The role of acetic acid bacteria in brewing and their detection in operation

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Abstract

Acetic acid bacteria (AAB) are often considered a threat of the past because today's equipment allows performing post-fermentation processes under a greatly reduced level of oxygen. This paper deals with the current importance of AAB in brewing. The risk of contamination as well as the functional role in spontaneously fermented sour beers is reviewed. The main harmful effect of AAB lies in the direct spoilage of draft beer and the formation of biofilms, most often in dispensing systems. On the contrary, AAB seems to be indispensable in the case of sour beer production. A key issue of AAB in the brewing environment is their (early) detection and identification. Therefore, a part of this study is devoted to both, the latest sophisticated methods and primarily those of traditional cultivation which are still prevalent in operating laboratories due to their low cost and easy implementation. Finally, an experimental and pictorial material is added as a guide for operations that have less experience with AAB.

Keywords: aerobic bacteria, acetic acid bacteria, *Acetobacter*, *Gluconobacter*, biofilm, brewing

1 Introduction

A group of gram-negative bacteria capable of oxidizing ethanol or sugar to acetic acid is known as acetic acid bacteria (AAB). These bacteria were discovered more than 150 years ago by Louis Pasteur who first described these microorganisms as agents for vinegar production (Lynch et al., 2019; Hommel and Ahnert, 1999). AAB are widespread and abundant in the environment, especially where alcoholic fermentation takes place. Several species of AAB are used in food industry e.g. to produce vinegar, kombucha, kefir, acidic beer, cocoa-based products, coffee, etc. (Papalexandratou et al., 2009; Vuyst et al., 2008; González et al., 2005; Gupta et al., 2001). They are also employed in various pharmaceutical industry and biotechnological applications in which some of their metabolites such as D-gluconic acid, L-sorbose or dihrydroxyacetone can be used (Paradh, 2015; Wang et al., 2015; Vuyst et al., 2008; Gupta et al., 2001). Further, production of biopolymers such as cellulose or acetan should be mentioned as well as the significance of AAB in vitamin C production (Lynch et al., 2019; Papalexandratou et al., 2009).

On the other hand, AAB also present a risk of undesirable contamination in biotechnological processes, namely spoilage of beverages e.g., wine, cider or beer. AAB damage the drink quality due to the formation of acetic acid that causes unpleasant vinegary taste and flavour accompanied by turbidity and ropiness (Gomes et al., 2018; Bartowsky and Henschke, 2008).

Several tens' years ago, AAB presented a serious problem in brewing industry. However, an implementation of modern technologies and equipment that enable high performance sanitation and maintenance of low level of oxygen helped to radically solve the issue concerning AAB (Paradh, 2015). Nowadays, AAB are considered as low risk contaminants in breweries and thus their role has substantially changed.

This overview deals with the current role of AAB in breweries and beer-associated places including restaurants, pubs, bars, etc. The conditions under which AAB can still manifest themselves as unpleasant contaminants spoiling beer are described. The role of indicator bacteria

pointing to technological errors or hygienic deficiency is underlined. And their relevance in the production of spontaneously fermented sour beers is also mentioned. The emphasis of this work is put on the methods of detection and identification of AAB directly in the production process or in dispensing system. The literature review is thus supplemented by several experimental data supported by pictorial material which breweries could facilitate to understand the AAB issue.

2   General characteristic of acetic acid bacteria

The AAB belong to the family of *Acetobacteraceae* as a branch of acidophilic bacteria in the α-subdivision of the Proteobacteria which currently comprises 47 genera with validly published names according to the List of Prokaryotic names with Standing in Nomenclature (Parte et al., 2020) or NCBI taxonomy database (Schoch et al., 2020). So far, only two genera have been important for the brewing environment. Namely *Acetobacter* including 39 species and *Gluconobacter* with its 20 species validly published under the International Code of Nomenclature of Prokaryotes (ICNP) up to date. However, due to a constantly changing taxonomic classification of AAB, the genera *Gluconoacetobacter* and *Komagataeibacter* are also likely to become known as significant in the brewery environment.

AAB are gram-negative, non-sporulating bacteria which usually have strictly aerobic metabolism with oxygen as a terminal electron acceptor. They are catalase-positive, oxidase negative cocci or rod-shaped cells of various lengths occurring individually or in chains. They belong to mesophilic microorganisms whose optimal growth temperature lies in the range of 25–30 °C. The maximum temperature at which they are able to grow is below 37 °C. The optimal pH for growth is between 5.0–6.5 (Paradh, 2015; Sievers and Swings, 2015; Wang et al., 2015; Sengun and Karabiyikli, 2011). In terms of nutrient requirements, AAB are classified as nutritionally undemanding microorganisms. They are able to use glucose, ethanol, arabinose, fructose, galactose, mannitol, mannose, ribose, sorbitol and xylose as carbon sources.

One of the most important metabolic characteristics is an ability to oxidize ethanol, sugars or sugar alcohol to corresponding organic acids, aldehydes or ketones under aerobic conditions. This oxidation can occur in neutral or acidic pH around the value 4.5 (Lynch et al., 2019; Wang et al., 2015; Papalexandratou et al., 2009). Produced organic acids are released and accumulated in external space which leads to acidification of the environment in order to prevent the growth of competitors. AAB are equipped with several mechanisms to cope with this acidity. These

mechanisms are described in detail in e.g. Wang et al. (2015). Moreover, these accumulated acids can be, after depletion of other carbon sources, completely oxidized to carbon dioxide and water to sustain the growth (De Roos and De Vuyst, 2018; Sievers and Swings, 2015).

Genus *Acetobacter* – ellipsoidal to rod-shaped cells with a size of 0.6–1.0 × 1.0–4.0 μm occurring as single cells, in pairs and in chains. They are either non-motile or motile due to peritrichous flagella. Non-spore-forming, gram-negative, obligately aerobic, catalase positive, oxidase negative. They are classified among chemoorganotrophic organisms with the best utilization of ethanol, glycerol and glucose. Optimal conditions for their growth are temperature around 30 °C and pH in the range 4–6. They are ubiquitous and can be isolated for example from flowers and fruits. They are a common contaminant of fermented meat, wine, beer, sake or cider. A pathogenic effect on human bodies has not been described (Sievers and Swings, 2015; Hommel and Ahnert, 1999). In breweries, the genus *Acetobacter* was isolated from wort, beer line, KEGs, biofilms and beer that was aged in KEGs for a long period of time. Contaminated beers were cloudy, with increased viscosity and exhibited a sour acetic taste and odour due to production of acetic acid (González et al., 2005; Van Vuuren and Priest, 2003; McDonnell and Russell, 1999; Ingledew, 1979).

Genus *Gluconobacter* – ellipsoidal to rod-shaped cells with a size of 0.5–1.0 × 2.6–4.2 μm occurring as single cells and/or in pairs, occasionally in chains. They are either non-motile or motile due to 3–8 polar flagella. Non-spore-forming, gram-negative, obligately aerobic, catalase positive, oxidase negative. Most strains are able to utilize ethanol, mannitol, fructose, glucose, maltose, glycerol and xylose. The members of this genus are capable of ketogenesis that means the formation of ketone compounds from polyols. optimal conditions for their growth are temperature around 25–30 °C and pH in the range 5–6. However, most strains are able to grow even at pH 3.5. The strains were isolated for example from flowers, fruits, wine, cider or beer (Sievers and Swings, 2015). In breweries, the genus *Gluconobacter* was isolated from wort, beer line and KEGs. Contaminated beers were cloudy, with a sour acetic taste and odour due to production of acetic acid, which can dissociate into acetate (González et al., 2005; Van Vuuren and Priest, 2003; McDonnell and Russell, 1999; Ingledew, 1979). The basic differences between both genera are summarized in Table 1.

Due to metabolic characteristics, the genus *Acetobacter* thrives in the brewing environment better than members of the genus *Gluconobacter*.

Table 1   *Basic characteristics of AAB important in brewing (Gomes et al., 2018; Paradh, 2015)*

Selected characteristic	*Acetobacter*	*Gluconobacter*
motility	non-motile	non-motile
flagella arrangement	peritrichous	polar
ox. of ethanol to acetic acid	+	+
ox. of acetic acid to CO_2 and H_2O	+	–
ox. of lactic acid to CO_2 and H_2O	+	–
formation of acid from raffinose	–	–
production of water-soluble brown pigment	–	+/–
major metabolites produced in beer	acetic acid	acetic acid/acetate

3   The role of acetic acid bacteria in brewing

The members of genera *Acetobacter* and *Gluconobacter* used to be feared contaminants in breweries due to conversion of ethanol to acetic acid or acetates resulting in vinegary off-flavours and formation of turbidity and slime (Sakamoto and Konings, 2003). AAB characteristics such as ability to survive in environments with high ethanol concentration (> 10% v/v) and low pH (< pH 3.8) and the resistance to bitter hop substances allowed them to thrive in the brewing environment. Nevertheless, nowadays beer production takes place with an emphasis on the elimination of oxygen in (post)-fermentation processes and thus the growth of AAB is suppressed by low concentration of oxygen. They are not currently considered as risky contaminants, especially in large breweries (Vriesekoop et al., 2012).

Despite the high modernization of beer production described above and strong antimicrobial properties of beer itself, AAB should be still considered as significant member of potentially occurring microbial communities in breweries. An overview of AAB recently isolated from brewing environment is listed in Table 2.

AAB can be divided into three groups according to their specific activity in a brewery:
- contaminants that spoil beer or a semi-finished product;
- contaminants as indicator microorganisms;
- production microorganisms – part of production consortium.

Beer spoilers. AAB can enter into breweries with raw materials (water, malt, wort extract, yeast) (Back, 2005) or via returning contaminated empty KEGs from consumers (Paradh, 2015; Back, 2005). Their spoiling activity results in the formation of slime and turbidity, decrease of alcohol content due to undesirable production of acetic acid. Such beer can be ropy because of bacterial polysaccharides and is characterized by an unpleasantly changed taste and flavour (Paradh, 2015; Storgårds, 2000).

Ploss et al. (1979), who studied 1203 samples taken from brewing production (from raw materials to finished beer), found 153 samples positive for AAB. The authors concluded that AAB are more common in lager cellars, filtration processes and filling plants. The most frequent member (70 %) belonged to *A. pasteurianus* subsp. *pasteurianus* (Ploss et al., 1979). A lower incidence of AAB was noted in samples from the main fermentation and storage tanks of input raw materials (Hill, 2015). Cases of beer contamination in KEG barrels were described as well (Ingledew, 1979). The cause of bacterial contamination in KEGs can occur when using air as a pressure medium without an integration of a suitable microbial filter behind compressor outlet.

It is necessary to mention that mini-breweries are more susceptible to AAB contamination as sanitation does not take place under such strict conditions, and modern bottling technologies are usually not installed.

Another critical area from the contamination point of view including AAB incidents is a dispensing system in restaurants, pubs, bars, etc. While AAB are already pretty rare in beer production, they can be captured in draught beer in relatively high amounts. The problem of tap equipment together with beer lines is presence of oxygen and higher temperature in some parts of this system in combination with insufficient hygiene (Jevons and Quain, 2021). These cases have led to a repeatedly confirmed finding that some AAB strains are able to survive under limitation of oxygen (De Roos et al., 2018; Van Vuuren and Priest, 2003), in other words the traces of oxygen are enough to maintain viability. The most problematic points are poorly accessible and thus insufficiently sanitized places with air access such as taps, valves, space under seal, cavities, beer lines, tap stools. The growth of few attached bacteria occurs very fast either as single cells or with the formation of a highly resistant biofilm. It is very difficult to remove such a biofilm from an invaded place (Back, 2005).

Table 2   *The acetic acid bacteria (AAB) detected in breweries*

Current name of AAB	Alternative name	Source	References
unspecified AAB	–	fermentation of Belgian red-brown ale	Snauwaert et al. (2016)
		collection vessel fermentation and storage tanks	van Vuuren et al. (1979)
genus *Acetobacter*			
A. aceti	–	fermentation of lambic beer	De Roos and De Vuyst (2018)
A. cerevisiae	–	spoiled beer	Wieme et al. (2014) Cleenwerck et al. (2002)
A. fabarum	–	drought beer	Jevons et al. (2021)
		spoiled beer	Wieme et al. (2014)
		fermentation of American coolship ale	Bokulich et al. (2012)
A. indonesiensis	–	fermentation of lambic beer	De Roos and De Vuyst (2018)
		spoiled beer	Wieme et al. (2014)
A. lambici	–	fermentation of lambic beer	De Roos and De Vuyst (2018) Spitaels, Li et al. (2014)
A. lovaniensis	A. pasteurianus subsp. lovaniensis	fermentation of lambic beer	De Roos and De Vuyst (2018)
		fermentation of American coolship ale	Bokulich et al. (2012)
A. orientalis	–	fermentation of lambic beer	De Roos and De Vuyst (2018)
A. orleanensis	A. aceti subsp. orleanensis	spoiled beer	Wieme et al. (2014) Cleenwerck et al. (2002)
A. pasteurianus	A. ascendens	(spoiled) beer, microbrewery surface	De Roos and De Vuyst (2018) Gonzáles et al. (2004)
		microbrewery surface	Maifreni et al. (2015)
		fermentation of Belgian red-brown ale	Snauwaert et al. (2016)
		fermentation of lambic beer	De Roos et al. (2020)
A. pasteurianus subsp. pasteurianus	–	cellar, filtration, filling	Vriesekoop et al. (2012) Ploss et al. (1979)
A. persici	–	spoiled beer	Wieme et al. (2014)
A. pomorum	–	fermentation of lambic beer	De Roos et al. (2020)
not specified *Acetobacter*	–	wort, beer dispenses, cask-conditioned beer, barrel-aged beer, biofilms in brewery	Paradh (2015) Vriesekoop et al. (2012)
		spoiled beer	Wieme et al. (2014)
		fermentation of lambic beer	De Roos et al. (2020)
genus *Gluconobacter*			
G. cerevisiae	–	fermentation of lambic beer	Spitaels, Wieme et al. (2014) De Roos and De Vuyst (2018)
		spoiled brewer's yeast	Spitaels, Wieme et al. (2014)
		spoiled beer	Wieme et al. (2014)
G. cerinus	–	spoiled beer	Wieme et al. (2014)
G. japonicus	–	spoiled beer	Wieme et al. (2014)
G. oxydans	–	brewing environment, beer	Paradh (2015) Gonzáles et al. (2004)
		spoiled beer	Wieme et al. (2014)
G. wancherniae	–	fermentation of lambic beer	De Roos and De Vuyst (2018)
not specified *Gluconobacter*	–	wort, beer dispenses, cask-conditioned beer	Paradh (2015) Vriesekoop et al. (2012)
		spoiled beer	Wieme et al. (2014)

Indicator bacteria. We can talk about indicator microorganisms (MOs) when they do not cause spoilage but they appear as a consequence of insufficient cleaning or errors in production. Thus their presence is often associated with the occurrence of current frightening beer spoilers and indicates deteriorated hygienic level or technological errors (Paradh, 2015; Back, 2005; Storgårds, 2000). The real harmfulness of detected AAB lies mainly in the formation of biofilms as they are one of the first microorganisms attaching to various types of surfaces. Critical points in terms of a microbial biofilm formation in brewing operations are those places (e.g. corners, folds, niches, etc.) that are hard to access while sanitary cleaning (mechanical and/or chemical). Residues of process intermediates, finished beer or other liquids are remains in such places and thus present a favourable environment for gradual development of a microbial biofilm. The first step of the biofilm formation is a colonization of a suitable surface under aerobic conditions by AAB together with some enterobacteria. These bacteria are not considered harmful in beer and may not even be detected. Nevertheless, their long-term biological activity causes slime coating of the surface (Storgårds, 2000) because many of AAB members are able to produce high levels of polysaccharides, among which the main are cellulose, dextran and levan (Paradh, 2015; Gullo and Giudici, 2008). In this manner, AAB create a suitable and protected environment for other MOs such as yeasts. Thus, yeasts start to grow together with AAB in the environment of polysaccharides and other nutrients, and produce other essential factors promoting the growth of lactic acid bacteria (LAB). LAB on the other hand accumulate lactic acid which can serve as a carbon and energy source for anaerobic bacteria, e.g. *Pectinatus* sp. or *Megasphaera* sp. (Maifreni et al., 2015; Timke et al., 2004; Storgårds, 2000).

The ability to form a biofilm on the number of surface materials has so far been proved in *Acetobacter* sp. (e.g. *A. aceti* or *A. pastorianus*), and *Gluconobacter oxydans* (Storgårds, 2000).

As mentioned above, the biofilm initiated by AAB frequently appears at dispensing lines.

Functional role. AAB communities can be detected during fermentation and maturation of spontaneously fermented sour beers such as Belgic lambic beer or American coolship ale, etc. The production of these acidic ales lies in a long term (up to 3 years) microbial process which proceeds in wooden barrels. The basic sensorial characteristic of spontaneously fermented beers is their pleasant/refreshing acidity (De Roos et al., 2020; De Roos et al., 2018; Snauwaert et al., 2016; Spitaels, Wieme, et al., 2014; Bokulich et al., 2012). A part of this acidity is caused

by AAB. The role of AAB was until recently considered limited because they were only rarely isolated from sour beers production most likely due to the following reasons:

- They are not detectable in wort, their noticeable development starts after the first week of the fermentation process;
- Their amount and species diversity change over time (De Roos et al., 2018);
- Strictly aerobic AAB are more concentrated on the surface of wort where the oxygen is abundant. Hence, they are omitted due to typical submerged sampling of wort/beer (De Roos et al., 2018; De Roos and De Vuyst, 2018);
- Occurrence of AAB in a viable but not culturable state (De Roos and De Vuyst, 2018);
- Their count and species diversity are specific for each brewery and the character of surroundings, moreover spontaneous fermentation is natural, variable and hardly predictable (Snauwaert et al., 2016).

Exploration of the spontaneous microbial consortia during acidic beer fermentation led to an isolation and detection of two new species of AAB, namely *Acetobacter lambici* (Spitaels, Li, et al., 2014) and *Gluconobacter cerevisiae* (Spitaels, Wieme, et al., 2014).

Also De Roos et al. (2018) dealt with the role and occurrence of AAB in lambic beer production and identified 359 AAB isolates in beer samples taken from two wooden casks where a 24-month fermentation/maturation took place. They found that species of genus *Acetobacter*, including *A. orientalis*, *A. pasteurianus*, or *A. lambici*, predominated over *Gluconobacter* species such as *G. cerevisiae*. The monitored metabolic products were acetic acid, little of gluconic acid, ethyl acetate and acetoin. This study looked at the AAB counts as a function of fermentation/maturation time.

The authors also confirmed that AAB can survive under limitation of oxygen.

Recently, this research team has published temporal metagenomic analysis which found AAB, represented by genera *Acetobacter* and *Komagataeibacter*, as the most abundant bacteria at the beginning and during the first 3 months of fermentation. Lower amount of AAB has proved to persist in the fermenting beer until the late stages of maturation (De Roos et al., 2020).

Although AAB play an essential role in acidic beer production, their excessive development is not desirable as too high concentration of acetic acid and acetoin can lead to a disharmonious sensory profile of the final product. Due to natural regulation of AAB caused by a limited access of oxygen, it is sufficient not to miss the routine control of AAB during processing.

4   Detection and Identification Methods of Acetic Acid bacteria

There are many traditional and modern techniques of AAB detection and identification that are crucial mainly in food processing, either to solve contamination incidents or to monitor microbial community during spontaneous fermentation. Traditional methods are based on physiological and chemotaxonomical properties, e.g. growth at low pH, production of acetic and/or gluconic acid from ethanol and/or glucose, or growth in the presence of 0.35% (v/v) acetic acid etc. (Vuyst et al., 2008). These cultivation techniques are inexpensive and require only common laboratory equipment. However, they are quite time-consuming and their reliability is not 100% (González et al., 2004; Storgårds, 2000). Established culture media often underestimate microbial populations in many systems, and furthermore a large proportion of the microbial cells can be in a non-culturable state caused by environmental stress such as oxygen deprivation (González, Hierro, et al., 2006; Storgårds, 2000). However, the cultivation method is still widely used for the enumeration of microbes (Storgårds, 2000) as well as a routine detection technique. In order to overcome these disadvantages, the cultivation methods have been complemented or replaced by modern molecular or instrumental techniques, see several examples stated in Table 3.

Genera *Acetobacter* and *Gluconobacter* are not nutritionally demanding. However, the most suitable carbon sources for *Acetobacter* are ethanol, glycerol and D-lactate, while for *Gluconobacter* it is D-mannitol, sorbitol, glycerol, D-fructose and D-glucose (Gullo et al., 2006). The choice of an appropriate medium depends on a particular goal e.g. strain isolation from spontaneous fermentation, study of a biofilm in operation or control of potential contamination. A number of diagnostic culture media are mentioned in scientific literature. The basis of these diagnostic media can be ethanol or acetic acid together with ethanol as well as diverse media containing various combinations of glucose, sorbitol together with mannitol or glucose medium with CaCO₃ and ethanol, etc. The nutritional supplement is usually peptone and/or yeast extract (Gomes et al., 2018; Sievers and Swings, 2015; Sengun and Karabiyikli, 2011). A number of media including their composition are mentioned for instance in Lynch et al. (2019), Wieme et al. (2014) or Hommel and Ahnert (1999).

The AAB strains can be isolated from complex matrices containing other microorganisms by reducing pH of the culture medium to the value 4.4 and/or by adding antimicrobial agents e.g. cycloheximide to inhibit yeasts or penicillin to inhibit lactic acid bacteria (Gullo et al.,

2006). Media relevant for the isolation of AAB strains are described for example in Lynch et al. (2019).

The main aim of this article is to offer a guide for an ordinary brewing laboratory in order to help them with a basic AAB diagnosis. The so-called chalk-ethanol test can be used for this purpose. This medium is among others used for phenotypic differentiation of AAB – cultivated AAB dissolves added CaCO₃ which results in the formation of transparent zones in the medium. Subsequent oxidation of acetic acid, which only some members are capable of, leads to a gradual re-formation of solid CaCO₃, which results in the formation of milky turbidity in the medium (Sievers and Swings, 2015). Calcium carbonate is a part of Frateur's medium and GYC agar.

The production of acetic acid during the bacterial growth and its subsequent oxidation considerably changes the pH of the culture medium. This ability is used to rapid phenotypic differentiation of AAB on Carr's medium with the addition of bromcresol blue.

The detection of AAB is not performed routinely in operational brewing laboratories because their harmfulness is not so serious and lies mainly in their ability to form a biofilm. Their identification is therefore carried out especially in situations where the overall microbiological quality of beer is deteriorated due to a technological error or insufficient sanitation. The most critical point is filling equipment such as impact heads or taps.

5   Experimental supplement materials

The recommended detection of AAB useful for brewing laboratories is described above, however, the authors believe that specific examples accompanied by pictorial documentation from our laboratory could be useful.

5.1   Culture Media
- **Meat Peptone Agar (MPA)**, preparation: Dissolving 20 g of dehydrated nutrient agar (Merck) in 1,000 ml of distilled water. The prepared soil is light yellow.
- **Carr Medium**, preparation: Dissolving 30 g of a yeast extract (Merck) + 20 ml ethanol (Lach:mer) + 20 g bacteriological agar (Oxoid) + 0.022 g bromcresol green (Lachema) in 1,000 ml of distilled water. The prepared soil is green-blue.
- **Frateur Medium**, preparation: Dissolving 30 g of a yeast extract (Merck) + 20 ml of ethanol (Lach:mer) + 20 g bacteriological agar (Oxoid) + 20 g calcium carbonate (Sigma) in 1,000 ml of distilled water. The prepared soil is light yellow and turbidity appears due to insoluble CaCO₃.
- **GYC Agar**, preparation: Dissolving 10 g of a yeast extract (Merck) + 100 g glucose (Merck) + 15 g bacteri-

Table 3   *An overview of modern detection and identification methods suitable for AAB*

Method	Level of differenciation	Source	References
DNA/RNA-dependent molecular techniques			
DNA:DNA hybridizations	species	fermentation of lambic beer	Spitaels, Li et al. (2014)
		brewery environment	Spitaels et al. (2014)
		fruits, flowers and related material	Tanasupawat et al. (2009)
		fermented cocoa beans	Vuyst et al. (2008)
		apple juice	Dellaglio et al. (2005)
		collection strains	Cleenwerck et al. (2002) Lisdiyanti et al. (2000)
PCR–RFLP 16S rRNA	species	grapes, fresh grape must, wine fermentation	Gonzáles et al. (2005)
		wine fermentation	Gonzáles, Guillamón et al. (2006) Ruiz et al. (2000)
		collection strains	Gonzáles, Hierro et al. (2006) Gonzáles, Guillamón et al. (2006) Poblet et al. (2000)
PCR–RFLP 16S rDNA	species	fresh grape must,	Gonzáles et al. (2004)
		red wine fermentation	
PCR–RFLP 16S-23S rRNA/ rDNA	species	fruits, flowers and related material	Tanasupawat et al. (2009)
		collection strains	Tanasupawat et al. (2009) Gonzáles, Guillamón et al. (2006) Trcek (2005) Trcek and Teuber (2002) Ruiz et al. (2000)
PCR–RFLP 16S-23S-5S rDNA	species	traditional balsamic vinegar	Gullo et al. (2006)
PCR–TRFLP	species	fermentation of American coolship ale	Bokulich (2012)
RAPD fingerprinting	strain	brewery environment	Spitaels et al. (2014)
		bottled wine	Bartovsky et al. (2003)
		spirit vinegar	Trcek et al. (1997)
		rice vinegar	Nanda et al. (2001)
MLST/MLSA	not specified	brewery environment	Spitaels et al. (2014)
PCR-DGGE	species	wine fermentation	Andorra et al. (2008)
		traditional balsamic vinegar	De Vero et al. (2006)
ERIC-PCR	strain	grape surface grape must wine fermentation	Gonzáles et al. (2005)
		rice vinegar	Nanda et al. (2001)
REP-PCR	strain	grape surface grape must wine fermentation	Gonzáles et al. (2005)
(GTG)5-rep-PCR fingerprinting	strain	collection strains	Papalexandratou et al. (2009)
		fermented cocoa beans	Vuyst et al. (2008)
454 Pyrosequencing	species	fermentation of Belgian red-brown ale	Snauwaert et al. (2016)
methagenomic DNA analysis	genus/species	lambic beer fermentation	De Roos et al. (2020)
Other non-DNA/RNA-dependent techniques			
MALDI-TOF-MS	species	lambic beer fermentation	De Roos et al. (2018)
		fermentation of lambic beer	Spitaels, Li et al. (2014)
		brewery environment	Spitaels et al. (2014)
		collection strain originating from brewery environment, spoiled beers	Wieme et al. (2014)

PCR – Polymerase Chain Reaction; RFLP – Restriction Fragment Length Polymorphism; RAPD – Ramdomply Amplified Polymorphic DNA; MLST – Multilocus Sequence Typing/Analysis; DGGE – denaturating gradient gel electrophoresis; ERIC – Enterobacterial Repetitive Intergenic Consensus; REP – Repetitive Extragenic Palindromic; MALDI – Matrix-Assisted Laser Desorption/Ionization; TOF – Time-of-Flight; MS – Mass Spectrometry

ological agar (Oxoid) + 20 g calcium carbonate (Sigma) in 1,000 ml of distilled water. The prepared soil is light yellow and turbidity appears due to insoluble $CaCO_3$.

- **WLN medium**, preparation: Dissolving 75 g of dehygrated WL nutrient agar (Merck) in 1,000 ml of distilled water.

The all-above-mentioned media were sterilized for 20 min at 121 °C.

5.2 Selected acetic acid bacteria and their culture conditions

The bacterial strains used in this work come from the Czech Collection of Microorganisms (CCM) in Brno, Czech Republic. The list of selected strains, their designation and origin are given in Table 4.

The strains were incubated under aerobic conditions on an MPA medium at 28 °C for 48 h before inoculation on a specific testing media. The cultures were then inoculated by cross-spreading on the testing media. Incubation was performed repeatedly under aerobic conditions at the optimal growth temperature (Table 4). The time of cultivation was 3 to 5 days and the bacterial growth was monitored on a daily basis.

5.3 Pictorial material

Diagnostic media. On the basic of culture medium MPA, all selected strains of AAB grew in the form of flat round smooth glossy colonies of white to slightly cream colour as stated by Back (2005). The fundamental diagnostic characteristic of AAB is an ability to oxidize ethanol to acetic acid, which is released into the medium, as mention above. The genera *Acetobacter* and *Gluconacetobacter* can further oxidize the acetic acid to CO_2 and water after depletion of ethanol (Sievers and Swings, 2015) while *Gluconobacter* lacks this ability and acetic acid is thus the final product of its metabolism (Kersters et al., 2006). Phenotypic differentiation of the genera *Acetobacter* and
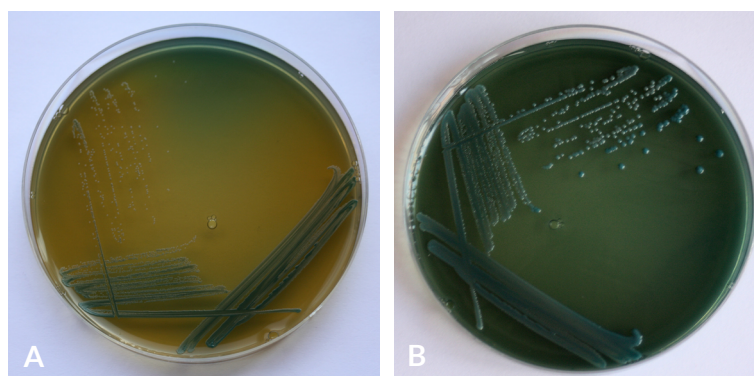

Figure 1 *A – Discoloration of Carr's medium at the growth site of Acetobacter pastorianus CCM 2374[T] after 48 hours incubation due to acetic acid formation; B – Subsequent darkening of agar after prolonged incubation to 120 hours due to oxidation of acetic acid to CO_2 and water.*

Gluconobacter on Carr's medium is based on this metabolic variance. The Carr's medium contains bromcresol green as a pH indicator. The formation of acetic acid is then indicated by a colour change from the originally blue-green to yellow where the subsequent re-bluing indicates re-increased pH due to the production of CO_2 and water (Sievers and Swings, 2015).

This apparent discoloration of Carr's agar appeared also during the cultivation of all selected AAB strains. The colour change was observable at the bacterial growth site as a result of acetic acid production after 48 hours of incubation (Figure 1A and 2A). The medium re-darkened to the original blue-green coloration after prolonged incubation of strains belonging to genera *Acetobacter* (Figure 1B) and *Gluconacetobacter*. As previously mentioned, the representatives of the genus *Gluconobacter* do not have the ability of acetic acid oxidation, therefore this acid remains in the medium, hence the change of pH and re-coloration of agar to blue-green did not appear (Figure 2B).

It was also mentioned that calcium carbonate ($CaCO_3$) can be used to detect acetic acid production during cultivation of AAB. $CaCO_3$ is decomposed due to the effect of acetic acid which leads to the formation of CO_2, water and soluble calcium acetate. This detection method was tested on GYC and Frateur's agars containing $CaCO_3$. Glucose is

Table 4 *The list of used strains of AAB, their collection, designation and origin.*

Bacterial Name according to CCM	Strain Designation	Origin	Optimal Growth Temperature
Acetobacter aceti	CCM 3620[T]	wine	30
Acetobacter pasteurianus	CCM 2374[T]	beer	25
Gluconacetobacter hansenii	CCM 1808	unknown	25
Gluconacetobacter liquefaciens	CCM 3621[T]	dried fruit	30
Gluconobacter oxydans	CCM 3607[T]	beer	25

CCM – Czech Collection of Microorganisms, Brno, Czech Republic

a source of carbon and energy in GYC agar, while in Frateur's agar it is ethanol. Before inoculation, both media were light yellow accompanied by milky turbidity caused by undissolved $CaCO_3$. Acetic acid produced during incubation dissolves $CaCO_3$ and thus clarifies the culture medium. A so-called halo effect appears around the colonies (Lisdiyanti et al., 2000).

Only slow growth of the examined AAB was noticed on Frateur's agar during 120 hours of incubation, and no clear zones formed around the colonies, an exception were the areas with a dense culture. Cream-colored colonies were hard to detect (Figure 3A).

The composition of Frateur's agar is relatively unfavourable for rapid growth of AAB due to the absence of sugars. Hence, it is evaluated as less suitable for using in an operating laboratory. On GYC agar, growth of AAB was accompanied by the formation of more pronounced clarified zones, but again only in the areas with a dense culture (Figure 3B).

AAB can be caught also on WLN agar during routine microbiological analyses in a brewery. An example is the determination of the total number of aerobic germs or total number of aerobic bacteria when actidion is added. The growth of AAB is then manifested by a colour change of originally blue-green medium to bright yellow around the colonies (Figure 4).

AAB can grow on a Chromocult Coliform medium that is intended for the determination of coliform bacteria. AAB forms colourless to creamy colonies so they cannot be confused with coliform bacteria, whose colonies are pink-purple in this medium. However, the capture of AAB in this medium can occur only at cultivation temperatures up to 34 °C as AAB are mesophilic microorganisms with an optimal growth temperature of about 30 °C. They do not grow when the temperature is above 34 °C (Lynch et al., 2019; Saichana et al., 2015).

Figure 2 *A – Discoloration of Carr's agar at the growth site of Gluconobacter oxydans CCM 3607^T after 48 hours incubation due to acetic acid formation; B – No re-coloration after prolonged incubation – acetic acid remains the final product of bacterial metabolism.*

Figure 3 *Cell growth of Gluconobacter oxydans CCM 3607^T on Frateur's agar (A) and GYC agar (B) after 120 hours of incubation. The so-called halo effect, i.e. the zone where $CaCO_3$ was dissolved by produced acetic acid, is visible only at the site of thicker culture coating.*

Figure 4 *Cell growth of Gluconacetobacter hansenii CCM 1808 (A) and Acetobacter pasteurianus CCM 2374^T (B) on WLN agar after 72 hours of incubation.*

Biofilms in breweries. Fig. 5–8 document unacceptably poor hygiene of brewery operation. The growth of a biofilm is extensive in the shown places and the risk of contact with beer or it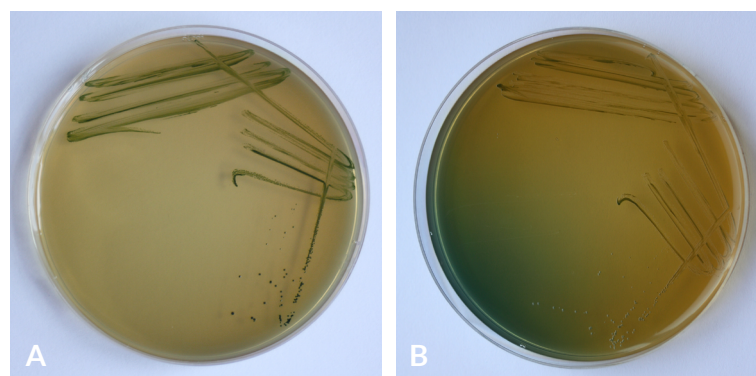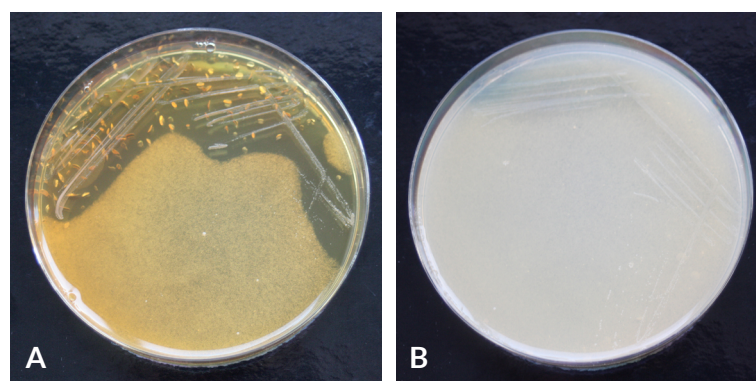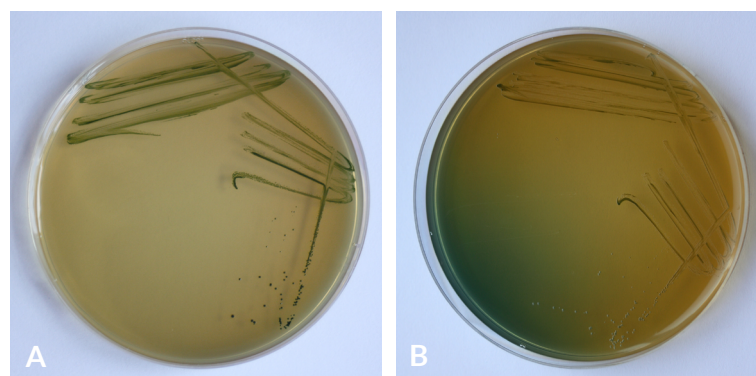s intermediate product is high. These appalling conditions indicate that these places have not been sanitized for a long time and that regular sanitation is ineffective as usually only microorganisms on the surface of the biofilm are killed. The only effective method for a biofilm disposal is perfect mechanical cleaning just before sanitation itself.

Biofilm inside the sterilization tank at a yeast propagation plant (Fig. 5) is already starting to dry and microbes

Figure 5 *Biofilm inside a sterilization tank at a yeast propagation plant*

Figure 6 *Flange on the pipe overgrown with active biofilm*

of contamination that causes microbial spoilage of beer.

6 Conclusion

A diverse role of AAB supplemented by modern and traditional methods for their detection and identification is discussed in this paper. Although AAB pose only a small risk of contamination compared to wild yeasts, lactic acid bacteria or strict anaerobes, their presence in brewing environment cannot be overlooked. Contamination by these bacteria is not so rare in draft beer and long-aged beers in KEGs. Their harmfulness lies mainly in an ability to form a biofilm which provides suitable conditions for the growth of truly harmful microorganisms. These bacteria should also be considered in the case of mini-breweries which usually do not have any modern bottling technologies installed and do not have such high demands on sanitation. Moreover, the occurrence is associated with tap equipment and beer lines in restaurants and pubs where they indicate an insufficient level of hygiene together with an inappropriately selected pressure medium. Due to a low risk of AAB contamination, they are not routinely monitored in beer and brewing operations. If their presence is suspected, Carr's medium can be used for detection and at the same time for rapid phenotypic differentiation of AAB.

do not have a sufficient source of energy and water. Complete removal of this biofilm will be very difficult because it is a hard-to-reach interior place. Chemical washing and subsequent disinfection will certainly not be enough. Figure 6 shows a flange on the pipe overgrown with an active biofilm. Contamination of sealing elements, interior and damper itself is clearly distinguishable. The biofilm already penetrates through its seal into the pipe where it comes into contact with beer or its intermediate products. Although the biofilm is outside the production facility, it easily becomes a part of an internal/closed system. If a hose or elbow is fitted to such a contaminated flange without proper mechanical cleaning of the biofilm complemented by subsequent sanitation, impurities and primarily beer spoiling microorganisms living in biofilm layers will enter the product. Such a place is definitely a source

7 Acknowledgement

Table 5 *An overview of bacterial growth on tested culture media after 72 and 120 hours of incubation*

Bacterial name according to CCM	Strain designation	Culture medium					
		Carr's medium		Frateur's medium		GYC-agar	
		72 hrs	120 hrs	72 hrs	120 hrs	72 hrs	120 hrs
Acetobacter aceti	CCM 3620ᵀ	+	+	~	~	+	+
Acetobacter pasteurianus	CCM 2374ᵀ	+	+	+	+	+	+
Gluconacetobacter hansenii	CCM 1808	+	+	~	~	+	+
Gluconacetobacter liquefaciens	CCM 3621ᵀ	+	+	~	~	+	+
Gluconobacter oxydans	CCM 3607ᵀ	+*	+*	+	+	+	+
	CCM 3617	+*	+*	~	+	+	+

+ growth; – no growth; ~ slight growth
* no re-coloration

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