KVASNÝ PRŮMYSL

Kvasny prumysl (2021) 67: 435–441
https://doi.org/10.18832/kp2021.67.435

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

Karim C. Piacentini[1], Sylvie Běláková[2]*, Liliana O. Rocha[3], Geovana D. Savi[4], Karolína Benešová[2], Marek Pernica[2], Ivo Hartman[2], Josef Čáslavský[5], Rastislav Boško[2,6], Benedito Corrêa[1]

[1] *Biotechnology Department, University of Sao Paulo, Av. Professor LineuPrestes, 2415, Sao Paulo, Brazil*
[2] *Research Institute of Brewing and Malting, Mostecká 971/7, 614 00 Brno, Czech Republic*
[3] *Department of Food Science, Food Engineering Faculty, University of Campinas, Av. Monteiro Lobato, 80, Brazil*
[4] *University of the Extreme Southern Santa Catarina, Av. Universitaria, 1105, Criciuma, Santa Catarina, Brazil*
[5] *Global Change Research Institute, Czech Academy of Sciences, Bělidla 986/4a, 603 00 Brno, Czech Republic*
[6] *Department of Crop Science, Breeding and Plant Medicine, Mendel University in Brno, 10 Zemědělská 1, 613 00 Brno, Czech Republic*

corresponding author: belakova@beerresearch.cz

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 linages. 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

Research Institute of Brewing and Malting, Plc.
Published online: 15 April 2021

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 at 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; Lutterschmid 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. graminerarum*, *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′- TTCCTTAGTCCTGGACACCA -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 $MgCl_2$, 0.2 mM dNTPs, 0.2 U/µL 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. 1.83 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 *EF-1α* 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

tree with all main clades containing at least one positive isolate for gushing. As mentioned previously, *Hyd5* phylogeny seems to follow the species tree (Piacentini et al., 2019a), therefore it is expected that the positive gushing samples will not cluster together.

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.

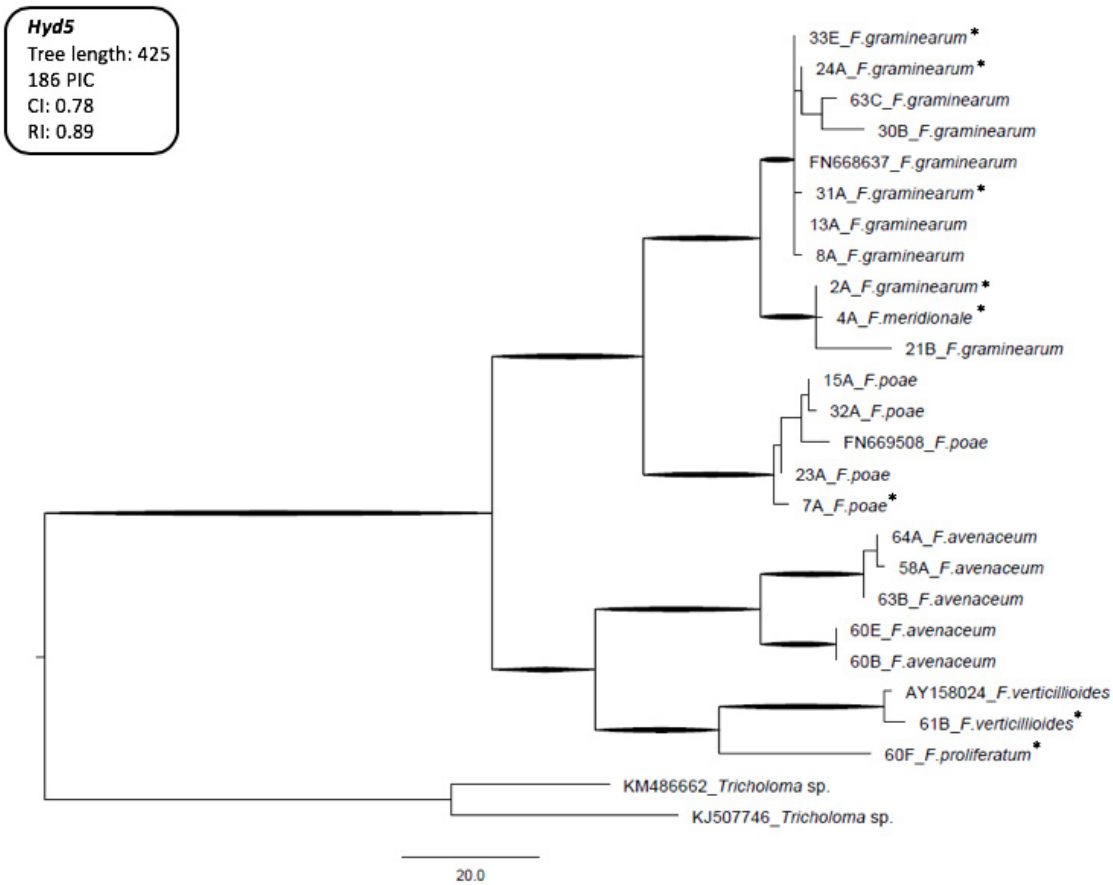
The brewing industry considers gushing phenomenon in beer to be a quality issue as it can lead to considerable economic losses. Several breweries in Europe have reported such problems, however, in Brazil the gushing phenomenon is not known. Excessive foaming has always been explained as a bacterial contamination 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. poae, F. culmorum, F. avenaceum, F. verticilioides, 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. Boostrap 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.

Table 1 *Positive gushing samples and* Fusarium *species isolated*

Barley variety	Sample	Fungal isolates	*Fusarium* species	Modified Carlsberg Test (MCT)	
				Gushing (g/bottle)	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.

6 Acknowledgements

The article was supported by the Ministry of Agriculture of the Czech Republic within the Institutional Support MZE-RO1918 and FAPESP (Sao Paulo Research Foundation) grant numbers 2016/01798-4, 2018/01795-0.

7 References

Běláková, S., Benešová, K., Čáslavský, J., Svoboda, Z., Mikulíková, R. (2014). The occurrence of the selected *Fusarium* mycotoxins in Czech malting barley. Food Control, 37, 93–98. https://doi.org/10.1016/j.foodcont.2013.09.033

Bennett, J.W., Klich, M. (2003). Mycotoxins. Clinical Microbiology Reviews, 16, 497–516. https://doi.org/10.1128/CMR.16.3.497-516.2003

Cox, A.R., Cagnol, F., Russell, A.B., Izzard, M.J. (2007). Surface properties of class II hydrophobins from *Trichoderma reesei* and influence on bubble stability. Langmuir, 23, 7995–8002. https://doi.org/10.1021/la700451g

Deckers, S.M., Vissers, L., Gebruers, K., Shokribousjein, Z., Khalesi, M., Riveros-Galan, D., Schönberger, C., Verachtert, H., Neven, H., Delcour, J., Michiels, C., Ilberg, V., Derdelinckx, G., Titze, J., Martens, J. (2012). Doubly Modified Carlsberg Test combined with Dynamic Light Scattering allows prediction of the primary gushing potential of harvested barley and malt. Cerevisia, 37, 77–81. https://doi.org/10.1016/j.cervis.2012.10.001

Denschlag, C., Vogel, R.F., Niessen, L. (2012). Hyd5 gene-based detection of the major gushing-inducing *Fusarium* spp. in a loop-mediated isothermal amplification (LAMP) assay. International Journal of Food Microbiology, 156, 189–196. https://doi.org/10.1016/j.ijfoodmicro.2012.03.009

Denschlag, C., Vogel, R.F., Niessen, L. (2013). Hyd5 gene based analysis of cereals and malt for gushing-inducing *Fusarium* spp. by real-time LAMP using fluorescence and turbidity measurements. International Journal of Food Microbiology, 162(3), 245–251. https://doi.org/10.1016/j.ijfoodmicro.2013.01.019

Flannigan, B. (2003) The microbiota of barley and malt. In: Priest, F.G., Campell, I. (eds.) Brewing Microbiology, 3rd ed., Chapman & Hall, Kluwer Academic/Plenum, New York, pp. 113–180.

Geiser, D.M., del Mar Jiménez-Gasco, M., Kang, S., Makalowska, I., Veeraraghavan, N., Ward, TJ., Zhang, N., Kuldau, G.A., O'Donnell K. (2004). *FUSARIUM*-ID v. 1.0: A DNA sequence database for identifying *Fusarium*. European Journal of Plant Pathology, 110, 473–479. https://doi.org/10.1023/B:EJPP.0000032386.75915.a0

Haikara, A. (1983). Malt and beer from barley artificially contaminated with *Fusarium* in the field. In: Proceedings of the European Brewery Convention Congress, Edited by Oxford, 401–408.

Christian, M., Titze, J, Ilberg, V., Jacob, F. (2010). New cognitions on gushing in the wort production process and in quantifying the gushing potential of malt. Cerevisia, 35, 35–37. https://doi.org/10.1016/j.cervis.2010.06.005

Kim, S., Ahn, I.P., Rho, H.S., Lee, Y.H. (2005). MHP1, a *Magnaporthe grisea* hydrophobin gene, is required for fungal development and plant colonization. Molecural Microbiology,57, 1224–1234. https://doi.org/10.1111/j.1365-2958.2005.04750.x.

Laitila, A. (2007). Microbes in Tailoring of Barley Malt Properties. VTT Publications, Espoo, Finland.

Lutterschmid, G., Muranyi, M., Stübner, M., Vogel, R.F., Niessen, L. (2011). Heterologous expression of surface-active proteins from barley and filamentous fungi in *Pichia pastoris* and characterization of their contribution to beer gushing. International Journal of Food Microbiology, 147, 17–25. https://doi.org/10.1016/j.ijfoodmicro.2011.02.030

Mastanjavic, K., Velic, N., Krstanovic, V., Novak, M., Marcek, T., Santek, B. (2015). Causes, consequences and prevention of beer gushing. Croation Journal Food Technology, Biotechnology Nutrition, 10, 14–22. Available from: https://hrcak.srce.hr/file/217371

MEBAK (2011). Raw Materials: Barley, Adjuncts, Malt, Hops and Hop Products; Collection of Brewing Analysis Methods of the Mitteleuropäische Brautechnische Analysenkommission. MEBAK, Freising-Weihenstephan, Germany.

MEBAK (2018). Raw Materials: Adjuncts, Barley, Malt, Hops and Hop Products; Collection of Brewing Analysis Methods of the Mitteleuropäische Brautechnische Analysenkommission. MEBAK, Freising, Germany. ISBN 978-3-9815960-3-8

Neme, K., Ibrahim, A.M. (2017). Mycotoxin occurrence in grains and the role of postharvest management as a mitigation strategies. A review. Food Control, 78, 412–425. https://doi.org/10.1016/j.foodcont.2017.03.012

Niu, B., Wang, D., Yang, Y., Xu, H., Qiao, M. (2012). Heterologous expression and characterization of the hydrophobin *HFBI* in *Pichia pastoris* and evaluation of its contribution to the food industry. Amino Acids, 43, 763–771. https://doi.org/10.1007/s00726-011-1126-5

O'Donnell, K., Ward, T.J., Geiser, D.M., Kistler, H.C., Aoki, T. (2004). Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. Fungal Genetics and Biology, 41, 600–623. https://doi.org/10.1016/j.fgb.2004.03.003

Oliveira, M.S., Rocha, A., Sulyok, M., Krska, R., Mallmann, C.A. (2017). Natural mycotoxin contamination of maize (*Zea mays* L.) in the South region of Brazil. Food Control, 73, 127–132. https://doi.org/10.1016/j.foodcont.2016.07.033

Piacentini, K.C., Savi, G.D., Pereira, M.E.V., Scussel, V.M. (2015). Fungi and the natural occurrence of deoxynivalenol and fumonisins in malting barley (*Hordeum vulgare* L.). Food Chememistry, 187, 204–209. https://doi.org/10.1016/j.foodchem.2015.04.101

Piacentini, K.C., Rocha, L.O., Savi, G.D., Carnielli-Queiroz, L., Almeida, F.G., Minella, E., Corrêa, B. (2018). Occurrence of deoxynivalenol and zearalenone in brewing barley grains from Brazil. Mycotoxin Research, 34, 173–178. https://doi.org/10.1007/s12550-018-0311-8

Piacentini, K.C., Rocha, L.O., Savi, G.D., Carnielli-Queiroz, L., De Carvalho Fontes, L., Correa, B. (2019a). Assessment of Toxigenic *Fusarium* Species and Their Mycotoxins in Brewing Barley Grains. Toxins (Basel), 11, 1–14. https://doi.org/10.3390/toxins11010031

Piacentini, K.C., Běláková, S., Benešová, K., Pernica, M., Savi, G.D., Rocha, L.O., Hartman, I., Čáslavský, J., Corrêa, B. (2019b). *Fusarium* mycotoxins stability during the malting and brewing processes. Toxins (Basel), 11, 257. https://doi.org/10.3390/toxins11050257

Sarlin, T. (2012). Detection and characterisation of *Fusarium* hydrophobins inducing gushing in beer. Dissertation. VTT Science 13, VTT, Espoo, Finland.

Sarlin, T., Kivioja, T., Kalkkinen, N., Linder, M.B., Nakari-Setälä, T. (2012). Identification and characterization of gushing-active hydrophobins from *Fusarium* graminearum and related species. Journal of Basic Microbiology, 52, 184–194. https://doi.org/10.1002/jobm.201100053

Sarlin, T., Nakari-Setala, T., Linder, M., Penttila, M., Haikara, A., (2005). Fungal hydrophobins as predictors of the gushing activity of malt. Journal of the Institute of Brewing, 111, 105–111. https://doi.org/10.1002/j.2050-0416.2005.tb00655.x

Sarlin, T., Vilpola, A., Kotaviita, E., Olkku, J., Haikara, A. (2007). Fungal hydrophobins in the barley-to-beer chain. Journal of the Institute of Brewing, 113, 147–153. https://doi.org/10.1002/j.2050-0416.2007.tb00271.x

Savi, G.D., Piacentini, K.C., Tibola, C.S., Scussel, V.M. (2014). Mycoflora and deoxynivalenol in whole wheat grains (*Triticum aestivum* L.) from Southern Brazil. Food Addit Contaminants: Part B, 7, 232–237. https://doi.org/10.1080/19393210.2014.898337

Shokribousjein, Z., Deckers, S.M., Gebruers, K., Lorgouilloux, Y., Baggerman, G., Verachtert, H., Delcour, J.A., Etienne, P., Rock, J.M., Michiels, C., Derdelinckx, G. (2011). Hydrophobins, beer foaming and gushing. Cerevisia, 35, 85–101. https://doi.org/10.1016/j.cervis.2010.12.001

Stübner, M., Lutterschmid, G., Vogel, R.F., Niessen, L. (2010). Heterologous expression of the hydrophobin *FcHyd5p* from *Fusarium culmorum* in *Pichia pastoris* and evaluation of its surface activity and contribution to gushing of carbonated beverages. International Journal of Food Microbiology, 141, 110–115. https://doi.org/10.1016/j.ijfoodmicro.2010.03.003

Swofford, D.L. (2002a). PAUP* phylogenetic analysis using parsimony (*and other methods). Version 4.0b10. Sinauer Associates. https://doi.org/10.1159/000170955

Swofford, D.L. (2002b). Phylogenetic Analysis Using Parsimony. Options, 42, 294–307. https://doi.org/10.1007/BF02198856

Tralamazza, S.M., Bemvenuti, R.H., Zorzete, P., De Souza Garcia F., Corrêa, B. (2016). Fungal diversity and natural occurrence of deoxynivalenol and zearalenone in freshly harvested wheat grains from Brazil. Food Chemistry, 196, 445–456. https://doi.org/10.1016/j.foodchem.2015.09.063

Virkajärvi, V., Sarlin, T., Laitila, A. (2017). *Fusarium* profiling and barley malt gushing propensity. Journal of the American Society of Brewing Chemists, 75, 181–192. https://doi.org/10.1094/ASBCJ-2017-3321-01

Wessels, J.G.H. (1994). Developmental Regulation of Fungal Cell Wall Formation. Annual Review of Phytopathology, 32, 413–437. https://doi.org/10.1146/annurev.py.32.090194.002213

Wösten, H.A.B., Schuren, F.H.J., Wessels, J.G.H. (1994). Interfacial self-assembly of a hydrophobin into an amphipathic protein membrane mediates fungal attachment to hydrophobic surfaces. EMBO J, 13, 5848–5854. https://doi.org/10.1002/j.1460-2075.1994.tb06929.x

Wösten, H.A.B., Van Wetter, M.A., Lugones, L.G., Van der Mei, H.C., Busscher, H.J., Wessels, J.G.H. (1999). How a fungus escapes the water to grow into the air. Current Biology, 9, 85–88. https://doi.org/10.1016/S0960-9822(99)80019-0

Zapf, M.W., Theisen, S., Vogel, R.F., Niessen, L. (2006). Cloning of wheat LTP1500 and two *Fusarium* culmorum hydrophobins in *Saccharomyces cerevisiae* and assessment of their gushing inducing potential in experimental wort fermentation. Journal of the Institute of Brewing, 112, 237–245. https://doi.org/10.1002/j.2050-0416.2006.tb00719.x