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Review: Gram Negative Bacteria in Brewing

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Abstract

Gram negative aerobic bacteria such as Acetic Acid Bacteria, which include *Acetobacter* and *Gluconobacter*, have historically caused significant problems to brewers. Although incidences of spoilage have recently reduced as a result of improvements in beer packaging, these bacteria are still a concern in dispense systems in pub breweries, public houses and cask conditioned beers. Gram negative facultative bacteria of the genus *Zymomonas* can spoil primed cask conditioned beer and cider. There is a wide range of *Enterobacteraeceace* which are found within brewery environments and they serve as indicator microorganisms for hygiene and sanitation. Gram negative strictly anaerobic bacteria such as *Pectinatus* and *Megasphaera* have recently emerged as a significant threat due to the improvement in reduction of oxygen levels in beer and an increase in production of unpasteurised beer. *Pectinatus* and *Megasphaera* are sensitive to routine cleaning agents used in breweries, but they can survive and proliferate in biofilms eventually causing spoilage of beer. This review focuses on Gram negative aerobic, facultative anaerobic and strictly anaerobic brewery related spoilage bacteria.

Keywords

Gram Negative Bacteria, Beer Spoilage, Pectinatus, Megasphaera, Acetic Acid Bacteria

1. Introduction

Beer is microbiologically stable due to several intrinsic microbiological properties. Firstly, it has low pH (pH 3.8 - 4.7) and the ethanol content can vary from 0.5% to 10% (w/w). Ethanol causes cellular membrane damage in addition to denaturation of proteins, interfering with metabolism and causing cell lysis of bacteria [1] [2]. Hop bitterness compounds (iso-α acids) are present at approximately 17 - 55 ppm and they can cross the cytoplasmic membrane of bacteria in their intact form. These compounds act as a protonophores dissipating the transmembrane pH gradient, which inhibits growth of hop sensitive microorganisms [3] [4]. The presence of low oxygen

concentration (less than 0.1 - 0.3 ppm) and relatively high CO_2 (0.5% w/v), makes beer almost anaerobic [5]. Beer also has extremely low levels of nutrients as most of the fermentable sugars are utilised by brewing yeast during fermentation. All of these factors make propagation of bacterial contaminants difficult in beer [6].

In addition, hurdles for bacterial contaminants are provided by process conditions such as wort boiling, pasteurisation and sterile filtration [7]-[9]. In some exceptional cases survival of some food spoilage microorganisms such as *Bacillus cereus* and *Bacillus licheniformis* has been reported in home brewed beer [10]. However such incidents in commercial brewing have not been reported.

Beer spoilage microorganism can be broadly classified into Gram positive bacteria, Gram negative bacteria and wild yeasts. Gram positive beer spoilage bacteria are regarded as the most hazardous for modern breweries [11]-[13], which mainly include lactic acid bacteria belonging to the genera *Lactobacillus* and *Pediococcus* [14]. Other less significant Gram positive bacteria capable of growth in beer include species belonging to genera *Leuconostoc*, *Micrococcus* and some *Staphylococcus* species [15] [16].

Gram negative beer spoilers mainly include anaerobic bacteria belonging to genera *Pectinatus*, *Megasphaera*, *Selenomonas* and *Zymophilus.* Other significant Gram negative aerobic and facultative anaerobe beer spoilers belong to genera *Acetobacter*, *Zymomonas*, *Selenomonas*, and *Obesumbacterium.* Certain *Enterobacteriaceae* such as *Rahnella* and *Hafnia* have also been reported in brewing environments [15]-[17]. Wild yeasts in brewing are generally described as "yeast strains which are not deliberately introduced and grow uncontrolled in the brewing process" [18]. Microbial contaminants exposed to brewing raw materials and beer at different stages are shown in **Figure 1**.

This review focuses on Gram negative aerobic, facultative anaerobic and strictly anaerobic brewery related spoilage bacteria. Important categories of Gram negative beer spoilage bacteria are comprehensively described in this review. The review deals with current taxonomic status, metabolic aspects, beer spoilage ability and detection methods utilised for these bacteria.

2. Gram Negative Aerobic and Facultative Anaerobic Bacteria in Brewing

Only a few Gram negative bacteria have been found to be responsible for beer spoilage and these bacteria can be

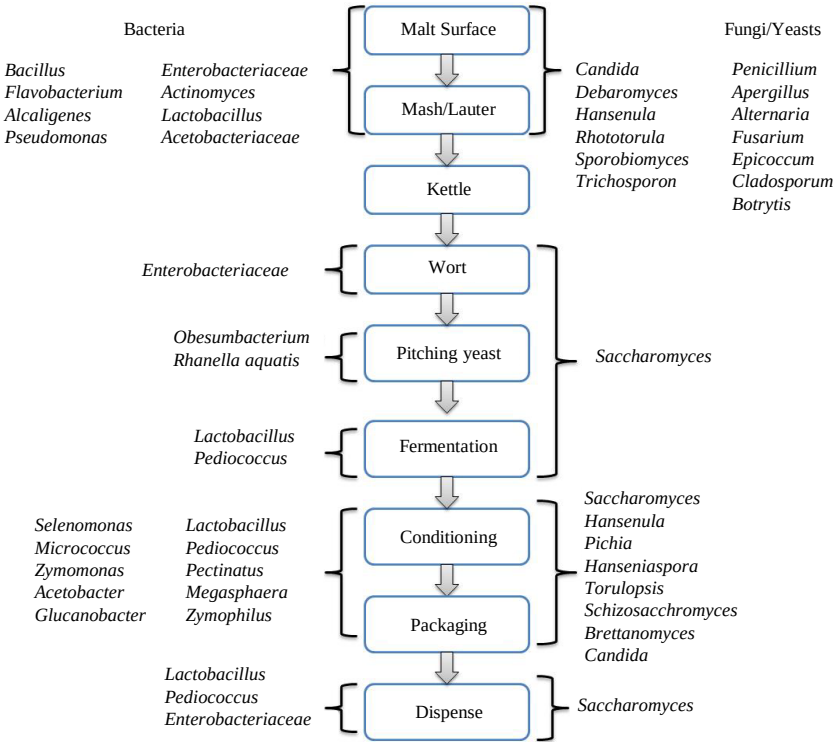

Figure 1. Microbial contaminants exposed to brewing raw material and beer at different stages (Originally adapted from [19] [20] [112]).

divided into two categories. The first category includes aerobic and facultative anaerobic bacteria such as Acetic Acid Bacteria, *Zymomonas* and certain *Enterobacteriaceae* species. The second category is the anaerobic beer spoilers belong to genera *Pectinatus*, *Megasphaera*, *Zymophilus*, *Selenomonas* and *Propionispora*. *Pectinatus* and *Megasphaera* are regarded as the most important beer spoilage bacteria, mainly in unpasteurised beer. Spoilage effects of Gram negative bacteria are shown in **Table 1**.

2.1. Acetic Acid Bacteria

Acetic Acid Bacteria (AAB) are industrially important as they can produce organic acid by oxidising sugar to ethanol then to organic acid, principally acetic acid. *Gluconobacter* are used for production of vinegar commercially. AAB bacteria are also used in various biotechnological applications [21] [22]. AAB are Gram-negative, aerobic, non-spore forming bacteria having ellipsoidal to short rod-shaped cell morphology. They occur individually, in pairs or in chains. AAB can be motile in nature and flagella arrangement may vary from peritrichous to polar [21]. AAB bacteria are also important due to their spoilage effect on alcoholic beverages such as wine and beer [23]. Beer spoilage AAB form a pellicle on the surface with cloudiness in beer containing oxygen. Due to formation of acetic acid, beer tastes sour to vinegary [24] [25]. AAB are strictly aerobic bacteria but some of the AAB isolated from draught beer have been reported to be micro-aerotolerant [26].

At present AAB taxonomically belongtofamily *Acetobacteraceae* [27] of class *Alpha Proteobacteria*. Two genera out of 15 validated AAB, namely *Acetobacter* and *Gluconobacter*, are reported to be associated with brewery environments [26]. Amongst the validated species of AAB, ten species of *Acetobacter*have been associated with brewing environmentsand *A. aceti*, *A. liquefaciens*, *A. pastorianus* and *A. hansii* are frequently found inbreweries [26] [28]. Only one species of *Gluconobacter* (*G. oxydans*) has been reported to be regularly associated with brewing environments [26] [29] [30]). However *Gluconobacter cerevisiae* has also been reported [31].

Production of acetic acid from oxidation of ethanol is asignificant characteristic of *Acetobacter* and *Gluconobacter*. The process is catalysed by cytoplasmic membrane bound enzymes *alcohol dehydrogenase* and *aldehyde dehydrogenase* for production of acetaldehyde from ethanol and acetaldehyde to acetic acid respectively [32]. Under acidic conditions the *alcohol dehydrogenase* activity of *Acetobacter* is comparatively more stable to the activity in *Gluconobacter* which results in more acetic acid production by *Acetobacter* [33]. A variety of carbohydrate sources such as arabinose, fructose, galactose, mannitol, mannose, ribose, sorbitol and xylose are utilised by AAB through the hexose monophosphate pathway [34], Embden-Meyerhof-Parnas (EMP) and Entner-Doudoroff (ED) pathway [35].

AAB occur throughout the brewing process (see **Figure 1**). But due to elimination of oxygen throughout, there has been significant reduction in spoilage incidents due to AAB. AAB are highly tolerant to hop bitterness compounds and can survive in high concentrations of ethanol (>10% v/v) [28]. ABB prevail in initial stages of biofilm formation in brewery environments [36]. AAB are more commonly associated with dispense lines in pubs and public houses due to higher oxygen and high temperature at some stages in beer dispense [37] [38]. Frequent incidents of beer spoilage in draught beer kegs have been reported [24]. *Acetobacter* and *Gluconobacter* have also been occasionally found in samples from beer fermentation and storage tanks [39]. AAB are still prevalent in cask conditioned and barrel aged beers [40].

As described, AAB bacteria produce sourness in beer due to formation of acetic acid. *Gluconobacter* in the beer leads to formation of a pellicle on the surface with cloudiness in beer containing oxygen. Some strains of *Gluconobacter* produced extran and levan leading to formation of ropiness in the beer with high viscosity [41]. *Gluconobacter oxydans* contains various membrane-bound *dehydrogenases*, these enzymes rapidly metabolise sugars or sugar acids from the sugar rich substrate and can even survive in high sugar substrates [42]. *Gluconobacter* are often isolated from soft drinks and various fruit based products [42] [43].

2.2. Genus Zymomonas

Zymomonas are short plump rods which occur singly, in pairs and sometimes in chains or rosettes [26]. These bacteria are Gram-negative, non-endospore forming and catalase positive. *Zymomonas* are aerotolerant and facultatively anaerobic in nature. *Zymomonas* are ethanol tolerant (below 10% ethanol v/v) and grow optimally at pH above 3.4 and temperature of 25˚C - 30˚C [44]. These bacteria can utilise monomer sugars such as glucose and fructose but are not able to metabolise maltose and maltotriose [26] [45]. *Zymomonas* species are often isolated as a source of spoilage microorganisms from various traditional alcoholic beverages all over the world.

Table1. Spoilage effects and metabolic products produced by gram negative beer spoilage bacteria.

Bacteria	Occurrence in brewery environments	Off flavour/aroma and odour	Visual poilage effects	Metabolic products
Acetic acid bacteria [2] [21] [24] [26]				
Acetobacter	Wort, beer dispenses and cask condition ales and barrel aged ales, brewery biofilm.	Sour, vinegar	Haze, Ropiness	Acetic acid
Glucanobacter	Wort, beer dispense and cask condition ales	Sour, vinegary	Haze	Acetic acid, acetate
Zymomonas	Primed beers (not found in lagers)	Fruity, rotten apple, rotten egg, sulphudic	Haze Ropiness	Acetaldehyde and H_2S
Enterobacteriaceae [26]				
Obesumbacterium	Pitching yeast and fermenting wort	Parsnip, sulphury	Haze	Dimethyl sulphides (DMS), diacetyl, higher alcohols and N-nitrosamines, acetoin
Citrobacter	Brewing liquor , fermenting wort	Parsnip, sulphury		Dimethyl sulphides (DMS), diacetyl, lactic acid, acetaldehyde
Rahnella	Pitching yeast, Early stages of fermentation (wort)	Fruity, sulphury,	-	Dimethyl sulphides (DMS), diacetyl, methyl acetate, ethyl acetate
Klebseilla	Fermenting wort, biofilm	Unpleasant odour	-	4-vinylguaicol, Dimethylsulphides (DMS), diacetyl.
Obligatory anaerobes [72] [76] [77] [99]				
Pectinatus	Low alcohol unpasteurised beer, beer filling area, biofilm	Rotten egg, unpleasant odour	Turbidity	Acetic acid, propionic acid, lactic acid, succinic acid, H_2S, acetoin, methyl mercaptan and other sulphur compounds
Megasphaera	Low alcohol, unpasteurised beer, beer filling area, biofilm	Unpleasant odour	Turbidity	H_2S, butyric acid, isobutyric acid, caprioc acid, valeric acid, isovaleric acid.
Selenomonas	Pitching yeast	Unpleasant odour	Turbidity	Acetic, lactic, and propionic acids.
Zymophilus	pitching yeast or brewery waste	Unpleasant odour	Turbidity	Acetic acid and propionic acid

References: [21] [24] [26] [72] [76] [77] [99].

These bacteria are found on the glucose rich sugarcane juice, agavesapand palm trees as a naturally occurring fauna [46]. *Zymomonas* is a biotechnologically important microorganism for industrial production of fuel ethanol [47] [48]. Genus *Zymomonas* belong to family: *Sphingomonadaceae* in Phylum: *Proteobacteria*. *Zymomonas* has only one species, cited as *Zymomonasmobilis* formerly known as *Achromobacter anaerobium*, isolated from beer [49]. *Zymomonas* has also been synonymously described as *Saccharomonaslindneri* and *Pseudomonaslindneri* [41]. At present, *Z. mobilis* has three sub species namely: *Z. mobilis subsp. Pomaceae* [50], *Z. mobilis subsp. Mobilis* [51] [52] and Z. *mobilis subsp. francensis* [46]. Out of the three validated species only *Z. mobilis* subsp. *mobilis* is reported to be a beer spoiler [26].

Spoilage due to *Zymomonas* is quite a common problem in ciders; a motile rod shape bacterium responsible for sick cider has been well studied. The original source of contamination by *Zymomonas* species in the brewery and cider house is still unknown. Soil is suggested to be the possible source of contamination in beer [24] [53], as incidents of *Z. mobilis* contamination are linked to times of construction of new facilities and excavation in breweries [24]. *Z. mobilis subsp. mobilis* has also been reported to prevail in public houses, well water sources, soil from brewery environments and bottling lines [51]. *Z. mobilis* contaminated beer has a fruity aroma (rotten apple due to production of acetaldehyde) which rapidly progresses to sulphidic and rotten egg aroma in spoiled beer.

The contamination incidents due to *Zymomonas* are limited to ales supplemented with primed sugar and spoilage problems due to these bacteria have never been encountered in lager beers [19] [54]. *Z. mobilis*is a distinctive aerobic microorganism as it utilises the Entner Doudoroff (ED) pathway anaerobically instead of the Embden Meyerhof Parnas (EMP) pathway. Z. *mobilis* uses the pathway to ultimately ferment glucose, fructose and sucrose to ethanol and CO_2 [51] [55]. *Zymomonas* is unable to utilise lactose, maltose and cellobiose due to the lack of genes responsible for production of enzymes necessary for metabolism of these sugars [55].

2.3. *Enterobacteriaceae* Related to Brewery Environments

The *Enterobacteriaceae* [56] [57] is a large family of Gram negative facultatively anaerobic bacteria belonging Class: *Gammaproteobacteria*of Phylum *Proteobacteria*. Within the family Coli form bacteria broadly comprise *Enterobacteriaceae* species belonging to genera *Enterobacter*, *Klebsiella*, *Escherichia*, *Hafnia* and certain strains of *Citrobacter* which are able to utilise lactose with gas and acid formation at 35˚C - 37˚C within 48 hours [58]. Coli form bacteria are indicators of the hygienic conditions and level of sanitation in breweries. Presence of Coli forms in water is related toincompetence in process water treatment. These bacteria can be introduced into wort through contaminated water or contact with external fluids through connecting pipes [16].

2.3.1. *Obesumbacterium* spp.

Obesumbacterium proteus belongs to the family *Enterobacteriaceae* and is an extensively studied beer spoilage microorganism. *O. proteus* shows negative reaction to Gram staining and is aerobic or facultatively anaerobic. Morphologically it is a short rod but it has also been reported to show pleomorphic rod morphology in the presence of yeast in fermenting wort [59]. *O. proteus* shows a negative reaction to the oxidase test and a delayed and weak positive reaction to the catalase test. The bacterium can reduce nitrate to nitrite in fermenting wort [60].

The genus *Obesumbacterium* contains only one species, *O. proteus*, and it was isolated as a contaminantwithin the brewing yeast culture. Formerly *O. proteus* was classified as *Flavobacterium proteus* [49]. Later this bacterium was assigned to the genus *Obesumbacterium* and *O. proteus* as a sole type strain within the genus [61] [62]. As a result of detailed taxonomic studies conducted by Priest *et al*. [63], the genus *Obesumbacterium* was assigned to the family *Enterobacteriaceae*. Further *O. proteus* biogroup-1 are supposed to be more closely related to *Hafnia alvei*, a common pathogenic bacterium, while the strains from biogroup-2 are commonly encountered in brewery environments and have not been reported from any other source [60] [63] [64]. Further, *O. proteus* biogroup-2 was assigned to a new genus *Shimwellia* and the species as *Shimwellia pseudoproteus* [60].

O. proteus is found in pitching yeast and fermenting wort, and it has never been reported in beer due to its inability to grow below pH 3.9. *O. proteus*is encountered in initial stages of fermentation where it competes with yeast for nutrients resulting inaslower rate of fermentation. *O. proteus* also produces metabolites such as dimethyl sulfoxide (DMS), acetoin, lactic acid, propanol, isobutanol and 2, 3-butandiaol. DMS imparts parsnip flavour to contaminated beer [59]. The threshold of detection of DMS is lower than 30 µg of DMS/L while *O. proteus* produce 14 - 18 µg of DMS/L in single pitching. Due to the practice of re-pitching, the concentration of these bacteria will eventually rise to produce off flavour above threshold levels [42]. Some *Enterobacteriaceae* especially *O. proteus* can produce N-nitrosamine compounds which are carcinogenic in nature [65]. Concentration of N-nitroso compounds (ATNCs) should be monitored in beer to less than (20 µg/L)and as these compounds pose a health risk [66] it is important to monitor levels of *Enterobacteriaceae* species related to brewery environments.

2.3.2. Other Brewery Related Coliforms

Brewery related *Enterobacteriaceae* serve as hygiene indicator microorganisms and are not normally able to grow in finished beer. They may, however, grow during the initial stages of the brewing process, causing unwanted off-flavours in the final product [67]. Coli forms such as *Citrobacter freundii*, *Rahnella aquatilis*, *Klebsiella oxytoca* and *Klebsiella terrigena* have been reported in unfermented and fermenting wort [16]. *Citrobacter freundii* is a facultative anaerobe, morphologically motile, slender, short rod occurring singly and in pairs and is catalase positive [26]. These bacteria are inhibited by ethanol and only occur during early stages of fermentation and rarely occur in beer. The effect is reported to produce an enhanced fermentation rate and production of diacetyl, lactic acid, acetaldehyde and dimethyl sulphide (DMS). *K. terrigena* and *K. oxytoca* have been reported in brewery environments [16]. *Klebsiella* species are important as they produce phenolic off flavours due to formation of 4-vinylguaiacol produced from decarboxylation of ferulic acid present in the wort similar to some wild yeast. *K. terrigena* also produces high concentrations of acetoin and 2, 3-butanediol through the 2, 3 butanediol pathway by enhanced formation of α acetolactate. All genes for the 2, 3 butanediol pathway in *K. terrigena* are located on a single operon and production of 2, 3 butanediol is related to amino acid synthesis, pH and presence of oxygen [68]. *Rahnella aquatilis* (formerly *Enterbacteragglomer*) has been isolated from various sources such as soil, water, food, plant material and occasionally from clinical specimens [69]. In brewing environments it has been reported as a contaminant in top fermenting yeast and fermented wort [70] [71]. *R. aquatilis*

has been reported to affect the fermentation rate initially but its growth is effected by ethanol during later stages of fermentation. The aroma and flavour of contaminated beer has been typically described as fruity, milky and sulphury due to production of dimethylsulphide (DMS), acetaldehyde, methyl acetate and diacetyl in fermenting wort [71]. Due to its ability to survive through the beer fermentation process and accumulate in pitching yeast *R. aquatilis* can be termed as a potential beer spoiler [70].

3. Gram Negative Strictly Anaerobic Bacteria in Brewing

Due to implementation of effective cleaning and sanitation procedures in modern breweries and effective removal of oxygen from post fermentation processes, spoilage due to aerobic Gram negative bacteria such as *Acetobacter* and *Glucanobacter* has been significantly reduced [6]. However the strictly anaerobic bacteria such as *Pectinatus* and *Megasphaera* have emerged as a potential spoilage threat to microbiological stability of beer. General characteristics of *Pectinatus* and *Megasphaera* are given in **Table 2**.

3.1. Pectinatus

Pectinatus was reported as a new genus of Gram negative, catalase negative, motile, obligate beer spoilage bacteria in the 1970s when it was first isolated from a brewery in the United States in unpasteurized beer stored at 30˚C [72]. *P. cerevisiiphilus* was later isolated from breweries in Finland, Germany, Norway, Japan, Spain, Netherlands, Sweden and France [73]-[75]. During the 1990s in an extensive taxonomic study of anaerobic rods isolated from breweries, a second species of the genus *Pectinatus* was identified as *P. frisingensis* [76].

Pectinatusfrisingnesis can fermentcellobiose, inositol and N-acetyl glucosamine but it cannot utilise xylose and melibiose which can be utilised by *P. cervisiiphilus* [76]. A third brewery related *Pectinatus* species, *P. haikarae* was identified on the basis of 16S rRNA gene sequence analysis and differences in sugar utilization, catalase activity, antibiotic resistance and temperature tolerance compared to the two previously characterised species [77]. *P. portalensis* was also proposed as a relatively fast growing, coccoid shaped, new species isolated from the waste water treatment plant of a winery [78], but 16S RNA gene sequencing analysis and phenotypical characteristics of *P. portalensis* type strains CECT 5841^T and LMG 22865^T did not validate as a new species and these strains were identified as cocci shaped *Enterococcus faecalis* [79].

The genus *Pectinatus* currently comprises three brewery related species: *P. cerevisiiphilus* [72], *P. frisingensis* [76] and *P. haikarae* [77]. The growth of *Pectinatus* species is accompanied by extensive turbidity and an offensive aroma similar to rotten eggs due to the production of various fatty acids, hydrogen sulphide and methyl mercaptan [72] [73]. All three species have been isolated from brewery environments and hence the genus

Table 2. General characteristics of beer spoilage *Pectinatus* and *Megasphaera* species.

Characteristics	Bacterial species					
	1	2	3	4	5	6
Inhabit	spoiled beer	brewery bottling hall	spoiled beer	spoiled beer	spoiled beer	spoiled beer
G + C Content (%)	38.6	39.1	38.4	42.4 - 4.8	40.5	43.1
Width x Length (µm)	0.7-1.0-30	0.6 - 0.8 × 3 - 50	0.7 - 0.9 × 3 - 50	1.5 - 2.1	1.2 - 1.9 × 1 - 1.4	1 - 1.4 × 0.8 - 1.2
Temperature (˚C)						
Range	10 - 45	15 - 30	15 - 37	10 - 37	10 - 30	10 - 30
Optimum	30	30	30	30	30	30
pH						
Range	3.5 - 8.5	4.0 - 8.0	3.5 - 8.0	-	-	-
Optimum	6.5	7	6.5	-	-	-
catalase activity	-	+	-	-	-	-
Spoilage ability	absolute beer spoiler	potential beer spoiler	absolute beer spoiler	potential beer spoiler	potential beer spoiler	potential beer spoiler

1) *Pectinatus cerevisiiphilus*; 2) *Pectinatus frisingensis*; 3) *Pectinatus haikarae*; 4) *Megasphaera cerevisiae*; 5) *Megasphaera paucivorans*; 6) *Megasphaera sueceinsis*; References: [77] [88].

Pectinatus was considered to be brewery specific. Recently two new species of *Pectinatus* have been recovered from salty pickle waste water, namely *P. brassicae* [80] and *P. sottacetonis* [81]. *P. brassicae* may be differentiated from other *Pectinatus* species based on high salt tolerance [80]. The non-beer *Pectinatus* such as *P. brassicae* and *P. sottacetonis* have not been studied for beer spoilage ability.

Previously, Gram negative anaerobic bacteria belonging to the genus *Pectinatus* were affiliated to sub branch *sporomusa* in the family *Acidamincocaceae* of class *Clostridia* [82]-[84]. However in 2010, a new class *Negativicutes* bacteria having a Gram negative cell wall, was proposed within the phylum *Fermicutes* along with a new order, *Selenomonadales* [85] which has changed the taxonomic status of the genus *Pectinatus* affiliating it to class-*Negativicutes* [85], Order-*Selenomonadales* [85], Family-*Veillonellaceae* [85] [86], Genus *Pectinatus* [72] [76] [77] [80]. *P. cerevisiiphilus*, even though *P. frisingensis* is suggested to be descended from the latter based on cross reactivity experiments of flagella antibodies [87]. *P. haikarae* which is capable of growing at slightly lower temperature than the other *Pectinatus* species is suggested to be diverged from *P. cerevisiiphilus* as a result of better acclimatisation to brewery environments. *P. haikarae* is also catalase positive unlike *P. cerevisiiphilus* and *P. frisingensis* which may provide better survival in aerobic brewery environments [88]. Scanning Electron Micrographs (SEM) of *Pectinatuscerevisiiphilus* and *Pectinatusfrisingensis* and *Megasphaera cerevisiae* are shown in **Figure 2**.

Most *Pectinatus* species have been isolated from beer and brewery environments but their natural environment and source of contamination are not well understood [89]. It has been found that several sources of contamination can be identified in the same brewery. *P. cerevisiiphilus* and *P. frisingensis* have been extensively studied and *P. frisingensis* has been more frequently held responsible for beer spoilage incidents compared to *P. cerevisiiphilus* in unpasteurised beer [75] [90] [91]. Along with unpasteurised beer *Pectinatus* species have also been isolated from drainage systems, water pipe systems, various equipment in bottling halls, air of bottling halls, conveyors belts and oil lubricants, cracked floors and tiles of the filling hall [11] [92].

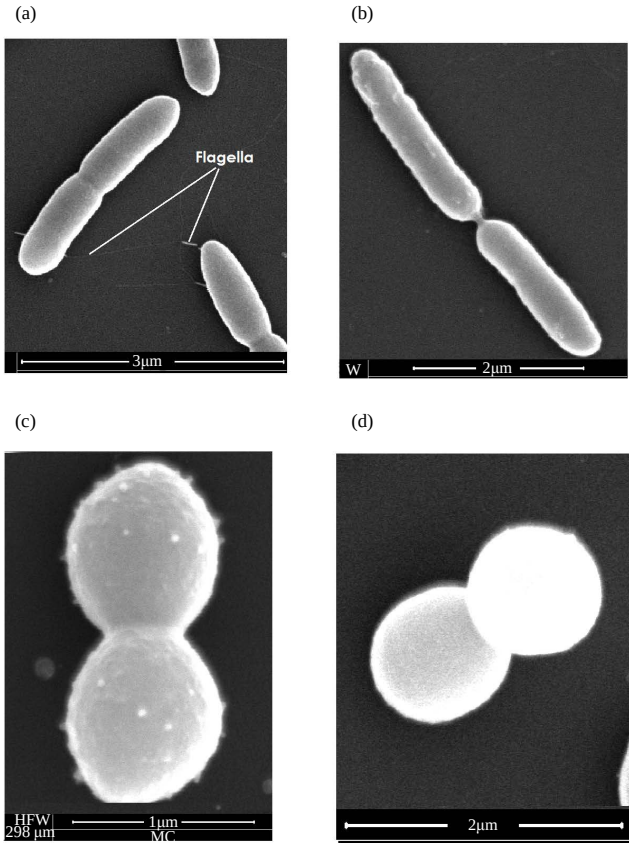

Figure 2. SEM Images of (a)—*Pectinatus cerevisiiphilus*, (b)—*Pectinatus frisingensis* and (c) & (d)—*Megasphaera cerevisiae* brewery isolates (images courtesy of A. Paradh).

Pectinatus have also been reported in pitching yeast and CO_2 recovery systems [91]. The isolation of *Pectinatus* has been mainly from beer filling halls and filling machines and prolonged survival of *Pectinatus* in biofilms formed in beer filling areas indicates that water may be a possible source of contamination [11]. Viable *Pectinatus* strains, although being anaerobic bacteria, have been found in aerosols around fillers of bottling machines indicating that air or other aerosols around fillers could be a possible source of contamination [91]. Survival of *Pectinatus* in aerobic environments of beer filling halls can be possible due to formation of biofilms with mixed populations of various micro-flora commonly occurring in brewery environments [93]. *P. portalensis* has been isolated from waste water of a winery [78] and recently *P. brassicae* has been isolated from a pickle waste water plant [80], suggesting that occurrence of *Pectinatus* species can be broadened from brewery environments to anaerobic and organic matter rich niches in food production and other beverage production environments.

Brewery related *Pectinatus species* are non-spore forming, motile rods with flagella attached laterally to one side of the cells. Young cells show an X shaped pattern formation during movement and old cells show slow snake like movement [72] [73] [76] [77]. For *P. cerevisiiphilus* and *P. frisingensis* growth occurs between 15˚C - 40˚C and optimum growth occurs at 30˚C - 32˚C [72] [76] [77]. Growth of *P. haikarae* is inhibited at temperatures above 37˚C and optimum growth occurs between 20˚C - 30˚C. *P. frisingensis* can maintain cellular homeostasis during sudden changes in temperature [94]. *P. cerevisiiphilus* when co-cultured with *S. cerevisiae* showed growth at 8˚C and it also affects the growth of *S. cerevisiae* [95]. The pH range for growth of these bacteria lies between 3.5 and 8.0 and optimum growth occurs at 6.5 - 7.0 [72] [76] [77]. *Pectinatus species* can tolerate ethanol concentration up to 3.7% - 4.4% (w/v) and growth is completely inhibited at ethanol concentration of 5.5% (w/v) [73] [91]. *P. cerevisiiphilus* and *P. frisingensis* can grow at a dissolved oxygen concentration of 0.4 - 0.8 mg/L and *P. frisingensis* showed better tolerance to dissolved oxygen compared to *P. cerevisiiphilus* [95]. The oxygen tolerance of *P. cerevisiiphilus* has been reported to improve with a decrease in temperature [96]. *P frisingensis* is better adapted to acidic and thermal environments compared to other *Pectinatus* species [88]. *P. frisingensis* can metabolise a wider range of fermentable sugars but it cannot utilise ethanol, maltose and essential amino acids [76] [97]. *P. cerevisiiphilus*, *P. frisingensis* and *P. haikarae* are reported to have strong beer spoilage ability mainly in unpasteurised and low alcohol content beer [91]. The spoilage effects mainly include production of propionic acid, acetic acid, H_2S, dimethyl sulphide (DMS), and methyl mercaptan. The rapid cell growth makes beer turbid and beer typically smells like rotten eggs due to production of sulphur compounds [88] [91].

3.2. *Megasphaera*

Genus *Megasphaera*, originally described by Rogosa [98], consists of five validly published species; *M. elsdenii* [98], *M. cerevisiae* [99], *M. micronuciformis* [84], *M. paucivorans* and *M. sueciensis* [77]. *Megasphaera* species have been isolated from a variety of different environments such as human clinical specimens, rumen gut flora and brewery environments [84] [100]. Important characteristics of beer spoilage *Megasphaera* species are shown in **Table 2**. At present the genus *Megasphaera* is comprised of three brewery associated species. *Megasphaeracerevisiae* [99] was the first brewery associated species, mainly representing low-alcohol beer spoiling cocci. *M. cerevisiae* was responsible for 3% - 7% of beer spoilage cases in Europe during the period 1980 to 2002, mainly in unpasteurised beer [93]. Later, two novel coccoid shaped bacteria were identified associated with beer spoilage and named *M. paucivorans* and *M. sueciensis* [77]. Spoilage effects of *M. cerevisiae* include turbidity and unpleasant odour, due to production of H_2S and short chain fatty acids. All *Megasphaera* species related to the brewery environment are strictly anaerobic, Gram negative, non-spore forming and non-motile [77] [99].

Brewery related *Megasphaera* species share common ecological niches with *Pectinatus* but are less widespread [77] [88] [89]. *M. cerevisiae* has been extensively studied as a contaminant of unpasteurised beer. M. *cerevisiae* has also been reported from brewery bottling hall biofilms and occasionally from pitching yeast and CO_2 recovery systems [77]. Occurrence of *M. paucivorans* and *M. sueceinsis* has not been studied well but these species have been reported to be isolated from unpasteurised beer and other brewery environments [88].

Growth occurs in the temperature range 15˚C - 37˚C and optimum growth is reported to be at 28˚C [101]. No growth is observed at 10 and 45˚C [77]. *Megasphaera cerevisiae* is limited to ethanol concentration of 2.1% (w/v) and its growth completely inhibited at a concentration of 4.2% (w/v) [101]. Growth at normal beer pH has

been detected but its growth is completely inhibited at pH 4.1 and above [77]. Beer spoilage ability of *Megasphaera* species is not as extensively studied compared to *Pectinatus*. *Megasphaera* species mainly affect low alcohol and unpasteurised beer producing turbidity and metabolic products such as butyric acid and minor amounts of acetic acid, valeric acid, caprioc acid and acetoin [88]. Considerable amounts of H_2S are produced in spoiled beer giving a very unpleasant odour [72] [101].

3.3. Other Strictly Anaerobic Bacteria in Brewery Environments

Other Gram negative, anaerobic beer spoilers phylogenetically related to *Pectinatus* and *Megasphaera* belong to genera *Zymophilus*, *Selenomonas* and *Propionispira*. *Selenomonaslacticiflex* and *Propionispira* are non-spore forming, motile rods and may lose mobility on repeated culturing *Selenomonaslacticiflex* and *Propionispira* are more sensitive to acidic environments than *Pectinatus* and *Megasphaera* and has been isolated from pitching yeast in Germany and Finland [89]. *Selenomonaslacticiflexis* have relatively high alcohol tolerance and can grow in beer at 4.5% (w/v) alcohol. *Selenomonaslacticiflexis* can also grow at lower temperature of yeast storage [102]. *Propionispira* species are considered as potential beer spoilage bacteria [88]. Brewery related *Propionispira* have been reported in brewery waste lines and drainage systems which could be suggested as a source of contamination [88]. There is limited data available on beer spoilage ability of *Propionispira* species [102]. *Z. raffinosivorans* and *Z. paucivorans* have been isolated from pitching yeast but have never been implicated as causative agents for beer spoilage due to their inability to grow in beer [89].

4. Detection of Gram Negative Beer Spoilage Bacteria

Conventional methods for detection of spoilage microorganisms in beer and other beverages generally involves pre-enrichment of the sample with a non-selective medium, followed by enrichment on selective or differential agar [17].

For AAB bacteria Frateur's differential medium, AE medium, Reinforced AE medium and YPM medium have been described in the literature [20]. No single medium has been found to be effective in supporting growth of AAB. Rapid detection of AAB using real time PCR [103] Restriction fragment length polymorphism (RFLP) [104] [105], Amplified fragment length polymorphism (AFLP) [106], Denaturing gradient gel electrophoresis (DGGE) [106] and Fluorescent *in situ* hybridisation (FISH) [106] have been utilised for detection and characterisation.

Detection of *Zymomonas* in the brewery using MYPG (malt yeast extract glucose and peptone) agar supplemented with 50 ppm actidione and 3% ethanol or beer with 100 ppm actidione has been reported [5]. For detection of *Zymomonas* in beer media supplemented with lead acetate (producing of black colonies) and Schiffs reagent (producing of purple colonies) has been documented [107]. PCR, Amplified ribosomal DNA restriction analysis (ARDA) method for rapid detection of *Zymomonas* at sub species level and primers specific for 23S rRNA gene for detection *Zymomonas* species has also been developed [46].

For detection of *Enterobacteriaceae* in wort and yeast slurries the use of MacConkey agar supplemented with actidione (10 ppm) for suppression of yeasts is recommended by the European Brewing Convention [5]. However *O. proteus* grow comparatively slower on MacConkey agar [28]. Universal beer agar (UBA) with actidione has been used for wort samples and WLN agar has been used for enrichment of beer [28]. A PCR based method for specific detection and discrimination of *O. proteus* biogroup-2 strains from *O. proteus* biogroup-1 andother related microorganisms has been documented [66]. Characterisation of *O. proteus* biogroup-1 strains using automated ribotyping and PCR based methods has also been reported.

For Gram negative strictly anaerobic bacteria such as *Pectinatus* and *Megasphaera*, SMMP (Selective Medium for *Megasphaera* and *Pectinatus*), NBB medium, and MRS medium with several modification have been described [5] [88]. There are several rapid detection methods available for detection of these microorganisms based on techniques such as Immunoassay, Ribotyping, PCR based methods, RT PCR based methods, Florescence and Luminescence based molecular probes [17] [88] [108] [111]. Although several methods are available in brewing literature, the actual use in commercial brewing microbiological labs is still limited to conventional plating and a few rapid detection methods.

5. Conclusions

There is a small range of non-pathogenic beer spoilage bacteria which can survive, grow and spoil beer. Very

few cases of beer spoilage have been reported in recent years due to high standards of hygiene and technological improvements within the brewing industry. However, due to food and beverage safety concerns, strict regulations regarding food and beverage production and maintaining high quality of products, beer spoilage microorganisms are of severe concern to breweries worldwide. Acetic Acid Bacteria such as *Acetobacter* and *Gluconobacter* were important beer spoilers. Due to implementation of effective cleaning and sanitation procedures in modern breweries and effective removal of oxygen from post fermentation processes, these bacteria are of minor importance in commercial brewing. However these bacteria are concern to dispense systems in pub breweries, public houses and cask conditioned beers [6]. *Zymomonas* is still a concern in primed cask condition beer and cider production. Brewery related Enterobactericeace serve as indicator microorganisms for the level of hygiene and sanitation.

Pectinatus and *Megasphaera* have been postulated to emerge due to high levels of hygiene and significant reduction in oxygen levels in beer and increased production of unpasteurised beer [102]. These microorganisms can cause serious damage to the brand image of the breweries as they are often detected sporadically in small packages (bottles, cans) and often in kegs, resulting in total recall of the batch in the supply chain. *Pectinatus* and *Megasphaera* are sensitive to routine cleaning agents used in breweries but they can survive and proliferate in biofilms dwelling the brewery environment eventually causing spoilage of beer. Detailed chapters on beer spoilage bacteria and technologies to reduce microbial spoilage can be found in the recently published book on Brewing Microbiology [112].

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