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Genotypic diversity of *Saccharomyces cerevisiae* spoilers in a community of craft microbreweries

In brewing, the yeast chosen to conduct the fermentation is one of the key factors that influences the flavor profile. Yeasts that are deliberately not under the control of the brewer are referred to as "wild yeasts". The growth of wild yeasts at any stage of beer production process can cause defects that negatively impact beer quality. Wild yeasts belonging to the *Saccharomyces* genus present the greatest risk, given their physiological and morphological similarity to the inoculated yeast. The production of craft beer in Andean Patagonia of Argentina has grown considerably and a large community of microbreweries co-exist. Most of these have strong interactions and share many raw material suppliers. They have not yet installed proper microbial quality control strategies and wild yeast contamination is challenging. The aim of this article was to genetically characterize for the first time a high number of *S. cerevisiae* wild yeasts isolated from craft beer that originated from Andean Patagonia, and to study the incidence of *S. cerevisiae* var. *diastaticus* contamination. The genetic distinctiveness of 32 wild *Saccharomyces* was determined using multiple real-time PCR systems and PCR-amplicon capillary electrophoresis of IGS2 region. All isolates were positive for *Saccharomyces cerevisiae*, and 66 % were var. *diastaticus* (*STA1* positive). Intriguingly, a single *STA1* positive isolate was also positive for *Saccharomyces bayanus*/*pastorianus*/*uvarum* and deserves further investigation. Strain level typification showed a large diversity even in isolates from the same brewery and also permitted the detection of well-established strains within single breweries. It also evidenced possible cross contaminations among breweries. This study provides the first insight into the genetic diversity and distribution of a large set of *S. cerevisiae* var. *diastaticus* in a community of microbreweries and provides important information on how to tackle this problem in the most efficient way and thereby help improve the quality of craft beer.

Keywords: craft beer, Patagonia, spoiler yeast, *Saccharomyces cerevisiae* var. *diastaticus*

1 Introduction

Recently, the consumption and production of craft beer has grown rapidly worldwide and in some regions the growth of this industry has reached 20–35 % annually during the past few years [1]. As a result, it is common to see multiple independent but considerably interconnected microbreweries coexist in certain geographical regions, however, these possess limited microbial quality control strategies. One example is the Andean region of Argentinean Patagonia where by 2018 there were more than 200 microbreweries and more than 40 only in the city of Bariloche (with a local population of 108,000 people). [1 and unpublished data]. Patagonia has unique conditions for brewing as it offers all the ingredients needed for brewing: I) Fresh meltwater from Patagonian glaciers offer exceptional quality for brewing [3]; II) Barley is largely produced in La Pampa province and small-scale plantations (and posterior malting) are also being developed in Patagonia with promising results; III) Argentina is the only producer of hops in Latin America and the principal South-American region of cultivation is El Bolsón (near Bariloche) [2]; IV) The discovery and identification of the *Saccharomyces eubayanus* yeast species, missing parent of the Lager hybrid [4, 5] in that geographic region, generated great interest in the study and characterization of its fermentative aptitude and its potential application in the beer industry as a tool for productive diversification and added value. Fast growth in volume and the number of craft breweries as well as the increased consumer demand for quality beers force brewers to constantly search for efficient and affordable quality control strategies. Microbial contamination has proven to be one of the most important limiting factors for quality improvement in beer in general and in craft beers in particular [6–8]. The most frequent microbial spoilers are some specific bacteria and wild yeasts [9]. Growth of wild yeasts during fermentation and/or in the packaged product may lead to multiple defects, including the formation of phenolic, acidic, fatty acid and estery off-flavors, excess of attenuation as well as haze and turbidity [10]. Wild yeasts can be divided into two groups: those belonging to the *Saccharomyces* genus and those that are not, the former represent the greatest risk and

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Table 1 ***Saccharomyces*** **isolates from Patagonian craft beer during 2016-2019 from 17 microbreweries using traditional methods of cultivation**

# Isolate	Sample format	Brewery	Location	Year	Isolation culture medium
1	BBT	BRY 1	Bariloche	2019	YM+$CuSO_4$ 500ppm
2	BBT	BRY 1	Bariloche	2019	YM+$CuSO_4$ 500ppm
3	BBT	BRY 1	Bariloche	2019	YM+$CuSO_4$ 500ppm
4	BBT	BRY 1	Bariloche	2019	YM+$CuSO_4$ 500ppm
5	BBT	BRY 2	Bariloche	2019	YM+$CuSO_4$ 500ppm
6	BBT	BRY 3	Bariloche	2019	LCSM
7	Bottle	BRY 3	Bariloche	2016	LCSM
8	Bottle	BRY 4	Bariloche	2016	LCSM
9	Bottle	BRY 5	Bariloche	2016	LCSM
10	BBT	BRY 6	Bariloche	2019	YM+$CuSO_4$ 500ppm
11	Bottle	BRY 6	Bariloche	2016	LCSM
12	Bottle	BRY 6	Bariloche	2016	LCSM
13	Bottle	BRY 7	Bariloche	2016	LCSM
14	BBT	BRY 8	Bariloche	2019	LCSM
15	BBT	BRY 8	Bariloche	2019	YM+$CuSO_4$ 500ppm
16	BBT	BRY 8	Bariloche	2019	YM+$CuSO_4$ 500ppm
17	BBT	BRY 9	Bariloche	2019	LCSM
18	BBT	BRY 9	Bariloche	2019	LCSM
19	BBT	BRY 9	Bariloche	2018	LCSM
20	Bottle	BRY 10	Bariloche	2016	LCSM
21	BBT	BRY 11	Bariloche	2017	LCSM
22	BBT	BRY 11	Bariloche	2017	LCSM
23	Bottle	BRY 12	El Bolson	2016	LCSM
24	Bottle	BRY 13	El Bolson	2016	LCSM
25	Bottle	BRY 14	El Bolson	2016	LCSM
26	Bottle	BRY 14	El Bolsón	2016	LCSM
27	Bottle	BRY 14	El Bolsón	2016	LCSM
28	Bottle	BRY 14	El Bolsón	2016	LCSM
29	Bottle	BRY 15	Epuyen	2016	LCSM
30	Bottle	BRY 15	Epuyen	2016	LCSM
31	Bottle	BRY 16	Epuyen	2016	LCSM
32	Bottle	BRY 17	Córdoba	2017	LCSM

its detection and control is challenging due to their physiological and morphological similarity to the inoculated yeast. In particular, diastatic strains of *Saccharomyces cerevisiae* [11], also referred as *S. cerevisiae* var. *diastaticus* in many publications for super-attenuating/highly fermentative yeasts [12, 13]. These strains are able to metabolize residual carbohydrates in naturally conditioned beers such as complex dextrins and starches due to the presence of *STA* genes encoding for the enzyme glucoamylase [14]. As the *STA* genes are not present in *S. cerevisiae* brewing strains, these genes can be used for specific identification of *S. cerevisiae* var. *diastaticus* [15]. Recently, it was demonstrated that the sole detection of the *STA* gene is not diagnostic of diastatic activity [17] given that alleles with certain deletions might affect the synthesis of the enzyme [16]. *S. cerevisiae* var. *diastaticus* occurs as a primary contaminant in yeast/fermentations and as a secondary contaminant in the filling process. A primary contamination can lead to a competition with the applied culture yeast during main fermentation and to a strong increase in the spoilage yeast cell counts in the fermentation substrate. Usually occurring as secondary contaminants derived from residues in bottles or in the formation of biofilms, this super-attenuating yeast can contaminate the finished product directly via contact with the product through beer lines, by air circulation in the area of the filling machine and the capper [17]. In contrast to most brewing strains, *S. cerevisiae* var. *diastaticus* frequently sporulates [17], providing opportunities for an efficient spread within and between breweries. To the best our knowledge, there are no previous works dealing with the incidence and distribution of different strains of *S. cerevisiae* var. *diastaticus* in microbrewery communities. As a case study, we selected the region of Andean Patagonia constituted by a heterogeneous group of small-scale microbreweries with very different levels of experience and size, but with a certain level of relationship due to common raw materials and suppliers, sharing of materials (including yeast slurries), and collaborative joint projects. Previous studies showed a high incidence of microbial contamination in this community as 69.3 % of the beers analyzed showed significant growth of bacteria and/or wild yeasts [18]. The aim of this study was to genetically characterize suspected *Saccharomyces* wild yeasts at the strain level, isolated from craft beers of Andean Patagonia in Argentina with a special focus on *S. cerevisiae* var. *diastaticus* diversity and distribution.

2 Materials and methods

2.1 Yeast isolates and strains

From 2016–2019, a total of 32 *Saccharomyces* spoilage yeast isolates were obtained from 120 samples of finished beer taken from bright beer tanks (BBT) and bottles (Table 1). All of them were isolated from ale type beers brewed in 17 craft microbreweries from different locations in Andean Patagonia (Argentina) with the exception of one strain from the province of Cordoba (Argentina). Lin's Cupric Sulfate Medium (LCSM) [19] or YM with 500 ppm of cupric sulfate was used for detection and isolation. These were preliminary identified as members of the *Saccharomyces* genus using MALDI-TOF according to previous reports [20, 21].

2.2 Genetic identification and typification of isolates

The genetic distinctiveness of each isolate was studied using qualitative real-time PCR according to *Hutzler* et al. [22]. For strain

Table 2 Qualitative real-time PCR systems for differentiation of brewing-related yeast species [22–24]

System name	Real-time PCR systems, primer and probe sequences (5´-->3´)	Reference	*S. cer.*	*S. past.*	*S. cer. var. dia.*
Sce	Sc-f CAAACGGTGAGAGATTTCTGTGC Sc-r GATAAAATTGTTTGTGTTTGTTACCTCTG Sc FAM-ACACTGTGGAATTTTCATATCTTTGCAACTT-BHQ1	[25, 26]	+	+	+
TF-COXII	TF-f TTCGTTGTAACAGCTGCTGATGT TF-r ACCAGGAGTAGCATCAACTTTAATACC TF-MGB FAM-ATGATTTTGCTATCCCAAGTT-BHQ1	[23]	+	–	+
BF-300	BF300E CTCCTTGGCTTGTCGAA BF300M GGTTGTTGCTGAAGTTGAGA BF300 FAM-TGCTCCACATTTGATCAGCGCCA-BHQ1	[25]	–	+	–
Sbp	Sbp-r1 TTGTTACCTCTGGGCGTCGA Sbp-r2 GTTTGTTACCTCTGGGCTCG Sbp ACTTTTGCAACTTTTTCTTTGGGTTTCGAGCA	[25, 26]	–	+	–
Sdia	Sd-f TTCCAACTGCACTAGTTCCTAGAGG Sd-r GAGCTGAATGGAGTTGAAGATGG Sdia FAM-CCTCCTCTAGCAACATCACTTCCTCCG-BHQ1	[25]	–	–	+

typification, a method based on a PCR-amplicon capillary electrophoresis of partial intergenic spacer 2 (IGS2) fragment (IGS2-314 PCR-capillary electrophoresis) was employed [17].

2.2.1 DNA extraction

To extract the DNA from each investigated yeast isolate these were cultured in YM agar slants for 48 hrs and an inoculation loop was used to transfer this to a 1.5 mL tube. It was mixed with an aliquot of 200 µL InstaGeneTM Matrix solution (Biorad, Munich, Germany). Each tube was vortexed for ten seconds and incubated at 56 °C for 30 minutes, followed by another ten seconds of vortexing and incubation at 96 °C for eight minutes. The incubation steps occurred in a Thermomix 5436 (Eppendorf, Hamburg, Germany). After incubation, the tubes were centrifuged at 13,000 g for two minutes then a 100 µL aliquot of the supernatant containing the DNA was transferred to a new 1.5 mL tube [17].

2.2.2 Real-time polymerase chain reaction (RT-PCR)

Real-time PCR (Light Cycler® 480 II. Roche Diagnostics Deutschland GmbH, Mannheim, Germany) was used to taxonomically classify the isolates. The primer and TaqMan® probe sequences used are listed in Table 2 and the RT-PCR procedure followed that of Hutzler [23]. All RT-PCR systems listed in Table 2 are compatible and were performed with 10 µL 2x Mastermix (Light Cycler® 480 Probe Master, Roche, Germany), 1.4 µL ddH2O PCR water, 0.8 µL (400 nM) of each primer (Biomers, Ulm, Germany), 0.4 µL (200 nM) probe (Biomers, Ulm, Germany; MGB probe from ThermoFisher scientific, Applied Biosystems®, USA), 0.5 µL IAC135-f (250 nM), 0.5 µL IAC135-r (250 nM), 0.4 µL IAC135-S (HEX) (200 nM), 0.1 µL IAC135 (dilution 1: 10 – 13), 0.1 µL IAC135 rev (dilution 1: 10 – 13) and 5 µL template DNA with a total reaction volume of 20 µL, using the same temperature protocol: 95 °C / 10 min; 40 cycles of 95 °C / 10 s, 60 °C / 55 s; 20 °C. IAC135 was developed by Riedl at the Research Center Weihenstephan for Brewing and Food Quality of the Technical University Munich. IAC (internal amplification control) is a control to confirm that the PCR reaction itself took place. The yeast strains *S. cerevisiae* (LeoBavaricus - TUM 68®) and *S. pastorianus* (Frisinga - TUM 34/70®) were used as a positive and negative control according to the RT-PCR system tested. The signal was considered to be positive when the Ct value (cycle threshold), defined as the number of cycles required for the fluorescent signal to cross the threshold, was less than 30.

2.2.3 PCR-DNA sequencing (D1/D2 26S rRNA gene and ITS)

The identity of isolates with ambiguous results obtained by RT-PCR were confirmed by sequence analysis of the D1/D2 26S and ITS1-5.8S-ITS2 ribosomal DNA according to previous reports [27].

2.2.4 DNA fingerprinting (PCR-capillary electrophoresis of the IGS2-314 fragment)

For strain typification, genetic fingerprints were generated, based on the study of the. IGS2 spacer region within the ribosomal gene cluster, using the IGS2-314 method [23]. For partial sequencing of the intergenic spacer 2 (IGS2-314) the specific primers IGS2-314f(5'-CGGGTAACCCAGTTCCTCACT-3') and IGS2-314r(5'-TAGCATATATTTCTTGTGTGAGAAAGGT-3') [28] were used at a concentration of 600 nM as described by Hutzler, Geiger and Jacob [23]. PCR was performed with 22.5 µL RedTaq Mastermix (2 x) (Genaxxon, Ulm, Germany) and 2.5 µL template DNA with a total reaction volume of 25 µL. The Mastermix contained 12.5 µL buffer solution (RedTaq Mastermix), 7.0 µL DNA-free PCR water and 1.5 µL of each primer (Biomers, Munich, Germany). Cycling parameters were: A pre-denaturing step at 95 °C for 300 s, then 35 cycles for denaturing at 95 °C for 30 s, for annealing and elongation at 54 °C for 30 s and 72 °C for 40 s and for final elongation at 72 °C for 300 s. PCR was performed using a SensoQuest LabCycler48s (SensoQuest GmbH, Gottingen, Germany). Amplified fragments were analyzed using a capillary electrophoresis system (Agilent DNA 1000 kit) following the manufacturer`s recommendations (lab on a chip, Bioanalyzer Agilent 2100, Agilent Technologies, Santa Clara, CA, USA).

2.2.5 Dendrogram analysis of the IGS2-314 fingerprint patterns

Based on the specific capillary electrophoresis IGS2-314 rDNA patterns, a dendrogram was built using the Bionumerics program 7.6 (Applied Maths, Belgium) to show the relationship between

Table 3 Qualitative real-time PCR systems for *Saccharomyces* spp. differentiation [22–24]

Strain #	Sce (Ct value)	BF300 (Ct value)	TF (Ct value)	Sbp (Ct value)	Sdia (Ct value)
1	+ (20.82)	–	+ (24.16)	–	–
2	+ (18.50)	–	+ (21.95)	–	–
3	+ (20.49)	–	+ (22.18)	–	–
4	+ (20.59)	–	–	–	–
5	**+ (26.41)***	**– ***	**– ***	**+ (22.22)***	**+ (27.04)***
6	+ (20.38)	–	+ (23.62)	–	+ (25.30)
7	+ (18.98)	–	+ (21.11)	–	–
8	+ (19.84)	–	+ (21.59)	–	+ (26.44)
9	+ (18.55)	–	+ (18.85)	–	+ (23.35)
10	+ (19.07)	–	–	–	+ (26.06)
11	+ (18.98)	–	+ (21.89)	–	+ (26.54)
12	+ (21.31)	–	+ (24.98)	–	+ (26.84)
13	+ (19.58)	–	+ (22.44)	–	+ (26.23)
14	+ (19.43)	–	+ (23.88)	–	+ (24.92)
15	+ (17.62)	–	–	–	+ (22.16)
16	+ (18.76)	–	+ (23.22)	–	+ (23.69)
17	+ (18.11)	–	+ (23.02)	–	+ (27.88)
18	+ (19.07)	–	+ (22.24)	–	+ (25.57)
19	+ (22.09)	–	–	–	+ (27.97)
20	+ (20.07)	–	+ (23.26)	–	+ (25.57)
21	+ (20.94)	–	+ (22.04)	–	+ (25.70)
22	+ (18.78)	–	+ (20.09)	–	–
23	+ (20.88)	–	+ (23.55)	–	–
24	+ (20.08)	–	+ (22.99)	–	+ (25.61)
25	+ (20.51)	–	+ (22.06)	–	+ (25.45)
26	+ (17.38)	–	+ (18.34)	–	–
27	+ (18.04)	–	+ (19.23)	–	–
28	+ (17.82)	–	+ (19.80)	–	–
29	+ (19.68)	–	+ (22.94)	–	+ (24.46)
30	+ (20.11)	–	–	–	+ (26.43)
31	+ (21.14)	–	+ (23.88)	–	+ (26.80)
32	+ (19.93)	–	+ (24.06)	–	–

(*) Average results of two replicates

the investigated yeast isolates and reference top-fermenting strain *Saccharomyces cerevisiae* SafAle S-04 and SafAle US-05 of Fermentis®. The clustering was built using a Dice similarity coefficient and the methodology applied was UPGMA. The patterns were optimized using an optimization degree of 0.3 % and a tolerance set of 1 % with a tolerance change of 0.2 %.

3 Results and discussion

All 32 yeast isolates that were confirmed as belonging to the genus *Saccharomyces* by the MALDI-TOF approach were analyzed using five specific *Saccharomyces* real-time polymerase chain reaction (RT-PCR) systems (Table 2) and the results are shown in table 3. The RT-PCR results showed that all isolates were positive for Sce locus which detects ITS1-5.8S-ITS rDNA present in *S. cerevisiae*, *S. pastorianus*, *S. paradoxus* and *S. cariocanus* [26], in line with MALDI-TOF data. Unsurprisingly, all isolates were negative for *Saccharomyces pastorianus* BF 300 system (BF = bottom fermenting). The BF300 Primer system was generated via subtractive hybridization and subsequent design of a real-time PCR system and is also a marker for *S. pastorianus* and some *S. bayanus* strains [25]. Twenty-eight isolates were positive for the PCR system TF-COXII based on the mitochondrial COXII genetic marker. This primer system is used to differentiate *Saccharomyces cerevisiae* from *S. pastorianus*, *S. uvarum* and *S. bayanus* [23]. Isolate 5 represented a unique case given it was the only positive yeast for the Sbp system (located on the ITS1-5.8S-ITS2) which detects species belonging to either *S. bayanus*, *S. pastorianus*, *S. uvarum* or *S. eubayanus*. Twenty-one isolates were positive for the *STA1* gene marker which was designed to specifically detect *S. cerevisiae* var. *diastaticus*, given this gene is responsible for the synthesis of glucoamylase enzyme that allows dextrin assimilation leading to super-attenuated beers. The remaining 11 isolates that were negative for Sdia were positive for TF-COXII system, suggesting that they could be a brewing ale strain or a *STA1* negative *Saccharomyces cerevisiae* spoiler.

The isolates 4, 5, 10, 15, 19 and 30 showed ambiguous RT-PCR results. For this reason, one additional system was used according to Salinas et. al. [22, 29] to detect *S. cerevisiae* top-fermenting yeast. With the exception of isolate 5 all the isolates had positive signals confirming their *S. cerevisiae* identity: 4 (Ct value = 27.01), 10 (Ct value = 25.26),15 (Ct value = 23.26), 19 (Ct value = 27.99), and 30 (Ct value = 27.14). It is probable that these isolates have nucleotide differences at the COXII target sequences of the TF Real-Time PCR system, given a considerable variability in this sequence for *S. cerevisiae* has been reported previously [5, 30]. Due to the Sdia system (*STA1* +) positive result these 5 isolates were considered to be *S. cerevisiae* var. *diastaticus*. Taking into account all the positive results for Sdia loci, 66 % of the total isolates can be assigned to *S. cerevisiae* var. *diastaticus*, representing 17.5 % of the beer samples analyzed (N = 120) and 47 % of the breweries studied (N = 45). Thus, this species becomes the most relevant yeast contaminant of the southern Argentina craft beer industry. Given that there not many studies focusing on the incidence of *S. cerevisiae* var. *diastaticus* in brewing, these values are hard to compare and discuss. For example, Meier-Dörnberg et al. [11] found higher values given that between 2008 and 2017, 56 % of the 100 samples of beer tested contained *S. cerevisiae* var. *diastaticus*. Isolate 5 represents a salient case given it was positive for the Salinas et al. system (Ct

value = 26.74) and Sdia system but was also positive for Sbp. Consequently, the ITS and D1/D2 regions were sequenced. The results showed 100 % identity with *Saccharomyces uvarum* CBS 395 (accession number NR153310.1). Further studies are needed to clarify the situation of this particular strain, including WGS in order to detect a possible hybrid scenario.

The PCR amplification of the IGS2-314 locus was used to typify the isolates and the two main top-fermenting yeasts used by craft breweries in Argentina were included in the analysis as references (Fig. 1). The results show a great genetic diversity among the isolates for the IGS2-314 locus and all isolates had different banding patterns to the reference strains, strain 27 being the most similar to strain S-04 (81.8 % of similarity). The reference strain US-05 had very low similarity (< 21 %) with the rest of the studied isolates. Eight of the 17 breweries from which isolates were studied here had multiple isolates and for 75 % of these cases (6 out of 8 breweries) the isolates from the same brewery did not share similar banding patterns (e. g BRY 1, BRY 3, BRY 6, BRY 11, BRY 14 and BRY 15). For example, breweries 6 and 14 had three isolates each and based on our typification approach all belong to distinct strains. The genetic heterogeneity found between strains from the same brewery suggest diversity of spoiler yeasts possibly associated with different contamination events and sources. On the other hand, for breweries with multiple isolates such as BRY 1, 8 and 9 identical (100 % similarity) or almost identical (88.9 % for BRY 1) it was also possible to detect genetic fingerprints for 3 isolates in each case (Fig. 1). This is an indication that those strains are well established in one particular brewery and is spread to the different batches by contaminated re-pitching brewing yeast, other process pathways or improperly sanitized equipment and surfaces. An interesting case is that of BRY 9 where *STA*-positive isolate 19 was obtained almost one year before the genetically identical isolates 18 and 17. The brewery moved their facilities to a new location in between the collection of the isolates, which suggests that this strain is strongly established in the brewery equipment.

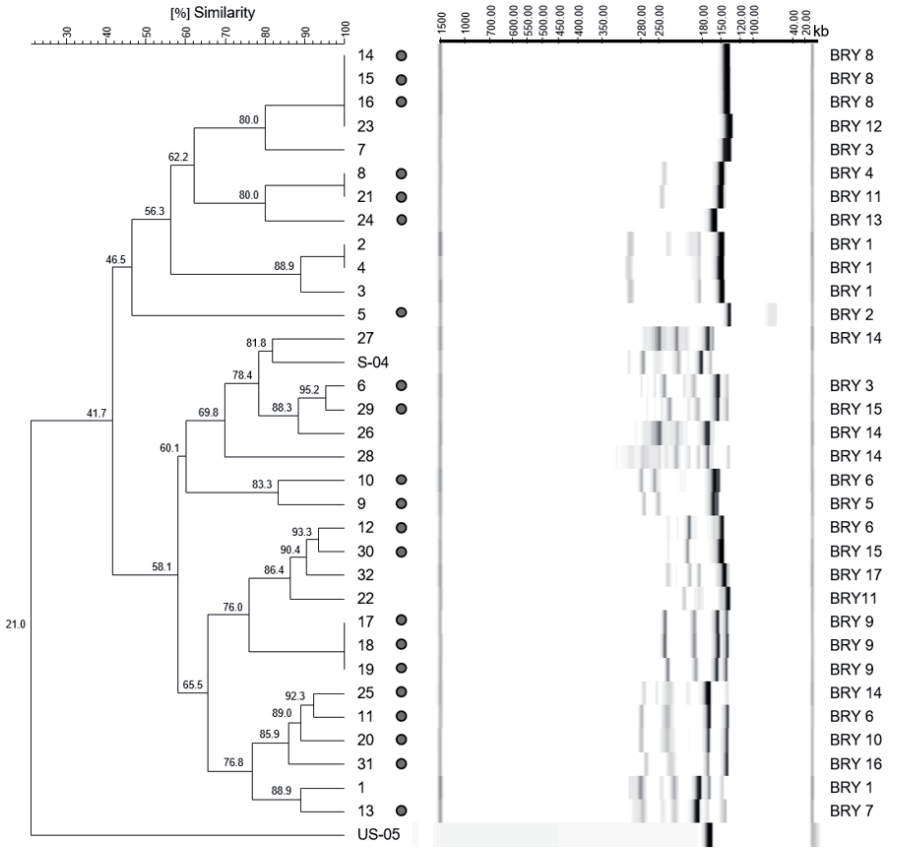

Fig. 1 **IGS2-314 rDNA band-based genetic relationship between *Saccharomyces cerevisiae* spoiler isolates (dendrogram built with Bionumerics 7.6). The dots indicate *STA*1-positive strains thus *S. cerevisiae* var. *diastaticus* identity. The code of the brewery (BRY) is in the right side of the figure**

Another interesting finding is the high number of cases (5) in which similar genetic patterns (> 89.0 % of similarity) and *STA* condition were obtained for isolates of different breweries. For example, isolates 14, 15 and 16 (BRY 8) are identical to isolate 23 (BRY 12), also isolates 8 (BRY 4) and 21 (BRY 11); 6 (BRY 3) and 29 (BRY 15); 12 (BRY 6) and 30 (BRY 15); and isolates 11 (BRY 6), 25 (BRY 10) and 20 (BRY 14). These results suggest potential cases of cross contamination between breweries due to some kind of interaction like contract filling, beer or yeast slurry exchange, or even collaborative beer projects. Another explanation could be that the breweries share the same provider for raw materials or equipment. The possible connection of contamination pathways is indeed an important and interesting aspect. The craft beer industry in this region has only started in the last few years, before that there were almost no small local breweries. Nevertheless, similar beer spoilers have settled in the breweries, which are also located in different geographic areas. This shows that the substrate and the respective environments are very similar. As far as the substrate is concerned, it is obvious that water, malt (probably mainly pale barley malt), were used in similar grist portions and similar hop products with similar hopping techniques. In any case, there are many possibilities that beer-spoiling yeasts can find niches. In addition, the breweries certainly have connection points and likely help each other out with raw materials. One alternative reason for the similar wild yeasts found in different breweries could be the use of the same dry yeast strains and suppliers given contaminated dry yeast and more likely re-pitched dry yeast can be a potential source of wild yeast contaminations.

In terms of the distribution of *STA*+ condition and thus var. *diastaticus* identity among the dendrogram, we observed that this trait is considerably widespread among most of the groups with a few exceptions of clusters of isolates that have either positive and negative *STA* cases. Probably the most notable case is that of the cluster of isolates 14, 15, 16 and 23 with 100 % similarity (Fig. 1), the latter being *STA*- and the rest positive. It should be noted that isolate 23 comes from a different brewery and a close inspection of the banding patterns suggests the existence of a slight difference in the size of the unique diagnostic band that should be further

studied to confirm if it belongs to the same strain as the other 3 isolates. It was observed that 4 of the 8 breweries with more than 1 isolate analyzed, showed strains that were positive for *STA1* and negative to this gene. Given the lack of information of how the *STA* gene is spread among brewing-related *S. cerevisiae* strains and within the brewery environment, it is difficult to draw more conclusions from the correlation of the presence of the *STA* gene and the isolates genetic typification.

The genetic typification again showed isolate 5 as a unique case, given it displays a banding pattern very different from the others (only 46.5 % similarity) confirming that it is a very interesting strain that requires a deeper analysis.

4 Conclusions

The identity and genetic distinctiveness of 32 suspected *S. cerevisiae* isolates regarded as beer contaminants from craft beers produced in Argentinian Patagonia was determined using a combination of methodologies including MALDI-TOF, real-time PCR and PCR-amplicon capillary electrophoresis. The real-time PCR approach proved useful for a rapid confirmation of *S. cerevisiae* identity and the detection of *STA*-positive isolates hence assignment to *S. cerevisiae* var. *diastaticus*, which was the majority of the isolates (66 %). The IGS2-314 analysis provided the right level of genetic variability among the isolates to allow finding very interesting cases such as 3 different breweries with multiple isolates of the same strain, and also several different breweries with putative identical contaminant strains. Both studies agreed on the genetic uniqueness of isolate 5, which deserves further attention. A working hypothesis could be that it represents a hybrid of *S. uvarum* and a *STA*+ *S. cerevisiae* strain, for which genome sequencing analysis will be applied in the future. The large genetic diversity of strains (and *STA* gene condition) found even among isolates of some breweries suggests that craft breweries in Patagonia, Argentina, are in contact with a large diversity of *S. cerevisiae* beer spoilers possibly associated with distinct contamination events/sources. It is worth mentioning that is almost impossible to find wild *S. cerevisiae* strains in the natural environment of Andean Patagonia [4, 31]. Thus, most probably these contaminants arrive to the brewery with raw material and establish there when possible, as seen in a few breweries in this study where the same strain appears several times, even after moving to a different location. In addition, more than a few breweries shared contaminant strains, which is indicative of cross contamination maybe arising from several type of interactions and exchanges that are common to the craft industry. *Saccharomyces* var. *diastaticus* (*STA1*-positive) was the most relevant case in this study and showed a large genetic heterogeneity, indicating the existence of multiple strains for this yeast. Hopefully, additional WGS studies will allow us to gain a better understanding of genomic basis of this strain differentiation and the mechanisms by which *STA* genes spread among spoiling strains. Overall, the data shows that *S. cerevisiae* var. *diastaticus* is an important contaminant in the brewing industry and is clearly one of the most frequent yeast spoilers in the craft industry based in the south of Argentina. Despite the rapid growth of the craft breweries in Patagonia, Argentina, our findings indicate that more attention should be paid to the microbiological quality of the beers produced, and the need to eliminate non-sanitary equipment or accessories, implement proper cleaning and sanitizing protocols and microbial quality control strategies in the breweries. This is the first study that provides source tracking profiles at a strain level of a high number of *S. cerevisiae* var. *diastaticus* isolates that show strain distribution within one specific brewery and across different breweries in one geographical region. There are some recent *S. cerevisiae* var. *diastaticus* studies but their focus is more on strain characterization and statistical distribution than strain tracking across different connected breweries [16]. The applied methods in this article and the data collected have the potential to assess different contamination sources and routes in order to improve the quality of Argentine craft beers. The strains studied in this work were cryo-preserved and incorporated into the IPATEC microbial collection given that they will be useful as a reference for designing strategies for the detection and control of spoilage microorganisms in micro-breweries, the study of their physiological and genetic characteristics, as well as their potential application in brewing innovation.

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