Occurrence of Pectinatus and Megasphaera in the major UK breweries

Occurrence of *Pectinatus* and *Megasphaera* in the Major UK Breweries

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ABSTRACT

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The occurrence of beer spoilage bacteria belonging to the genera *Pectinatus* and *Megasphaera* in ten major UK breweries was investigated. The sampling points were selected from fermentation areas, beer conditioning areas and beer bottling and canning sites. Multiplex PCR methodology was used for detection of three *Pectinatus* and three *Megasphaera* species using species-specific primers. The presence of six *Lactobacillus* species was also examined. Overall, 117 samples were analysed from ten breweries; six samples were positive for the presence of *Pectinatus* species and three samples were positive for the presence of *Megasphaera* species, while 34 samples were positive for the presence of *Lactobacillus* species. *Lactobacillus* species appeared to be the major potential spoilage microorganisms. Although none of the actual beer samples were found to be positive for *Pectinatus* and *Megasphaera* species, their occurrence in aerobic brewery environments indicates sanitation problems and revealed the presence of highly established biofilms in some breweries.

Key words: beer spoilage bacteria, *Megasphaera,* multiplex PCR, *Pectinatus*

INTRODUCTION

Gram positive lactic acid bacteria of the genera *Lactobacillus* and *Pediococcus*[40] are considered to be the most hazardous beer spoilage bacteria. *Lactobacillus brevis*, *Lactobacillus lindneri* and *Pediococcus damnosus* are reported to be responsible for approximately 70–80% of microbial beer spoilage incidents in Europe during the period 1980 to 2002[2,6]. *L. brevis* has been implicated in more than half of beer spoilage incidents within the same period[3,6,21] while a further 15–20% have been caused by *L. lindneri*[2,6]. *L. coryniformis*, *L. casei and L. plantarum* are other important *Lactobacillus* species which have been reported to spoil beer[6,38] with a frequency of beer spoilage incidents of 3, 2 and 1% respectively[2,47,48]. *Lactobacillus* species cause high turbidity, hazy appearance, unpleasant flavours and a high level of diacetyl in beer[42].

During the 1990s beer spoilage due to Gram negative bacteria belonging to the genera *Pectinatus* and *Megasphaera* increased due to significant reduction of the oxygen content in the final product - a result of improvement in filling technology[18]. However, since then there has been a decrease in spoilage incidences due to these bacteria[2]. *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[31]. *P. cerevisiiphilus* was later isolated from breweries in Finland, Germany, Norway, Japan, Spain, Netherlands, Sweden and France[15,29,45,50]. In an extensive taxonomic study of anaerobic rods isolated from breweries, a second species of the genus *Pectinatus* was identified as *Pectinatus frisingensis*[44]. *P. frisingensis* differs from *P. cerevisiiphilus* on the basis of growth rate and substrate utilization. In recent studies, a third species, *Pectinatus 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[25]. The growth of *Pectinatus* species is accompanied by extensive turbidity and an offensive aroma similar to rotten egg due to the production of various fatty acids, hydrogen sulphide and methyl mercaptan[15,31].

At present the genus *Megasphaera* is comprised of three brewery associated species. *Megasphaera cerevisiae,* originally described by Engelmann and Weiss[12] was the first brewery associated species, mainly representing low-alcohol beer spoiling cocci. *M. cerevisiae* has been responsible for 3–7% of beer spoilage cases in Europe during the period 1980 to 2002, mainly in non-pasteurised beer[2,3,6]. Later, two novel coccoid shaped bacteria were identified associated with beer spoilage and named *M. paucivorans* and *M. sueciensis*[25]. 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[12,25].

Pectinatus and *Megasphaera* are a major problem from the brewer's point of view as they mainly spoil the beer in the later stages of processing causing financial losses. The contamination causes high turbidity in beer and formation of by-products that cause off-flavours and sour tastes making the beer unsuitable for consumption. This secondary contamination results from ineffective sterilization and pasteurization techniques, hence suitable measures are needed to reduce the incidence of these beer spoilage bacteria. As contamination is caused in the late stages of processing in packaged products, the financial loss is high.

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Lactobacillus species are found in almost every stage of the brewing process[21,54]. *Pectinatus* and *Megasphaera* spp. have been reported mainly from spoiled beer and pitching yeast[16]. They have also been isolated from drainage and water pipe systems of beer filling halls, parts of filling machines and the air and floor of filling halls, condensed water on ceilings, loose tiles and in cracks of damaged floors[3,7,32,35]. Plate counting and enrichment remain the principal methods for detection of microbial contamination in breweries during the brewing process and in final products[8]. In recent years, various new methods have been adopted in the brewing industry based on cell and microcolony visualisation and extensive analysis of cellular and genetic content[39,41,46]. PCR based methods have been widely evaluated in brewing laboratories in recent years[1,22–24,27,28,34,37,43,49].

In the current study, multiplex PCR methodology originally described by Asano et al.[1] and later modified by Iijima et al.[23] was used to detect *Pectinatus, Megasphaera* and *Lactobacillus* species. The current literature has no reports on the occurrence of *Pectinatus* and *Megasphaera* species in the UK brewing industry and hence the main objective of this study was to investigate scope and occurrence of these microorganisms in brewery environments in the UK.

MATERIALS AND METHODS

Pure cultures and culture conditions

Species and strains used in this study are shown in Table I. *Pectinatus* and *Megasphaera* were maintained on PYF agar (peptone-yeast extract-fructose)[12] and *Lactobacillus* and *Pediococcus* species were maintained using MRS agar (Oxoid)[10]. Working cultures were obtained by inoculating 10 µL of pure culture onto the specified agar plates and incubating in anaerobic conditions under an atmosphere of N_2:H_2:CO_2 (80:10:10) using a Don Whitley Mac-500 anaerobic cabinet for 4 days at 30°C. A single colony was picked and inoculated into 50 mL of specified broth and incubated as described above.

Sample collection

Based upon information on occurrence and survival sites of the microorganisms in brewery environments, all the sampling points were selected from the fermentation area, conditioning tanks and packaging sites, where anaerobic conditions could prevail or the sites are prone to biofilm formation. A schematic diagram of sample points is shown in Fig. 1. All the samples were taken in the form of sterile swabs, rinse liquor or beer samples.

The pre-reduction of autoclaved medium in aliquots of 62.5 mL in 250 mL bottles was carried out by purging with anaerobic gas mixture N_2:H_2:CO_2 (80:10:10) using Don Whitley Mac 500 anaerobic cabinet followed by incubation of media in anaerobic conditions under an atmos-

Table I. Reference strains of beer spoilage bacteria.

Bacteria	Strains
Pectinatus cerevisiiphilus	ATCC 29359, DSM 20467
Pectinatus frisingensis	VTT E 79100, DSM 6306
Pectinatus haikarae	VTT E 88330, DSM 16980
Megasphaera cerevisiae	ATTC 43254, DSM 20461
Megasphaera sueciensis	DSM 17042
Megasphaera paucivorans	DSM 16981
Lactobacillus brevis	ICBD culture collection strain[a]
Lactobacillus casei	ICBD culture collection strain[a]
Lactobacillis paracollinodes	ICBD culture collection strain[a]
Lactobacillus plantarum	ICBD culture collection strain[a]
Lactobacillus corynformis	ICBD culture collection strain[a]
Pediococcus damnosus	ICBD culture collection strain[a]
Pediococcus inopinatus	ICBD culture collection strain[a]
Pediococcus pentosaceus	ICBD culture collection strain[a]

[a] Culture collection strain from the International Centre for Brewing and Distilling (ICBD), School of Life Sciences, Heriot Watt University, Edinburgh, UK.

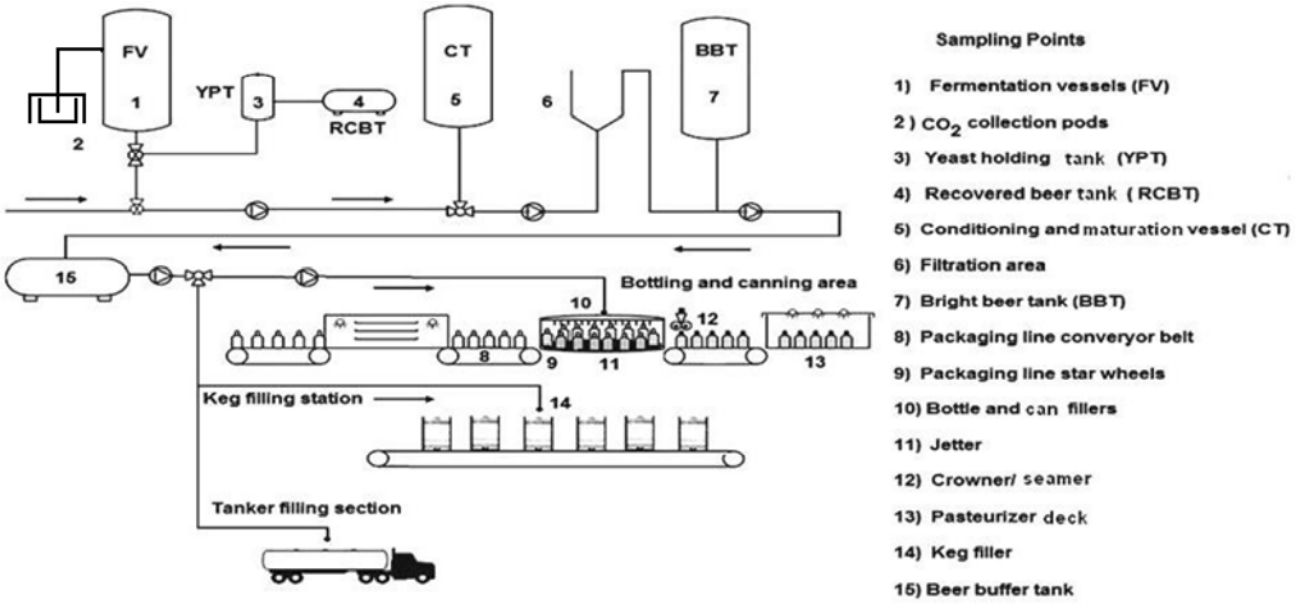

Fig. 1. Schematic representation of sampling points used in the present study.

phere of N_2:H_2:CO_2 (80:10:10) overnight at 30°C. Pasteurised commercial lager containing 4% ABV was degassed in a sterile container by heating at 60°C for 15 min and reduced by purging with anaerobic gas mixture N_2:H_2:CO_2 (80:10:10) using Don Whitley Mac 500 anaerobic cabinet followed by incubation of media in anaerobic conditions under an atmosphere of N_2:H_2:CO_2 (80:10:10) overnight at 30°C.

Swab samples were taken mainly from beer bottling lines and canning lines, specifically from equipment and sites which come into direct contact with packaging materials or finished products. Swabs from bottle conveyor belts, in-feed and outlet star wheel, jetters, crowner and filler tubes were taken. Swabs were taken using sterile swabs and immediately inoculated into a pre-reduced mixture of a 250 mL volume of MRS broth + 1% fructose: pasteurised beer with 4% ABV (1:4) ratio (v/v). Bottles were sealed with parafilm and maintained under anaerobic conditions using an anaerogen kit (Merck) in an anaerobic jar (Merck) at room temperature and transferred to an anaerobic chamber within 12 h for further incubation.

Rinse samples mainly included samples from fillers and wash liquid from fermentation CO_2 collecting pods. Beer samples were selected from fermentation tanks, yeast holding tanks, bright beer tanks and beer buffer tanks. For rinse liquor and direct beer samples, liquid was directly poured into a sterile 250 mL bottle containing 62.5 mL pre-reduced MRS broth +1% fructose. Bottles were sealed with parafilm and maintained under anaerobic conditions using an anaerogen kit (Merck) in an anaerobic jar at room temperature and transferred to an anaerobic chamber within 12 h. All samples were incubated at 30°C for 14 days prior to DNA extraction. Fructose was utilised to enhance the growth of *Megasphaera* species. For samples containing brewing yeast cells, 50 ppm cycloheximide was used to suppress the growth of yeast[26,33].

Cell harvesting and DNA extraction

All the enriched samples were centrifuged at 12,000 rpm for 5 min to concentrate cells. A 500 µL aliquot of concentrated cell suspension was transferred to a 1.5 mL tube and repeatedly washed with sterile deionised water before being used for DNA isolation. Alternatively for some of the samples, 50 µL of concentrated cell suspension was inoculated onto MRS agar + 1% fructose and incubated for 4 days under anaerobic conditions at 30°C and DNA was extracted from representative colonies picked up and resuspended aseptically into 500 µL of sterile deionised water. DNA extraction was carried out using a Qiagen/Gentra-Puregene® kit according to the manufacturer's instructions. Successful DNA extraction was confirmed by running 5 µL of DNA sample on a 1.5% agarose gel.

Primer selection

All the primers were based on rRNA gene sequences and in some species the internal transcribed spacer (ITS) region. The details of primer sequences, target DNA and predicted product sizes are shown in Table II. All primers were purchased from Eurofins MWG Operon (UK). Solution of primers was carried out according to the manufacturer's instructions (to obtain a concentration of 100 pmol/µL) using sterile deionised water and they were then stored at –20°C.

PCR and gel electrophoresis

The multiplex PCR reactions were set up in three reaction formats for each of the three *Pectinatus* species (*Pectinatus* multiplex), three *Megasphaera* species (*Megasphaera* multiplex) and six main beer spoilage *Lactobacillus* species (*Lactobacillus* multiplex), as previously described by Asano et al.[1] and Iijima et al.[23] Certain modifications were made in the multiplex PCR method to ensure specificity and reactivity in order to overcome false positive or false negative results. All three multiplex PCR mixes were specific when checked against closely related species as shown in Table I. The sensitivity of all three PCR multiplexes was determined using serially diluted genomic DNA from target species and positive results were evaluated based on a visible band being obtained on

Table II. List of primers used for detection of *Pectinatus, Megasphaera* and *Lactobacillus* spp. by multiplex PCR[a].

Method	Primer	Direction	Primer sequence (5′ to 3′)	Target species	Target DNA	Product size (bp)
Pectinatus multiplex	16C-F	Forward	CGTATGCAGAGATGCATATT	*P. cerevisiiphilus*	16S-rDNA	621
	IC-R	Reverse	CACTCTTACAAAGTATCTAC	*P. cerevisiiphilus*	ITS region	
	16F-F	Forward	CGTATCCAGAGATGGATATT	*P. frisingensis*	16S-rDNA	701, 883
	IF-R	Reverse	CCATCCTCTTGAAAATCTC	*P. frisingensis*	ITS region	
	Phf1	Forward	AATACCGAATGTTGTAAGAG	*P. haikarae*	16S-rDNA	508
	Phr2	Reverse	CTCTCCTGCACTCAAGACAT	*P. haikarae*	16S-rDNA	
Megasphaera multiplex	mc-f4	Forward	ACCGAATACGATCTAAAG	*M. cerevisiae*	16S-rDNA	452
	mc-rf	Reverse	TTAAGACCGACTTACCGA	*M. cerevisiae*	16S-rDNA	
	Msp-f	Forward	TATGGCCAATACCCATAGAT	*M. sueciensis*	16S-rDNA	155
	Msp-r	Forward	CACTTTTAAGACAGACTTGA	*M. paucivorans*	16S-rDNA	
Lactobacillus multiplex	LBP2	Forward	CTGATTTCAACAATGAAGC	*L. brevis*	16S-rDNA	861
	L74P1	Forward	GGATTTTAACATCGGATGAG	*L. paracollinoides*	16S-rDNA	854
	LCP11	Forward	GAACCGCATGGTTCTTGGC	*L. casei*	16S-rDNA	729
	LOP4	Forward	GGGACTAGAGTAACTGTTAGTCC	*L. corynformis*	16S-rDNA	453
	LPP7	Forward	GTTGTTAAAGAAGAACTTATC	*L. plantarum*	16S-rDNA	490
	LLITSF8	Forward	AACTTACACCGATCAAAATC	*L. lindneri*	ITS region	850
	LL23SR12	Reverse	CTTAACCTTGCATGCAACT	*L. lindneri*	16S-rDNA	-----
	UNP1	Reverse	CCGTCAATTCCTTTGAGTTT	*Lactobacillus* spp. (consensus primer)	23S-rDNA	[a]

[a] Primer UNP1 is shared as a common reverse primers by all five *Lactobacillus* species except *L. lindneri.* Source: Iijima et al.[23]

the agarose gel. Optimization was also necessary to overcome certain variable components such as primer concentration, the nature of the DNA template, quality of Taq polymerase, concentration of the buffer components.

For each reaction mixture 0.5 µL (2.5 units) of BIOTAQ™ DNA Polymerase (BIOLINE) was used. Standard reaction buffer containing a final concentration of 0.8 mM $(NH_4)_2SO_4$, 3.5 mM Tris-HCL, 1.5 mM $MgCl_2$ and 0.2 mM of each of the four dNTPs was used. For *Pectinatus* and *Megasphaera* multiplexes, 1 µL of each primer (100 pmol/µL) was used, for *Lactobacillus* multiplex primer concentrations were as previously described by Asano et al.[1] A 1 µL aliquot of extracted DNA solution was used as a template and the final volume of the reaction mixture was made to 50 µL using sterile deionised water. PCR reactions were performed using BIORAD and Applied Biosystem thermal cyclers. Positive controls were maintained by using a 1 µL DNA template of *P. frisingensis, M. cerevisiae* and *L. brevis* for *Pectinatus, Megasphaera and Lactobacillus* multiplex PCR respectively. Negative controls were maintained using the reaction mixture as described above, but with no DNA template.

The PCR amplification was carried out with an initial denaturation for 4 min at 95°C followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec and primer extension at 72°C for 1 min. Final primer extension was carried out for 4 min at 72°C followed by an end hold at 4°C. PCR products were stored at 5–6°C before analysis by gel electrophoresis using 2% agarose gels in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) containing ethidium bromide for DNA staining. A 5 µL aliquot of PCR product was used for analysis and a 100 bp ladder (Hyper ladder IV- BIOLINE) was used as the molecular size marker.

For *Lactobacillus* multiplex, certain similar size amplified fragments were confirmed using simplex PCR as described above, except 1 µL each of species specific primer was used.

RESULTS AND DISCUSSION

Optimization of culture enrichment and multiplex PCR method

Collection and enrichment of samples were important tasks during the study. All the samples were treated on site soon after collection and anaerobic conditions were maintained during transportation of samples to the laboratory by using an anaerogen kit (Merck) and anaerobic jars. PCR is a highly sensitive method for detection of even low levels of contaminants in samples, but for detection of highly anaerobic bacteria, enrichment of samples was carried out for 14 days. For strict anaerobes, culture enrichment is needed to achieve detectable numbers of cells in samples. In addition sometimes the high volume of sample is more important than incubation time to achieve detectable growth of target microorganisms[26] and the volume of samples was 250 mL to overcome this limitation of the enrichment method.

Multiplex PCR methodology was used to detect *Pectinatus*, *Megasphaera* and *Lactobacillus* beer spoilage species, as it was recently used successfully for the comprehensive detection of major beer spoilage bacteria[1,23]. The optimisation of the multiplex PCR method was carried out according to a stepwise protocol described by Henegariu et al.[19] Genomic DNA isolated from pure cultures was used to test Multiplex PCR regimes. Modifications in the multiplex PCR method were made to determine the amplification of weak loci by modifying the primer concentration and optimizing PCR cycles[19]. PCR reactions were optimized and successfully used for further detection of real brewery samples. The original multiplex protocols[1,23] comprised of 30 cycles of denaturation, annealing and extension and the 15 sec, 15 sec and 30 sec respectively, was modified to 30 cycles of 30 sec, 30 sec and 1 min respectively, for all three multiplex PCR methods. It was verified that *Pectinatus*, *Megasphaera and Lactobacillus* species were detected with high specificity and selectivity.

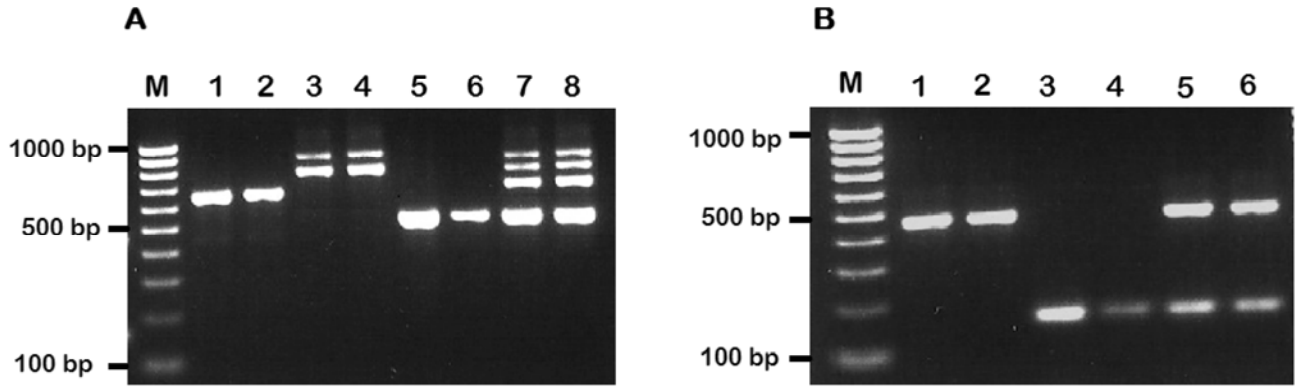

Fig. 2. Specificity of *Pectinatus* and *Megasphaera* multiplex primers was evaluated. **A,** *Pectinatus* multiplex PCR was carried out using different combinations of target bacterial species, 1 and 2 represent *Pectinatus* multiplex results for *P. cerevisiiphilus;* 3 and 4 represent *Pectinatus* multiplex for *P. frisingensis;* 5 and 6 represent *Pectinatus* multiplex for *P. haikarae;* 7 and 8 represent *Pectinatus* multiplex for all three *Pectinatus* species. **B,** *Megasphaera* multiplex PCR was carried out using different combinations of target bacterial species, 1 and 2 represent *Megasphaera* multiplex results for *M. cerevisiae;* 3 and 4 represent *Megasphaera* multiplex for *M. paucivorans;* 5 and 6 represent *Megasphaera* multiplex for *M. cerevisiae* and *M. paucivorans;* M represents 100 bp DNA ladder (Hyper ladder IV Bioline).

Table III. Multiplex PCR results for the brewery samples.

Brewery number		1	2	3	4	5	6	7	8	9	10
Total no. of samples		10	10	10	10	15	15	7	10	10	20
No. of positive samples through multiplex PCR	*P. cerevisiiphilus*	1[a]		1[a]							
	P. frisingensis		2[a]			2[a]					
	P. haikarae										
	M. cerevisiae		1[a]	1[a]							
	M. paucivorans & *M. sueciensis*		1[a]								
	L. brevis	5[ab]	3[ab]	2[a]	1[a]	2[a]	2[a]			1[b]	
	L. lindneri			2	3[a]			1[b]	1[b]	4[ab]	2[a]
	L. casei									1[b]	2[a]
	L. corynformins								1[b]		
	L. plantarum							1[b]			
	L. paracollinoides										

[a] Samples collected from indirect sampling points – swabs and rinse samples from vessels and packaging equipment.
[b] Samples collected from direct beer samples – beer sample/fermenting wort and yeast slurry.

Specificity of *Pectinatus* and *Megasphaera* multiplexes has been illustrated in Fig. 2. All three multiplex PCR were found to be able to detect less than 100 fg of target DNA, where positive results were concluded based on visibility of an amplified band on an agarose gel (data not shown).

Multiplex PCR results

During the investigation of anaerobic beer spoilage bacteria in major UK breweries, 117 samples from ten major breweries were analysed. Of these 117 samples, two samples were positive for *P. cerevisiiphilus,* four samples were positive for the presence of *P. frisingensis,* two samples showed the presence of *M. cerevisiae* and one sample was found positive for the presence of *M. sueciensis* and *M. paucivorans* (detected by the same pair of primers). PCR positive samples for *Pectinatus, Megasphaera* and *Lactobacillus* multiplexes are shown (Table III). *L. brevis* and *L. lindneri* were found to be the most frequently occurring *Lactobacillus* species with 16 and 13 positive samples respectively, while *L. casei, L. plantarum* and *L. corynformis* were found in three, one, and one samples respectively. Ten actual beer samples were positive for the presence of *Lactobacillus* species, mainly from conditioning areas and filtration units.

Pectinatus multiplex PCR samples from star wheels of bottling lines from breweries one and three were positive for the presence of *P. cerevisiiphilus,* while for *P. frisingensis,* two conveyor belt sterile swab samples from brewery two and two samples both from the CO_2 collecting bubble pods of fermenters from brewery five were positive. All six positive samples for *Pectinatus* multiplex were from indirect sampling points and none of the isolates from direct beer samples were found to be positive. It was interesting to find that the samples from the star wheels and conveyor belts, samples which are highly aerobic in nature, showed the presence of strictly anaerobic beer spoilage bacteria. The liquid rinse samples from the CO_2 bubble pods were also of note, as the presence of *Pectinatus* species in the fermentation area is considered to be rare, but the isolation of anaerobic beer spoilage bacteria from CO_2 recovery systems has been frequently reported from the breweries in UK (brewery personal communication). The samples from breweries two and three were each positive for presence of *Megasphaera cerevisiae* and one sample from brewery two taken from the conveyor belt swab of the canning lines was positive for the presence of *M. paucivorans* and *M. sueciensis*. None of the samples from the other breweries (brewery four, six, seven, eight, nine and ten) showed the presence of *Pectinatus* or *Megasphaera* by multiplex PCR. On the other hand *Lactobacillus* species were found to be distributed among the samples from all of the breweries.

Survival of strictly anaerobic bacteria in this aerobic environment can possibly be due to biofilm formation[6,47]. Instruments used in the filling process are prone to formation of biofilms, which are a niche for various beer spoiling microorganisms. The slime produced by these biofilms can protect microbes from routine cleaning procedures. Yeast and *Lactobacillus* species can dwell in these slimes, while the lactic acid produced by *Lactobacillus* species can be metabolized to propionic acid by anaerobic bacteria such as *Pectinatus* species, which can cause undesirable changes to final products[52]. Detection of low levels of *Pectinatus* from biofilms on a conveyor belt in a beer bottling line based on fatty acid profiles has previously been reported[53]. The presence of *Pectinatus* and *Megasphaera* species in fermentation areas and bottling lines of the four major breweries in the UK (breweries one, two, three and five) shows that *Pectinatus* and *Megasphaera* species are natural inhabitants of the breweries in the UK and not infrequent invaders. Breweries one to five were sampled during the months of March to August and breweries six to ten were sampled during the months of September to February. The concentration of *Pectinatus* and *Megasphaera* in brewery environments, in hotter months of the year, could be estimated to be higher than in the cooler months of the year.

In all ten breweries, conventional microbiological practices were adopted for the detection of beer spoilage contaminants based on plate count methods. For the detection of beer spoilage anaerobes, Raka Ray medium has been recommended by European Brewing Convention (EBC)[42] and this medium supplemented with cycloheximide and 2-phenyl ethanol was utilized in all of the ten breweries. In addition, two breweries utilised NBBC broth for the detection of anaerobes. None of the breweries use SMMP medium (Selective Medium for detection of *Megasphaera* and *Pectinatus*)[33] for detection of *Pectinatus* and *Megasphaera* in brewery samples. The Raka Ray medium has the limitation of detecting only facultatively anaerobic bacteria belonging to *Lactobacillus* species and the recov-

Table IV. Summary of hygiene monitoring, inspection and microbial methods utilized in the breweries.

Brewery no.	Capacity (Hl)	Hygiene certification[a]	Packaging facilities	Microbial detection methods/media used for detection of anaerobes	Hygiene inspection	CIP formulation used for packing lines
1	1,900,000	No data	bottling, canning, kegging	Plate count method, Raka Ray	No data	Automatic caustic CIP (1–2%) twice weekly
2	4,000,000	ISO-9001	canning, kegging, casking	Plate count method, Raka Ray	ATP bio-luminescence	Automatic caustic CIP (1–2%) twice weekly
3	9,000,000	ISO-9001	bottling, canning, kegging	Plate count method, Raka Ray	ATP bio-luminescence	Automatic caustic CIP (1–2%) twice weekly
4	4,000,000	ISO-9001	bottling, kegging, casking	Plate count method, Raka Ray	No data	Automatic caustic CIP (1–2%) twice weekly
5	4,000,000	ISO-9001	bottling, canning, kegging	Plate count method, Raka Ray	ATP bio-luminescence	Automatic caustic CIP (1–2%) + combination of para acetic acid (PAA) and chlorine (Cl_2), twice weekly
6	3,800,000	BRC, HACCP	bottling, canning, kegging	Plate count method, Raka Ray, NBBC broth	ATP bio-luminescence	Automatic acid CIP commercial formulation (Johnson Diversey Chemicals, UK)
7	1,900,000	ISO-9001, BRC	kegging	Plate count method, Raka Ray	ATP bio-luminescence	Automatic caustic CIP (1–2%) after every use
8	1,100,000	ISO-14000, BRC	kegging	Plate count method, Raka Ray	ATP bio-luminescence	Automatic caustic CIP twice weekly
9	1,100,000	ISO-9001, ISO-22000, ISO-14000	kegging	Plate count method, Raka Ray	ATP bio-luminescence	Automatic caustic CIP twice weekly
10	No data	BRC	bottling, canning, kegging	Plate count method, Raka Ray, NBBC broth	ATP bio-luminescence	Automatic caustic CIP every 48 h, acid CIP occasionally

[a] ISO: International Organization for Standardization. BRC: British Retail Consortium. HACCP: The Hazard Analysis and Critical Control Point certification.

ery rate on this medium is not good (brewery personal communication), hence it can be confirmed that except NBBC, no effective medium is utilized to specifically detect *Pectinatus* and *Megasphaera* in the UK breweries. The identification of brewery contaminants is mainly based on microscopic analysis. Thus it can be concluded that microbial spoilage due to anaerobic bacteria cannot be specified by the conventional methods used in these breweries unless NBBC is used. The summary of hygiene monitoring and microbial methods adopted in the studied breweries is given in Table IV.

Cleaning and hygiene validation of fermentation tanks, beer storage tanks and packaging lines was carried out by using an ATP bioluminescence method in eight out of the ten breweries. The sensitivity of the ATP method is not suitable for detection of low levels of contaminants; moreover some residues of cleaning agents and disinfectants could affect the enzyme reaction causing light production thus giving non-specific results[30]. ATP bioluminescence is not suitable for the actual detection of contaminants in breweries, as the results are often not similar to those obtained by conventional methods for the same samples[36].

It has been observed that beer with a low alcohol content is more prone to spoilage by *Pectinatus* and *Megasphaera* species. *Pectinatus* species are more resistant to acidic pH and can survive at a pH of 4.1[17]. The pH tolerance of these anaerobic bacteria is influenced by the presence of ethanol[47]. *Pectinatus* and *Megasphaera* species are tolerant to hop bitter substances and can grow in beer with bitterness ranging between 33–38 EBC bitterness[5,29]. *P. frisingensis* shows significant ability to maintain internal homeostasis to mild heat treatment[51] and also its thermal resistance is high compared to *P. cerevisiiphilus*[13]. The growth of *Pectinatus* species is affected significantly by the oxygen content of the beer and has been observed at a dissolved oxygen content of 1.91 mg/L[45]. Modern filling techniques have limited the oxygen content of beer to 0.4–0.8 mg/L, which makes the growth and proliferation of *Pectinatus* in beer possible[9]. The growth of *Megasphaera* in beer with 3.5% ethanol (w/v) is completely restricted[14].

A routine pasteurisation of beer (27–30 PU) is sufficient to inhibit all microorganisms in the beer[4]. *Pectinatus* can be inhibited by a heat treatment of 58–60°C for 1 min, which is less than routine pasteurisation treatment[14]. Aseptic sterilisation using 0.45 μm filters is as effective as flash pasteurisation[4]. It has been reported that *Pectinatus* and *Megasphaera* are susceptible to most of the disinfectants used in the breweries such as iodine, chlorine, and formaldehyde[14]. *Pectinatus* and *Megasphaera* are easy to control via thermal and disinfectant treatment, but these microorganisms still survive in hard to access corners or in biofilms, which are not easy to access and disinfect[47].

In recent years compared to premium lager, there has been development of sub-premium lager brands with a low alcohol content and also mid-strength lager. These brands are at an increased risk from secondary contaminants including *Pectinatus*, primarily due to the low alcohol content of the beer. If these brands are brewed and packaged in the same conditions observed during the current study, the potential risk of contamination in the final packaged product cannot be denied.

The presence of *Pectinatus* species on conveyor belts and star wheels of beer filling lines signifies a higher risk

for packaged beer[18]. *Pectinatus* can be transmitted to fillers and subsequently to packaged beer via aerosols produced during the filling process[11] and cleaning procedures[20,47]. CO_2 recovery systems are never subjected to cleaning regimes as this involves intensive dismantling of equipment (brewery personal communication), hence the bacteria can prevail in this part of the brewery throughout the year, creating a potential threat to packaged beer products in several ways.

The presence of *Pectinatus* and *Megasphaera* in highly aerobic brewery environments can be due to the formation of biofilms and symbiotic associations of microorganisms survive within them[47]. Their presence in highly aerobic conditions provides basic knowledge about the complexity of these biofilms. It is thought that anaerobic bacteria dwell in well established biofilms[6,53]. Contamination could also occur in drainage areas and in floors with defects, areas which are often anaerobic. Even though extensive cleaning procedures are adopted periodically in all of the breweries, the cleaning procedures are not effective enough to completely remove attached biofilms and thus strictly anaerobic beer spoilage bacteria can propagate and be dispersed in packaging plants. The hygiene around the filling machine is also important. The lack of any complaints of spoilage signifies that these secondary contaminants in bottling lines are still in their lag phase of adaptation, due to periodic cleaning regimes ensuring hygienic operating conditions as described by Back in 1994[6]. However ineffective cleaning procedures (as we have found in the breweries sampled), allow the continued presence of these microbes in the filling hall, resulting in their concentration approaching a culmination point. Subsequently some breweries can suffer severely from secondary contaminants without any noticeable prior warning[6]. Other possible reasons that there are no reports on anaerobic bacterial contamination in these breweries could be that most of the premium lagers (5% ABV) brewed do not support the growth of *Pectinatus* and *Megasphaera*. However, the presence of these anaerobic beer spoilers in aerobic brewery environments means that there is a very real risk of contamination of unpasteurised or flash pasteurised beers with a low alcohol content.

At present, automated CIP (Cleaning in Place) with a varying concentration of sodium hydroxide (NaOH; 1–2%); cold and hot CIP, once or twice a week is utilised in most breweries. In general, filling equipment is cleaned using automated caustic CIP and foam cleaning after every use. Brewery six utilizes an acid based commercial formulation (Johnson Diversey Chemicals, UK); this brewery showed comparably better hygienic conditions in the brewery equipment and canning lines and none of the samples were positive for *Pectinatus* and *Megasphaera,* and only two samples were positive for *Lactobacillus*.

Brewery five utilizes disinfectants, such as PAA (paraacetic acid) and Cl_2 (chlorine) in addition to caustic CIP, for the cleaning of bottling and canning lines respectively. In some breweries, the practice of increasing caustic concentration (1.5–4%) along with increased temperature in hot CIP is also employed in cases of severe problems of secondary contaminants, but this practice seems to be unnecessary as there is a need for a modification in detergent formulation rather than using a high concentration of caustic CIP, which could be cost intensive.

It may be concluded that alternation in caustic CIP with the use of modified detergent formulations can be beneficial to achieve satisfactory hygiene conditions in breweries and packaging facilities. There is scope for development of modified detergent formulations, as the trend in shifting caustic CIP to alternate formulations can be seen in major lager breweries in the UK.

CONCLUSIONS

This study found the presence of *Pectinatus* and *Megasphaera* spp. from indirect sampling points in four out of ten breweries. Although none of the direct beer samples were found to test positive for anaerobic beer spoilage bacteria, the presence of *Lactobacillus* species in direct beer samples indicates sanitation problems in these breweries. The record of anaerobic microbes and their sampling sites can provide beneficial data for further studies and the experimental results are useful in designing improvements in the UK breweries.

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