting angiotensin-converting enzyme (ACE) which
1370 reduces plasma levels of the strong vasoconstrictive, angiotensin II (Samad et al., 2016).

1371 Few studies on the anticancer properties of vinegar or acetic acid are available. The effect of
1372 kurosu, a Japanese rice vinegar on the proliferation of a number of human cancer cell lines,
1373 including colon adenocarcinoma, lung carcinoma, breast adenocarcinoma, bladder carcinoma,
1374 and prostate carcinoma cells has been studied. It was reported to up-regulate the expression of
1375 enzymes involved in DNA repair and cell apoptosis in cells, and to inhibit the growth of all
1376 tested cell lines in a dose-dependent manner (Nanda et al., 2004). Kurosu was also shown by
1377 Baba et al. (2013) to inhibit the proliferation of human squamous cell carcinoma cells via
1378 programmed necrosis (Baba, Higashi, & Kanekura, 2013). The antioxidative nature of certain
1379 types of vinegar is also postulated to have a role in anticancer activity. Kibizu, a Japanese
1380 vinegar made from sugar cane, inhibited the growth of human leukaemia cells due to its high
1381 radical-scavenging capacity (Mimura et al., 2004).

1382 While there is significant evidence linking vinegar consumption to the modulation of blood
1383 glucose levels, scientific studies linking vinegar intake to other health benefits such as control
1384 of lipid levels and anticancer effects remain equivocal (Johnston & Gaas, 2006). As shown in
1385 Table 7, the majority of studies have been performed in animal models; therefore, more human
1386 studies, including large-scale and long-term clinical trials are required before any definitive
1387 health claims can be made (Karabiyikli & Sengun, 2017).

1388

1389 *Health benefits associated with kombucha*

1390 As reported by Dufresne and Farnworth (2000), a myriad of health benefits have been attributed
1391 to the consumption of kombucha, from protecting against diabetes to counteracting ageing and
1392 improving eyesight, mostly based on drinkers testimonials, and few of which have been
1393 investigated scientifically (Dufresne & Farnworth, 2000). Those health benefits that have been
1394 studied have primarily been performed in animal models, with very little data related to studies
1395 on humans (Jayabalan et al., 2014). Many of the claimed beneficial effects of kombucha have
1396 been correlated with its antioxidant activity. This has been mainly attributed to the tea substrate
1397 and the presence of tea polyphenols, Vitamin C and D-saccharic acid-1,4-lactone (DSL).
1398 Kombucha tea has a higher polyphenol content than un-fermented tea which is hypothesised to
1399 be due to structural modification of tea polyphenols by enzymes produced during fermentation
1400 by bacteria and yeast (Jayabalan et al., 2014). The polyphenol content of kombucha, and thus
1401 its antioxidant capacity can be dependent on a number of factors, such as, the fermentation time
1402 and conditions, variety of tea substrate, and the kombucha culture microbiota, which itself can
1403 vary depending of the substrate and fermentation conditions, and which, in turn determines the
1404 nature of the metabolites produced and present in the final beverage. In kombucha prepared
1405 with different types of tea, reducing power, hydroxyl radical scavenging ability, and anti-lipid
1406 peroxidation were decreased, while total phenolic compounds and scavenging activity against
1407 DPPH and the superoxide radical increased with a prolonged fermentation time (Jayabalan,
1408 Subathradevi, Marimuthu, Sathishkumar, & Swaminathan, 2008). Preparation of kombucha
1409 with different starter cultures of mixed AAB and a single yeast species on green tea had lower
1410 antioxidant capacity compared to a native (complex culture) kombucha (Malbaša, Lončar,
1411 Vitas, & Čanadanović-Brunet, 2011). In a recent study, the polyphenol content of kombucha
1412 was shown to be dependent on fermentation temperature, with a higher level at low (20°C)

1413 compared to high (30°C) temperatures. This was linked to the varying species dominance at
1414 the different temperatures (De Filippis, Troise, Vitaglione, & Ercolini, 2018).

1415 Investigations in cell lines animal models found that kombucha can protect against
1416 hepatotoxicity caused by several toxicants such as paracetamol, carbon-tetrachloride, aflatoxin
1417 B1 and acetaminophen (Jayabalan, Baskaran, Marimuthu, Swaminathan, & Yun, 2010; Pauline
1418 et al., 2001; Wang et al., 2014). Bellassoued et al. (2015) demonstrated that the high
1419 thiobarbituric acid reactive substances (TBARS) concentration was significantly reduced in the
1420 liver and kidney of rats fed with cholesterol-rich diets after the treatment with fermented tea
1421 (Bellassoued et al., 2015). The anti-toxigenic effect of kombucha has been attributed to its
1422 antioxidant activity and the hepatoprotective effects against acetaminophen were primarily
1423 attributed to the presence of DSL (Wang et al., 2014). Early investigations of the potential
1424 health effects and detoxifying capacity of kombucha had primarily attributed the effects to its
1425 acidic composition; in particular, the ability of glucuronic acid to bind toxic components
1426 (glucuronidation) and to increase their excretion from the body was cited. However, the
1427 possibility that what was actually being measured in those early studies as glucuronic acid was
1428 in fact 2KGA, has been debated (Dufresne & Farnworth, 2000). Indeed, the concentration of
1429 glucuronic acid in kombucha may be as much as ten thousand times lower than that of GA
1430 (Lončar, Petrovič, Malbača, & Verac, 2000).

1431 Given that acetic acid is a primary metabolite of the microbial consortia in kombucha, it could
1432 be hypothesised that those health benefits attributed to the presence of acetic acid, as observed
1433 in vinegar, may also contribute to the potential health effects of kombucha, notwithstanding
1434 the difference in acetic acid concentration between both fermented products. Srihari et al.
1435 (2013a) observed that the daily administration for 45 days, of a kombucha extract, reduced
1436 glycated haemoglobin and increased the plasma insulin level, thus demonstrating an
1437 antiglycaemic effect. However, the authors attributed the effect mainly to the polyphenolic

1438 component of kombucha and non-specifically to the presence of organic acids and B-complex
1439 vitamins (Srihari, Karthikesan, Ashokkumar, & Satyanarayana, 2013a).

1440 Kombucha has for many decades been claimed by drinkers to have anticancer effects, even in
1441 a population study conducted in 1951 in Russia by the “Central Oncological Research Unit”
1442 and the Russian Academy of Sciences in Moscow. The antiproliferative activity of kombucha
1443 produced from black tea has been demonstrated against a number of cancer cell lines, such as
1444 HeLa (cervix epithelial carcinoma) and HT-29 (colon adenocarcinoma) cells (Cetojevic-Simin,
1445 Bogdanovic, Cvetkovic, & Velicanski, 2008). In addition, an ethyl acetate extract of black tea
1446 kombucha caused cytotoxic effects on 786-O (human renal carcinoma) and U2OS (human
1447 osteosarcoma) cells and significantly reduced cell invasion and motility of these cells in
1448 addition to A549 (human lung carcinoma) cells (Jayabalan et al., 2011). Srihari et al. (2013b)
1449 demonstrated that a lyophilized extract of kombucha significantly reduced the survival of
1450 prostate cancer cells via down regulation of angiogenesis stimulators (Srihari, Arunkumar,
1451 Arunakaran, & Satyanarayana, 2013b). Again, the presence of polyphenols and their phenolic
1452 degradation products, as a consequence of fermentation, have been generally attributed as the
1453 anticancer bioactive compounds in kombucha.

1454 Similar to those studies on the health effects of vinegar, many of those investigating similar
1455 benefits associated with the consumption of kombucha tea have relied on in vitro and animal
1456 model investigations. Therefore, clinical trials and further in vivo evaluations are necessary in
1457 order to confirm the claimed health benefits of kombucha tea. In particular, it is questionable,
1458 and data is necessary to support such claims, as to whether the efficacious concentrations for
1459 the positive effects observed through in-vitro studies would be achievable in-vivo in humans.

1460 **Regulatory aspects on the use of AAB as food cultures**

1461 The application of microbial food cultures in the production of fermented foods, used in both
1462 traditional backslopping practices and in recent decades as defined starter cultures, has an
1463 important role in ensuring the quality and safety of these products, in addition to imparting
1464 desirable flavour, aroma and textural properties (Bourdichon et al., 2012a). Today, with the
1465 large and every expanding variety of fermented foods and beverages that exists, and the
1466 possibility of applying new strains and species in foods, where they previously may not have
1467 been used, ensuring consumer safety is of prime importance. Following a brief description of
1468 the regulations pertinent to microbial food cultures in the European Union (EU) and the United
1469 States of America (USA), aspects related to the application of AAB will be considered.

1470

1471 *Microbial Food Cultures and Regulation in the European Union*

1472 In the EU, the European Food Safety Authority (EFSA) operates the Qualified Presumption of
1473 Safety (QPS) approach (European Food Safety Authority, 2007). Hereby, a taxonomic unit
1474 (usually species) that is notified to the EFSA is pre-assessed for its safety based on aspects such
1475 as the associated body of knowledge, a history of apparent safe use in food, scientific literature,
1476 clinical aspects and industrial application. In this approach, familiarity is a critical aspect to
1477 support evidence of a microbial presumption of safety (Russo, Spano, & Capozzi, 2017).
1478 Familiarity, as a concept, is “taken to include practical experience of use of the organism(s)
1479 including its history of use for particular purposes and any body of literature on the biology of
1480 the taxonomic unit” (European Food Safety Authority, 2005), which could be translated to the
1481 body of knowledge supporting evidence for the historical use or consumption of such
1482 microorganisms in fermented foods, with no apparent adverse effects. For those
1483 microorganisms which have not traditionally been significantly consumed within the EU (prior

1484 to May 1997), or, are not generally associated with foods, the concept of a “novel food” has
1485 been devised and is regulated separately (European Commission, 2015).

1486 If a species is deemed safe by the EFSA Panel on Biological Hazards it is placed on the QPS
1487 list which is published by EFSA (Ricci et al., 2017a). However, individual strains must still
1488 satisfy certain criteria, or qualifications, before being applied in food (and beverages) or feed.
1489 As an example, a generic qualification for all bacterial taxonomic units on the QPS list is that
1490 strains should not harbour any acquired antimicrobial resistance genes to clinically relevant
1491 antimicrobials (Ricci et al., 2017a). In addition, as an alternative to exclusion from the list,
1492 certain bacterial species may have specific qualifications placed on them, which give them QPS
1493 status, but only when used for a defined application (Leuschner et al., 2010). Thus, for those
1494 species that are on the QPS list, they may be permitted for use in food or feed once the strain(s)
1495 being applied satisfy the attached qualifications. All microorganisms not on the QPS list remain
1496 subject to a full safety assessment (European Food Safety Authority, 2007). However, it is not
1497 entirely clear, at least from documents and literature from EFSA, what constitutes a full safety
1498 assessment. To this end, Pariza et al. (2015) proposed a decision tree that could be used for the
1499 safety evaluation of both non-QPS and QPS strains (Pariza, Gillies, Kraak-Ripple, Leyer, &
1500 Smith, 2015) (Figure 8). This will be discussed further below with relevance to AAB. In
1501 addition, microbial cultures assessed under the novel foods regulation (and not on the QPS list)
1502 must undergo a full safety assessment (Laulund, Wind, Derkx, & Zuliani, 2017).

1503 The absence of a species from the QPS list does not necessarily imply a risk associated with its
1504 use. Individual strains may be safe, but the body of knowledge may not be sufficient to exclude
1505 any potential risk. In addition, the EFSA may not have been requested to date to evaluate the
1506 taxonomic unit in question (Bourdichon et al., 2012a). It is also possible that, for some
1507 microorganisms on the QPS list, EFSA may have been requested to evaluate a taxonomic unit
1508 for use in a defined application, and the scope of the evaluation may have been narrow, to

1509 exclude only the potential risks associated with the application for which the microorganism
1510 was notified. Such microorganisms are included on the list, with a qualification of a specific
1511 use (e.g. QPS applies only when used for vitamin production).

1512 There remains some ambiguity and uncertainty around the QPS approach and regulation, as
1513 outlined by Laulund et al. (2017) (Laulund et al., 2017). It appears that microbial food cultures
1514 with a history of safe use in food are considered as traditional food ingredients and are legally
1515 permitted for use in food in the EU without pre-market authorisation. On the other hand, those
1516 with no history of use in foods would be considered as novel food, and require full safety
1517 assessment, as mentioned above. This seems to suggest that it would be possible to place a
1518 product on the market that uses microbial cultures that have not undergone any safety
1519 assessment, if the manufacture considers that such cultures have a historical safe use in food.
1520 It also raises two questions. Firstly, what exactly defines microbial food cultures? Given the
1521 use of the wording “history of safe” and “traditional food ingredients”, does this suggest
1522 complex, multi-strain cultures? What about single strain starter cultures? Secondly, how is a
1523 history of safe use defined and how can it be proven? To address these questions and the gaps
1524 in the regulation, the European Food and Feed Cultures Association (EFFCA) have proposed
1525 a definition of food cultures, which outlines what constitutes a microbial food culture. Their
1526 2015 definition defines microbial food cultures as “safe live bacteria, yeasts or moulds used in
1527 food production, and they are in themselves a characteristic food ingredient. FC [food culture]
1528 preparations are formulations, consisting of concentrates ($>10^8$ CFU per g or mL) of one or
1529 more live and active microbial species and/or strains, including unavoidable media components
1530 carried over from the fermentation and components, which are necessary for their survival,
1531 storage and to facilitate their application in the food production process, and are in some cases
1532 standardised to a low count with carriers” (European Food and Feed Cultures Association,
1533 2018). In addition, to address the question of cultures with a history of safe use in food, EFFCA

1534 have, in association with the International Dairy Federation (IDF), compiled an “Inventory of
1535 Microorganisms with a documented history of use in food”, the first inventory of FC with a
1536 documented significant use in food production before 1997 (Laulund et al., 2017). The first
1537 IDF/EFFCA Inventory, published in 2002, and primarily addressing cultures used in the dairy
1538 fermentation industry, was updated in 2012 with an expanded scope to include microbial
1539 cultures used in a wider range of food products (including meat, vegetable, cereals, beverages
1540 and vinegar) (Bourdichon et al., 2012b). One of the main criteria for inclusion in the inventory
1541 is a documented presence in fermented foods, and not just an incidental isolate (Bourdichon et
1542 al., 2012c).

1543 Therefore, it appears that a food producer could conceivably place a product on the market
1544 that uses a single microbial culture or a number of cultures, which have an associated history
1545 of safe use in food, proven due to their inclusion in the IDF/EFFCA Inventory, and therefore
1546 without the need for pre-authorisation or safety assessment of the employed strains. However,
1547 it is likely that food producers would preferably opt to assess the safety of any strains employed
1548 as much as possible, primarily to ensure the safety of consumers, but secondly, to minimise
1549 liability, especially if the species is not on the QPS list.

1550

1551 *Microbial Food Cultures and Regulation in the United States of America*

1552 In the USA, the Food and Drug Administration (FDA) operates the Generally Recognised as
1553 Safe (GRAS) system. This system is applied to a wide range of ingredients and is not specific
1554 to microbial FCs in the way that the EU QPS system is. Substances (including microbial
1555 cultures) added to food can be considered either as additives or GRAS substances. If they are
1556 considered as additives, then a pre-market authorisation by the FDA is required. However, if
1557 their use pre-dates 1958, or they have GRAS status, then no pre-market approval is required

1558 (Russo et al., 2017). Achieving GRAS status is, similar to the QPS system, built on evidence
1559 of safety; in the case of GRAS, this must be guaranteed, with reasonable certainty, through
1560 examination of the body of knowledge (typically scientific publications) and the assessment
1561 and consensus of a panel of experts as to the safety of the substance or FC under its conditions
1562 for use (Bourdichon et al., 2012a). In contrast to QPS, the GRAS designation encompasses the
1563 substance / microbial culture and its specific application or usage (e.g. in a particular type of
1564 product), rather than applying solely to the microorganism itself; thus, while for QPS the
1565 evaluation is at the level of the taxonomic unit (species), and applies to that unit independent
1566 of application, GRAS designation can be at the species or strain level because the specific
1567 application (including formulation, dosages etc.) is considered (Ricci et al., 2017b).

1568 From a legal standpoint, with a GRAS determination the onus and liability are placed on the
1569 food company, as a GRAS designation is issued based on evidence evaluated by the food
1570 manufacturer and the panel of experts engaged by that company. This is in contrast to the food
1571 additive designation, where the onus is placed to a greater degree on the FDA, where a full
1572 safety assessment by the authority is required. However, by law, neither a GRAS determination
1573 nor the notification to the FDA of the new use of a microbial culture is mandatory for a food
1574 company (Russo et al., 2017). Nevertheless, performance of a safety evaluation and the
1575 attainment of GRAS status would serve to reduce the liability on the food manufacturer.

1576

1577 *Safety aspects of AAB, with emphasis on G. oxydans*

1578 Unlike LAB which are today primarily applied as single, defined starter cultures, the
1579 application of AAB is still via undefined, mixed or complex cultures, where the mode of
1580 transfer of the microorganisms to the next fermentation is through the traditional process of
1581 “backslopping”. This is, for example, the case for both traditional and industrial methods of

1582 acetous vinegar fermentation. This has been described to be, firstly, due to the nutritionally
1583 fastidious nature of AAB, which are difficult to preserve as a dried starter; and, secondly,
1584 vinegar is generally viewed as an inexpensive commodity and its production has therefore not
1585 warranted the development or use of expensive starter cultures (Solieri & Giudici, 2009). This
1586 is also the case for other fermented foods in which AAB play a dominant role; thus, as their
1587 use has been seen as traditional and viewed as safe through a long history of use, this group of
1588 microorganisms have not been considered to a large extent with regard to the regulations
1589 surrounding microbial FCs. However, with the increasing consumer interest in fermented food
1590 products it is foreseeable that the industry will require the future development of defined AAB
1591 starter cultures, which will necessitate the safety evaluation of such strains.

1592 The only AAB (and one of only two Gram-negative bacteria) listed on the QPS list is
1593 *Gluconobacter oxydans* (EFSA Panel on Biological Hazards, 2018). *G. oxydans* was assessed
1594 by the EFSA Panel on Biological Hazards for the first time in 2013 and recommended for the
1595 QPS list. The species was subject to a qualification, however; QPS only applies when the
1596 species is used for vitamin production (EFSA Panel on Biological Hazards, 2013). It is not
1597 stated in the Scientific Opinion exactly why the qualification was put in place; the panel noted
1598 the general non-pathogenic nature of the genus and species to humans and animals (De Muynck
1599 et al., 2007), and a review of over 5,000 references raised no human or animal safety concerns.
1600 The possible pathogenic effect of [unidentified or unstated] *Gluconobacter* species was
1601 mentioned by two studies, however, these cases involved individuals with compromised
1602 immune systems (Alauzet et al., 2010; Bassetti et al., 2013). While the panel noted the reported
1603 rare occurrence of infections, as well as colonization with AAB in patients with underlying
1604 chronic diseases and/or indwelling devices, and the potential of some *Gluconobacter* species
1605 (although not *G. oxydans*) to be opportunistic pathogens (Alauzet et al., 2010), no article
1606 reported safety concerns related to consumption of foods and feed. The potential for

1607 monobactam antibiotic production by strains of *G. oxydans*, as reported in one study in 1982
1608 was noted (Wells et al., 1982), with a decision to follow this aspect in future QPS reviews.
1609 Nevertheless, the Panel placed *G. oxydans* on the QPS list with a qualification that it applies
1610 only when the species is used for vitamin production. While it was not stated why the panel
1611 placed this qualification on the taxonomic unit, it was noted that the qualification was “relevant
1612 for the intended use for which the species was notified”. Whether the Panel placed this
1613 qualification on the taxonomic unit because of unanswered questions around the safety of the
1614 species, or, considered the safety of the species only in the context of the notified (narrow)
1615 application is not known (EFSA Panel on Biological Hazards, 2013). In the intervening time
1616 period to the present (August 2018), no new safety concerns were raised by EFSA and *G.*
1617 *oxydans* has been maintained on the QPS list (EFSA Panel on Biological Hazards, 2018). In
1618 the USA, to date, no products using *Gluconobacter* species have been submitted to the FDA
1619 for GRAS designation (Food and Drug Administration, 2018).

1620 The 2012 IDF/EFFCA Inventory lists 3 genera of AAB, encompassing 18 species. Species
1621 from the genera *Acetobacter*, *Gluconacetobacter* (some of which would now be
1622 *Komagataeibacter*) and *Gluconobacter* are included for their roles in vinegar, vegetable, coffee
1623 and cocoa fermentations (Bourdichon et al., 2012b). Thus, the historic and safe use of these
1624 species, with relative certainty, is unquestionable. With regard to any future safety assessment
1625 of these species, the following paragraphs will outline some important considerations, using
1626 the decision tree outlined in Figure 8 to guide the discussion, and with emphasis on *G. oxydans*.

1627 Strain characterisation and genome sequencing: It is generally accepted today that bacterial
1628 isolates are identifiable to species level using molecular approaches such as 16S rRNA gene
1629 sequencing (Weisburg, Barns, Pelletier, & Lane, 1991). In addition, whole genome sequencing
1630 of strains is now commonly performed and can prove indispensable for the safety assessment,
1631 providing additional insights into the genetic basis of strain safety. Different bioinformatics

1632 tools and databases can be used to screen for the presence of virulence factors and for antibiotic
1633 resistance genes e.g. microbial virulence factors can be screened for using the MvirDb database
1634 (Zhou et al., 2006), while the Comprehensive Antibiotic Resistance Database (McArthur et al.,
1635 2013) and ResFinder (Zankari et al., 2012) can be used to search for antibiotic resistance
1636 determinants (Laulund et al., 2017). Currently, in the National Center for Biotechnology
1637 Information (NCBI) database there are approximately 170 sequenced genomes of AAB strains
1638 of the genera *Acetobacter*, *Gluconacetobacter*, *Gluconobacter* and *Komagataeibacter*, with 14
1639 *G. oxydans* genomes, 5 of which are complete (NCBI, 2018).

1640 Screening for undesirable attributes and metabolites: With regard to the AAB strain producing
1641 virulence factors or toxins, a whole genome sequence can be used to screen for genetic elements
1642 encoding such traits, as described above. Besides those studies detailed in the 2013 QPS update,
1643 which highlighted the rare occurrence of AAB as potential opportunistic pathogens, such cases
1644 related to individuals with underlying chronic diseases and/or indwelling devices (EFSA Panel
1645 on Biological Hazards, 2013). In the case of *G. oxydans*, no further studies in the intervening
1646 time period raised any new concerns (EFSA Panel on Biological Hazards, 2018). In addition,
1647 their long history of safe use and the presence of many AAB species on the 2012 IDF/EFFCA
1648 Inventory is testament, within the limits of reasonable certainty, to their safety when consumed
1649 by healthy individuals. Unlike the LAB, some species of which are biogenic amine producers,
1650 AAB have not been found to produce these toxigenic compounds (Landete, Ferrer, & Pardo,
1651 2007). However, in-vitro tests can be performed on strains to identify biogenic amine
1652 producers; in addition, the genome sequence of strains can be screened for putative responsible
1653 amino acid decarboxylase genes (or their homologues). The question of antibiotic resistance is
1654 fundamental in assessing the safety of a strain. Chiefly, the strain must be free of functional
1655 and transferable antibiotic resistance genes. Proven intrinsic (natural) resistance is generally
1656 acceptable; the resistance determinant must not be transferable – this is to prevent the

1657 horizontal or lateral transfer of (acquired) resistance to antimicrobials of human and veterinary
1658 importance from FC microorganisms to (potentially pathogenic) commensal microorganisms
1659 in the gut (Pariza et al., 2015). Unlike LAB, for which guidelines and procedures have been
1660 developed by the regulatory authorities to inform the testing and interpretation of antibiotic
1661 susceptibilities of this microbial group (EFSA FEEDAP, 2012; International Organization for
1662 Standardization, 2010), no such distinct guidelines are in place for AAB. In this case, it is
1663 recommended that methods described by the Clinical and Laboratory Standard Institute be
1664 used. Again, the genome sequence can also be useful to search for genes conferring antibiotic
1665 resistance, with particular focus on genes associated with mobile genetic elements (plasmids,
1666 conjugative transposons) that would be potentially transferrable (Laulund et al., 2017). In the
1667 2013 QPS update when *G. oxydans* was admitted to the QPS list, the Panel reported that no
1668 evidence of resistance to antibiotics was found in any of the papers screened. According to
1669 only one publication, strains of some *Gluconobacter* species, although not *G. oxydans*, may be
1670 multi-resistant to some antimicrobial agents. In addition, such strains were isolated from
1671 hospitalised patients and the nature of the resistance (intrinsic or acquired) was not investigated
1672 (Alauzet et al., 2010). When performing antibiotic susceptibility testing of strains, it is
1673 important to differentiate between intrinsic and acquired antibiotic resistance and have
1674 knowledge of the potential for intrinsic resistance in the strains being assessed; for example,
1675 bacteria differ in their susceptibility to penicillin G; Gram-positive bacteria are generally
1676 sensitive, while most Gram-negative bacteria are naturally resistant because this compound
1677 cannot penetrate the outer cell membrane (Madigan et al., 2015). Gram-negative bacteria are
1678 also resistant to glycopeptide antibiotics (such as vancomycin) for the same reason. Although
1679 arguable, for food products where the microbial culture has been killed or inactivated (through
1680 an intense pasteurisation, for example), it appears, at least in the past, that the presence of
1681 acquired microbial resistance was not considered to be an issue and was acceptable (Russo et

1682 al., 2017). However, if these dead cells are damaged or lysed in some way, either because of
1683 food processing steps or passage through the gastrointestinal tract, it should be considered
1684 whether mobile genetic elements carrying antibiotic resistance genes could still be released
1685 into the gut environment and taken up, for example, via transformation by other
1686 microorganisms present (European Food Safety Authority, 2008).

1687 The ability of microbial food cultures to produce antimicrobial agents is also appreciable
1688 because such agents could select for resistance in the host bacterial population (Bourdichon et
1689 al., 2012a). As mentioned above, the potential for monobactam antibiotic production by strains
1690 of *G. oxydans* was reported in a 1982 study (EFSA Panel on Biological Hazards, 2013; Wells
1691 et al., 1982). The Panel stated the decision to follow this aspect in future QPS reviews; however,
1692 up to and including the latest QPS update, no further information has emerged.

1693 In general, there is only a very small body of knowledge and few studies related to such safety
1694 aspects of AAB - antimicrobial susceptibility and potential pathogenicity. More research is
1695 required, research which will also inform regulatory authorities and enable the development of
1696 guidelines which should 1) guide academia and industry on the safety testing of AAB strains
1697 and 2) allow streamlining of the pre-market approval process for the use of such strains as
1698 starter cultures in both traditional and novel food products.

1699 Genetic modification: If a strain has been isolated from a natural environment or system (e.g.
1700 a fermented food product produced by traditional methods), it is unlikely to have been
1701 genetically modified. If the strain has been genetically modified, regulatory approval and an
1702 assessment of the safety of the expressed product is required in many countries (Pariza, 2007).

1703 Strain origin: Consideration of the origin of the microbial FC focuses on its isolation from a
1704 food with a demonstrated history of safe consumption and its significant role in the production
1705 and characteristics of that food. As outlined in previous sections in this review, AAB, in

1706 particular members of the genera *Acetobacter*, *Gluconobacter*, *Gluconacetobacter* and
1707 *Komagataeibacter*, are key members in fermented food products such as vinegar and
1708 kombucha (Table 1), which are often sources for the isolation of new strains. Such fermented
1709 foods have a long history of safe consumption, particularly in Eastern Europe, Russia and Asia.
1710 Therefore, the long history of safe consumption of members of the AAB is self-evident.
1711 Nonetheless, non-food products such as flowers can also be sources of new AAB strains; the
1712 acceptability of the use of such strains, not strictly satisfying the guidelines of having been
1713 isolated from food, could be an important topic for discussion.

1714 The safety of a strain can only be assessed based on the existing body of scientific knowledge.
1715 In addition, this knowledge should be assessed by an authoritative group and/or a group of
1716 qualified scientific experts (Pariza et al., 2015). While *G. oxydans* is on the QPS list, with
1717 qualifications, no other AAB has yet been placed on the list. However, as outlined above, 8
1718 species, including *G. oxydans*, are included in the 2012 IDF/EFFCA Inventory (Bourdichon et
1719 al., 2012b). Thus, the IDF/EFFCA Panel would be considered as an authoritative group of
1720 scientific experts, and their inclusion of these AAB in the Inventory should be considered as
1721 an affirmation, with reasonable certainty, of their safety. Nevertheless, such expert evaluations
1722 should not be taken alone to constitute the absolute safety of a species and it is important to
1723 consider, particularly when dealing with individual strains, the safety of a culture with respect
1724 to the results of in-vitro tests and an analysis of the genome. In addition, it is noteworthy that
1725 the term safe or safety, in this context, means that that there is a reasonable certainty in the
1726 minds of competent scientists that a substance or microorganism is not harmful under the
1727 intended conditions of use. However, as observed by Laulund et al. (2017), it is impossible in
1728 the present state of scientific knowledge to establish with complete certainty the absolute
1729 harmlessness of the use of any microorganism (Laulund et al., 2017). It is also important to

1755 the same time, the EFSA Panel on Biological Hazards carried out a QPS assessment of the
1756 species; however, while no safety concerns could be identified the panel found that the
1757 published studies about *B. xylanisolvens* were too few and not sufficient to definitively exclude
1758 safety issues. Therefore, the microorganism was not included on the QPS list. The fact that the
1759 strain under investigation had no history of use in the food industry, and no strain in the genus
1760 *Bacteroides* has a proven history of use in food production was also considered. In addition, a
1761 gene encoding β -lactam antibiotic resistance was found in the genome. However, no mobile
1762 genetic elements were found, and the Panel considered the transfer of genes therefore unlikely
1763 due to this fact, in addition to the heat inactivation of the cells (Brodmann et al., 2017; EFSA
1764 Panel on Biological Hazards, 2015). Even though *B. xylanisolvens* was not placed on the QPS
1765 list, the positive outcome in terms of designation as novel food opens the possibility of using
1766 other species or strains of bacteria as microbial FC, even if the body of knowledge is
1767 insufficient and/or safety concerns cannot be fully excluded; however, with the qualification
1768 that no viable cells remain in the product. This is noteworthy if the strain has been applied for
1769 reasons beyond its fermentative capacity – for example, as viability is an essential condition
1770 for probiotic activity, inactivated cells should not be promoted for their potentially probiotic
1771 properties, and statements such as “contains live and active cultures” should not be made if the
1772 microbial FC has been inactivated. Nevertheless, non-viable cells may still elicit immunogenic
1773 effects and may have health benefits, hence, the term “paraprobiotic” has been coined to
1774 describe such cultures (Taverniti & Guglielmetti, 2011).

1775 As an aside, one commercial beverage product which is produced via a *Gluconobacter oxydans*
1776 fermentation of malt-base (wort, or similar sugar source) is Bionade® [patent: DE4012000A1].
1777 Following the fermentation, the *G. oxydans* cells are stated to be removed via filtration
1778 (www.bionade.de/en/production-process).

1779 In conclusion, in light of their long history of safe consumption, it appears unlikely that the
1780 application of certain species of AAB (e.g. *Acetobacter*, *Gluconobacter*) would pose a safety
1781 risk. However, in the interest of consumer safety it would be prudent that any strains of AAB,
1782 applied as microbial FC in the future, would be thoroughly evaluated for their safety using a
1783 QPS approach or following a decision tree similar to that shown in Figure 8. However,
1784 relatively few studies have been conducted with regard to the safety of this microbial group,
1785 which means that the body of scientific knowledge in the area is lacking, especially when
1786 compared to the LAB (for example, prevalence and breadth of resistance to antimicrobials,
1787 production of toxins etc.). In addition, distinct guidelines from the responsible regulatory
1788 authorities (e.g. EFSA) are required, that deal with this important microbial group; however, it
1789 is clear and understood that such guidelines can only be built on a pre-existing body of scientific
1790 knowledge.

1791 **Conclusion**

1792 AAB are most commonly known for their role in vinegar production, yet, this diverse group of
1793 bacteria play an important part in the production of other fermented products such as kombucha
1794 and water kefir. Their highly efficient oxidative metabolism is unique and can be harnessed for
1795 the production of compounds that find application not only in the food and beverage area e.g.
1796 GA, ascorbic acid, cellulose. The diversity of uses for this group of bacteria is particularly
1797 highlighted by *Gluconobacter oxydans*. However, an understanding of their characteristics and
1798 metabolism is critical to maximising the potential of these bacteria, especially with respect to
1799 their strict oxidative requirements. Therefore, such an understanding of their metabolism can
1800 be applied to enable process optimisations for decreased process times or increased product
1801 yields, for example. In addition, further research may likely uncover other additional metabolic
1802 transformations performed by these bacteria which may have as yet unknown important
1803 applications.

1804 With rising consumer interest in fermented foods and beverages, linked to purported health
1805 benefits (both scientifically proven and anecdotal) and an increasing strive by consumers
1806 towards a more health-conscious lifestyle, the AAB, like the LAB, are well poised for future
1807 exploitation – both in the re-imagining of traditional foods or beverages, such as kombucha,
1808 and in the development of new types of products. However, further and more extensive studies
1809 and trials are needed with regard to the health benefits related to the consumption of AAB-
1810 fermented products. In addition, guidance needs to be put forward by the relevant regulatory
1811 authorities, regarding the safety aspects of the application of this important group of
1812 microorganisms. However, it is clear that more fundamental studies on the safety aspects of
1813 this microbial group are required, upon which guidance from regulatory authorities can be
1814 based.

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1820 www.youtube.com/watch?v=cbJf0MuWbJw

1821

1822

1823 **Author Contributions**

1824 Kieran M. Lynch and Emanuele Zannini wrote the manuscript with critical input and
1825 corrections by Elke K. Arendt. Kieran M. Lynch did the final editing. Stuart Wilkinson and
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2794 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)	<i>Acetobacter pasteurianus</i> , <i>A. aceti</i> , <i>Komagataeibacter xylinus</i> , <i>K. europaeus</i> , <i>K. hansenii</i>	(De Vero et al., 2006), (Gullo, Caggia, De Vero, & Giudici, 2006),
Kombucha	<i>K. xylinus</i> , <i>A. pasteurianus</i> , <i>K. hansenii</i> , <i>A. aceti</i> , <i>Ga. saccharivorans</i> , other <i>Acetobacter</i> , <i>Gluconobacter</i> , and <i>Gluconacetobacter spp.</i>	(Jayabalan et al., 2014), (Tan et al., 2012), (Liu et al., 1996), (Wang et al., 2014), (Marsh et al., 2014)
Water kefir	<i>A. lovaniensis</i> , <i>A. fabarum</i> , <i>A. cerevisiae</i> , <i>A. aceti</i> , <i>A. ghanensis</i> , <i>A. lovaniensis/fabarum</i> , <i>A. sicerae</i>	(Magalhaes et al., 2010), (Miguel, Cardoso, Magalhães, & Schwan, 2011), (Gulitz et al., 2013) (Stadie, Gulitz, Ehrmann, & Vogel, 2013), (Laureys & De Vuyst, 2014)
Lambic beer	<i>A. lambici</i> , <i>G. cerevisiae</i>	(De Roos & De Vuyst, 2018a, 2018b; Spitaels et al., 2014b; Spitaels et al., 2014c)

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

<i>Characteristic</i>	<i>Acetobacter</i>	<i>Gluconobacter</i>	<i>Gluconacetobacter</i>	<i>Komagataeibacter</i>
Type strain	<i>A. aceti</i> NBRC 14818 ^T	<i>G. oxydans</i> NBRC 14819 ^T	<i>Ga. liquefaciens</i> NBRC 12388 ^T	<i>K. xylinus</i> JCM 7644 ^T
Production of acetic acid from ethanol	+	+	+	+
Flagellation	Per	Pol	Per	N
Oxidation of (to CO ₂ and H ₂ O)				
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	+	+	+	+

2809 Per: peritrichous; Pol: polar; N: none; +, positive; -, negative; nd, not determined; b: some
2810 strains in the genus are positive; w, weakly positive; vw, very weakly positive. Modified from
2811 Yamada, 2016.
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2832 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

2833 GYC, glucose-yeast extract-calcium carbonate; AE, acetic acid-ethanol; YPM, yeast extract-peptone-mannitol;
 2834 MYA, malt extract-yeast extract-acetic acid; DMS, deoxycholate-mannitol-sorbitol; mDMS, modified
 2835 deoxycholate-mannitol-sorbitol.

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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-sorbose	Potential Vitamin C production	(Sugisawa, Hoshino, Nomura, & Fujiwara, 1991)
L-sorbose dehydrogenase*	SNDH _{ai}	PQQ	L-sorbose	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-myoinositol	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-1-sorbose	Production of the antidiabetic drugs, 1-deoxynojirimycin and miglitol	(Schedel, 2000)

2850 *Note: Another enzyme of the same name, SNDH-FAD, is located in the cytoplasm and
2851 converts L-sorbosone to 2-keto-L-gulonic acid.

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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)
<i>Codex Alimentarius</i> 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	<ol style="list-style-type: none"> 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)	<p>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</p> <p>2) Synthetic vinegar that is manufactured by diluting glacial acetic acid or acetic acid with drinking water.</p> <p>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</p>	(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)

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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	Jujube 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		
					Kakisui	Japan	
Animal	Whey	Whey	Fermented whey	Lactose	Whey vinegar	Europe	
	Honey	Honey	Diluted honey win, tej	Fructose, glucose	Honey vinegar	Europe, America, Africa	

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^aVegetable 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. ^bObtained by bamboo sap fermentation (González & De Vuyst, 2009).

^cUmeboshi 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. ^dListed in order, from the largest to the smallest amount.

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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	<i>E. coli</i> 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 otorrhea, 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 otorrhea 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- <i>O</i> - tetradecanoylphorbol-13-acetate induced myeloperoxidase activity and H ₂ O 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	(Tagliacruzchi 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)

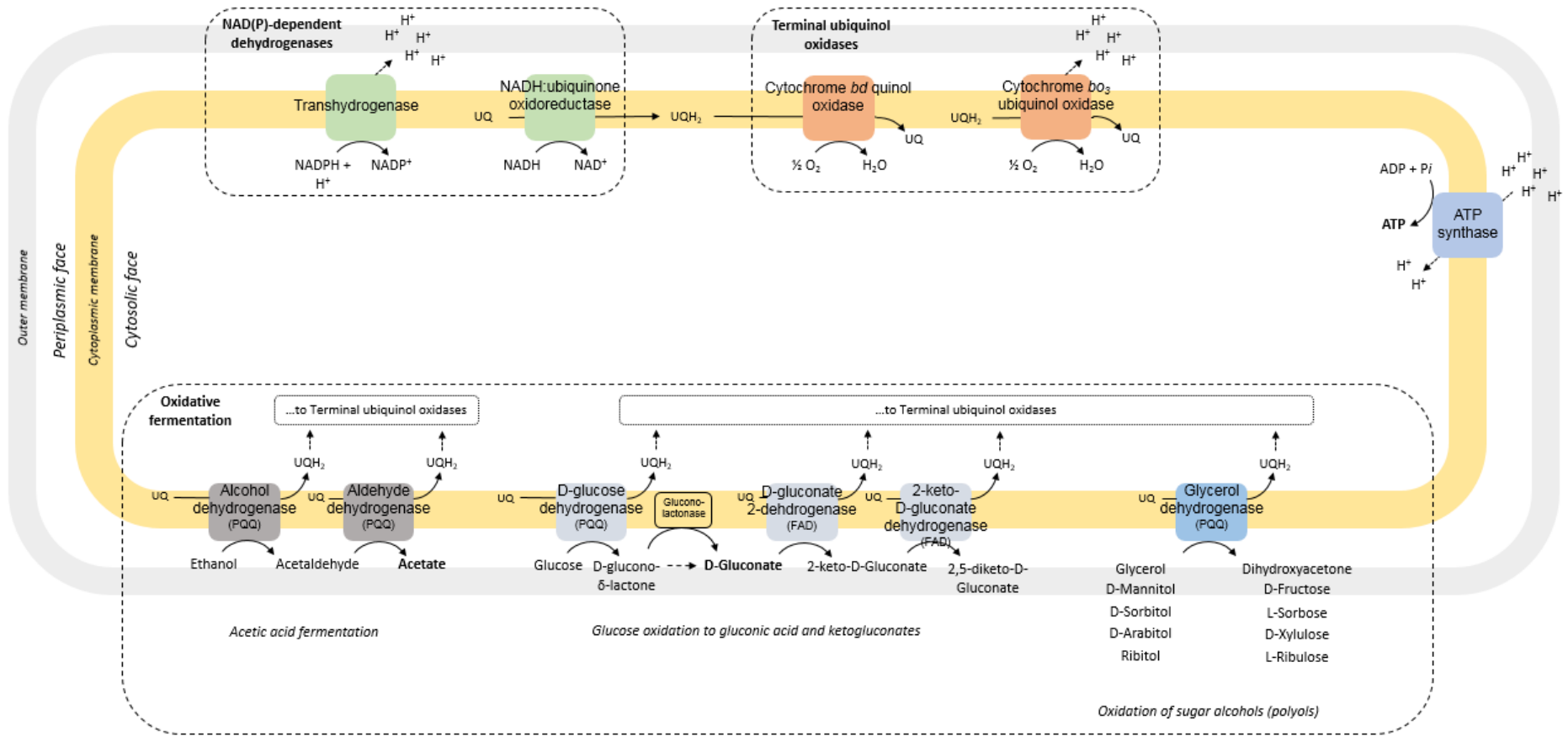
Apple vinegar	Blood glucose control	America	27 patients with type 2 diabetes	Oral intake of vinegar (1.4g/d) for 12 weeks significantly reduced haemoglobin A1c values in individuals with type 2 diabetes mellitus.	(Johnston et al., 2009)
Apple vinegar	Blood glucose control	America	8 healthy volunteers	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 <i>in vitro</i> .	(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 <i>in vitro</i> .	(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 <i>in vitro</i> .	(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 <i>in vitro</i> .	(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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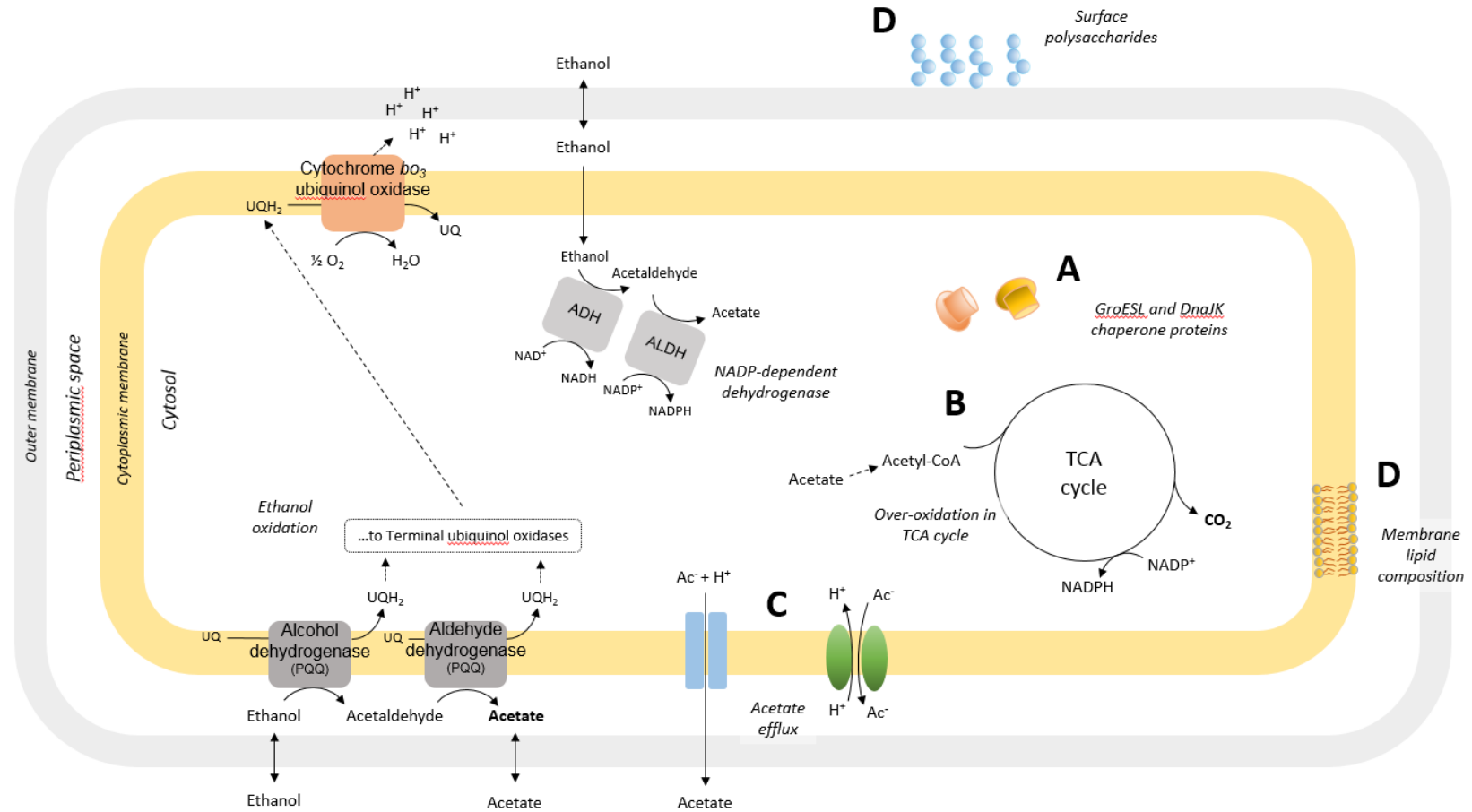


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2878 Figure 1. Respiratory and oxidative fermentation chains and associated dehydrogenases in Acetic acid bacteria.

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2882 Figure 2. Strategies of acetic acid resistance employed by Acetic acid bacteria. a) adaption of and protection of intracellular proteins to and
 2883 against acid stress, b) metabolism (overoxidation) of intracellular acetic acid, c) efflux of acetic acid from the cell and d) prevention of acetic
 2884 acid from entering the cell.

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2888 Figure 3. The process of wine vinegar-making from grape juice.

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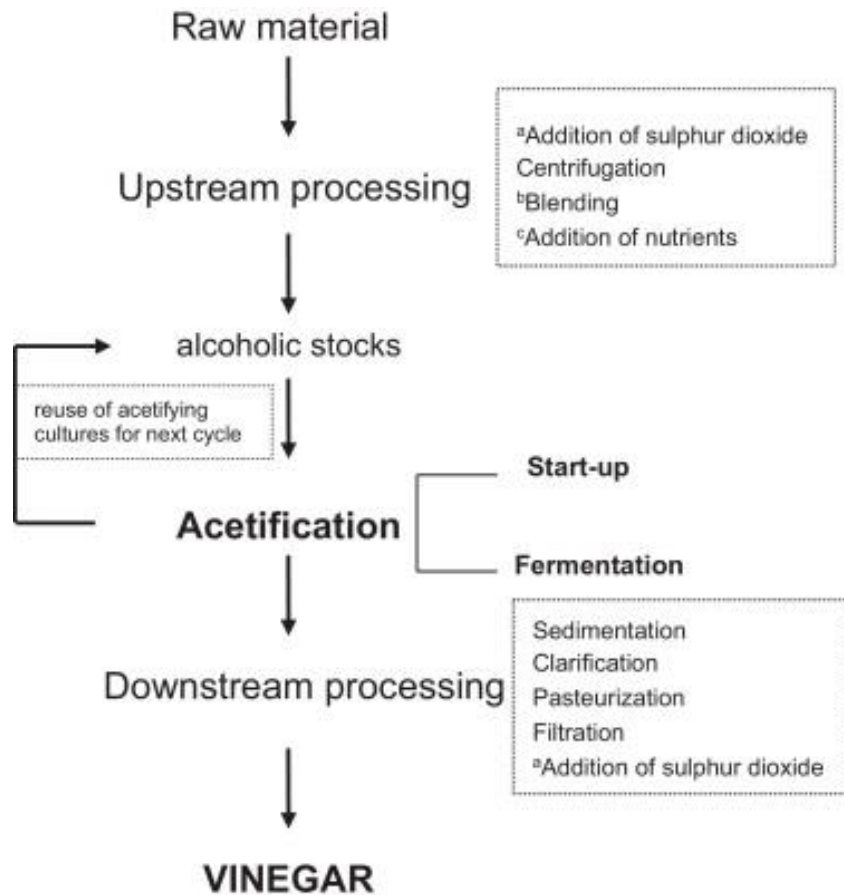
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2905 Figure 4. Schematic representation of vinegar production in submerged system. ^aAt concentrations
 2906 specified by legislation; ^b Blending with high acidity vinegar, to block undesired alcoholic fermentation;
 2907 ^c nutrients containing carbon and nitrogen sources, vitamins and minerals are supplemented especially
 2908 to produce high acidity vinegar (>12% of acetic acid) from alcoholic stocks containing no carbon
 2909 sources except for ethanol. Reprinted with permission from Gullo et al., 2014.

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2918 Figure 5. Kombucha cellulose pellicle (SCOBY).

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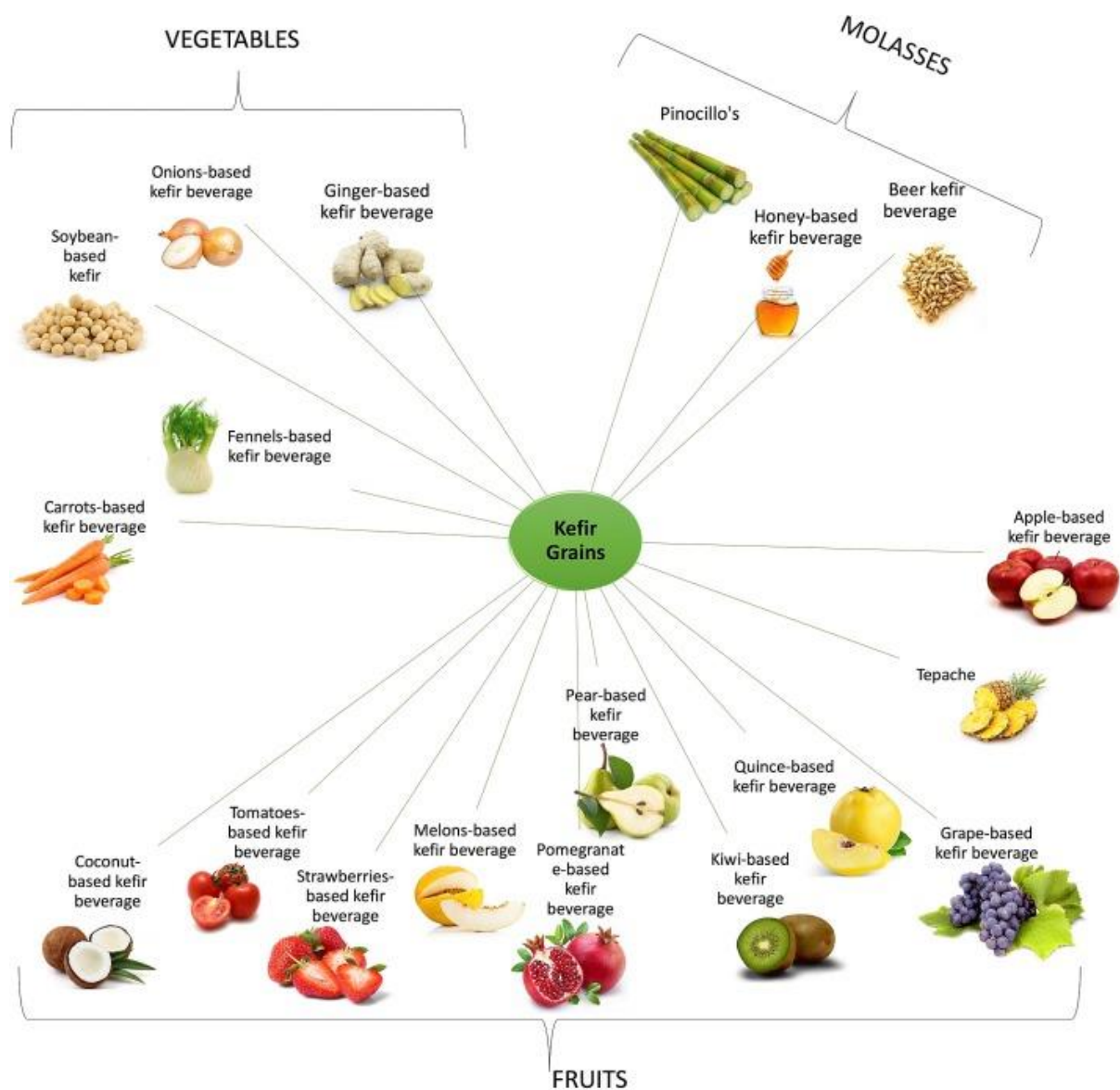
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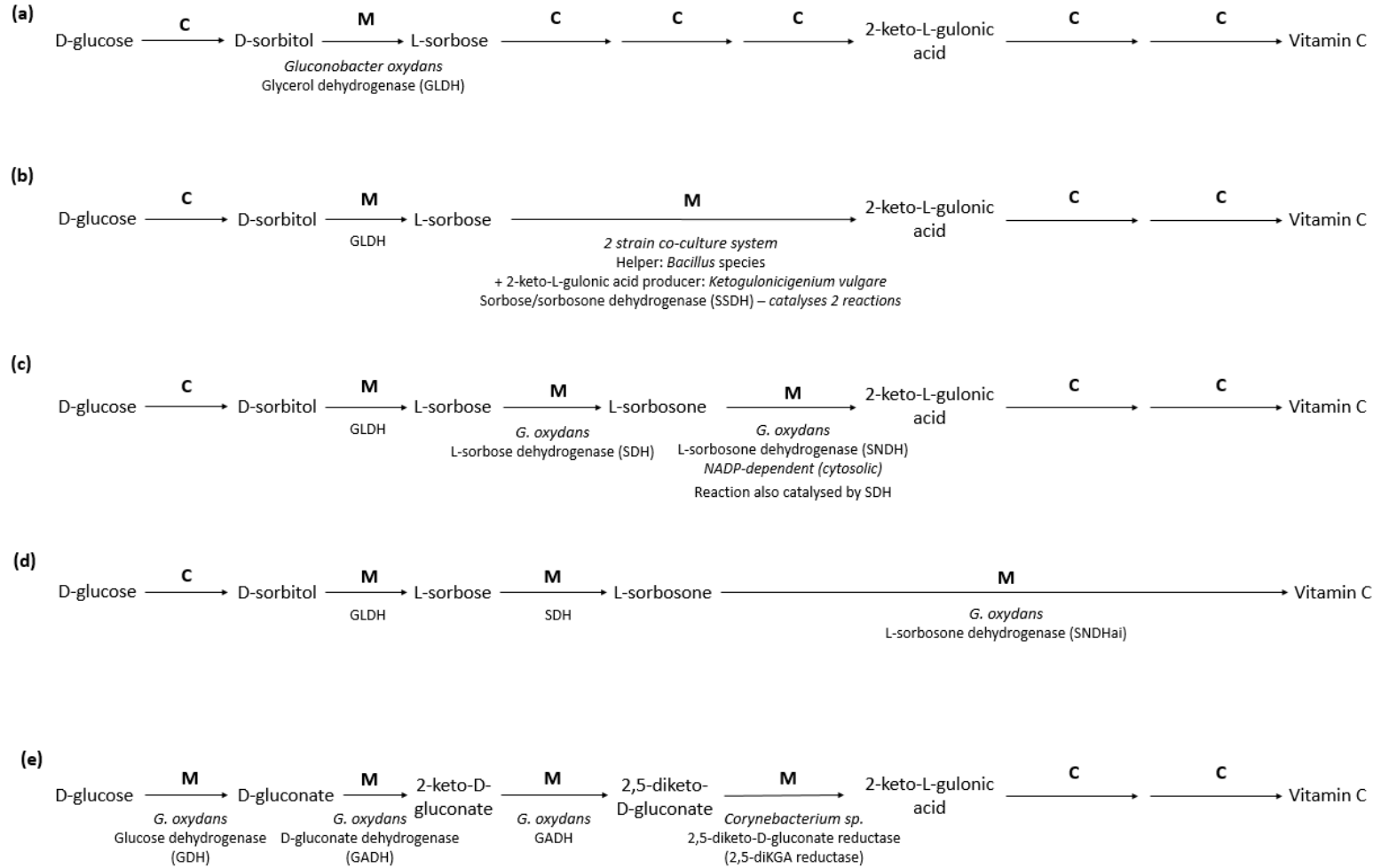
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2930 Figure 6. Different non-dairy products from sugary kefir fermentation. Reprinted with permission from
 2931 Fiorda et al., 2017.



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2933 Figure 7. Processes used in, or having the potential for, Vitamin C production. (a) The Reichstein Process, microbial production of L-sorbose from
 2934 D-sorbitol; (b) The Two-Step Fermentation Process, microbial production of 2-KLGA from L-sorbose via a 2-step fermentation; (c) Microbial
 2935 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
 2936 2,5-diKGA pathway. C: chemical reaction, M: microbial bioconversion.

Strain characterization and genome sequencing

Has the strain been characterized to genus and species level?
AND
Has the strain been completely genome sequenced?

If NO do it
If YES go on

Screening for undesirable attributes and metabolites

Is the strain free from genetic elements encoding known virulence factors and/or toxins associated with pathogenicity?
AND
Is the strain free of functional and transferable antibiotic resistance gene DNA?

Any NO: additional safety studies are required
If YES go on

Does the strain produce antimicrobial substances useful in human medicine?

↓
If YES: strain is not appropriate for human consumption
If NO go on

Genetic modification

Has the strain been genetically modified?

If YES: additional safety studies are required (+regulatory approval required in most countries)
If NO go on

Strain origin

Was the strain isolated from a food that has a history of safe consumption for which the species is a substantial and characterizing component (not simply an incidental isolate)?
AND
Has the species undergone comprehensive peer reviewed safety evaluation (e.g. QPS) or has it been affirmed safe by authoritative group/qualified scientific experts?
AND
Do findings published since completion of the peer review continue to support safety?

Any NO: additional safety studies are required
If YES go on

Exposure levels

Will the intended use of the strain expand exposure to the species beyond the group(s) that typically consume the species in traditional foods (in which it is typically found)?
AND
Will the intended use of the strain expand overall intake of the species (e.g. increasing the number of foods beyond traditional foods in which the species is found or using the strain as probiotic rather than starter culture)?

If NO: Strain is considered safe for human consumption
Any YES: additional safety studies are required

Additional safety studies

Does the strain induce undesirable physiological effects in appropriately designed safety studies (e.g. animal models or clinical trials)?

If NO: Strain is considered safe for human consumption
If YES: strain is not appropriate for human consumption

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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