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Bavarian Wheat Beer, an Example of a Special Microbe Habitat – Cultivation, Detection, Biofilm Formation, Characterization of Selected Lactic Acid Bacteria Hygiene Indicators and Spoilers

For the food industry, hygiene conditions within production plants are of high relevance to product quality. Most microbiological quality issues can be traced back to inadequate plant hygiene. In particular, the formation of mature biofilms is highly connected with product spoilage. The formation of biofilms depends on the provision of nutrients and therefore on the product. With a wider range of beer types and beer-like products, new spoilage organisms are becoming relevant. For Bavarian Wheat Beer types, other low-hopped beer types and beer mix beverages, the potential beer-spoiling bacteria *Lactobacillus acetotolerans*, *Lactobacillus rossiae*, *Lactococcus lactis* and *Leuconostoc mesenteroides* can be critical, either because of the spoilage potential or because of the biofilm formation potential. The majority of strains of the above-mentioned species proved that they could develop biofilms de novo in MRS, which makes them important hygienic indicator germs. An adapted media to detect Bavarian Wheat Beer spoiling bacteria (Wheat Beer media by Hutzler and Riedl (WBM-HR)) was developed. For rapid detection and identification, real-time PCR systems with compatible standard protocols were developed for the specified species. The detection of obligate slow-growing Bavarian Wheat Beer-spoiling species *Lactobacillus acetotolerans* was modified. The developed methods can be applied to specific contamination tracking and to evaluating the hygiene status of breweries that produce Bavarian Wheat Beer.

Descriptors: Bavarian Wheat Beer spoilage, *Lactobacillus rossiae*, *Lactobacillus acetotolerans*, *Lactococcus lactis*, *Leuconostoc mesenteroides*

1 Introduction

The overall beer consumption in Germany has decreased by 18 % in recent years [20]. While global beer sales are stagnating, even decreasing by about 0.6 % in 2014 [25], consumer preferences are changing. While the export lager beer sector in Germany is diminishing, other sectors are flourishing. The Bavarian Wheat Beer sector has increased its share of the German beer market to 8.8 % since 1970 when 98 % of the beer market was dominated by bottom fermented beer types. Beer mix beverages increased to 6.8 % and alcohol-free beer to 3.6 % of the market in 2010 [21]. In 2015, the Bavarian Wheat Beer sector alone increased to 7.2 % [23]. While beer mix beverages decreased by 2.9 % in 2015, alcohol-free beer-based beverages are still popular in Germany [22].

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The changing product portfolios in breweries present new challenges for microbiological quality control. In comparison with other beverages and foods, beer is a very stable product in terms of its microbiology. The spectrum of microorganisms able to grow in beer is reduced by the presence of ethanol (0.5–10 % w/w), hop bitter compounds (approx. 17–55 ppm of iso-α-acids), high levels of carbon dioxide (approx. 0.5 % w/w), low pH (3.8–4.7) and (at least for industrially produced and filled beer) very low amounts of oxygen (<0.1 ppm), as well as traces of fermentable carbohydrates and amino acids [32]. Due to the high microbial selectivity of beer, non-culture organisms inhabiting breweries are categorized according to their spoilage potential as absolute harmful organisms, potentially harmful organisms, indirectly harmful organisms and indicator germs [4]. There are only a few bacteria that can grow in beer. The most prominent are gram-positive lactic acid bacteria of the genus *Lactobacillus* and *Pediococcus*, as well as gram-negative bacteria *Pectinatus* and *Megasphaera*, and some super-attenuating yeasts [2, 17, 18].

Producing Bavarian Wheat Beer or beer mix beverages involves varying the parameters that protect the product against microbio-

logical spoilage as mentioned above. In Bavarian Wheat Beer the content of hop bitter acids is reduced compared to most bottom fermented beer types (10–14 IBU in Bavarian Wheat Beer to 15–55 IBU in bottom fermented beer types [13, 27]). This beer type also contains a higher level of nutrients, – amino acids and fatty acids in particular in the case of unfiltered Bavarian Wheat Beer – and can therefore be spoiled by a higher number of species, which are normally in the category of potentially harmful organisms (*Lactobacillus rossiae*, *Lactobacillus acetotolerans*) [18]. Beer mix beverages, alcohol reduced and alcohol-free beer have even greater variety in these parameters.

In industrial settings the Bavarian Wheat Beer spoiling bacteria *L. rossiae* and *L. acetotolerans* are being found more often in Bavarian Wheat Beer samples than in previous years. The former was first isolated in 2005 from sourdough [6] and can often be found as persistent contamination of bottling plants. It is considered biofilm relevant due to its slime-forming potential [18]. The latter was originally found as a spoilage organism in rice vinegar broth [12]. It was later described as a spoilage organism in low-hopped Chinese beer and alcohol-free and regular Bavarian Wheat Beer [8; 16; 31]. In 2016 this species even attracted the attention of non-scientific media in reports of a massive beer spoilage in the US caused by *L. acetotolerans* [28]. This species is mostly found as a spoiler in fermentation vessels and pitching yeast and is hard to detect due to its ability to enter a viable, but putatively non-culturable (VPNC) state in beer. In addition, under cold stress *L. acetolerans* grows slowly and requires an incubation time of more than two weeks using classic media until detection [8, 9]. *Leuconostoc mesenteroides* and *Lactococcus lactis* are common in the filling area. They are considered to be potential beer-spoilage bacteria [4, 17, 34] and are known to be able to spoil non-alcoholic beverages [4]. There are no recently documented cases of these species spoiling beer, but they can be widespread in the filling area [3, 4]. They are considered to be biofilm relevant due to the production of exopolysaccharides (EPS), which also makes them potential biofilm initiators [4, 10, 29, 34]. The production of EPS is not necessarily required for initial cell-surface attachment, but is essential for the growth of mature biofilms [1, 19]. *L. lactis* strains, isolated from dairy plants were described as having biofilm-producing potential [10]. Besides the spoilage risk for beer mix and non-alcoholic beer types, this makes them important as organisms that indicate hygiene problems, since biofilms containing non-product-spoiling lactic acid bacteria can provide habitats for strictly anaerobic, obligate beer spoiling *Acidaminococcaceae* such as *Pectinatus* sp. and *Megasphaera* sp. [34]. Real-time PCR-based detection systems have already been designed for most known beer-spoiling bacteria and yeasts by *Brandl* and *Hutzler* [5, 14, 15], but no rapid detection and identification methods have been published for *L. acetotolerans*, *L. rossiae*, *L. lactis* or *L. mesenteroides*.

The aim of this study was to improve the detection of bacteria that are relevant to Bavarian Wheat Beer as an example of beer types with reduced selectivity by developing an adapted enrichment medium. To date, the modular and multiplex compatible, TaqMan based real-time PCR systems developed by Brandl [5] and Hutzler

Table 1 Strain set of selected obligate and potential beer spoilage organisms and brewery related non-target microorganisms, used to validate WBM-HR Medium against NBB

Species	Strain	Origin	Properties
Acetobacter pasteurianus	DSM 3509 T	Type strain, DSMZ culture collection, originally isolated from beer	aerobic background flora, non-target germ, most common slime-forming species at critical points with direct product contact [3]
Lactobacillus acetotolerans	TUM BP 111012019-2001	Brewery, Bavarian Wheat Beer, culture collection of the Research Center Weihenstephan BLQ	target germ, potential beer spoiler/ Bavarian Wheat Beer spoiler, slow growth [18]
Lactobacillus brevis	TUM BP 120816044-2790	Brewery, rinsing water, dealcoholizing plant, culture collection of the Research Center Weihenstephan BLQ	target germ, obligate beer spoiler
Lactobacillus rossiae	TUM BP 130607017-2573	Brewery, Bavarian Wheat Beer light, culture collection of the Research Center Weihenstephan BLQ	target germ, potential beer spoiler/ Bavarian Wheat Beer spoiler [18]
Lactococcus lactis	TUM BP 120611046-8446	Brewery, beer, culture collection of the Research Center Weihenstephan BLQ	target germ, potential Bavarian Wheat Beer spoiler (only when severe production errors occur), biofilm potential, common background flora
Leuconostoc mesenteroides	TUM BP 130927040-0820	Brewery, swab sample filler, culture collection of the Research Center Weihenstephan BLQ	target germ, potential Bavarian Wheat Beer spoiler (only when severe production errors occur), biofilm potential, common background flora
Pseudomonas fluorescens	DSM 50090 T	Type strain, DSMZ culture collection	aerobic background flora, water- and soil bacterium, non-target germ, non-pathogenic representor of *Pseudomonas* sp., *Pseudomonas* sp. common in biofilms at secondary critical points in filling area [3, 34, 35]
Saccharomyces cerevisiae	TUM 68 (FZW-BLQ)	most common top fermenting Bavarian Wheat Beer yeast, culture collection of the Research Center Weihenstephan BLQ	top fermenting culture yeast, non-target germ
Saccharomyces pastorianus var. carlsbergensis	TUM 34/70 (FZW-BLQ)	most common bottom-fermenting lager yeast, culture collection of the Research Center Weihenstephan BLQ	bottom-fermenting culture yeast, non-target germ

[14, 15] were not able to detect *L. acetotolerans*, *L. rossiae*, *L. lactis* and *L. mesenteroides* . As a result, new, compatible specific systems for those species were developed. The biofilm formation potential of strains of the four target species was also evaluated.

2 Materials and methods

2.1 Biofilm formation potential in stationary phase

For testing the biofilm formation potential of the used microorganisms (Table 6) an adapted test design according to *Kolari* et al. [24] and modified by *Timke* [36] in a 96-well microtiter format was used. This test was selected as it is described as straightforward and usable for quantifying a broad range of microbial biofilms with the exception of *P. aeruginosa* biofilms [30]. The test was carried out in sterile, black, flat-bottomed polystyrene microtiter 96 well plates with polymeric optical bottoms for fluorescence applications (Thermofisher Scientific, Rochester, USA). Each well was filled with 250 µl MRS-broth (DeMan-Rogosa-Sharpe broth) [7] with 0.02 µg/ml resazurin as a redox indicator for growth and cysteine hydrochloride 0.006 µg/ml as an oxidative quencher. Overnight cultures of the test strains were washed by suspending and centrifuging in sterile distilled water and adjusted to 10*E6 cells/ml. The filled wells were incubated with 2.5 µl of the washed and adjusted cultures. Afterwards, the microtiter plates were sealed with gas permeable foil and anaerobically incubated at 28 °C without disturbance for 24 hours. The fluorescence at 530 nm extinction and 590 nm emission (Multi-Detection-Reader Synergy 2, BioTek, Bad Friedrichshall, Germany) was measured directly after sealing the plates and after incubation as indirect growth control. The plates were emptied and rinsed afterwards, using sterile distilled water. 300 µl of crystal violet solution (4 g/l in 20 % vol ethanol) was added to stain residing cells, forming a biofilm in the wells for 5 minutes at room temperature. All wells are emptied and rinsed 3 times using 400 µl sterile distilled water. The remaining crystal violet that was still bound to the cells was dissolved in 300 µl 96 % vol ethanol at 10 °C overnight and A590 was measured using the Synergy 2 Multi-detection reader. Means were calculated using four independent measurements of four biological replicates per strain and normalized against the mean of four independent measurements of four blank samples. All wells were counter-checked and documented microscopically for adherent cells and trub particles using a Nikon inverted research microscope Ti-E, using a CFI S P-Fluor ELWD ADM 60x C air objective for phase contrast microscopy.

2.2 Test strains used for media validation

For media validation, 9 representative species were selected to cover the possible spectrum from obligate beer spoiling bacteria

Table 2 Formulation of developed Wheat Beer media

	WBM-HR-Broth (WBM-HR-B)	WBM-HR-Agar (WBM-HR-A)
	concentration [g/L]	concentration [g/L]
Chlorophenol red	0.04	0.04
D(–)fructose	2.00	2.00
D(–)ribose	0.54	0.54
D(+)glucose	15.99	15.99
Diammonium hydrogen citrate	1.20	1.20
Dipotassium hydrogen phosphate	1.20	1.20
Dipotassium hydrogen phosphate trihydrate	0.80	0.80
Meat peptone	1.99	1.99
Meat extract	4.80	4.80
Yeast extract	10.38	10.38
L-arginine mono hydrochloride	0.08	0.08
L-cysteine mono hydrochloride	0.20	0.20
Magnesium sulfate hexahydrate	0.12	0.12
Maltose monohydrate	1.50	1.50
Manganese sulfate1hydrate	0.03	0.03
Natamax®	0.05	0.05
Sodium acetatetrihydrate	3.00	3.00
Casein peptone	6.00	6.00
Sucrose	1.00	1.00
Trisodium citratedihydrate	0.16	0.16
Tween 80	0.24	0.24
Agar-Agar		18.00
Decarbonized Bavarian Wheat Beer (alcohol free)	600 ml	600 ml
H_2O	400 ml	400 ml

(*Lactobacillus. brevis*), potential beer spoiling bacteria (*L. rossiae*, *L. acetotolerans*, *L. lactis* and *L. mesenteroides*) to non-spoilage bacteria (*Acetobacter pasteurianus* and *Pseudomonas fluorescens*), as well as the omnipresent brewing yeasts (*Saccharomyces cerevisiae* and *Saccharomyces pastorianus* var. *carlsbergensis*). The exact test strains used are shown in table 1, which also provides origin and property information.

2.3 Media properties for enrichment cultures

To improve the enrichment of Bavarian Wheat Beer spoiling *Lactobacillus* sp. a new media, Wheat Beer media by Hutzler and *Riedl* (referred to as WBM-HR in the following) was developed by Hutzler and Riedl primarily to provide a medium with properties closer to those of Bavarian Wheat Beer with a known composition for the detection of *L. acetotolerans* at the Forschungszentrum Weihenstephan für Brau- und Lebensmittelqualität. Deng described catalase as an appropriate additive to overcome the VPNC state of *L. acetotolerans* by reducing oxidative damage of the non-media-adapted cells [8; 9]. Using an alternative method, the medium was created using L-cysteine monohydrochloride as an antioxidative agent. The medium was designed as a broth medium and as an agar plate medium. For validation, NBB (Doehler, Darmstadt Germany) was used as a comparable reference medium according to the instructions for use. The detailed formulations of the developed media are listed in table 2.

2.4 Media validation

The media were validated by using five target germ species, which are supposed to grow on the developed media. Those target germs are namely *L. brevis* as obligate beer spoilage organism the four potential beer spoiling organisms *L. rossiae*, *L. acetotolerans*, *L. lactis* and *L. mesenteroides*. For the non-target species, which are not supposed to grow under the intended conditions, *A. pasteurianus*, *P. fluorescens*, *S. cerevisiae* and *S. pastorianus* var. *carlsbergensis* were used. The detailed strain numbers and properties are listed in table 1.

All strains were cultivated in liquid culture and standardized to 1000 cells/ml. All media were inoculated with about 100 cells, the liquid media (WBM-HR-B and NBB-B) with 100 µl of the standardized cell suspension, the agar media (WBM-HR-A and NBB-A) as a pour plate culture with 1 ml of a 1:10 dilution with sterile ringer solution of the standardized cell suspension.

The broth media and pour plate agar cultures were inoculated and incubated at 28 °C for 7 days.

To validate the growth rate in WBM-HR-B, 25 µl of an MRS culture, which was inoculated from a cryogenic storage culture and incubated for 48 h at 28 °C, was inoculated in tubes with 10 ml WBM-HR-B and NBB-B in parallel and incubated anaerobically for 14 days at

Table 3 TaqMan® based real-time PCR detection systems for potential Bavarian Wheat Beer spoilage bacteria

Target organism	System Name	Probe	Primer	Target Area	Nucleotide sequences (5' → 3')	Reference
internal amplification control	IAC135	IAC135-S	IAC135-f	IAC135	TGGATAGATTCGATGACCCTAGAAC	This study
			IAC135-r		TGAGTCCATTTTCGCAGATAACTT	This study
Lactobacillus acetotolerans	Lac	Lac-S	Lac-f	16S rDNA	CGAGCCGAACCAATTGATTAC	This study
			Lac-r		TGTGATCTCTCCTTTTATCCGGTAT	This study
Lactobacillus rossiae	Lro	Lro-S	Lro-f	16S rDNA	GGCGTGCCTAATACATGCAAR	This study
			Lro-r		TGTCTCGTCAATCTGGTGCAA	This study
Lactococcus lactis	iLacLa	i200	Llac-f	16S rDNA	GAAAGATGCAATTGCATCACTCAA	This study
			LP-r		ATTCCCTACTGCTGCCTCCC	[5]
Leuconostoc mesenteroides	iLeu	i200	Leu-f	16S rDNA	GCGCTTCGGCGTCACC	This study
			LP-r		ATTCCCTACTGCTGCCTCCC	[5]

Table 4 Probes used for TaqMan® real-time PCR detection systems for potential Bavarian Wheat Beer spoiler bacteria

Probe	Reporter	Quencher	Nucleotide sequence (5' → 3')	Reference
i200	FAM	BHQ-1	CCACATTGGGACTGAGACACGGCC	[5]
Lro_S	FAM	BHQ-1	TCGAACGCACTTCGGKTTTTGATTGA	This study
Lac_S	FAM	BHQ-1	CCTACCCTATAGTCTGGGATACCACTTGGAAACAG	This study
IAC135-S	HEX	BHQ-1	TGGGAGGATGCATTAGGAGCATTGTAAGAGAG	This study

Table 5 Artificial target sequence nucleotide IAC135 for internal amplification control

Nucleotide	Nucleotide sequence (5' → 3')	Reference
IAC135	TGCTAGAGAATGGATAGATTCGATGACCCTAGAACTAGTGG GAGGATGCATTAGGAGCATTGTAAGAGAGTCGGAAGTTA	This study
IAC135-rev	TGCGACACCTTGGGCGACCGTCAATAGGCCACTCGAAT GAGTCCATTTTCGCAGATAACTTCCGACTCTCTTACAATGCT	This study

Table 6 Biofilm formation potential of selected obligate and potential Bavarian Wheat Beer spoiling lactic acid bacteria

Species	Test strain	$\bar{E}_{590} - \overline{SD}_{590}$	Biofilm formation potential[1]	observable microscopical cell adhension to surface
Lactobacillus acetotolerans	TUM BP 111012019-2001	0,188	–	yes
Lactobacillus acetotolerans	TUM BP 120706025-2505	0,290	–	yes
Lactobacillus brevis	TUM BP 120816044-2790	0,745	++	yes
Lactobacillus brevis	TUM BP 120816004-2781	0,385	–	yes
Lactobacillus brevis	TUM BP 120827005-2823	5,00	+++	yes
Lactobacillus rossiae	TUM BP 131022000-2858	0,302	–	no
Lactobacillus rossiae	TUM BP 131022011-2866	0,357	–	yes
Lactobacillus rossiae	TUM BP 131011001-2846	0,345	–	yes
Lactobacillus rossiae	TUM BP 130607017-2573	1,00	++	yes
Lactococcus lactis	DSM20481T	0,826	+	yes
Lactococcus lactis	TUM BP 120611046-8446	0,619	+	yes
Leucocostoc mesenteroides	TUM BP 130927040-0820	1,13	+	yes
Leuconostoc mesenteroides	TUM BP 130920005-0816	1,81	++	yes

[1] Biofilm formation according to Diaz [10]

- : no biofilm formation $\bar{E}_{590} - \overline{SD}_{590} < E_{590\ cutoff}$

+ : weak biofilm formation $E_{590\ cutoff} < \bar{E}_{590} - \overline{SD}_{590} < 2 * E_{590\ cutoff}$

++ : moderate biofilm formation $2 * E_{590\ cutoff} < \bar{E}_{590} - \overline{SD}_{590} < 4 * E_{590\ cutoff}$

+++ : strong biofilm formation $\bar{E}_{590} - \overline{SD}_{590} > 4 * E_{590\ cutoff}$

28 °C in duplets. The time of the pH-indicator change was documented visually against blind samples of a non-inoculated medium.

2.5 DNA-extraction

For the DNA extraction, a modified protocol using the InstaGene Matrix™ (Bio-Rad, Hercules, CA, USA) was used [26]. Hence 200 µl of a dense liquid enrichment culture was transferred into a 1.5 ml Eppendorf reaction tube and centrifuged for 1 minute at 15,114 x g (Hettich Mikro 200). The supernatant was discarded and 200 µl InstaGene™ DNA isolation buffer was added to the residing cell pellet and incubated at 56 °C for 30 minutes (Eppendorf Thermomix comfort). After a 10 second vortex step, the tube was incubated for another 8 minutes at 95 °C then centrifuged for 1 min at 15,114 x g. The DNA concentration was measured using a Nanodrop ND 2000 (Thermofisher Scientific, Rochester, USA). For the validation, the DNA concentration of the test strains was adjusted with PCR-clean ddH2O to 5 ng/µl. The DNA sample material and sample strain set were stored at –20 °C.

2.6 Real-time-PCR

For the rapid detection of the potential beer-spoiling target species *L. lactis*, *L. mesenteroides*, *L. rossiae* and *L. acetotolerans*, TaqMan® real-time PCR assays were designed. The target sequence for all four species is located in the 16S rDNA region. To identify usable, specific primer binding areas, the 16S rDNA-sequences of 99 strains of common brewery microorganisms collected from the NCBI-Database (http://www.ncbi.nlm.nih.gov) were aligned (DNASTAR, MegAlign, Lasergene, Version 11). To achieve the greatest specificity possible, primer target areas were selected that had specific polymorphisms. Primers and probes were designed using Primer Express (Primer Express 1.5, Applied Biosystems, Thermofisher Scientific). For internal amplification control, a synthetic, random DNA sequence was generated and a specific TaqMan real-time PCR system with this sequence as target area was designed (Table 3, Table 4 and Table 5). All primers and probes were tested in silico using BLAST (Basic Local Alignment Search Tool) for homologies with other sites or species. All real-time PCR systems were designed to be compatible with other systems for spoilage organism detection, designed by Brandl [5] and Hutzler [14, 15]. The real-time PCR parameters used for the design were therefore:

- Annealing temperature primers: 60 °C.
- Annealing temperature probes: 10 °C above annealing temperature primers.
- Maximum amplicon length: 200 bp.

All nucleotides were synthesized by biomers.net GmbH, Ulm, Germany. All PCR-runs were carried out on a LightCycler® 480 Instrument II (Roche Diagnostics Deutschland GmbH, Mannheim, Germany), using 10 µl LightCycler® 480 Probes Master in a 20 µl volume assay with a 5 µl sample. The real-time PCR was performed using 400 nmol l^{-1} of forward and reverse primer and 200 nmol l^{-1} TaqMan probe of the main PCR system. The Primers were added in aliquots of 0.8 µl and 10^{-5} mol l^{-1}, the probes were added in 0.4 µl aliquots of 10^{-5} mol l-1.

The internal amplification control IAC135 was added using 250 nM of IAC135-f and IAC135r, 200 nM and IAC135-S. The target DNA of the internal amplification control IAC135 and IAC135-rev was used at a concentration of $5*10^{-20}$ mol l^{-1}. The primers were added in aliquots of 0.5 µl and 10^{-5} mol l^{-1}, the probes were added

Table 7 **Quantitative validation WBM-HR-A according to DIN EN ISO 11133:2015-01 [11] after 7 days incubation at 28 °C with selected obligate and potential Bavarian Wheat Beer spoiling bacteria and brewery related non-target microorganisms**

	WM-A				NBB-A					
	#1 [CFU]	#2 [CFU]	#3 [CFU]	Ø	#1 [CFU]	#2 [CFU]	#3 [CFU]	Ø	Productivity P_R=WM-A/NBB-A (WBMHR-A versus NBB-A) $P_R \geq 0.5$	Selectivity (growth level)
Acetobacter pasteurianus	0	0	0	0	0	0	0	0	complete inhibition	0
Lactobacillus acetotolerans	235	230	245	237	257	277	284	273	0.87	2
Lactobacillus brevis	49	42	44	45	46	50	53	50	0.90	2
Lactobacillus rossiae	78	72	78	76	82	92	76	83	0.92	2
Lactococcus lactis	88	76	52	72	57	81	96	78	0.92	2
Leuconostoc mesenteroides	79	82	84	82	102	91	75	89	0.92	2
Pseudomonas fluorescens	0	0	0	0	0	0	0	0	complete inhibition	0
Saccharomyces cerevisiae TUM 68	5	7	7	6	0	0	0	0	partial inhibition	1
Saccharomyces pastorianus var. carlsbergensis TUM 34/70	16	13	11	13	0	0	0	0	partial inhibition	1

Selectivity (growth levels): 0 = no growth, 1 = weak growth, 2 = good growth
CFU = Colony Forming Units

Table 8 **Qualitative validation with selected obligate and potential Bavarian Wheat Beer spoiling bacteria and brewery related non-target microorganisms of WBM-HR-B, incubated at 28 °C for 7 days (4-field test) [33]**

Medium	WBM-HR-B (7 d)			NBB-B (7 d)				
	#1	#2	#3	#1	#2	#3		
Acetobacter pasteurianus	–	–	–	–	–	–		
Lactobacillus acetotolerans	+	+	+	+	+	+		
Lactobacillus brevis	+	+	+	+	+	+		
Lactobacillus rossiae	+	+	+	+	+	+	**a:** number of positive analysis results in both methods:	30
Lactococcus lactis	+	+	+	+	+	+	**b:** number of wrong negative analysis results versus the reference method (NBB-B):	0
Leuconostoc mesenteroides	+	+	+	+	+	+	**c:** number of wrong positive analysis results versus the reference method (NBB-B):	0
Pseudomonas fluorescens	–	–	–	–	–	–	**d:** number of overall negative results in both methods:	24
Saccharomyces cerevisiae TUM 68	–	–	–	–	–	–	**n:** overall analysis results:	54
Saccharomyces pasteurianus var. carlsbergensis TUM 34/70	–	–	–	–	–	–	**relative specificity WBM-HR-C (d/(c+d))*100%**	100 %
							relative accuracy WBM-HR-C ((a+d)/n)*100 %	100 %
							relative sensitivity (a/(a+b))*100%	100 %

- : no visible indicator color change + : visible color change/growth

Table 9 Detection speed validation of WBM-HR-B with selected obligate and potential Bavarian Wheat Beer spoiling bacteria, incubated anaerobically at 28 °C for 14 days

Species	Medium	Day 1	Day 2	Day 3	Day 4	Day 7	Day 10	Day 14
Lactobacillus acetotolerans TUM BP 120706025-2967	WBM-HR-B	–	–	–	+/–	+	+	+
	NBB-B	–	–	–	–	+	+	+
Lactobacillus backi TUM BP 140407001-2242	WBM-HR-B	–	+	+	+	+	+	+
	NBB-B	–	+	+	+	+	+	+
Lactobacillus brevis TUM BP 120711011-2578	WBM-HR-B	–	+	+	+	+	+	+
	NBB-B	–	+	+	+	+	+	+
Lactobacillus casei TUM BP 120509129-2360	WBM-HR-B	–	+	+	+	+	+	+
	NBB-B	+	+	+	+	+	+	+
Lactobacillus collinoides TUM BP 000-2061	WBM-HR-B	–	–	+	+	+	+	+
	NBB-B	–	–	+	+	+	+	+
Lactobacillus lindneri TUM BP 121213056-2397	WBM-HR-B	–	–	–	+/-	+	+	+
	NBB-B	–	–	–	–	+	+	+
Lactobacillus parabuchneri TUM BP 121008043-2282	WBM-HR-B	–	+	+	+	+	+	+
	NBB-B	–	+	+	+	+	+	+
Lactobacillus perolens TUM BP 130000240-2596	WBM-HR-B	–	+	+	+	+	+	+
	NBB-B	–	+	+	+	+	+	+
Lactobacillus plantarum TUM BP 121121170-2217	WBM-HR-B	–	+	+	+	+	+	+
	NBB-B	+	+	+	+	+	+	+
Lactobacillus rossiae TUM BP 130806019-2754	WBM-HR-B	–	+	+	+	+	+	+
	NBB-B	–	+	+	+	+	+	+
Leuconostoc mesenteroides TUM BP 000-0983	WBM-HR-B	–	–	–	–	–	+/–	+/–
	NBB-B	–	+	+	+	+	+	+
Megasphaera cerevisiae TUM BP 121011015-5986	WBM-HR-B	–	–	+/–	+	+	+	+
	NBB-B	–	–	–	–	+	+	+
Pectinatus frisingensis TUM BP 000-4327	WBM-HR-B	–	–	+	+	+	+	+
	NBB-B	–	–	–	–	–	+	+
Pediococcus damnosus TUM BP 140313142-2243	WBM-HR-B	–	–	+	+	+	+	+
	NBB-B	–	–	+	+	+	+	+

- : = no color indicator change, + : = color indicator change from red to yellow, +/- : = incomplete, partial color indicator change from red to yellow

in 0.4 µl aliquots of 10^{-5} mol l^{-1}, the internal amplification control target DNA was added in 0.1 µl aliquots of 10^{-17} mol l^{-1}.The initial heating at 95 °C was held for 10 minutes, then 40 cycles were performed at 95 °C for 10 seconds and 60 °C for 30 seconds. The fluorescence was measured at the end of the 60 °C step of each cycle (modified [5, 14, 15]).

2.7 Validation real-time-PCR

As a strain set to validate the PCR specificity, 99 representative species, known to be common in the brewing process were selected. All strains listed in table 10 (see page 46) were grown as dense three-day cultures in WBM-HR-B, the DNA isolated according to section 2.5 and adjusted to 5 ng/µl.

3 Results and discussion

3.1 Biofilm formation

To validate the biofilm formation potential, cut-off values were used to differentiate between biofilm-forming strains and non-biofilm-forming strains. The biofilm-forming cut-off for non-biofilm forming organisms $(E_{590\ cutoff})$ was defined as the mean of the measured optical density of four independent negative samples $(\overline{E}_{590\ Blank})$ plus three times the mean standard deviation $(\overline{SD}_{590\ Blank})$ and the mean measurement of the blank value $(\overline{E}_{590\ Blank})$ plus the mean standard deviation $(\overline{SD}_{590\ Blank})$. The mean of four independent measurements of a strain minus the standard deviation of these measurement $\overline{SD}_{590}$ equal or below this threshold was defined as no biofilm formation, values between $E_{590\ cutoff}$ and $2 * E_{590\ cutoff}$ were defined as weak biofilm formation, between $2 * E_{590\ cutoff}$ and $4 * E_{590\ cutoff}$ as moderate biofilm formation and above $4 * E_{590\ cutoff}$ as strong biofilm formation according to *Diaz* [10]. All wells in the microtiter plate were checked microscopically for cell adhesion.

As shown in table 6, two of the three tested *L. brevis* showed biofilm formation in MRS, of which TUM BP 120827005-2823 showed very strong biofilm formation. Of the four tested strains of *L. rossiae*, only the strain TUM BP 130607017-2573 showed biofilm formation. All test strains of the species *L. lactis* and *L. mesenteroides* were able to build biofilm in MRS.

Table 10 Results of real-time PCR specificity validation with DNA isolates adjusted to 5 ng/µl of target species and brewery related non-target microorganisms

Species	Strain	Real-time PCR-identification *L. rossiae*	Real-time PCR-Screening *L. acetotolerans*	Real-time PCR-Screening *L. lactis*	Real-time PCR-Screening *L. mesenteroides*
Acetobacter aceti	TUM BP 000-1991	negative	negative	negative	negative
Acetobacter pasteurianus	TUM BP 000-1990	negative	negative	negative	negative
Asaia lannensis	TUM BP 000-0994	negative	negative	negative	negative
Bacillus subtilis	TUM BP 000-0980	negative	negative	negative	negative
Candida boindinii	TUM YP 000-6007	negative	negative	negative	negative
Cryptococcus laurentii	TUM YP 000-0011	negative	negative	negative	negative
Debaryomyces hansenii	TUM YP 000-0006	negative	negative	negative	negative
Dekkera anomala	TUM YP 000-3040	negative	negative	negative	negative
Dekkera bruxellensis	TUM YP 000-3096	negative	negative	negative	negative
Enterobacter sp.	TUM BP 000-6088	negative	negative	negative	negative
Enterococcus sp.	TUM BP 111206005-0075	negative	negative	negative	negative
Escherichia coli	TUM BP 000-0981	negative	negative	negative	negative
Gluconacetobacter liquefaciens	TUM BP 000-0105 (DSM 5603 (BS 279))	negative	negative	negative	negative
Gluoconobacter oxydans	TUM BP 000-0078	negative	negative	negative	negative
Hafnia alvei	TUM BP 000-0993	negative	negative	negative	negative
Hanseniaspora uvarum	TUM YP 000-0054 (CBS 5074)	negative	negative	negative	negative
Kazachstania exigua	TUM YP 000-337	negative	negative	negative	negative
Kluyvera ascorbata	TUM BP 131213038-0099	negative	negative	negative	negative
Kluyveromyces marxianus	TUM YP 000-0005	negative	negative	negative	negative
Kocuria kristinae	TUM BP 000-0083 (DSMZ 22032)	negative	negative	negative	negative
Lactobacillus acetotolerans	TUM BP 120706025-2967	negative	positive (ct 24.35)	negative	negative
Lactobacillus acidophilus	TUM BP 000-2081 (DSMZ 20079)	negative	negative	negative	negative
Lactobacillus alimentarius	TUM BP 000-2979	negative	negative	positive (ct 28.89)	negative
Lactobacillus amylolyticus	TUM BP 000-2969	negative	negative	negativ	negative
Lactobacillus amylophilus	TUM BP 000-2068	negative	negative	negativ	negative
Lactobacillus amylovorus	TUM BP 000-2080 (DSMZ 20531)	negative	negative	negativ	negative
Lactobacillus backi	TUM BP 140407001-2242	negative	negative	negativ	negative
Lactobacillus bifermentans	TUM BP 000-2014 (DSMZ 20003)	negative	negative	negativ	negative
Lactobacillus brevis	TUM BP 120711011-2578	negative	negative	negativ	negative
Lactobacillus brevis (formerly Lactobacillus brevisimilis)	TUM BP 000-2976	negative	negative	negativ	negative
Lactobacillus buchneri	TUM BP 000-2060	negative	negative	negativ	negative
Lactobacillus casei	TUM BP 120509129-2360	negative	negative	negativ	negative
Lactobacillus collinoides	TUM BP 000-2061	negative	negative	negativ	negative
Lactobacillus coryniformis	TUM BP 000-2978	negative	negative	negativ	negative
Lactobacillus curvatus	TUM BP 000-2977 (BS 218)	negative	negative	positive (ct 28.83)	negative
Lactobacillus delbrueckii ssp. delbrueckii	TUM BP 000-2968	negative	negative	negative	negative
Lactobacillus dextrinicus	TUM BP 000-2987	negative	negative	positive (ct 28.96)	negative
Lactobacillus fermentum	TUM BP 000-2069	negative	negative	negative	negative
Lactobacillus frisingensis	TUM BP 130919043-2789	negative	negative	negative	negative
Lactobacillus fructivorans	TUM BP 000-2038	negative	negative	negative	negative
Lactobacillus gasseri	TUM BP 000-2970	negative	negative	negative	negative
Lactobacillus ghanensis	TUM BP 000-2931	negative	negative	negative	negative
Lactobacillus harbinensis	TUM BP 120906016-2993	negative	negative	negative	negative
Lactobacillus helveticus	TUM BP 000-2971	negative	negative	negative	negative
Lactobacillus hilgardii	TUM BP 000-2975	negative	negative	negative	negative
Lactobacillus johnsonii	TUM BP 000-2972 (BS 224)	negative	negative	negative	negative
Lactobacillus kefiri	TUM BP 000-2037	negative	negative	negative	negative

Lactobacillus lindneri	TUM BP 121213056-2397	negative	negative	negative	negative
Lactobacillus malefermentans	TUM BP 000-2974	negative	negative	positive (ct 28.83)	negative
Lactobacillus parabrevis T	TUM BP 000-2080 (DSMZ 20531)	negative	negative	negative	negative
Lactobacillus parabuchneri	TUM BP 121008043-2282	negative	negative	negative	negative
Lactobacillus paracollinoides	TUM BP 150113003-2371	negative	negative	negative	negative
Lactobacillus perolens	TUM BP 130000240-2596	negative	negative	negative	negative
Lactobacillus plantarum	TUM BP 121121170-2217	negative	negative	negative	negative
Lactobacillus reuteri	TUM BP 000-2055 (BS 227)	negative	negative	negative	negative
Lactobacillus rhamnosus	TUM BP 000-2996	negative	negative	negative	negative
Lactobacillus rossiae	TUM BP 130806019-2754	positive (ct 22.82)	negative	negative	negative
Lactobacillus salivarius	TUM BP 000-2997	negative	negative	negative	negative
Lactobacillus sanfranciscensis	TUM BP 000-2982	negative	negative	negative	negative
Lactococcus lactis	TUM BP 000-8973	negative	negative	positive (ct 16.43)	negative
Leuconostoc mesenteroides	TUM BP 000-0983	negative	negative	negative	positive (ct 22.43)
Megasphaera cerevisiae	TUM BP 121011015-5986	negative	negative	negative	negative
Meyerozyma guilliermondii	TUM YP 000-0041	negative	negative	negative	negative
Micrococcus luteus	TUM BP 000-0995	negative	negative	negative	negative
Oenococcus oeni	TUM BP 000-0013	negative	negative	negative	negative
Pantoea dispersa	TUM BP 000-0992	negative	negative	negative	negative
Pectinatus cerevisiiphilus	TUM BP 120919033-4402	negative	negative	negative	negative
Pectinatus frisingensis	TUM BP 000-4327	negative	negative	negative	negative
Pectinatus haikarae	TUM BP 120919239-4404	negative	negative	negative	negative
Pediococcus clausenii	TUM BP 000-3986	negative	negative	negative	negative
Pediococcus damnosus	TUM BP 140313142-2243	negative	negative	negative	negative
Pediococcus inopinatus	TUM BP 000-3984	negative	negative	negative	negative
Pediococcus pentosaceus	TUM BP 000-3985	negative	negative	negative	negative
Pichia membranifaciens	TUM YP 000-2009	negative	negative	negative	negative
Pseudomonas poae	TUM BP 000-7057	negative	negative	negative	negative
Pseudomons fluorescens	DSM 50090 (BS236)	negative	negative	negative	negative
Rhodosporidium toruloides	TUM YP 000-0110	negative	negative	negative	negative
Rhodotorula mucilaginosa	TUM YP 120306011-7159	negative	negative	negative	negative
Saccharomyces bayanus	TUM YP 000-1999	negative	negative	negative	negative
Saccharomyces cer. var. diastaticus	TUM YP 000-1042 (DSM 70487)	negative	negative	negative	negative
Saccharomyces cerevisiae TUM 184	TUM YP 000-1001	negative	negative	negative	negative
Saccharomyces cerevisiae TUM 66/70	TUM YP 000-1044	negative	negative	negative	negative
Saccharomyces cerevisiae TUM 68	TUM YP 000-1045	negative	negative	negative	negative
Saccharomyces paradoxus	TUM YP 000-1043 (BS 11 (WYSC 63))	negative	negative	negative	negative
Saccharomyces pastorianus	TUM YP 000-1010	negative	negative	negative	negative
Saccharomyces pastorianus TUM 34/70	TUM YP 000-1008	negative	negative	negative	negative
Saccharomyces pastorianus TUM 34/78	TUM YP 000-1010	negative	negative	negative	negative
Saccharomyces uvarum	TUM YP 000-1090	negative	negative	negative	negative
Saccharomycodes ludwigii	TUM YP 000-0046 (SL17)	negative	negative	negative	negative
Schizosaccharomyces pombe	TUM YP 000-0039	negative	negative	negative	negative
Selenomonas lacticifex	TUM BP 000-0998	negative	negative	negative	negative
Torulaspora delbrueckii	TUM YP 000-0003	negative	negative	negative	negative
Weissella paramesenteroides	TUM BP 000-0988	negative	negative	negative	negative
Weissella viridescens	TUM BP 000-0989 (BS 198)	negative	negative	negative	negative
Wickerhamomyces anomalus	TUM YP 000-2004	negative	negative	negative	negative
Zygosaccharomyces bailii	TUM YP 000-5094	negative	negative	negative	negative
Zygosaccharomyces rouxii	TUM YP 000-5092	negative	negative	negative	negative
Zymomonas mobilis	TUM BP 000-0036 (DSMZ 424)	negative	negative	negative	negative

Biofilm formation could not be measured in either of the tested strains of *L. acetotolerans* under the described standardized experiment parameters, but cell adhesion could be observed microscopically The appearance of this species in fermentation tanks and vessels [8, 9] may be linked to its adhesion to surfaces in substrates with good growth factor distribution such as MRS. Further tests with standardized Bavarian Wheat Beer wort and finished Bavarian Wheat Beer should be conducted and the incubation time should be increased for this species, to evaluate the potential risk of this species permanently inhabiting this production area.

The *L. rossiae* species includes very heterogenic strain types that differ physiologically and in their genetic profile [6]. It is therefore only logical that these have different biofilm building potentials. The experimental procedure was designed to analyze the biofilm formation potential of lactic acid bacteria in general, using MRS as the universal medium for this group. As *L. rossiae* is a bigger problem in the filling and packaging area of breweries [17], the biofilm-forming potential in media that are closer in composition to fermented beer/ diluted beer will be tested in addition to conducting the standard test with MRS. As EPS development is a reaction of microorganisms to their environment and correlated to the composition of their substrates, lower distribution of growth factors and higher concentrations of growth inhibitors can lead to different biofilm-building potentials.

The tested *L. lactis* and *L. mesenteroides* strains could all develop biofilms in MRS. The ability to form initial biofilms correlates with the appearance of both species in brewery-related biofilms has already been described [4, 34].

3.2 Media validation

The agar medium (WBM-HR-A) was validated using the modified method according to the DIN ISO 11133 [11] as shown in table 7. The target organisms *L. acetotolerans*, *L. rossiae*, *L. brevis*, *L. mesenteroides* and *L. lactis* showed good growth on WBM-HR-A. The productivity of all 5 species was above 0.7 compared with NBB-A. *A. pasteurianus* and *P. aeruginosa* were both completely inhibited by WBM-HR-A and NBB-B. The two culture yeast strains *S. cerevisiae* TUM 68 and *S. pastorianus* TUM 34/70 showed partial inhibition in WBM-HR-A.

The minimum productivity proportion PR is set as 0.7 according to the DIN EN ISO 11133 for the validation of alternative media in comparison with a similar reference medium for target germs. This criterion was fulfilled by WBM-HR-A. The partial inhibition of *S. cerevisiae* TUM 68 and *S. pastorianus* TUM 34/70 can relate to a process step in the preparation of the agar medium. The sole yeast-inhibiting agent in all three new developed media is Natamax® (Natamycin) which is not heat stable in solution. For pouring the agar plates, the medium has to be heated for melting the agarose, which can lead to reduced antimicrobial effectiveness. Adding further antimycotics such as actidione (cycloheximide) can easily mitigate this disadvantage.

As shown in table 8 and table 9, the new broth medium was evaluated using the 4-field test [33]. All target organisms (*L. acetotolerans*, *L. brevis*, *L. rossiae*, *L. lactis* and *L. mesenteroides*) were able to grow in WBM-HR-B. The non-target organisms (*A. pasteurianus*, *P. fluorescens*, *S. cerevisiae* TUM 68 and *S. pastorianus* TUM 34/70) did not grow. The relative specificity, the relative accuracy and the relative sensitivity according to the 4-field test [33] are therefore 100 %.

To inhibit the dilution effects, it is possible to use the medium with higher amounts of sample volume, for example with 50 % medium using a double concentrated medium, or 10 % medium with a 10-fold concentrated medium. In that case, the final pH and bitter units in the mixture of sample and concentrated medium have to be checked.

The detection speed of WBM-HR-B was checked against NBB-B with a selection of target organisms as shown in table 9 in duplets. The detection speed of both media is comparable. *L. acetotolerans* TUM BP 120706025-2967, *Lactobacillus lindneri* TUM BP 121213056-2397, *Megasphaera cerevisiae* TUM BP 121011015-5986 and *Pectinatus frisingensis* TUM BP 000-4327 show an earlier detectable change of the indicator color in WBM-HR-B than in NBB-B. The test strains *L. casei* TUM BP 120509129-2360, *L. plantarum* TUM BP 121121170-2217 and *L. mesenteroides* TUM BP 000-0983 can be detected earlier in NBB-B.

3.3 Real-time-PCR

The real-time PCR systems for *L. acetotolerans*, *L. rossiae*, *L. lactis* and *L. mesenteroides* were validated against a strain set of 99 species that belong to the group of lactic acid bacteria or that are known to be present in breweries and beverage bottling plants (Table 10).

The identification systems for *L. acetotolerans*, *L. rossiae* and *L. mesenteroides* proved to be highly specific. Only the target species showed a significant signal within 40 PCR cycles. The relative specificity, the relative accuracy and relative sensitivity are therefore at 100 % [33].The identification system for *L. lactis* showed positive reactions after 28 cycles with the DNA of the following strains: *Lactobacillus malefermentans* TUM BP 000-2974, *Lactobacillus curvatus* TUM BP 000-2977 (BS 218), *Lactobacillus alimentarius* TUM BP 000-2979 and *Lactobacillus dextrinicus* TUM BP 000-2987. This means 4 cross reactions within the specificity evaluation and therefore a relative specificity of the *L. lactis* identification system of 96 % [33].

4 Conclusion/Summary

With the exception of some *L. rossiae* strains, all the tested bacteria could build biofilms in MRS, a nutrient rich medium, as used in the standard method to evaluate biofilm formation potential. While this medium is comparable in nutrient composition with wort and most alcohol-free beer types, most intermediate stages in the beer production offer quite different nutrient profiles. The missing biofilm formation of *L. acetotolerans* sustains the thesis that this germ mostly originates from the brewery sections that contain yeast such as fermentation tanks and vessels in the case of contamination cases as described by *Deng* [8].

L. rossiae is mostly described as a germ that originates in filling devices. Heterogenic phenotypes concerning biofilm formation potential in different strains of this species could be observed. *L. rossiae* has already been described as having a wide intra-species variation of biochemical properties and RAPD genotypes [6]. This high variance also seems to affect the biofilm-forming potential. The risk to breweries that are confronted with this species of building persistent biofilms on filling equipment can therefore vary. It is still possible for non-biofilm forming strains of *L. rossiae* to occur in symbiosis with other biofilm forming microorganisms.

All tested strains of the *L. lactis* and *L. mesenteroides* species showed biofilm-forming potential in MRS. There are no cases reported of these species having a product spoiling potential in Bavarian Wheat Beer under normal production conditions, but the de novo biofilm production by these species can generate a habitat for spoilage organisms.

The developed and validated WBM-HR media proved to be sufficient. Compared with the reference medium NBB, the broth variant showed comparable results. The advantage is that the formula/ recipe is known and that it can be modified in any direction (concentrate, double-concentration, beer content, brewery specific beer, etc.). It also detects slow-growing germs such as *L. acetotolerans* very rapidly compared with comparable enrichment media. The WBM-HR agar can be used after modifying the yeast inhibition substance. The addition of cycloheximide is a possible option that should be tested in the future.

The real-time PCR identification systems developed for *L. acetotolerans*, *L. rossiae* and *L. mesenteroides* proved to be highly specific with the target species. No cross reactions could be observed in vitro. The identification system for *L. lactis* showed a positive PCR reaction after 28 cycles with the strains of following species *Lactobacillus malefermentans*, *Lactobacillus curvatus*, *Lactobacillus alimentarius* and *Lactobacillus dextrinicus*. The species that showed cross reactions are not potential or obligate beer spoilers and are lactic acid bacteria that seldom occur or do not occur in a brewery environment. *L. lactis* pure culture DNA isolate was detected much earlier at a PCR ct value of 16 (16 PCR cycles). Cross reactions of the *L. lactis* could therefore additionally be excluded by checking the ct value of a pure culture or enriched culture analysis. No influence of the developed medium on the PCR reaction could be observed in any of the tests. A combined use of the WBM-HR with the PCR screening systems is of great value for quality management in breweries, especially Bavarian Wheat Beer breweries, confronted with the discussed species.

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6 Literature

1. Allison, D. G. and Sutherland, I. W.: The role of exopolysaccharides in adhension of fresh water bacteria, Journal of general microbiology **133** (1987), no. 5, pp. 1319-1327.
2. Back, W.: Sekundärkontaminationen im Abfüllbereich, BRAUWELT **133** (1994), no. 16, pp. 686-697.
3. Back, W.: Biofilme in der Brauerei und Getränkeindustrie, BRAUWELT **142** (2003), no: 24/25, pp. 766-777.
4. Back, W. (2005). Colour Atlas and Handbook of Beverage Biology. 1st edit, Fachverl. Hans Carl, Nuernberg. ISBN 978-3-418-00799-1.
5. Brandl, A.: Entwicklung und Optimierung von PCR-Methoden zur Detektion und Identifizierung von brauereirelevanten Mikroorganismen zur Routine-Anwendung in Brauereien, Fakultät Wissenschaftliches Zentrum Weihenstephan für Ernährung, Landnutzung und Umwelt, TU München, 2006.
6. Corsetti, A.; Settanni, L.; van Sinderen, D.; Felis, G. E.; Dellaglio, F. and Gobbetti, M.: *Lactobacillus rossii* sp nov., isolated from wheat sourdough, International journal of systematic and evolutionary microbiology **55** (2005), no. 1, pp. 35-40.
7. De Man, J. C.; Rogosa, M. and Sharpe, M. E.: A medium for the cultivation of *Lactobacilli*, Journal of Applied Bacteriology **23** (1960), no. 1, pp. 130-135.
8. Deng, Y.; Liu, J. Y.; Li, H. P.; Li, L.; Tu, J. X.; Fang, H. J.; Chen, J. and Qian, F.: An improved plate culture procedure for the rapid detection of beer-spoilage lactic acid bacteria, Journal of the Institute of Brewing **120** (2014), no. 2, pp. 127-132.
9. Deng, Y.; Liu, J. Y.; Li, L.; Fang, H. J.; Tu, J. X.; Li, B.; Liu, J.; Li, H. P. and Xu, Z. B.: Reduction and restoration of culturability of beer-stressed and low-temperature-stressed *Lactobacillus acetotolerans* strain 2011-8, International journal of food microbiology **206** (2015), pp. 96-101.
10. Diaz, M.; Ladero, V.; del Rio, B.; Redruello, B.; Fernandez, M.; Martin, M. C. and Alvarez, M. A.: Biofilm-Forming Capacity in Biogenic Amine-Producing Bacteria Isolated from Dairy Products, Frontiers in Microbiology **7** (2016), pp. 158-167.
11. DIN Deutsches Institut für Normung e. V., (2015). DIN EN ISO 11133:2014: Mikrobiologie von Lebensmitteln, Futtermitteln und Wasser - Vorbereitung, Herstellung, Lagerung und Leistungsprüfung von Nährmedien (ISO 11133:2014) Deutsche Fassung EN ISO 11133:2014. Berlin: Beuth, 11.01.2014
12. Entani, E.; Masai, H. and Suzuki, K. I.: *Lactobacillus acetotolerans* – A new species from fermented vinegar broth, International journal of systematic bacteriology **36** (1986), no. 4, pp. 544-549.
13. Geissler, A. J.; Behr, J.; von Kamp, K. and Vogel, R. F.: Metabolic strategies of beer spoilage lactic acid bacteria in beer, International journal of food microbiology **216** (2016), pp. 60-68.
14. Hutzler, M.: Entwicklung und Optimierung von Methoden zur Identifizierung und Differenzierung von getränkerelevanten Hefen, Fakultät Wissenschaftliches Zentrum Weihenstephan für Ernährung, Landnutzung und Umwelt, Technische Universität München, Dissertation, 2009.
15. Hutzler, M. (2010). Getränkerelevante Hefen- Identifizierung und Differenzierung: Wie können Hefen praxisrelevant unterschieden werden, und wie können Identifizierungsergebnisse technologisch bewertet

werden? 1st edit, Südwestdeutscher Verlag für Hochschulschriften AG Co. KG. ISBN 978-3-8381-1482-8.
16. Hutzler, M.: Brauereimikrobiologie - ausgewählte Befunde und Entwicklungen 2010, 44. Technologisches Seminar Weihenstephan, 2011, Freising, Weihenstephan,
17. Hutzler, M.; Koob, J.; Riedl, R. and Jacob, F.: Classification, Identification and Detection of Beer Spoiling Microorganisms - A Review, World Brewing Congress, 2012, Portland, Oregon,
18. Hutzler, M.; Riedl, R. and Koob, J.: Sinnvoller Einsatz moderner Analysenmethoden zur Untersuchung spezifischer brauereimikrobiologischer Problemstellungen, Technologisches Seminar Weihenstephan, 2012, Freising-Weihenstephan,
19. Ian W, S.: The biofilm matrix – an immobilized but dynamic microbial environment, Trends in Microbiology 9 (2001), no. 5, pp. 222-227.
20. Kelch, K.: Veränderungen im deutschen Brauereiwesen, BRAUWELT **149** (2010), no. 4, pp. 83-84.
21. Kelch, K. and Hohmann, C.: Die Entwicklung des Biermarktes von 1965 bis heute, BRAUWELT **151** (2012), no. 6, pp. 170-172.
22. Kelch, K. and Hohmann, C.: Leichter Abwärtstrend bei Biermischgetränken, BRAUWELT **155** (2016), no. 21/22, pp. 593-594.
23. Kelch, K. and Hohmann, C.: Positiver Weißbiermarkt 2015, BRAUWELT **155** (2016), no. 23, pp. 635-636.
24. Kolari, M.; Schmidt, U.; Kuismanen, E. and Salkinoja-Salonen, M. S.: Firm but slippery attachment of Deinococcus geothermalis, Journal of bacteriology **184** (2002), no. 9, pp. 2473-2480.
25. Meier, H.: The Barth report – Hops 2014/2015. Nürnberg, 1-32, Joh. Barth & Sohn GmbH & Co. KG, (2015).
26. Müller-Auffermann, K.; Hutzler, M.; Riedl, R.; Osnabrügge, F.; Caro, M. and Jacob, F.: Alternative, Biological Methods in the Flow Profile: Characterization of Bioreactors, BrewingScience – Monatsschrift für Brauwissenschaft **66** (2013), no. 9/10, pp. 123-134.
27. Narziss, L.; Back, W.; Gastl, M. and Zarnkow, M. (2017). Abriss der Bierbrauerei, Wiley. ISBN 9783527696727.
28. Noel, J.: Goose Island identifies bacteria that caused tainted beer, but not source, Chicago Tribune May 26th 2016 (2016),
29. Paulo, E. M.; Vasconcelos, M. P.; Oliveira, I. S.; Affe, H. M. D.; Nascimento, R.; de Melo, I. S.; Roque, M. R. D. and de Assis, S. A.: An alternative method for screening lactic acid bacteria for the production of exopolysaccharides with rapid confirmation, Ciencia E Tecnologia De Alimentos **32** (2012), no. 4, pp. 710-714.
30. Peeters, E.; Nelis, H. J. and Coenye, T.: Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates, Journal of Microbiological Methods **72** (2008), no. 2, pp. 157-165.
31. Qian, F.: Einfluss der Bierzusammensetzung auf die mikrobiologische Stabilität von Reisbier, 42. Technologisches Seminar Weihenstephan, 2009, Freising, Weihenstephan,
32. Sakamoto, K. and Konings, W. N.: Beer spoilage bacteria and hop resistance, International journal of food microbiology **89** (2003), no. 2-3, pp. 105-124.
33. Schweizerische Akkreditierungsstelle SAS, (2013). SAS, Dokument Nr. 328.dw: Leitfaden zur Validierung mikrobiologischer Prüfverfahren und zur Abschätzung der Messunsicherheit im Bereich Lebensmittel- und Umweltmikrobiologie. 04.2013
34. Timke, M.; Wang-Lieu, N. Q.; Altendorf, K. and Lipski, A.: Community structure and diversity of biofilms from a beer bottling plant as revealed using 16S rRNA gene clone libraries, Applied and environmental microbiology **71** (2005), no. 10, pp. 6446-52.
35. Timke, M.; Wang-Lieu, N. Q.; Altendorf, K. and Lipski, A.: Fatty acid analysis and spoilage potential of biofilms from two breweries, Journal of Applied Microbiology **99** (2005), no. 5, pp. 1108-1122.
36. Timke, M.; Wang-Lieu, N. Q.; Altendorf, K. and Lipski, A.: Identity, beer spoiling and biofilm forming potential of yeasts from beer bottling plant associated biofilms, Antonie van Leeuwenhoek **93** (2008), no. 1-2, pp. 151-61.

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