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Presence of *Saccharomyces cerevisiae* subsp. *diastaticus* in industry and nature and spoilage capacity of its vegetative cells and ascospores

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ABSTRACT

Saccharomyces cerevisiae sub-species *diastaticus* (*S. diastaticus*) is the main fungal cause of spoilage of carbonated fermented beverages in the brewing industry. Here, prevalence of *S. diastaticus* in nature and breweries was assessed as well as the spoilage capacity of its vegetative cells and spores. *S. diastaticus* could only be enriched from 1 out of 136 bark and soil samples from the Netherlands, being the first described natural isolate of this yeast outside South America. On the other hand, it was identified by PCR and selective enrichment in 25 and 21 out of 54 biofilm samples from beer filling halls in Asia, Africa, Europe and North America. ITS sequencing revealed that *S. cerevisiae* (including *S. diastaticus*) represented <0.05% of fungal DNA in 17 out of 20 samples, while it represented 0.1, 2 and 32% in samples VH6, VH1 and VH3 respectively. Next, vegetative cells and ascospores of the natural *S. diastaticus* isolate MB523 were inoculated in a variety of beer products containing 0.0–5.0% alcohol (*v*/v). Ascospores spoiled all beer products, while vegetative cells did not grow in Radler lemon 0.0, Radler lime mint 0.0 and Radler lemon lime 0.0. Notably, vegetative cells could spoil these Radlers when they first had been grown in alcohol free beer either or not mixed with Radler lemon lime 0.0. Conversely, vegetative cells that had been grown in Radler lemon lime lost their spoilage potential of this beer product when they had grown in YPD medium for more than 24 h. In addition, it was shown that cells grown in alcohol free beer were more heat resistant than cells grown in YPD (D_{52} 40 min and ≤ 10.3 min, respectively). Together, these data show that *S. diastaticus* is a less prevalent variant of *S. cerevisiae* in nature, while it accumulates in breweries in mixed biofilms. Data also show that both vegetative cells and spores can spoil all tested beer products, the latter cell type irrespective of its environmental history.

1. Introduction

Several yeast genera can spoil beer but *Saccharomyces cerevisiae* subsp. *diastaticus* (in short *S. diastaticus*) is the most dangerous spoilage yeast in breweries (Hutzler et al., 2012). In contrast to *S. cerevisiae, S. diastaticus* contains the extracellular amylase genes *STA1–3*, enabling it to degrade starch and dextrins (Andrews and Gilliland, 1952; Lambrechts et al., 1991; Tamaki, 1978; Yamashita et al., 1985a, 1985b; Yamashita and Fukui, 1983). As a result, the body of the beer is impacted. In addition, *S. diastaticus* produces phenolic off-flavor and causes haze formation and super-attenuation. The latter leads to increased alcohol content and over-carbonation of beer (Andrews and Gilliland, 1952; Priest and Campbell, 2003). It is assumed that costs associated with spoilage incidences due to *S. diastaticus* contamination range from millions to billions of euros annually in Europe alone (Hutzler et al., 2012; Stratford, 2006). The reported incidence of *S. diastaticus* spoilage in the brewing industry has increased from 1 to 19 cases each year between 2008 and 2016 (Meier-Dörnberg et al., 2017). *S. diastaticus* contamination had occurred during filling of the beer in about two-third of these cases, most likely originating from biofilms in the brewery (Timke et al., 2008). Cells in biofilms are known to have increased stress resistance (Quain and Storgards, 2009) and are not easily removed; some locations were recolonized in 2–12 h (Storgards et al., 2006).

S. cerevisiae is easily isolated from vineyards, wineries, breweries, bakeries and distilleries (Greig and Leu, 2009; Liti et al., 2009). In nature, it has been isolated from oak, other plants, soil, insects, insect nests

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and fruit, but also from humans as a commensal or even as a pathogen (Almeida et al., 2015; Angebault et al., 2013; Goddard et al., 2010; Goddard and Greig, 2015; Knight and Goddard, 2015; Sampaio and Gonçalves, 2008; Wang et al., 2012). Fermented fruit is currently the only natural habitat from which *S. cerevisiae* can be isolated without the need of enrichment (Goddard and Greig, 2015). Using a selective medium with raffinose and ethanol Sampaio and Gonçalves isolated 53 *Saccharomyces* isolates from 191 bark samples from 4 geographical origins, including 15 *S. cerevisiae* strains. It is not yet known whether these strains actively grow on bark or that they are present in a dormant vegetative stage or as ascospores (Greig and Leu, 2009; Knight and Goddard, 2015).

Recently, 54 out of 1169 *S. cerevisiae* strains were identified as *S. diastaticus* based on the presence of a 79 bp *STA1* specific sequence. These 54 strains are clustered in the "Beer 2 / Mosaic" and "French Guiana human" *S. cerevisiae* clades (Gallone et al., 2016; Krogerus et al., 2019; Peter et al., 2018). Of these 54 strains, only 5 strains originated from nature; 2 from Ecuador from water on a leaf and an insect, and 3 from French Guiana, from *Agouti paca* and fruit.

Selective enrichment or PCR based methods used for identification of *S. cerevisiae* or *S. diastaticus* does not reveal whether vegetative cells and/or ascospores are present in samples from nature or man-made environments. Ascospores represent the most resilient phase of *S. cerevisiae* (Coluccio et al., 2008; Milani et al., 2015; Reuter et al., 2007). For instance, they are more resistant to mild alkali and acid conditions and to digestive enzymes when compared to vegetative cells (Coluccio et al., 2008; Knight and Goddard, 2015; Reuter et al., 2007). Yet, stationary phase vegetative cells are similarly resistant to freeze-thaw and desiccation. In addition, stationary phase and quiescent cells have a similar heat resistance as compared to ascospores up to 42 °C but the latter cells can be more resistant at higher temperatures (Coluccio et al., 2008).

Here, the prevalence of *S. diastaticus* was studied in nature in the Netherlands and in the brewing industry at a global level. This resulted in the isolation of one isolate from bark and 21 strains from 6 breweries located in Europe, Asia, North America and Africa. Additionally, it was shown that ascospores of *S. diastaticus* strain MB523 spoil all tested beer products, while pre-culturing conditions determine whether vegetative cells spoil lemon or lime based beer products.

2. Material and methods

2.1. Isolation of strains from nature and the industry

Soil (~1 g) and bark (~3 cm^2) samples were used to inoculate 30 ml enrichment medium (Sampaio and Gonçalves, 2008) contained in 30 ml sterile infusion bottles. The selective medium consisted of 6.8 g/l yeast nitrogen base without amino acids (Difco, www.difco.nl) supplemented with 130 mg/l L-methionine, 130 mg/l L-tryptophan, 65 mg/l L-histidine, 11.79 g/l raffinose-5-H_2O, and 8% (*v*/v) ethanol. The flasks were tightly capped and incubated at 30 °C.

Swabs were taken from biofilms in filling halls of breweries (Table 1), collected in sterile tubes and shipped on ice to the Netherlands. Biofilm samples were still cold upon arrival. Samples were resuspended in 100–300 μl 0.9% NaCl, depending on the size of the sample, by vigorously vortexing, of which 10–50 μl was used for PALL PCR GeneDisc analysis using the Yeast ID disc (Pall Corporation, www.pall.com). The PALL PCR GeneDisc is a multiplex Real Time Quantitative PCR platform. The Yeast ID disc identifies major spoilage yeast species such as *Saccharomyces* spp. (including *S. cerevisiae*, *S. pastorianus*, *S. cerevisiae* var. *diastaticus*), *Brettanomyces / Dekkera* spp. (including *B. /D. bruxellensis*), *Candida* spp. / *Pichia* spp., *Zygosaccharomyces* spp. (including *Z. bailii*), *Saccharomycodes* spp. and *Schizosaccharomyces* spp. As the primer sets and targets are proprietary data, it is not known which sequences are used to distinguish between the species. The remaining sample was used to inoculate selective medium as described above. Cultures inoculated with soil, bark, or biofilm material were checked for turbidity during a 6 week period. Turbid samples were checked microscopically. Samples containing yeast were streaked on 50 g/l glucose - peptone - yeast extract (YPD; Sigma, www.sigmaaldrich.com) supplemented with 20 g/l agar and 50 μg/ml ampicillin. Colonies were analyzed by PALL PCR. *S. diastaticus* isolates were purified, enriched and stocked in 20% glycerol at −80 °C. YPD slopes with 20 g/l agar were inoculated with 30 μl of frozen stock cultures. Cultures were grown for 72 h at 30 °C and stored at 4 °C for a maximum of 2 months to use as inoculum.

A set of 20 samples from the brewery survey was used for metabarcoding analysis at BaseClear (www.baseclear.com) using Next Generation Sequencing of the internal transcribed spacer region (ITS) (Garnier et al., 2017; Schoch et al., 2012). The ITS target region of the samples was amplified using Phusion HF PCR mastermix (www.thermofisher.com) and the primer pair TCGTCGGCAGCGTCAGATGTGTATAAGA GACAGGCATCGATGAAGAACGCAGC/GTCTCGTGGGCTCGGAGATGTGTATAAGAGAC AGTCCTCCGCTTATTGATATGC. PCR amplification was performed according to the following program: 98 °C 30 s, 25 cycles of 98 °C 10 s, 55 °C 30 s, 72 °C 30 s, followed by 72 °C for 5 min and hold at 4 °C. Barcodes and adapter sequences were added to the amplicons, after which they were purified, normalised, pooled and used for sequencing on the MiSeq system (PE300 run) (www.illumina.com). The ITS primer sequence adapters were trimmed from the Illumina MiSeq reads using cutadapt version 1.13 (Martin, 2011). Usearch version 9.2 was used to create pseudoreads using the fastq_mergepairs command, followed by dereplication of pseudoreads with the usearch fastx_uniques command (Edgar, 2010). Chimera filtering of the pseudoreads was performed using usearch uchime2_denovo (Edgar, 2016). Raw data are available in the SRA database under bioproject accession number PRJNA715177. Classification of fungi was based on the UNITE ITS gene database version 20.11.2016 (Abarenkov et al., 2010). Usearch commands fastx_uniques, cluster_otus, usearch_global and otutab_norm, were used to create an OTU-table. The commands alpha_div, beta_div were used to calculate alpha and betadiversity (Edgar, 2010). Abundance of a fungal species was expressed as relative abundance of its ITS sequence.

2.2. Isolation of vegetative cells and asci with ascospore of S. diastaticus

S. diastaticus strain MB523 was grown for 20 h at 20 °C and 200 rpm in 50 g/l glucose - peptone - yeast extract (YPD broth; Sigma, www.sigmaaldrich.com). Cultures were inoculated with cells from colonies grown on the same medium with 20 g/l agar. Cells were harvested by centrifugation at 2000*g* for 10 min, washed twice with Milli-Q water, resuspended in physiological saline (PS; 9 g/l NaCl), and transferred ($5{\cdot}10^7$ cells per ml) to sporulation medium consisting of 10 g/l potassium acetate, 1 g/l yeast extract, and 0.5 g/l glucose (SPO1; Merck, www.merckmillipore.com). Vegetative cells and asci were harvested after 120 h of growth at 20 °C and 200 rpm as described above and stored in PS at 4 °C. Cells and asci were sorted twice by using a jet-in-air-based BD Influx flow cytometer (BD Biosciences, www.bdbiosciences.com) at a rate of 8000 particles per *sec*. Cells and asci were discriminated based on scatter and autofluorescence using a 488 nm laser at 100 mW. Forward scatter (FSC) and side scatter (SSC) were detected through a 488/5 bandpass filter and auto-fluorescence was measured with a 695/40 bandpass. Sort gates were set on scatter and fluorescence. Sorted fractions were sorted a second time to improve purity.

2.3. Staining of vegetative cells and ascospores

Cells and asci were dried on object glasses, heat fixed, and stained with 50 g/l malachite green (Sigma) for 3 min. This was followed by rinsing with demi water, destaining with 96% (v/v) ethanol for 20 s, rinsing with demi water, staining with 50 g/l safranin O (Sigma) for 30 s, rinsing with water, and drying at room temperature. Spore percentage

Table 1
Sample codes, origin of biofilms, yeast species identified by PCR, *S. diastaticus* identified by selective enrichment and relative abundance of *S. cerevisiae* in selected samples as assessed by meta-sequencing. Gray shading indicates positive identification of *S. diastaticus. S: Saccharomyces* spp., *S.c: S. cerevisiae, S.d: S. diastaticus, C/P: Candida* spp. */ Pichia* spp., and *B.b: Brettanomyces bruxellensis.* Note that the primer pair used cannot distinguish between *Candida* and *Pichia.*

Sample code	***Location***	***Yeasts identified by PCR***	***S. diastaticus identified by selective enrichment***	***Relative presence of S. cerevisiae (%) assessed by ITS metabarcoding***
Z M (ZW905)	Out crowner 12	*C/P*	YES	
Z N	Besides conveyor inlet Pasteur 12	-		
Z O	Side conveyor 12 cable underneath	*S - S.c - S.d - C/P*		
Z P (ZW906)	Closer line 11	*S - S.c - S.d - C/P - B.b*	YES	
Z Q	Outside pasteur inlet 12	*C/P*		
Z R (ZW907)	Conveyor drip bottle out 12	*S - S.c - S.d - C/P*	YES	
Z S	Conveyor after filler 12	-		
Z T (ZW912)	Conveyor 12	*S - S.c - S.d - C/P*	YES	
SL 1	Filler outlet star wheel guide	*S - S.c - S.d - C/P*	YES	
SL 2	Crowner	*S - S.c - S.d - C/P*	YES	
SL 3	Filling tubes	-		
SL 4	Star wheel inlet to crowner	*S - S.c - S.d - C/P*	YES	<0.05%
SL 5	Conveyor filler outlet	*S - S.c - S.d - C/P*	YES	<0.05%
SL 6	Filler out conveyor 2	*S - S.c - S.d - C/P*	YES	<0.05%
SL 7	Conveyer filler inlet	-		
SL 8	Star wheel guide for crowner	*S - S.c - S.d - C/P*	YES	<0.05%
M 1	Between starwheels	*S - S.c - S.d - C/P - B.b*		<0.05%
M 2	Crown head	*S - S.c - S.d - C/P*	YES	
M 3	Inside filling tube	-		
M 3.1	Outside filling tube	-		
M 4	Bottle holder	*S - S.c - S.d - C/P*		<0.05%
M 5	Filling conveyer	-		

M 6	Inside keg filling head	-		
M 7	Keg conveyor	*S - S.c - S.d - C/P*	YES	<0.05%
S 1	Filling heads (multiple)	-		
S 2	Filler area - hard to access place	*S - S.c - S.d - C/P*		<0.05%
S 3	HUB elements	*C/P*		<0.05%
S 4	Outlet conveyer from the filler	*C/P*		
S 5	HUB elements	*C/P*		
S 6	Filling heads and area (multiple)	-		
S 7	Filler exit conveyer area	-		
S 8	Exit conveyer from labeller	-		<0.05%
LR 1	Starwheel outlet	*S - S.c - C/P*	YES	<0.05%
LR 2	Capper head	-		
LR 3	Starwheel	-		
LR 4	Filler head	-		<0.05%
LR 5	Below inlet conveyor	-		
LR 6	Guide outlet filler	*S - S.c - S.d - C/P*	YES	<0.05%
VH 1	Around filling head	*S - S.c - S.d - C/P - B.b*	YES	2%
VH 2	Filling head	*S - S.c - S.d - C/P - B.b*		
VH 3	Near to CO2 distribution turret	*S - S.c - S.d - C/P*		32%
VH 4	Seamer	*S - S.c - S.d - C/P - B.b*		
VH 5	Door at seamer	*S - S.c - C/P*		
VH 6	Starwheel at crowner	*S - S.c - S.d - C/P - B.b*	YES	0.1%
VH 7	Crowner	*S - S.c - S.d - C/P - B.b*		
VH 8	Door at crowner	*S - S.c - C/P - B.b*	YES	
Z 1	Conveyor cleaned dry line filler 111	*S - S.c - S.d - C/P - B.b*		<0.05%
Z 2	Tube underneath conveyor filler 12	*S - S.c - S.d - C/P*	YES	<0.05%
Z 3	Conveyor out filler line 12	*S - S.c - S.d - C/P*	YES	<0.05%
Z 4	Under conveyor pasteur line12	-	YES	
Z 5	Outside filler 12	*S - S.c - S.d - C/P*	YES	<0.05%
Z 6	Can rinser under, line 62	-		
Z 8	Under watersensor rinser 61	-		
Z 9	Inlet cans 61 approach switch	-		

was determined after monitoring ~400 objects using light microscopy at 1000× magnification. Mature spores are bright blue (malachite green stains the spore, safranin O cannot pass the mature spore wall), immature spores are dark blue/purple (malachite green and safranin O stain the spores), while vegetative cells are red (malachite green but not safranin O is removed by washing).

2.4. Spoilage of beer products

Most asci were shown to contain 4 ascospores. Therefore, 100 cells or 25 asci were inoculated into 70 ml beer product in a 70 ml bottle. Bottles were incubated at 25 °C for a maximum of 6 months and checked for turbidity and/or pellets weekly. Samples were compared with non-inoculated bottles. Positive samples were analyzed microscopically to confirm that yeast had caused the turbidity or pellet formation.

To examine whether vegetative cells can adapt to the conditions in Radler lemon lime (Radler LL), its amount was stepwise increased in 0.0% beer (beer 0.0%), with both pure beer products serving as a control. To this end, cells from a YPD slope were resuspended in PS and 75 cells were inoculated into 70 ml Radler lemon lime 0.0 (Radler LL) and alcohol free beer (beer 0.0) in 70 ml bottles (Fig. 1). After growing for 1 week at 25 °C, 10^5 cells of the beer 0.0 culture were transferred to 70 ml Radler LL and to 70 ml beer 0.0 with 20% Radler LL. Again after 1 week, 10^5 cells of the 20% Radler LL culture were transferred to 70 ml Radler LL and to 70 ml beer 0.0 with 40% Radler LL. This procedure was repeated with stepwise increase of Radler LL to 60% and 80%. After growing in 80% Radler LL, 75, 10^3, 10^4, and 10^5 cells were added to 70 ml Radler LL. Moreover, a mix of 75 objects from sporulation medium (~50 cells and ~25 asci) were inoculated into 70 ml Radler LL (Fig. 2). After 7 weeks, 75 vegetative cells (resulting from germinated ascospores) were trasferred to 70 ml Radler LL. Furthermore, $2{\cdot}10^5$ vegetative cells from Radler LL were inoculated in 50 ml YPD in a 70 ml bottle and incubated at 25 °C for two days. After 24 and 48 h 5, 50 and 500 vegetative cells of this YPD culture were used to inoculate 70 ml Radler LL (Fig. 2). All experiments were performed in triplicate.

2.5. Heat resistance determined by pre-culturing conditions

The background of spoilage yeast in breweries may be beer related. Therefore, heat resistance of MB523 was assessed after growing in YPD as well as Heineken 0.0% alcohol (H0.0). Cells were grown for 40 h at 20 °C in 5 ml YPD and in 5 ml H0.0. Cells from YPD cultures were harvested and washed as described in 2.2. and resuspended in 1 ml PS. Cells from H0.0 cultures were concentrated by centrifugation at 2000*g* for 5 min, 4 ml of supernatant was discarded, the remaining 1 ml was the inoculum. 200 µl, containing 10^8–10^9 cells, was inoculated into 19.8 ml preheated PS and in H0.0 in 100 ml shake flasks in a shaking water bath at 52 °C and 100 rpm. 200 µl samples were taken at various timepoints and immediately chilled on ice. Viable cell concentrations before and after treatment were determined by a spot dilution assay. The cells were serially diluted up to 10^5-fold and 5 µl was plated on YPD medium supplemented with 30 g/l agar. Cultures were incubated at 25 °C for 5 days. Colony forming units (CFU) before and after treatment were compared and D_{52}-values were calculated. The D_{52}-value is the time needed at 52 °C to achieve a one $\log_{10}$ reduction, that is to kill 90% of the population.

3. Results

3.1. S. diastaticus in nature and industry

Bark and soil samples were collected from 111 locations across the Netherlands over a period of two years. *S. cerevisiae* could be enriched from 8 of these samples but *S. diastaticus* was not detected. In addition, 25 bark samples were collected from different trees in park de Gagel in Utrecht between January and April 2018. *S. cerevisiae* was enriched in 4 out of these 25 bark samples.

To assess presence of *S. diastaticus* in the filling halls of breweries, 54 biofilm samples were collected in Asia, Africa, Europe and North America (Table 1) and analyzed by PALL PCR and selective enrichment. PALL PCR on DNA extracted from the biofilms showed the presence of *S. cerevisiae* and *S. diastaticus* in 29 and 25 samples, respectively. *S. diastaticus* could be enriched in 21 out of 54 biofilms; 17 out of the 25 PALL PCR positive samples and 4 out of 29 PALL PCR negative samples. *S. diastaticus* was mainly cultured from biofilms isolated from conveyor belts, starwheels, crowners and filling heads (Table 1). PALL PCR showed the presence of *Candida* and/or *Pichia* in all samples that were also positive for *S. diastaticus* and/or *S. cerevisiae*, as well as in 5 other samples. 17% of the samples that were positive for *S. diastaticus* and/or *S. cerevisiae* was also positive for *Brettanomyces bruxellensis*. Other *Saccharomyces* spp., such as the brewing yeast *S. pastorianus*, were not detected in the 54 biofilm samples.

The relative abundance of *S. cerevisiae* was calculated based on ITS (including its subspecies; the ITS sequence does not distinguish *S. cerevisiae* and *S. diastaticus*) using a metabarcoding approach on 20 out of the 54 biofilm samples (Table 1; Fig. 3). In contrast to other breweries, *S. cerevisiae* was found in all three samples in brewery VH with an abundance of 0.1, 2 and 32% of the fungal community. *S. diastaticus* was

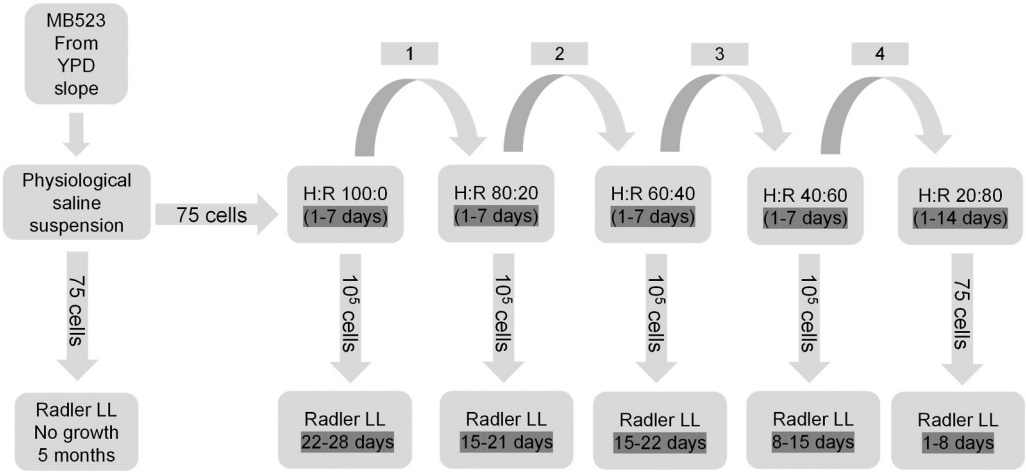

Fig. 1. Procedure to adapt YPD-grown *S. diastaticus* vegetative cells to enable colonization of Radler Lemon Lime 0.0 (Radler LL). Highlighted in gray: the number of days needed to detect growth at 25 °C. Arrows indicate the cell numbers that were transferred to the fresh medium. PS: physiological saline; R: Radler lemon and lime; H: beer 0.0. Ratio is in percentage products mixed.

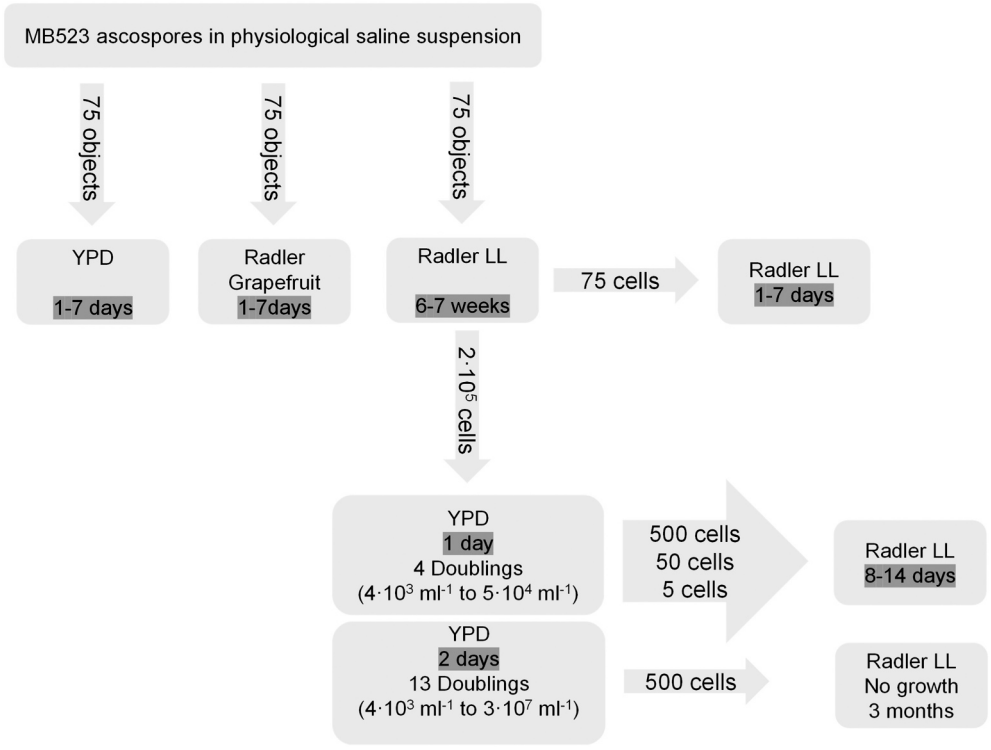

Fig. 2. Procedure to adapt *S. diastaticus* vegetative cells (resulting from germinated ascospores) to enable colonization of Radler lemon lime 0.0 (Radler LL). Highlighted in gray: the number of days needed to detect growth at 25 °C. Arrows indicate the numbers of cells that were transferred to the fresh medium.

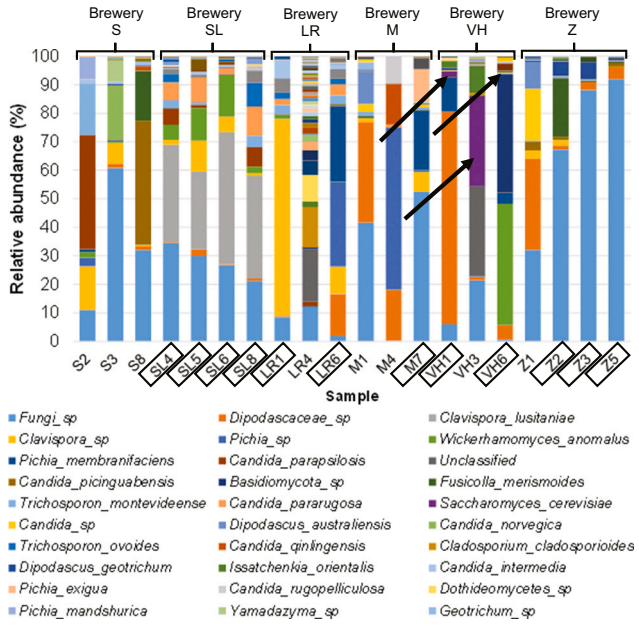

Fig. 3. Relative abundance of fungal species in biofilm samples based on ITS sequencing. *S. cerevisiae* is depicted in purple (indicated with arrows), while *S. diastaticus* was isolated from boxed samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

enriched from two of these samples (VH1 & VH6). In the 17 remaining samples the relative abundance of *S. cerevisiae* was <0.05% but *S. diastaticus* was isolated from 10 of these 17 samples (Table 1; Fig. 3).

3.2. Spoilage capacity of vegetative cells and asci

Vegetative cells and asci were sorted from a sporulating culture using fluorescence activated cell sorting (FACS) (Fig. 4). Sorting was based on the smaller size of the vegetative cells and the fact that they were less granular and auto-fluorescent when compared to asci. Sorting resulted in 67% vegetative cells and 33% asci. Sorting of these enriched fractions resulted in a purity of 99.99% vegetative cells or asci. Beer products were inoculated with these double sorted populations at 1.4 cells or ascospores per ml. The bottles were checked weekly for turbidity and/or pellet formation (Table 2). After 1 week of cultivation, both vegetative cells and spores had colonized YPD, beer 0.0, fruity rose 0.0, wheat rose beer 4.0, and wheat Radler 0.0. These products contain 13–75 g/l fermentable sugar, 0–4% (*v*/v) alcohol and have a pH between 3.1 and 6.4. After 4 weeks, vegetative cells and ascospores also showed visible growth in beer containing 5% (v/v) alcohol. Vegetative cells had not colonized Radler lemon 0.0, Radler lime mint 0.0 and Radler lemon lime 0.0 even after 6 months of incubation. In contrast, ascospores had germinated and colonized the products after 6, 7 and 9 weeks, respectively.

YPD-grown vegetative cells resuspended in PS did not colonize Radler lemon lime 0.0 (Radler LL) during a 5-month period (Fig. 1). In contrast, growth was observed after 22–28 days when Radler LL was inoculated with vegetative cells that were grown in beer 0.0. Colonization was faster when cells were transferred from a beer 0.0 – Radler LL mix; the higher the percentage of Radler LL in the mixture, the faster growth in pure Radler LL was observed. Transfer of cells pre-cultured in 80% Radler LL resulted in growth in Radler LL after 1–8 days incubation (Fig. 1).

Cells from a mixed inoculum (25 asci and 50 vegetative cells) obtained from sporulation medium showed visible growth in Radler LL after 7 weeks of incubation. When 75 of these cells were transferred to fresh Radler LL, visible growth was already observed after 1–8 days of incubation (Fig. 2). Next, $2{\cdot}10^5$ cells from Radler LL were inoculated into 50 ml YPD and incubated at 25 °C. Growth for 24 and 48 h resulted in 4

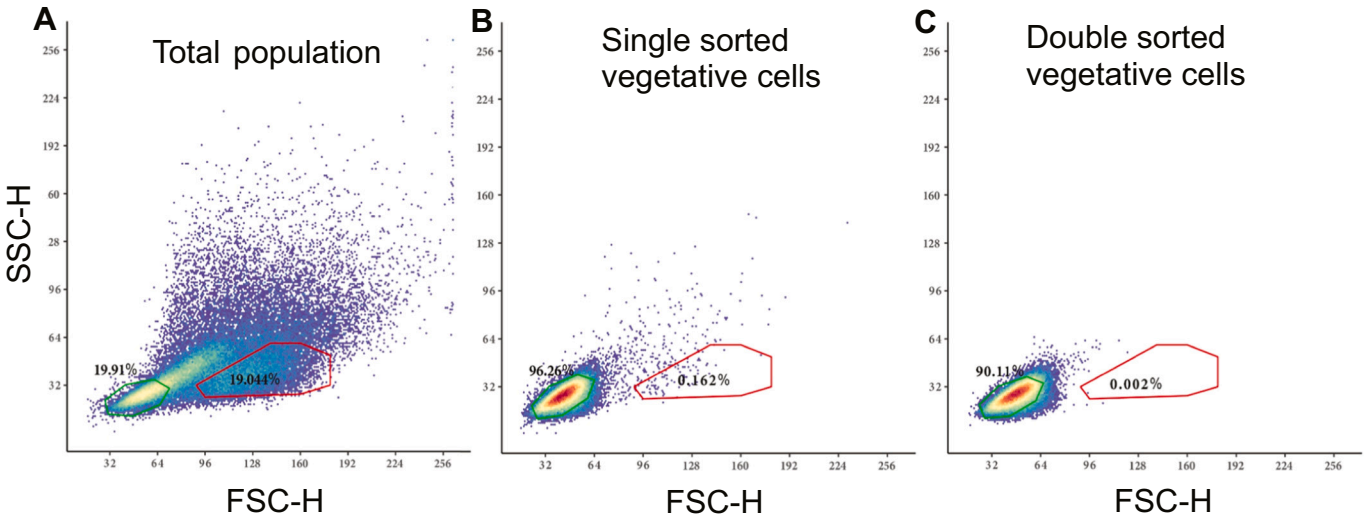

Fig. 4. Sorting of vegetative cells and asci from sporulating cultures. The green and red boxed areas represent the vegetative cells and asci, respectively. Total population (A) and single (B) and double (C) sorted vegetative cells. The same workflow was used for asci, except that the asci were selected and sorted. Percentages of cell types are indicated in the panels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
The number of weeks needed to detect growth in beer products after inoculation with vegetative cells and ascospores and incubation at 25 °C. Cultures were monitored for 6 months.

Product	Total carbohydrates (g/ l)	Fermentable sugar (g/ l)	Alcohol (% v/v)	pH	turbidity/pellet after (week) vegetative cells inoculated	turbidity/pellet after (week) ascospores inoculated
YPD (control)	20	20	0	6.4	1	1
Beer 0.0	48	13	0	4.3	1	1
Beer 5.0	32	0	5	4.3	4	4
Fruity rose 0.0	87	75	0	3.1	1	1
Wheat Rose beer 4.0	53	26	4	3.2	1	1
Wheat beer Radler 0.0	86	61	0	3.2	1	1
Radler lemon 0.0	80	70	0	3.1	–	6
Radler lime mint 0.0	88	80	0	3.2	–	7
Radler lemon lime 0.0	60	56	0	3.1	–	9

and 13 doublings (or generations), respectively, and a total of 5 10^4 and 1.6 10^7 cells per ml. Transferring 5, 50 and 500 of the cells of the 24-h-culture to 70 ml Radler LL resulted in visible growth after 8–14 days (Fig. 2). In contrast, no growth was observed for 3 months when 500 cells of the 48-h-culture were transferred to 70 ml Radler LL (Fig. 2). Together, data show that the ability of vegetative cells to grow in Radler LL depends on the pre-culture conditions.

3.3. Heat resistance determined by pre-culturing conditions

D_{52}-values were determined in PS and H0.0 for vegetative cells grown for 40 h in YPD and in Heineken 0.0 (H0.0). YPD grown cells had a D_{52}-value in PS of 10.3 min, which is significantly higher than the D_{52}-value of 3.5 min in H0.0 (Fig. 5). Cells grown in H0.0 showed almost no decrease in viable cell counts at 52 °C, neither in PS nor in H0.0. D_{52}-values were about 40 min in both menstruum fluids (Fig. 5).

4. Discussion

Prevalence of *S. diastaticus* was studied in nature and the brewing industry. In addition, the spoilage capacity of its vegetative cells and ascospores was assessed. This resulted in the first description of a natural isolate outside South America. In addition, 21 *S. diastaticus* strains were isolated from 54 biofilm samples from 6 breweries located in Europe, Asia, North America and Africa. Data also show that both vegetative cells and spores can spoil beer products, the latter cell type irrespective of its environmental history.

S. cerevisiae could be enriched in 12 out of 136 bark and soil samples, while *S. diastaticus* was detected in a single bark sample from de Gagel (Utrecht, the Netherlands). This indicates that *S. diastaticus* is a less prevalent subspecies of *S. cerevisiae* in nature. Indeed, only 54 out of 1169 *S. cerevisiae* strains were identified as *S. diastaticus* (Krogerus et al., 2019). Of these 54 strains, only 5 strains originated from nature. In contrast to natural environments, *S. diastaticus* was prevalent in breweries. *S. cerevisiae* and *S. diastaticus* were identified by PALL PCR in 25 out of 54 biofilms. In only 4 out of 54 samples *S. cerevisiae* was detected by PCR, while *S. diastaticus* was absent. Notably, the brewing yeast *S. pastorianus* was not detected in any of the biofilms. In contrast, all samples that were positive for *S. diastaticus* and/or *S. cerevisiae*, also contained *Candida / Pichia*. This indicates that these yeasts form mixed biofilms. Indeed, ITS sequencing indicated that the relative abundance of *S. cerevisiae* (and subsp.) was <0.05% of the fungal cells in most of the biofilms. Although the relative abundance of ITS sequences may not be directly related to the relative abundance of a species due to a slight bias in sequencing, we did find that the *Saccharomyces* spp. are a minority in the mixed biofilms. The primary colonizers of surfaces in brewery

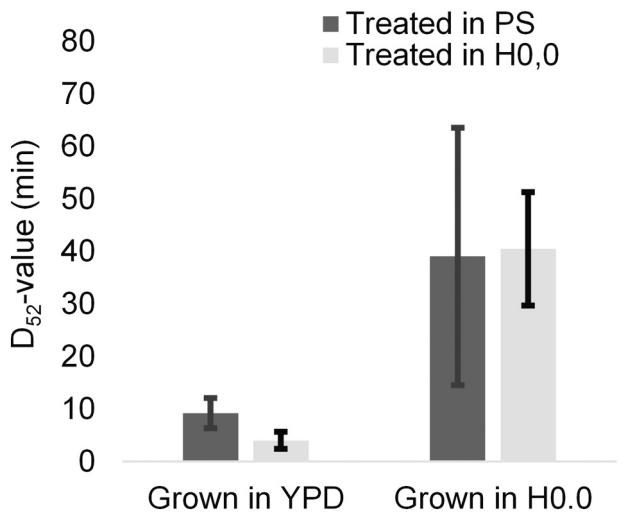

Fig. 5. D_{52}-values for MB523 vegetative cells, grown in YPD and in Heineken 0.0 (H0.0) for 40 h, and treated in physiological saline (PS) and in H0.0. Cells pre-cultured in YPD have different D_{52}-values in PS and in H0.0 of 9.2 and 4.1 min, respectively (p-value <0.037 by two-tailed Student's t-test). Cells precultured in H0.0 have similar D_{52}-values in PS and in H0.0 of 39.1 and 40.5 min, respectively (p-value >0.9 by two-tailed Student's t-test). Cells grown in H0.0 are 10 times more heat resistant than cells grown in YPD, when treated in H0.0 at 52 °C (green bars), with average D_{52}-values of 40.5 and 4.0 respectively (p-value: 0.004 by two-tailed Student's t-test).

bottling plants are gram-negative bacteria such as *Pseudomonas* spp. In the next step, yeasts (particularly *Pichia anomala*, *Candida sake* and *Debaryomyces hansenii*) will adhere (Storgards et al., 2006) followed by *S. cerevisiae*. The fact that *Candida* did show biofilm formation in a microplate assay, while *S. cerevisiae* did not, confirms that the former is among the first colonizers of surfaces in breweries (Timke et al., 2008).

PALL PCR analysis performed on DNA from the biofilms did not fully correlate with isolation of *S. diastaticus* by selective enrichment. The yeast could not be cultured in the case of 8 PCR positive samples. This may be explained by the presence of dead *S. diastaticus* cells in the biofilm or by cells that are not fit enough to grow. Such "false positive" biofilms would likely not be able to spoil beer. On the other hand, *S. diastaticus* was isolated by selective enrichment in 4 PCR negative samples. Possibly, the lysis method is not 100% effective in releasing genomic DNA or the PCR has a lower detection limit than growth in selective medium. In the latter case, one cell is sufficient to ultimately cause turbidity of the medium. These results are of interest for breweries that perform risk assessments by checking for the presence of *S. diastaticus*. It should be noted that PCR diagnosis is performed within 2 h, while selective enrichment takes up to 3 weeks.

The spoiling capacity of vegetative cells and asci of *S. diastaticus* MB523 was assessed. To this end, ascospores were used from sporulation medium, while vegetative cells orginated from sporulation medium, YPD, beer or mixtures of alcohol free beer (beer 0.0) and Radler lemon lime 0.0 (Radler LL). Ascospores were able to grow in all tested beer products, while the vegetative cells originating from the sporulation medium grew in all products except for lemon or lime based Radlers. Beer with 5% alcohol showed slower colonization when compared to YPD, beer 0.0, fruity rose 0.0, wheat rose beer 4.0, and wheat Radler 0.0. This is probably explained by the absence of fermentable sugars in this product, meaning that starch and dextrins have to be degraded to grow.

Like vegetative cells from sporulation medium, YPD pre-grown cells also did not colonize Radler LL. This may be explained by the low pH combined with other hurdles, such as the presence of D-limonene. The pH shock that these vegetative cells experience upon transfer to the Radlers (from pH 6.5 to 3.1) possibly prevents them from growing. The fact that ascospores do grow under these conditions may be explained by the fact that their dormancy enables them to adapt to the new medium. In fact, the acid environment of Radlers might initiate germination of ascospores. This was shown to occur in the acid environment of insect guts, allowing dispersal of yeast and promoting outcrossed mating, thereby enhancing genetic variance. Also in this case, vegetative cells do not survive insect guts possibly because the pH shock is lethal for a metabolically active cell (Knight and Goddard, 2015; Reuter et al., 2007). To test this hypothesis, vegetative cells were first pre-cultured in alcohol free beer or in mixtures of beer 0.0 and Radler LL. Preculturing in beer 0.0 already resulted in spoilage capacity. This may be due to the lower pH of beer 0.0 (pH 4.3) initiating a stress response (Causton et al., 2001; Gasch, 2007) that prepares the cells to be able to grow in Radler LL that has a pH of 3.1. Spoilage capacity was further increased by preculturing in increasing amounts of Radler LL. Reversely, spoilage capacity was lost when vegetative cells grown in Radler LL (resulting from germinated ascospores) were transferred and grown for 48 h in YPD resulting in 13 generations. In contrast, growth in Radler LL was still observed when inoculated with cells from 24-h-old YPD cultures that had 4 doublings. These findings likely are explained by cellular memory of acquired stress resistance. D-limonene is one of the components in Radler LL. This monoterpene damages cell walls, affects plasma membrane properties and triggers accumulation of reactive oxygen species, thereby causing oxidative stress (Bakkali et al., 2005; Liu et al., 2013; Lushchak, 2011; Prashara et al., 2003). Organisms have evolved physiological adaptations to cope with changing environments, such as cross-protection (Dhar et al., 2013). For example, yeast cells subjected to mild salt stress have acquired tolerance to severe doses of H_2O_2, meaning that they have improved fitness to oxidative stress for several generations (Guan et al., 2012). The gradual increase of Radler LL in the growth medium or the lower pH in beer 0.0 may be experienced as a mild stress by yeast cells, thereby acquiring cross-protection against a range of stresses and enabling them to grow in Radler LL.

Cells grown in YPD are more heat resistant when treated in PS when compared to treatment in H0.0 with D_{52}-values of 10.3 and 4.0 min, respectively. This may be explained by the lower pH of H0.0 (4.3) when compared to PS (~6.5). However, cells grown in H0.0 were 10 times more heat resistant than cells grown in YPD when treated in H0.0 at 52 °C, with average D_{52}-values of 40.5 and 4.0, respectively. This could be due to induced cross-protection against environmental stresses for instance by strengthening of the cell walls (Causton et al., 2001; Gasch, 2007).

Together, data show that ascospores as well as vegetative cells are capable of spoiling beer products. Data also imply that changing environmental conditions in breweries may reduce spoilage of lemon and lime based Radlers by vegetative cells of *S. diastaticus*.

CRediT authorship contribution statement

Inge M. Suiker: Conceptualization; Formal analysis; Investigation; Methodology; Writing - original draft; Writing - review & editing. Ger J. A. Arkesteijn: Methodology. Petra J. Zeegers: Investigation; Methodology; Writing - review & editing. Han A. B. Wösten: Supervision; Writing - original draft; Writing - review & editing.

Declaration of competing interest

All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.

This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.

The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript

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