Gushing phenomenon and gene Hyd5 from Fusarium species in barley samples from Brazil

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Abstract

Hydrophobins produced by the Fusarium genus in brewing barley in connection with gushing phenomenon have been studied in many different countries. However, the presence of proteins associated with gushing in grains harvested in Brazil is currently unknown. The surface active class 2 hydrophobin Hyd5 has been identified as a possible causative agent for gushing of beer. For this reason, the objective of this study was to evaluate the presence of Hyd5 in isolated Fusarium strains from Brazilian barley samples. This was followed by gushing tests. The Hyd5 gene was identified in all fungal isolates and phylogenetic analysis exhibited four main lineages. Gushing tests demonstrated that among the 21 analysed malt samples 8 were positive considering overfoaming at the bottle opening. Levels ranged from 8 to 124 g/bottle of gushing. No correlation between phylogenetic analysis and gushing was observed when they were evaluated together. This is due to positive isolates for gushing which are scattered throughout the phylogenetic tree. The other cause may be that all main clades contain at least one positive isolate for gushing. More research regarding hydrophobins and gushing should be carried out in the future due to the fungi isolated in Brazilian samples. These results offer excellent support that could help the brewing industry to prevent its losses and to provide further understanding of the gushing phenomenon in beer.

Keywords: barley, beer, Hyd5, gushing, quality

1 Introduction

The Fusarium genus is considered to be the most prevalent fungi found in barley, wheat, corn and oats (Oliveira et al., 2017; Piacentini et al., 2015; Savi et al., 2014). Contamination may lead to losses in agricultural yields as well as in the food industry, due to issues such as nutritional loss in food and significant risk to humans and the food chain (Neme and Ibrahim, 2017; Bennett and Klich, 2003).

In recent years, many studies have been carried out to increase our understanding of the occurrence of these contaminants and the metabolites produced (mycotoxins). This is because they are associated with health problems and the effect on the end product quality (Piacentini et al., 2018; Tralamazza et al., 2016; Běláková et al., 2014).

In the same context, other metabolites (non-mycotoxins) produced by this genus are also being widely studied for their ability to affect product quality. The primary metabolites known as hydrophobins are small proteins (common pattern of eight cysteines at conserved positions) produced in the cell wall of fungi and are characterised as low molecular weight proteins. They are able
to decrease the surface tension of water by self-assembling at water/air interfaces, thus enabling transition of this barrier during the production of aerial mycelia (Mastanjavic et al., 2015; Cox et al., 2007; Wösten et al., 1999). Moreover, their other role may be to establish the proper contact between fungal cells and host tissue during plant infection (Kim et al., 2005; Wösten et al., 1994).

Two subgroups of hydrophobins (Class 1 and Class 2) have been identified according to differences in spacing and sequence between cysteines, hydrophobicity patterns and solubility in organic solvents (Wessels, 1994). Nevertheless, only Class 2 have been shown to induce gushing in beer. The surface active Class 2 hydrophobin Hyd5 has been identified as a possible causative agent for the gushing of beer (Denschlag et al., 2013; Niu et al., 2012; Sarlin et al., 2012; Lutterschmidt et al., 2011; Stübner et al., 2010). Gushing is a phenomenon in which beer spontaneously overfoams out from its container immediately upon opening (Shokribousjein et al., 2011). It is considered a beer quality problem, associated mainly with the quality of malt. The phenomenon has been studied for decades, however its mechanism is not fully elucidated (Sarlin, 2012).

Several studies reported that contamination by Fusarium spp. such as F. graminearum, F. culmorum, F. poae in barley is highly correlated with gushing induction in beer (Sarlin et al., 2005). However, other genera such as Alternaria, Aspergillus, Nigrospora, Penicillium and Stemphylium have also been reported to induce this phenomenon (Flannigan, 2003).

For this reason, the objective of this study was to evaluate the presence of Hyd5 in isolated Fusarium strains. This was followed by gushing tests in barley samples from Brazil in order to correlate these two factors.

## 2 Material and Methods

### 2.1 Barley Samples

A total of 21 brewing barley (BRS Brau variety) samples were collected by Embrapa (Brazilian Agricultural Research Corporation) from the 2016 harvest in the state of Rio Grande do Sul, Brazil. Samples were collected from bulk batches after dirt removal and drying (up to 60 °C) in storage units. Sampling was performed using a grain auger from different points of the bulk batches with a minimum final weight of 5 kg. Each sample was homogenised and reduced into portions of 1.0 kg (Piacentini et al., 2019a). Then samples were malted in 2018 and used for gushing tests.

### 2.2 Fusarium Strains

The Fusarium strains isolated from barley samples used in this study were previously identified morphologically (Piacentini et al., 2019a), further by using the partial sequences of elongation factor (EF-1α) and the second fragment of RPB2 (7CF/11AR) (Geiser et al., 2004; O’Donnell et al., 2004).

### 2.3 Hyd5 Detection in Fusarium Strains

The detection of the Hyd5 gene in Fusarium isolates was performed with primers designed from the gene best matching the hydrophobin sequence found in the Fusarium genome database (NCBI - National Center for Biotechnology Information). The PCR primers were as follows: 5′-CACCATGMAGTTCTCACTCGC-3′ (forward) and 5′- TTCTTAAGTCCTGGACACCA -3′ (reverse).

PCR reactions were set up to a total volume of 25 μL by mixing 100 ng of target DNA per reaction with 5× PCR buffer containing 0.7 mM of each primer, 3 mM MgCl₂, 0.2 mM dNTPs, 0.2 μM Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA), and PCR grade water to the final volume.

The PCR protocol consists of an initial denaturing at 94 °C for 90 s, followed by 25 cycles of denaturing at 94 °C for 30 s, primer annealing at 55 °C for 90 s and elongation at 68 °C for 2 min, and a final extension at 72 °C for 5 min. PCR products were separated in 1.2% (w/v) agarose gels and visualised using UV light. Amplicons were purified with ExoSAP-IT (Affymetrix, Santa Clara, CA, USA). They were then sent to the Centre of Human Genome Studies, University of Sao Paulo, Brazil for sequencing in ABI PRISM 3130 DNA Analyser (Applied Biosystems, Foster City, CA, USA). Sequences were aligned using the multiple alignment software ClustalX v. 1.83 plug-in in the software Geneious v. 5.3.6 (Biomatters, Auckland, New Zealand). The alignments were edited using the sequence alignment-editing program Geneious v. 1.03 and each polymorphism was re-examined by checking the chromatograms. The sequences generated in this study were deposited in the GenBank.

### 2.4 Phylogenetic Study

Phylogenetic analysis was performed based on the Hyd5 dataset using the PAUP 4.0b10 (Swafford, 2002a). Phylogenies were obtained using unweighted parsimony analysis and a heuristic search option with 1000 random addition sequences and tree bisection reconnection branch swapping in PAUP 4.0b10 (Sinauer Associates, Sunderland, MA, USA) (Swafford, 2002b). Gaps were treated as missing data. The Consistency Index (CI) and the Retention Index (RI) were calculated to indicate the amount of homoplasy present.

Clade stability was assessed via bootstrap analysis in PAUP 4.0b10, using 1000 heuristic search replications with a random sequence addition. The data sets were rooted with Tricholoma sp. as it is considered a suitable out-group. The reference sequences for the Fusarium species used in this study were obtained from NCBI.
2.5 Malting Process

First, the barley samples were submitted to the malting process. On day 1, steeping water was added to the grains for 5 h followed by 19 h of air rest. On day 2, grains were subjected to 4 h in water followed by 20 h of air rest. Finally, on day 3 of steeping, the grains were submerged in water for 20 min and then subjected to air rest for 23 h and 40 min. The germination step was performed over 3 days (72 h), with a subsequent kilning step. The grains in the steeping and germination processes were maintained at a controlled temperature of 14 °C; their moisture content of 45% was controlled and measured in each step. The total kilning time was 22 h, with a pre-kilning temperature of 55 °C for 12 h and a kilning temperature of 80 °C for 4 h (Piacentini, 2019b).

2.6 Modified Carlsberg Test (MCT)

The gushing test was performed according to the Modified Carlsberg Test (MCT) (MEBAK, 2018). To accomplish the experiments, 100 g of malt was milled and carefully homogenised. Then 400 mL of distilled water was added and mixed in a laboratory mixer at the maximum speed (252 RCF), for 60 s to form a suspension. The suspension was transferred to a tube and centrifuged for 10 minutes at RCF of 4,500 g. And following centrifuging, 300 mL of the supernatant was transferred to an 800 mL glass beaker and boiled until (20–25 min) the volume reduced to 200 mL. Immediately after boiling the solution was filtered through a fluted filter into a volumetric flask with a rubber stopper and cooled down to 20 °C. 5 mL of sodium azide stock solution was added, followed by the addition of sterile water at 20 °C making up the volume up to 200 mL.

Consequently, carbonated water (Mattoni, Karlovarské minerální vody, a.s., Karlovy Vary, Czech Republic) in bottles (volume 0.33 L) at 5 °C was chosen with the same headspace. Using a graduated cylinder, 50 mL of water was removed from the bottles. The removed water was replaced by the same amount of filtrate. The bottles were corked immediately, rotated once through 180° along the longitudinal axis and weighed. Subsequently, they were fixed horizontally in a laboratory shaker and shaken for 72 h at a temperature of 20 °C at 75 rpm.

Later, the bottles were removed from the shaker and were put in an upright position for 10 min, followed by 180° rotation 3 times for 10 s. Finally, the bottles were opened and evaluated to determine if overfoaming occurred. Then, they were weighed individually, and the results were calculated. The difference between the weights before and after opening corresponded to the gushing volume in g/bottle.

The gushing positive malt was defined when the overfoaming volume of the carbonated water was in excess of 5 g. According to MEBAK, 0–5 g difference in weight equals no potential for gushing, 5–50 g equals a possible potential for gushing with 50% probability, > 50 g signifies a potential for gushing with 92% probability (MEBAK, 2011).

3 Results

The presence of Hyd5 gene was confirmed in all fungal isolates (Table 1). Additionally, the phylogenetic study was conducted for the species with the gushing potential. The Hyd5 dataset was composed of 25 taxa and 272 nucleotides, of which 186 were parsimony informative characters (PICs) and the phylogenetic analysis resulted in 10 most parsimonious trees (CI=0.78, RI=0.89). Topological differences were not detected between neighbour joining and maximum parsimony phylogeny inferences.

The phylogeny was composed of four main lineages and one Tricholoma sp. as an outgroup (Figure 1). The first lineage was subdivided into two main clades: one composed of F. verticillioides and the other composed of F. proliferatum. The second lineage composed of F. avenaceum only. The third lineage was composed of F. poae and the fourth lineage contained F. graminearum and F. meridionale, both species are part of the F. graminearum species complex. All of the main lineages presented MPBS (maximum parsimony bootstrap support). The phylogeny of the Hyd5 seems to be congruent with the species phylogeny, as all isolated species were clustering within its own group. These findings were consistent with the morphological characterization and with the phylogeny composed of the EP-1a and RPB2 loci (Piacentini et al., 2019a). However, further studies containing more Fusarium isolates should be conducted in order to check whether or not this locus is consistent with the species tree within the above mentioned genus.

The data obtained from the gushing tests demonstrated that among the 21 analysed malt samples 8 of them were overfoaming-positive at the bottle opening. The levels ranged from 8 to 124 g/bottle of gushing. The evaluation according to MEBAK methodology (MEBAK, 2011) was as follows: 62% of samples showed no potential for gushing, 28% of samples showed a possible potential for gushing with 50% probability and 10% of samples showed a potential for gushing with 92% probability (Table 1).

The results from both analyses were evaluated together in order to find the correlation between the presence of the Hyd5 gene and positive gushing samples. No connection was identified, because positive isolates for gushing were scattered throughout the phylogenetic
4 Discussion

Barley samples harvested in Brazil have always been monitored for fungal contamination (Piacentini et al., 2019a; Piacentini et al., 2018; Piacentini et al., 2015). However, this fungal contamination may also be associated with the presence of hydrophobins in the raw material and may cause problems such as beer gushing.

The brewing industry considers gushing phenomenon in beer to be a quality issue as it can lead to considerable economic losses. Several breweries in Europe have reported such problems, however, in Brazil the gushing phenomenon is not known. Excessive foaming has always been explained as a bacterial contamination or secondary gushing, i.e. faults in the beer production process or incorrect treatment of packaged beer.

The literature commonly explains that gushing is caused by Fusarium fungi, however other genera such as Alternaria, Aspergillus, Nigrospora, Penicillium and Stemphylium have also been revealed to induce gushing. This phenomenon is mainly caused by proteins (hydrophobins) with a very small amount, ppm or lower (0.4 ppm), able to induce this problem in beer (Sarlin, 2012; Sarlin et al., 2005).

According to Sarlin et al. (2007), the genus Fusarium is able to proliferate and produce hydrophobins during the malting process, especially during the maceration and germination stages. Ten times higher amounts of hydrophobins were found in malt compared to that of the corresponding barley. This fact may be explained by changes that occur during malting. During this stage there are highly favourable conditions for microbial growth in terms of available nutrients, temperature, humidity and gaseous atmosphere (Laitila, 2007).

Several Fusarium species, such as F. graminearum, F. proliferatum, F. paeae, F. culmorum, F. avenaceum, F. verticillioides, F. sporotrichioides, have been reported to produce hydrophobins and also to induce gushing (Sarlin et al., 2012). However, there are differences between

Figure 1  Inferred phylogeny of the HyD5 locus of Fusarium isolated from barley grains by maximum parsimony. Bootstrap values > 70% are indicated at the base of each clade (bold branches). Outgroup represented by Tricholoma spp.
Fusarium species in terms of how severely they affect the gushing potential of malt. Haikara (1983) observed that F. culmorum isolates induced a more vigorous gushing than F. avenaceum isolates.

In the present study, the species with the highest gushing production was F. meridionale, which was not reported in previous research. The other species that presented a considerable amount of gushing was F. graminearum. However, this species is already reported by several studies for producing such phenomenon in beer (Virkajärvi et al., 2017).

The study performed by Denschlag et al. (2012) associated the genes for the Class 2 hydrophobin Hyd5p with Fusarium spp. as a group-specific genetic marker which can improve the detection and identification of gushing in malt. In literature, the homologous sequence of the FcHyd5 gene has already been detected in F. graminearum (Zapf et al., 2006). The FcHyd5p could provide an interesting model for beer gushing due to the presence of the gene in species that have been closely associated with the induction of the phenomenon. FcHyd5p could also be an essential factor for gushing induction in carbonated aqueous liquids (Stübner et al., 2010).

The 2016 harvest samples used in this research showed a new profile of barley produced in the country. Therefore the study can be compared to other studies conducted in countries with this problem.

A Belgian study which focused on Cargill samples also performed gushing tests (Deckers et al., 2012). Wort was produced from the malt samples and the results were presented as a percentage. In the experiments, the samples were found to have an excessive foam production of up to 15%, considering the total volume of the bottle.

Although the present study shows the occurrence of gushing in malt samples contaminated with Fusarium, the presence of the Hyd5 gene is not a predictive factor of the fact that the species will induce gushing. This phenomenon may depend on certain conditions the gene requires to be expressed, as well as other possibilities unrelated to the gene, which require further elucidation.

| Barley variety | Sample | Fungal isolates | Fusarium species | Modified Carlsberg Test (MCT) | Evaluation (according to MEBAK, 2011) |
|----------------|--------|----------------|-----------------|-----------------------------|--------------------------------------|
|                |        |                |                 |                             |                                      |
| BRS Brau       | 1      | 33E            | F. graminearum  | 12                          | possible potential for gushing (50%) |
|                | 2      | 24A            | F. graminearum  | 24                          | possible potential for gushing (50%) |
|                | 3      | 63C            | F. graminearum  | < 5                         | no potential for gushing             |
|                | 4      | 30B            | F. graminearum  | < 5                         | no potential for gushing             |
|                | 5      | 60E            | F. avenaceum    | < 5                         | no potential for gushing             |
|                | 6      | 13A            | F. graminearum  | < 5                         | no potential for gushing             |
|                | 7      | 8A             | F. graminearum  | < 5                         | no potential for gushing             |
|                | 8      | 21B            | F. graminearum  | < 5                         | no potential for gushing             |
|                | 9      | 4A             | F. meridionale  | 124                         | potential for gushing (92%)          |
|                | 10     | 2A             | F. graminearum  | 38                          | possible potential for gushing (50%) |
|                | 11     | 15A            | F. poae         | < 5                         | no potential for gushing             |
|                | 12     | 32A            | F. poae         | < 5                         | no potential for gushing             |
|                | 13     | 31A            | F. graminearum  | 117                         | potential for gushing (92%)          |
|                | 14     | 23A            | F. poae         | < 5                         | no potential for gushing             |
|                | 15     | 7A             | F. poae         | 42                          | possible potential for gushing (50%) |
|                | 16     | 61B            | F. verticilioides| 10                         | possible potential for gushing (50%) |
|                | 17     | 60F            | F. proliferatum | 8                           | possible potential for gushing (50%) |
|                | 18     | 64A            | F. avenaceum    | < 5                         | no potential for gushing             |
|                | 19     | 58A            | F. avenaceum    | < 5                         | no potential for gushing             |
|                | 20     | 63B            | F. avenaceum    | < 5                         | no potential for gushing             |
|                | 21     | 60B            | F. avenaceum    | < 5                         | no potential for gushing             |
5 Conclusion

The present study found that! all Fusarium spp. isolates contained the Hyd5 gene and that F. meridionale is able to induce the gushing phenomenon which was unreported previously. Each identified species of Fusarium apart from F. avenaceum was able to induce gushing from the Brazilian barley samples, however not all individual samples displayed the phenomenon. This suggests that the presence of the Hyd5 gene is not a suitable predictor for gushing yet may be involved depending on specific conditions that facilitate expression of the gene.

More research regarding hydrophobins and gushing should be performed in the future due to the fungi found in the samples. The results will be an excellent support for the brewing industry to prevent losses and also to understand this beer quality problem.

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