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Lactobacillus sp. brewery isolate: A new threat to the brewing industry?

Only a restricted group of bacterial species is known to be capable to spoil beer. To maintain a good microbiological quality control it is important to know which microbes are hazardous for the brewing industry.
Three *Lactobacillus (L.)* isolates that could not be identified by a commercial realtime PCR system for the detection and identification of beer-spoilage bacteria (Foodproof® Beer Screening Kit, Biotecon Diagnostics, Germany) were obtained from brewery samples. A multivariate study was conducted on the basis of phenotypic, genotypic and beer-related characteristics. The tests were carried out with regard to the two most probable species, *L. brevis* and *L. parabrevis*.
The comparison of the 16S rRNA gene and the *pheS* housekeeping gene sequences revealed that the three isolates belong to one species or one operational taxonomic unit (OTU). Furthermore, the unknown brewery isolate could be differentiated from the two most probable species by gene sequence comparisons and DNA-DNA hybridizations. The evaluation of physiological characteristics of the unknown brewery isolate did not demarcate it clearly. The Lactobacillus isolate contained two hop resistance genes and was able to grow in four different beer types resulting in significant compound concentration changes.
It was determined that the unknown bacterium did not belong to the species *L. brevis* or *L. parabrevis.* In parallel, a species description is submitted to the International Journal of Systematic and Evolutionary Microbiology. Therein, further discrimination of the isolated bacterium from genetically related species (*L. yonginensis, L. koreensis, L. hammesii)* with no relation to the brewing sector is shown.

Descriptors: lactic acid bacteria, beer spoilage, brewery isolate, *Lactobacillus brevis*, *Lactobacillus parabrevis*, multivariate study

1 Introduction

Beer is known to be microbiologically stable. Only a few bacteria and yeasts are able to tolerate the hop and alcohol content, the anaerobic atmosphere and the altered nutrient composition compared to wort. The largest group of beer spoilage microorganisms belongs to the genus *Lactobacillus (L.)* with the most frequent spoilage species being *L. brevis.* In some years, *L. brevis* causes more than 40 % of all bacterial spoilage incidents in breweries [5, 3, 4, 26, 32].

Typical consequences of microorganism growth in beer are sediment formation, increased turbidity and acidity, off-flavors and, in the worst case, slime formation. Brewing microbiologists are still trying to develop new strategies to detect contaminations faster and to identify the spoiling bacterium down to strain level, if necessary. The characteristics a bacterium requires to be able to spoil beer are not yet fully understood. Every now and then new species show up in the brewing environment, which, after adapting to the adverse properties of beer, enlarge the group of beer-spoilage organisms. Recently included species in this restricted collection of bacteria are *L. backii, L. rossii, L. paucivorans* and *L. acetotolerans* [8, 11, 16, 17, 36, 56].

In 2013 and 2014, three bacterial isolates were obtained from turbid beer samples of a German brewery that could not be identified by the commercially available realtime PCR foodproof® beer screening kit of Biotecon Diagnostics (Potsdam, Germany). Subsequent 16S rRNA gene sequencing and BLAST (Basic Local Alignment Tool) analysis revealed the species with the highest values of sequence homology to be *L. koreensis* (99.45 %), *L. yonginensis* (98.82 %), *L. parabrevis* (98.71 %), *L. brevis* (98.69 %) and *L. hammesii* (98.24 %).

Except for *L. brevis*, none of these species was known to have beer-spoilage potential. Due to the fact that *L. parabrevis* was originally classified as *L. brevis* and the latter is the most dangerous and most common beer-spoilage bacterium [42, 57], it was assumed that the unknown Lactobacillus isolates would belong either to the species *L. brevis* or to *L. parabrevis*. Additional reasons for the inclusion of *L. parabrevis* in further analyses were that the type strain derived from wheat and its malt is a raw material used for wheat beer production in German breweries. Furthermore, the *L. parabrevis* type strain was isolated in Europe. In 2011, a contamination incident with *L. parabrevis* was reported to the Research Center Weihenstephan

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for Brewing and Food Quality, TU München, which was identified by another brewing microbiology laboratory. Unfortunately, the isolated beer-spoilage strain was not stored and the identification could not be reconfirmed by the Research Center Weihenstephan. In summary, these facts contributed to the decision that *L. brevis* and *L. parabrevis* were further analyzed for their beer-spoilage ability. To verify the brewery isolates' identity and to demarcate it from two different strains of *L. brevis* as well as the *L. parabrevis* type strain, physiological and molecular biological methods were executed in a multivariate study. By beer incubation test it was determined in which beer type the newly obtained brewery isolate was able to grow using a standard method of the Research Center. This beer incubation test was performed under standardized conditions minimizing the inoculation time when oxygen and carbon-dioxide concentrations of the bottles are manipulated and minimizing the oxygen uptake by manual stimulation to foam over. This method is state of the art to test if a product is susceptible for a certain microbe that is inoculated in a defined cell concentration. In case of growth, the impact of the bacterial metabolism on the respective beer type was evaluated by physico-chemical analyses. Additionally, the presence of genes was checked that were discussed to be related to hop resistance.

2 Material and methods

2.1 Strains

The three *Lactobacillus* brewery isolates were obtained from a lager tank sample, a beer sample just before filling and a bright beer tank sample of the same brewery at three different points in time within a period of eight months. Since the isolates showed 100 % similarity in 16S rRNA gene as well as in pheS (phenylalanyl-tRNA synthase alpha subunit) housekeeping gene sequence (Fig. 1 and 2), all further analyses were performed using only one of the isolates (L. sp. 2301; Table 1) which derived from the bright beer tank sample.

The *L. parabrevis* and *L. brevis* type strains as well as one highly beer-spoiling *L. brevis* brewery isolate were included in the analyses.

Table 1 List of species used in this study

Designation	Reference number	Characteristics
L. sp. 1872	TUM BP 130919043-2789[a]	Brewery isolate, lager tank sample
L. sp. 1921	TUM BP 131010000-2400[a]	Brewery isolate, sample before filling
L. sp. 2301	TUM BP 140423000-2250[a]	Brewery isolate, bright beer tank sample
L. parabrevis	LMG 11984^{T} [b]	Type strain
L. brevis	DSM 20054^{T} [c]	Type strain
L. brevis 31	TUM BP 111115005-2022[a]	Brewery isolate, finished Pilsener beer

[a] = Culture collection of the Research Center Weihenstephan, Freising, Germany; [b] = purchased from the Belgian Coordinated Collections of Microorganisms (BCCM™), Ghent, Belgium; [c] = purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany

2.2 Microorganism cultivation and DNA isolation

The brewery isolates and the strains of the culture collections were fractionated streaked on MRS agar and incubated anaerobically [13]. Ten colonies of each strain were picked and pooled to form the initial cultures. The pooled pure cultures were re-suspended in MRS broth and stored in glycerol at –80°C in a cryobank. Active strains were cultivated in MRS broth and weekly transferred into fresh medium. Strain identity was monthly checked using a GTG_5 PCR capillary electrophoresis typing with subsequent Bionumerics fingerprint analysis. The strain patterns remained stable over the period of the study (data not shown).

Bacterial DNA was extracted using the InstaGene™ matrix according to the manufacturer's instructions for bacteria (Bio-rad Laboratories, Munich, Germany). The DNA content was determined by spectrometric analysis using the NanoDrop ND 1000 (Thermo Scientific Fisher, Wilmington, USA) and adjusted with Ampuwa® (Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany) to the initial concentration of 100 ng/µL.

2.3 Molecular biological methods

The complete 16S rRNA gene sequence was determined using the primer pairs 27f / 1492r and 933f / 1541r (Table 2). The PCR procedure consisted of an initial denaturation step at 95 °C for 5 min 35 cycles of 95 °C/25 sec, 55 °C/40 sec, 72 °C/2 min and one final extension step at 72 °C for 5 min. The PCR reaction mixtures (50 µL in total) contained 25 µL 2-fold RedTaq mastermix containing

Table 2 Primers used in this study

Primer	Sequence (5' → 3')	Source
27f	AGA GTT TGA TCM TGG CTC AG	[44]
933f	GCA CAA GCG GTG GAG CAT GTG G	[31]
1492r	TAC GGY TAC CTT GTT ACG ACT T	[35]
1541r	AAG GAG GTG ATC CAG CCG CA	[33]
pheS-forward	CAS GAT ACS TTC TAC ATY AC	[15]
pheS-reverse	ACC ATA CCR GCA CCY ACT TC	[15]
rpoA-21-F	ATG ATY GAR TTT GAA AAC C	[34]
rpoA-23-R	ACH GTR TTR ATD CCD GCR CG	[34]
horA-F	GGT CAA GGA ACT GTT GGC CA	This study
horA-R	TAA GAC CAA TGC GCC AAC CA	This study
horA-P[a]	TTC GGT TCC CAA AAC CGC AAC TTC G	This study
horC-F	TGA ATG CTC AAA TAT CGC AAT TG	This study
horC-R	CAC TTT GTT GCT GTG CGC TAA	This study
horC-P[a]	TAT CCC AAG CAC TTC CTA AGA TTG CAA ATG C	This study
28F (*hitA*)	AGC GTA GCA GAA GAA CCT AAG	[20]
207R (*hitA*)	CAA TTA CCA GGA TCC ATG TAC C	[20]

[a] modified for realtime PCR application: 5' end reporter: 6-FAM, 3' end quencher: BHQ-1; P = probe

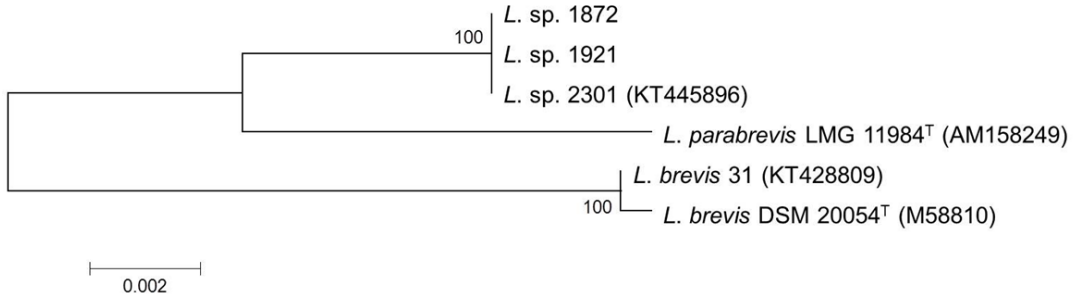

Fig. 1 **Neighbor-joining tree based on the 16S rRNA gene sequences of the three *Lactobacillus* sp. brewery isolates 1872, 1921 and 2301 (*L.* sp. 2301 GenBank accession no. KT445896), of the *L. parabrevis* type strain (AM158249), the *L. brevis* type strain (M58810) and the *L. brevis* brewery isolate 31 (KT428809). Bootstrap percentages > 50 % after 1000 simulations are shown [18]. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were executed using MEGA6 [53, 54]**

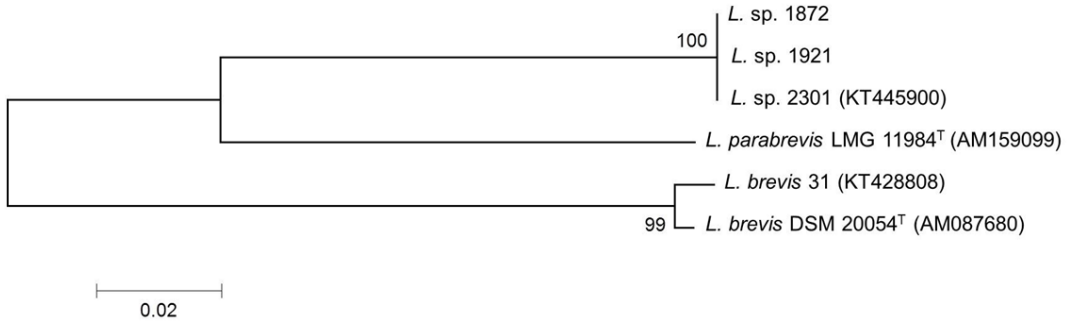

Fig. 2 **Neighbor-joining tree reconstructed from a comparative analysis of pheS gene sequences including the three *Lactobacillus* sp. brewery isolates 1872, 1921 and 2301 (L. sp. 2301 GenBank accession no. KT445900), *L. parabrevis* LMG 11984^T (AM159099), *L. brevis* DSM 20054 (AM087680) and the *L. brevis* beer-spoilage isolate 31. Bootstrap values (expressed as percentages of 1000 replicates) > 50 % are shown [18]. Distances were computed using the Maximum Composite Likelihood method [53]. Bar, 0.02 substitutions per nucleotide position**

Taq polymerase, $MgCl_2$ and dNTP's (Genaxxon Bioscience GmbH, Ulm, Germany), 20 pmol of each primer, 5 µL DNA extract (100 ng/µL) and water (Ampuwa®, Fresenius Kabi Deutschland GmbH, Bad Homburg, Germany).

The sequence of the housekeeping gene *pheS* (product: phenylalanyl-tRNA synthase alpha subunit) was obtained by PCR using the primer pair *pheS*-forward / *pheS*-reverse (Table 2) as postulated by *Ehrmann* et al. [16]. The PCR mix for *pheS* gene amplification was composed of 25 µL 2-fold RedTaq mastermix, 25 pmol of each primer, 2.5 µL DNA extract and PCR-clean water adding up to a total volume of 50 µL. The temperature protocol for *pheS* gene PCR adopted from *Naser* et al. [34] was modified by changing the annealing temperature to 54 °C [16].

The PCR mix for the amplification of the *rpoA* housekeeping gene (product: RNA polymerase alpha subunit) contained in a total volume of 100 µL per reaction: 50 µL 2-fold RedTaq mastermix, 25 pmol of each primer (Tab. 2), 5 µL DNA template and sterile water. The PCR protocol was adopted from Naser et al. [34].

The size of the amplified fragments was checked by capillary electrophoresis using the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). If electrophoresis displayed a single band of the expected size (for *pheS*: 300–350 bp; for *rpoA*: 700–800 bp), the PCR product was purified using the QIAquick® PCR purification kit (QIAGEN GmbH, Hilden, Germany) according to manufacturer's instructions. The sequencing was performed by GATC Biotech AG (Constance, Germany). The obtained 16S rRNA sequences were trimmed and combined using the EditSeq software (DNASTAR, Madison, USA) followed by the comparison with stored type strain sequences using the neighbor-joining method of the MEGA6 (Molecular Evolutionary Genetics Analysis) software [18, 53, 54].

DNA-DNA hybridization is a method to differentiate two microbial strains sharing more than 97 % 16S rRNA gene homology [43, 55]. According to the recommendation of *Wayne* et al., 70 % DNA-DNA homology represent the threshold value for the definition of a new species [59]. DNA-DNA hybridizations were carried out at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) considering the pairings: *L.* sp. 2301 x *L. parabrevis* LMG 11984^T and *L.* sp. 2301 x *L. brevis* DSM 20054^T [10, 12, 25].

2.4 Physiological characterization

Sugar fermentation patterns were analyzed using the API CHL 50 system with *Lactobacillus paracasei* (BAA-52TM*) as the quality control organism (Biomerieux, Nürtingen, Germany).

Additional physiological characteristics were analyzed in MRS broth inoculated with 100 µL of overnight bacterial suspension in triplicates [13]. Alcohol tolerance (2.5–8.0 % (v/v) undenatured ethanol), salt tolerance (4.0, 6.5 and 8.0 % (w/w) NaCl), acid tolerance (pH values 4.0, 5.0, 7.2 and 7.8, adjusted with lactic acid or sodium hydroxide, respectively) and temperature tolerance (10, 15, 40 and 45 °C) were evaluated by visual control of turbidity and sediment formation in the test tubes over a period of four weeks. Incubation was carried out anaerobically using the Anaerocult®

Table 3 Beer types and their properties for the beer incubation test

Beer type	Alcohol content [% (v/v)]	Bitter units [EBC units]	pH value	CO_2 content [% (v/v)]	Fermentable sugars[a] [g/100 mL]
Lager beer	5.10	19.9	4.46	0.53	0.28
Alcohol-free lager beer	0.38	19.2	4.41	0.57	0.27
Wheat beer	5.64	13.0	4.61	0.68	0.17
Alcohol-free wheat beer	0.47	12.6	4.51	0.63	0.16
Filtered wheat beer „Kristallweizen“	5.53	13.1	4.59	0.64	0.21

[a] sum of glucose, fructose, sucrose, maltose and maltotriose

system (Merck KGaA, Darmstadt, Germany) at 28 ± 1°C, if not indicated otherwise (i.e. temperature tolerance).

2.5 Genes associated with hop tolerance

The ability to tolerate the antibacterial properties of hop compounds is a main criterion for bacteria to grow in and spoil beer. Many studies have focused on the identification and application of different genes associated with hop resistance [6, 7, 19–24, 27–29, 38–41, 45–52, 58]. The plasmid-localized genes *horA* and *horC* that encode for multidrug transporters inserted into the cytoplasma membrane of gram-positive bacteria proved to be particularly good indicators for the ability to tolerate hop acids. The presence of *horA* and *horC* was analyzed by realtime PCR at the Research Center Weihenstephan for Brewing and Food Quality. The method was created to be compatible with the bacteria and yeast identification systems developed by *Brandl* and was modified using the *horA*- and *horC*-specific primers and probes listed in table 2 [9].

It was also proposed that the gene *hitA* confers hop tolerance to bacteria [24, 46]. Its product is homologous to divalent-cation transporters that can be found in many organisms. The endpoint PCR method for the detection of *hitA* was adopted from Haakensen and modified [20]. PCR mixes consisted of 12.5 µL 2-fold RedTaq mastermix, 5 pmol of the primers 28F and 207R (Table 2), 1 µL bacterial DNA and sterile water up to a final volume of 25 µL per reaction. The PCR procedure was composed of one cycle at 95 °C for 5 min, 35 cycles of 94 °C/45 sec, 52 °C/45 sec, 72 °C/50 sec and one final extension step at 72 °C for 5 min. Amplicons of the expected size (approx. 179 bp) were detected by capillary gel electrophoresis as described above.

2.6 Beer incubation test

To investigate the extent of the strain *L.* sp. 2301's beer-spoilage potential, five different beer types were inoculated with microorganisms, incubated at 28 ± 1 °C and visually evaluated for a six-week period with regard to turbidity or sediment formation. All strains were adapted to the different beers in flasks containing 75 % target beer and 25 % 2-fold MRS broth [13]. After determining the cell count using a Thoma counting chamber, each microorganism suspension was inoculated in three beer bottles resulting in a final concentration of $1x10^5$ cells per bottle (or 200 cells per mL). Table 3 shows the beer types with their physico-chemical properties that were chosen for the incubation test. Chemical analyses were carried out according to MEBAK (Central European Brewing Committee for Analysis) instructions [1, 14, 30].

The inoculated bacteria were checked for strain identity before and after incubation by 16S rRNA gene sequencing. After incubation, all bottles showing haze, turbidity or sediments were checked for the present bacterial cell count using the Thoma counting chamber and compared with the originally inoculated cell concentration. From all samples showing no signs of microorganism growth, 1 mL beer was poured into a petri dish, mixed with MRS agar and incubated anaerobically for 7 days at 28 ± 1°C.

2.7 Physico-chemical analyses

Physico-chemical analyses were executed at the Research Center Weihenstephan for Brewing and Food Quality in advance and after the six-week storage period at 28 ± 1°C with all beer samples of the incubation test that were positive for microorganism growth. In parallel, un-inoculated samples that were also opened, stimulated to foam over and exposed to the same storage conditions were additionally analyzed providing reference values with regard to amino acid, fermentation by-product, organic acid, vicinal diketone, fatty acid, and fermentable sugar content [1, 14, 30].

3 Results

3.1 Phylogeny

16S rRNA gene sequence comparison

On the basis of a multiple alignment similarity matrix, a phylogenetic tree (Fig. 1) was constructed using the neighbor-joining method in the MEGA6 software [37]. The statistical reliability of the tree was tested using 1000 bootstrap replicates [18]. NCBI (National Center for Biotechnology Information) accession numbers are shown in brackets.

pheS housekeeping gene sequence comparison

Both, the *pheS* and the 16S rRNA gene sequence comparison, revealed that the three isolates belong to one strain and are closer related to the species *L. parabrevis* than to the *L. brevis* branch. Further analyses were performed with only one of the isolates, *L.* sp. 2301.

rpoA housekeeping gene sequence comparison

The comparison between the *rpoA* housekeeping gene sequences shows the distance between the unknown isolate *L.* sp. 2301 and the two other branches (Fig. 3; accession numbers in brackets).

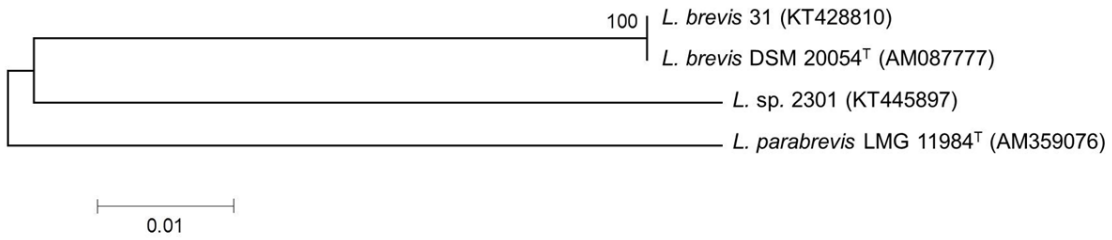

Fig. 3 Reconstruction of a phylogenetic tree based on the neighbor-joining method of MEGA6 software [37, 53, 54]. The obtained *rpoA* housekeeping gene sequences of *L. brevis* 31 and *L.* sp. 2301 were compared to the deposited type strain sequences. A bootstrap analysis with 1000 replicates was executed [18]. Bar, 0.01 substitutions per nucleotide position

No close relationship between the subgroups is recognizable.

DNA-DNA hybridization

DNA-DNA similarity values obtained at the DSMZ are shown in table 4. The values in brackets indicate the results of the measurement repetition.

Table 4 DNA-DNA similarity values [%]

	L. sp. 2301
L. parabrevis LMG 11984^T	50.5 (56.5)
L. brevis DSM 20054^T	55.3 (58.9)

3.2 Physiological characteristics

Carbohydrate utilization

The main results of API CHL 50 systems applied on the four tested strains are shown in table 5. All strains were positive for D-xylose, galactose, glucose, fructose, N-acetyl glucosamine and maltose.

Stress tolerance

The results of the stress tolerance tests after four weeks of anaerobic incubation at 28 ± 1°C in MRS broth are displayed in table 6 (see next page). All tested strains tolerated up to 8 % (v/v) ethanol in MRS broth and pH values between 4.0 and 7.8. At 10 and 15 °C, all strains were able to proliferate and cause a turbidity increase in the test tubes. None of the strains was able to grow at 40 °C or 45 °C in MRS broth. The differentiating stress factor seemed to be the salt concentration since the strains behaved heterogeneously if inoculated in MRS broth supplemented with different sodium chloride concentrations. *L. parabrevis* LMG 11984^T tolerated NaCl concentrations of up to 8.0 % (w/w), *L. brevis* DSM 20054^T up to 6.5 % (w/w). Both beer-spoilage isolates were more sensitive to salt. *L. brevis* TUM BP 111115005-2022 tolerated a NaCl concentration of 4.0 % (w/w) in MRS broth and the unknown isolate was not able to grow in any of the tested media supplemented with salt.

Table 5 Main results of carbohydrate fermentation test system API CHL 50

Acid production from:	*L.* sp. 2301	*L. brevis* DSM 20054^T	*L. brevis* 31	*L. parabrevis* LMG 11984^T
L-Arabinose	w	+	+	+
Ribose	w	+	+	+
Methyl β-xyloside	–	–	–	w
Mannitol	w	–	–	w
Methyl α-D-glucoside	w	+	+	+
Lactose	–	w	–	–
Melibiose	w	+	w	–
Sucrose	–	–	+	w
D-Turanose	–	–	–	w
D-Arabitol	–	–	–	+
Gluconate	+	w	w	+
5-Ketogluconate	–	+	–	–

+ = positive after 48 hours of incubation; w = weakly positive after 48 h and positive after 120 h; – = negative after 120 hours of incubation

3.3 Presence of genes associated with hop resistance

The realtime PCR for the presence of the hop resistance genes *horA* and *horC* as well as the endpoint PCR for the detection of *hitA* resulted in the scheme in table 7 (see next page). Both type strains did not contain any of the analyzed genes associated with hop resistance, in contrast to the isolates obtained from beer environment.

3.4 Results of beer incubation test

The isolate *L.* sp. 2301 could proliferate in four out of five tested beer samples up to a cell concentration of at least 2 x 10^7 cells per bottle or 40,000 cells per milliliter. The lager beer with a higher alcohol content of 5.1 % (v/v), a higher amount of hop bitter units (19.9 BU) and a pH value of 4.46 did not show any signs of microorganism growth.

3.5 Physico-chemical analyses of spoiled beer samples

The values of the physico-chemical analyses carried out with the four beer types that were positive for microbial growth were compared to those of the respective un-inoculated but equally treated beer samples which served as reference. The percentage deviations from the reference values were calculated for every spoiled beer type and every analyzed compound. If a compound concentration was increased or decreased by microorganism growth by more than 50 % in all analyzed samples (n = 12), it was regarded as significant. All significant changes obtained by physico-chemical analyses are shown in table 8.

4 Discussion

The question if the newly isolated strain *Lactobacillus* sp. 2301 belongs to any known beer-spoiling species has been the main

Table 6 Results of stress tolerance tests

Strain	Alcohol [vol. %]					NaCl [% (w/w)]			pH value				Temperature [°C]			
	2.5	3.5	5.0	6.5	8.0	4.0	6.5	8.0	4.0	5.0	7.2	7.8	10	15	40	45
L. sp. 2301	+	+	+	+	+	–	–	–	+	+	+	+	+	+	–	–
L. parabrevis LMG 11984^T	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
L. brevis DSM 20054^T	+	+	+	+	+	+	+	–	+	+	+	+	+	+	–	–
L. brevis 31	+	+	+	+	+	+	–	–	+	+	+	+	+	+	–	–

+ = growth (turbidity/sediments) visible after 4 weeks of incubation; – = no growth (turbidity/sediments) visible after 4 weeks of incubation; experiments were carried out in triplicate, anaerobically, in 10 ml MRS broth supplemented with the particular stress factor at 28 ± 1°C or at different temperatures, respectively

objective of this study. Regarding the postulation that two bacteria belong to the same species if the 16S rRNA gene homology displays values ≥ 97 %, *L.* sp. 2301 belongs to either the species *L. parabrevis* or *L. brevis*. In contrast, the sequences of the two analyzed housekeeping genes *pheS* and *rpoA* suggest that the unknown beer-spoiler does not belong to one of these species. Also, the DNA-DNA similarity values were far below the threshold of 70 % which would be necessary to assign this bacterium to a certain species according to Wayne et al. [59].

The pattern of carbohydrate utilization does not provide any relevant insights into the crucial question as the newly isolated bacterium has commonalities with both species it was compared to. The stress tolerance tests indicated that *L.* sp. 2301 is not affected by alcohol concentrations up to 8 % (v/v) or by deviations in pH value (4.0–7.8) in MRS broth. But it is influenced by temperature since growth was observable at low (10 °C and 15 °C), but not at higher temperatures (40 °C or 45 °C). It is noticeable that the unknown brewery isolate seemed to be highly salt-sensitive, which is probably the result of an adaptation to the low-salt medium beer. Another factor that confirms the adaptation to the brewing environment is the presence of two genes that are discussed to confer hop resistance to bacterial cells (*horA* and *horC*).

Table 7 Results of the screening for genes *horA*, *horC* and *hitA*

Strain	*horA*	*horC*	*hitA*
L. sp. 2301	+	+	–
L. parabrevis LMG 11984^T	–	–	–
L. brevis DSM 20054^T	–	–	–
L. brevis 31	+	+	+

+ = presence of selected gene; – = absence of selected gene; analyses carried out by realtime PCR (for *horA* and *horC*) or endpoint PCR and subsequent gelelectrophoresis (for *hitA*)

Table 8 Significant concentration changes (≥ ± 50 %) in beer induced by contamination with *L.* sp. 2301 compared to the un-inoculated beer samples

Compound	Group	*L.* sp. 2301
		Increase/Decrease [%]
Alanine	Amino acids	– 90.6 ± 2.73
Acetaldehyde	Fermentation by-product	– 62.8 ± 41.08
Pyruvate	Organic acid	– 97.8 ± 11.30
Acetic acid	Organic acid	90.1 ± 33.60
Lactic acid	Organic acid	133.5 ± 26.57
Citric acid	Organic acid	– 97.7 ± 1.07

Growth of *L.* sp. 2301 was detected in four of the five tested beer types. Only the lager beer with normal alcohol (5.1 % (v/v)) and hop bitter acid concentrations (19.9 BU) was negative for microorganism growth after six weeks of incubation at 28 ± 1 °C. The remaining beer types are classified as more sensitive to microorganism spoilage based on the reduction in alcohol and/or hop content resulting in the spoilage by the recently isolated beer-spoiler. According to the classes established by *Prof. Back*, *L.* sp. 2301 has to be classified as a potential beer-spoiler [2].

The growth of *L.* sp. 2301 resulted in a significant decrease of the amino acid alanine and of the fermentation by-product acetaldehyde. The two organic acids pyruvate and citric acid were reduced and the levels of acetic acid and lactic acid were raised. This indicates a heterofermentative metabolism of the unknown brewery isolate resulting in the production of lactic acid, acetic acid and CO_2 as main products from glucose. Diacetyl production was not observed.

In conclusion, it could be determined that the unknown brewery isolate did not belong to *L.* brevis or *L. parabrevis* which were the two most probable species from the brewing microbiology perspective. Further analyses confirmed that a new species within the genus *Lactobacillus* has to be described. In parallel, a species description is submitted to the International Journal of Systematic and Evolutionary Microbiology. Therein, further discrimination of the isolated bacterium from genetically related species (*L. yonginensis*, *L. koreensis*, *L. hammesii*) with no relation to the brewing sector is shown.

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Received 29 June 2016, accepted 2 August 2016