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Fundamental study on the improvement of the antifungal activity of *Lactobacillus reuteri* R29 through increased production of phenyllactic acid and reuterin

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- MRS was supplemented with phenylalanine or glycerol and fermented by *Lactobacillus reuteri* R29
- Accumulation of phenyllactic acid and reuterin was significantly increased
- Antifungal activity *in vitro* against common food spoilage fungi was significantly increased
- Antifungal performance of PLA was transferable in a bread system
- Antifungal activity of reuterin was not transferable in a bread system

1 **Fundamental study on the improvement of the antifungal**
2 **activity of *Lactobacillus reuteri* R29 through increased**
3 **production of phenyllactic acid and reuterin**

4

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12 ABSTRACT

13 Lactic acid bacteria have shown great potential as bio-preservative agents to
14 maintain high food quality and safety. The strain *Lactobacillus reuteri* R29 is reported
15 to have a broad spectrum of antifungal activity and suitability for application in food
16 systems. Its antifungal activity is predominantly based on phenyllactic acid (PLA).
17 Furthermore, it is potentially a producer of reuterin, a potent antimicrobial agent. This
18 study focused on increasing the antifungal activity *in vitro* by supplementation of the
19 growth medium with phenylalanine and glycerol to increase the yield of PLA and
20 reuterin, respectively. For PLA, the addition of 1.5% phenylalanine (w/v) to MRS,
21 resulted in significantly increased accumulation of PLA and antifungal performance
22 against *Fusarium culmorum*. Supplementation of MRS with 500mM glycerol
23 combined with a reduced glucose content (1.5%) showed the highest reuterin
24 accumulation combined with fungal inhibition. To investigate the antifungal
25 performance *in situ*, these cell-free supernatants (cfs) were applied in a bread
26 system. The application of PLA-enriched cfs resulted in significantly extended (4
27 days) microbial shelf life compared to the control. The reuterin-enriched medium did
28 not lead to significant shelf life extension. In conclusion, *Lactobacillus reuteri* R29
29 and its PLA-enriched cfs were found to be very promising alternatives for food bio-
30 preservation.

31 Keywords: *Lactobacillus reuteri* R29, phenyllactic acid, reuterin, antifungal, bread
32 making

34 **1 Introduction**

35 Lactic acid bacteria (LAB) are key microorganisms used for the production of various
36 bakery products. They substantially improve nutritional and technological properties,
37 as well as the flavour. However, current research is predominantly focused on their
38 antimicrobial properties and potential applications as food bio-preservatives (Dalié,
39 Deschamps, & Richard-Forget, 2010). The use of such LAB has the potential to
40 replace chemical preservatives, such as calcium propionate, which would allow a
41 “clean label” and hence lead to higher consumer acceptance (Pawlowska, Zannini,
42 Coffey, & Arendt, 2012). In recent years, several strains of different genera have
43 been found to express antimicrobial activity *in vitro* and *in situ* against food spoilage
44 fungi (Axel et al., 2016; Crowley, Mahony, & Van Sinderen, 2013).

45 The antifungal activity of LAB is predominantly based on complex synergistic effects
46 between several compounds. The production of these compounds is highly
47 dependent on the strain and growth substrate. The exact interactions between the
48 bacterial metabolites which create this synergistic effect are not yet fully understood.
49 However, several metabolites that contribute to the antifungal activity have been
50 identified and characterised. These metabolites, amongst others, include carboxylic
51 acids (Ryan, Dal Bello, & Arendt, 2008) and 3-hydroxypropionaldehyde (3-HPA) also
52 known as reuterin (Lüthi-Peng, Dileme, & Puhan, 2002).

53 Recently, the production and activity of 3-phenyllactic acid (PLA) has received great
54 attention (Mu, Yu, Zhu, Zhang, & Jiang, 2012). This broad-spectrum antimicrobial
55 compound originates in LAB fermented products from the catabolism of
56 phenylalanine (Phe). Thereby, the Phe first undergoes a transamination by
57 transferring the amino group onto a keto-acid acceptor. The synthesised

58 phenylpyruvic acid is then reduced to PLA by a dehydrogenase (Vermeulen, Gänzle,
59 & Vogel, 2006). The antifungal activity of PLA is also dependent on a synergistic
60 mechanism with other bacterial metabolites. Nevertheless, if the metabolic pathway
61 yielding PLA is promoted, the antifungal activity is likely to increase. This could
62 expand the field of application for LAB as bio-preservatives.

63 A very promising antimicrobial agent is the multicomponent system called reuterin,
64 which results from the conversion of glycerol to 3-HPA (Engels et al., 2016). The
65 antimicrobial activity of reuterin is, according to Schaefer et al. (2010), mainly based
66 on its reactivity with free thiol groups of proteins, inducing oxidative stress in the
67 target cells. It is understood that 3-HPA is released upon enzymatic dehydration of
68 glycerol. Recent research conducted by Engels et al. (2016) suggests a rapid *in situ*
69 conversion to acrolein, which is mainly responsible for the antimicrobial activity of the
70 reuterin system. However, if sufficient amounts of carbohydrates, in particular
71 glucose, are available, 3-HPA is further reduced to 1,3-propanediol which has no
72 antimicrobial activity (Gänzle, 2015). Unfortunately, the research on reuterin so far
73 has mainly focused on its industrial use as precursor for the synthesis of acrolein,
74 not on food applications. To date, there are only two studies conducted by Gomez-
75 Torres et al. (2014) and Ortiz-Rivera et al. (2017) available, investigating a possible
76 application of reuterin as an antibacterial agent in a food system. As Gänzle (2015)
77 observed, the applicability of reuterin, particularly in heat treated food systems,
78 remains unclear. Further research is required, in particular to elucidate the effect of
79 glycerol on bacterial growth and reuterin production.

80 A strain previously reported as demonstrating strong antifungal activity and suitability
81 for food applications is *Lactobacillus reuteri* R29 (Axel et al., 2016; Oliveira et al.,

82 2015). Oliveira et al. (2015) demonstrated that the cell-free supernatant (cfs) of
83 medium fermented for 48 h with this strain showed antifungal activity against *F.*
84 *culmorum*, with PLA being the predominant antimicrobial compound produced. As it
85 belongs to the species *L. reuteri*, the strain is likely to be a reuterin producer. To date
86 there have been no studies carried out investigating the ability of *L. reuteri* R29 to
87 produce and accumulate reuterin. Thus, the enhancement of the production and
88 accumulation of antifungal compounds, namely reuterin and PLA, in the bacterial cfs
89 represents a promising opportunity in particular, if this antifungal activity is applicable
90 in food systems.

91 Therefore, the aim of this study was to increase the *in vitro* antifungal activity of *L.*
92 *reuteri* R29 cfs. Different variations of the fermentation medium composition were
93 investigated to increase the accumulation of PLA and reuterin. *F. culmorum* was
94 used as indicator mould to determine the antifungal activity *in vitro*. Secondly,
95 characterisations regarding the heat stability of the most efficient cfs were evaluated.
96 Finally, antifungal cfs delivering the best results were applied in the bread system to
97 obtain information regarding suitability for application in a food matrix. This study
98 provides important information regarding the potential of *L. reuteri* R29 cfs as
99 antifungal agent and its application as a bio-preservative agent, in the bread system.

100 **2 Materials and Methods**

101 **2.1 Materials**

102 Ingredients for microbiological media, chemicals and analytical standards used in
103 this study were at least analytical grade and obtained from Sigma-Aldrich (Dublin,
104 Ireland), unless otherwise stated. The analytical standards had a purity of $\geq 95\%$.

105 Wheat flour (bakers flour, Odlums, Dublin, Ireland), dry yeast (Puratos, Groot-
106 Bijgaarden, Belgium), salt (Glacia British Salt Limited, Cheshire, UK), commercially
107 available sunflower oil, sodium stearoyl lactylate (SSL) (Danisco, Denmark) and
108 ascorbic acid (Storefast solutions, UK) were used for the baking trials.

109 **2.2 Cultures, media and growth conditions**

110 *Fusarium culmorum* TMW4.2043, *Aspergillus niger* FST4.21, *Penicillium expansum*
111 FST 4.22 originated from the culture collection of School of Food and Nutritional
112 Sciences, University College Cork (Cork, Ireland). Fungal spore suspensions were
113 prepared according to the method described by (Oliveira, Mauch, Jacob, Waters, &
114 Arendt, 2012).

115 *Lactobacillus reuteri* R29, originally isolated from human intestine, was obtained from
116 the UCC culture collection (School of Food and Nutritional Sciences, University
117 College Cork, Cork, Ireland). For long term storage, the culture was kept in
118 commercial de Man Rogosa and Sharpe (MRS) broth containing 40% glycerol at -
119 80°C. The culture was routinely refreshed on MRS agar plates (Fulka Chemie AG,
120 Buchs, Switzerland) under anaerobic conditions at 37°C for 48 h.

121 **2.3 Production of LAB cell-free supernatants**

122 For production of 1-step fermented LAB cfs, a single colony, grown as described in
123 2.2, was inoculated in 5 mL MRS broth for 24 h at 37°C. Subsequently, a 1%
124 inoculum into fresh fermentation medium (Table 1 and 2) was prepared and
125 incubated at 37°C for the designated time. After fermentation, cell cultures were
126 centrifuged (5,000 g, 15 min, 4°C) and the supernatant was sterile filtered through a
127 0.22 µm filter. The cfs obtained was stored at -20°C until further use.

128 The 2-step fermentation process was based on the method described by Doleyres et
129 al. (2005) with the following modifications. After the first fermentation step, cells were
130 washed and resuspended in sterile distilled water, containing 400 mM glycerol, at a
131 final concentration of 10^9 CFU/mL. The suspension was incubated for 2 h at 30°C
132 and the cfs collected as described above.

133 **2.4 Evaluation of *in vitro* antifungal activity and determination of minimal** 134 **inhibitory concentration (MIC₉₀)**

135 Pre-trials were carried out to determine the ideal fungal spore concentration to
136 investigate the increase of antifungal performance in the respective cell-free
137 supernatants. Thereby, a concentration of 10^6 spores/mL as applied in the assay
138 described were chosen for the subsequent experiments (data not shown). To
139 determine the *in vitro* antifungal activity, 1 mL of *Fusarium culmorum*, *Aspergillus*
140 *niger* or *Penicillium expansum* spore solution (containing approximately 10^6 spores)
141 was transferred into a 2 mL microcentrifuge tube and centrifuged at 3,000 g (10 min,
142 4°C). The supernatant was discarded and the spore pellet resuspended in 1.0 mL of
143 diluted LAB cfs (10%, v/v, in unfermented MRS). Subsequently, 200 µL aliquots were
144 pipetted into a 96-well microplate (Sarstedt AG and Co, Nurembrecht, Germany). To
145 control for the effect of condensation, each supernatant was also inoculated with 1
146 mL of sterile synthetic nutrient-poor bouillon, instead of fungal spore solution (blank).
147 The microplate was sealed with an optically clear seal for Q-PCR (Thermo Scientific,
148 Waltham, USA) and incubated in a Multiskan FC microplate-reader (Thermo
149 Scientific, Waltham, USA) for 5 days at 25°C. The optical density was recorded at
150 620 nm (OD₆₂₀) every 2 h, with agitation in 4 s intervals. Values of each cfs
151 inoculated with spore solution were corrected by the respective mediums blank

152 value. Antifungal activity was calculated as the percentage of OD reduction
153 compared to the respective 0 h fermented medium and expressed as “percentage of
154 growth inhibition”.

155 To determine the MIC₉₀ of PLA the substance was added to unfermented MRS-broth
156 to a final concentration of 20,000 ppm and the pH adjusted to match the fermented
157 medium. Due to the lack of a commercially available standard for reuterin, a serial
158 dilution of the 2-step fermented supernatant was used to determine the MIC₉₀. The
159 MIC₉₀ was defined as the minimal concentration of the respective antifungal
160 compound required to achieve at least 90% reduction of fungal growth after 120 h
161 under the assay conditions compared to a control medium without the antifungal
162 compound.

2.5 Determination of antifungal fermentation products

2.5.1 Determination of carboxylic antifungal compounds

163
164 An Agilent 1260 high-performance liquid chromatography system equipped with an
165 ultraviolet-diode array detector (UV/DAD) was used to quantify antifungal carboxylic
166 compounds. Standard calibration curves for lactic and acetic acid (2 – 32 mM), as
167 well as 13 phenolic compounds (catechol, hydroxyphenyllactic acid, 4-
168 hydroxybenzoic acid, hydrocaffeic acid, caffeic acid, phloretic acid, hydroferulic acid,
169 p-coumaric acid, ferulic acid, benzoic acid, salicylic acid, hydrocinnamic acid,
170 methylcinnamic acid, vanillic acid and 3-phenyllactic acid) were prepared, using five
171 different concentrations in duplicate. Calibration curves showed good linearity with
172 correlation coefficients of ≥ 0.999 for all compounds. Extraction of antifungal organic
173 acids (acetic acid and lactic acid) was carried out as described by Axel et al. (2016).
174 For separation of the acids a Hi-Plex H Column (300×7.7 mm, 8 μ m; Agilent, Cork,
175 Ireland), equipped with a guard column (50×7.7 mm, 8 μ m; Agilent, Cork, Ireland)

176 was used. Setting the UV/DAD at 210 nm, lactic and acetic acids in the cfs were
177 determined after elution with 0.004 M sulphuric acid at 65 °C with a flow of 0.5
178 mL/min. Injection volume used was 20 µL.

179 Antifungal phenolic compounds were extracted, using a QuEChERS approach, as
180 described by Peyer et al. (2016). Separation was carried out using a Gemini C₁₈
181 column (150 x 2.0 mm, 5 µm; Phenomenex, Macclesfield, UK), equipped with a
182 guard column (AF0-8497; Phenomenex, Macclesfield, UK). The mobile phase
183 consisted of A) H₂O with 0.1% FA and B) ACN with 0.1% FA. To ensure separation
184 from other compounds, a gradient flow was performed (0 – 5 min – 90% A, 30 min –
185 80% A, 35 min – 60% A, 45 min – 5% A, 45 – 70 min – 90% A), using a flow rate of
186 0.2 mL/min at a temperature of 30°C. The volume injected was 10 µL and detection
187 carried out at a wavelength of 210 nm.

188 If necessary, samples were diluted prior to extraction to ensure peak areas were
189 within the calibration range. Identity of the peaks evaluated in the cfs samples was
190 confirmed as PLA by comparison of the UV/VIS spectrum with the standard solution.
191 The limit of detection (LOD) and the limit of quantification (LOQ) were determined
192 from the signal/ noise (s/n) ratio. The LOD was set for s/n of 3:1 and the LOQ was
193 set for s/n of 10:1. Recovery rates of the phenolic compounds were done with 3.0
194 mg/L of each analyte added in MRS and in chemically acidified MRS (pH 3 with 0.1
195 N HCl), and ranged from 89.1% (hydrocinnamic acid) to 118.1% (vanillic acid) of the
196 total spiked amount.

197 **2.5.3 Colorimetric assay for reuterin quantification**

198 The concentration of reuterin in the cfs of the 1- and 2-step fermentations was
199 determined using the colorimetric assay described by Lüthi-Peng et al. (2002).

200 Calibration was carried out, using acrolein as standard in distilled water (0.1 – 6.0
201 mM) and MRS broth (0.2 – 10 mM). Both calibration curves showed good linearity
202 with correlation coefficients of >0.999.

203 **2.6 Heat stability of antifungal fermentation products**

204 Heat stability of the antifungal supernatants was determined by subjecting the cfs to
205 100°C for 1 h. Subsequently, samples were cooled immediately and kept in the
206 fridge until further use. Control samples (not heat treated) were kept in the fridge at
207 all times. Analysis of *in vitro* antifungal activity and quantification of antifungal
208 compounds was carried out as described in sections 2.4 and 2.5, respectively but
209 only *F. culmorum* was used as indicator mould.

210 **2.7 Application of cfs in bread system and shelf life analysis**

211 For baking trials, water in the recipe was replaced by the respective LAB cell-free
212 supernatant. For preparation of breads 63% cfs, 3% yeast, 3% oil, 2% salt, 0.5%
213 SSL and 0.1% ascorbic acid (each based on flour weight) were used. Reactivation of
214 the yeast and dough preparation was carried out according to Heitmann et al.
215 (2015). The dough was then divided into 65 g portions, placed in non-stick baking
216 tins (dimensions: top inside, 50 mm x 90 mm; bottom outside, 45 mm x 85 mm;
217 inside depth, 30 mm; Sasa UK, Enfield Middlesex, UK) and proofed for 75 minutes
218 (30°C, 85% RH). Doughs were transferred into the oven (MIWE, Condo, Arnstein,
219 Germany) and baked for 14 minutes at 210°C (top and bottom). Following baking the
220 bread loaves were removed from the tins immediately and cooled to room
221 temperature for at least 120 min before further use.

222 The microbial shelf life test was carried out according to Heitmann et al. (2015). The
223 amount of fungal spoilage was visually recorded over 13 days for each slice, using a
224 calliper to determine the fungal colony size. Based on the percentage of the total
225 surface area of the slice where fungal outgrowth occurred, each slice was
226 categorised every day as A) mould-free, B) <10% covered with mould, C) 11 – 25%
227 covered with mould, D) 26 – 50% covered with mould or E) >50% covered with
228 mould.

229 **2.8 Statistical analysis**

230 Baking trials and all analyses were carried out from three independent fermentations,
231 analysing each sample in duplicate. Statistical analysis was performed using Minitab
232 17 software. Data points were checked for outliers (Grubb's test) and evaluation of
233 significant differences was performed using one-way analysis of variances (ANOVA).
234 All differences were considered significant at $P < 0.05$. Where F-values were
235 significant, pairwise comparisons were carried out with the help of Tuckey Post Hoc
236 test to describe the statistical significance between the different fermentation media
237 and times.

238 **3 Results and Discussion**

239 **3.1 Effect of phenylalanine on antifungal activity and production of** 240 **phenyllactic acid**

241 Fungal isolates of *F. culmorum*, *Aspergillus niger* and *Penicillium expansum* were
242 used as indicator moulds to evaluate the impact of Phe supplementation on the
243 antifungal activity *in vitro* and PLA production. The results obtained are summarised
244 in Table 1. The sole addition of up to 2% Phe (w/v) to MRS broth had no noteworthy

245 effect on the fungal growth in the unfermented medium (0 h fermentation). The PLA
246 quantities for all 0 h fermented samples were below the limit of detection. Thus, all
247 media used for inoculation of the different fungal spores were generally suitable for
248 their growth and any inhibition obtained in the fermented samples was due to the
249 antifungal metabolites produced by *L. reuteri* R29. Concentrations of antifungal
250 carboxylic and phenolic acids were determined for all samples analysed. However,
251 no significant differences ($p < 0.05$), apart from the PLA concentration, was found
252 between any of the samples fermented for equal amounts of time (data not shown).

253 After 24 h of fermentation, all samples were found to contain substantial amounts of
254 PLA. In addition, the PLA quantities were in direct correlation to the amount of Phe
255 initially added. This confirms the findings of Rodríguez et al. (2012), that more PLA is
256 produced when more Phe is available during fermentation. The PLA level of the
257 unsupplemented MRS at this point (24 h) was 38.4 ± 1.3 ppm. When challenged with
258 the fungal spores (10^7 spores/mL) the antifungal activity of the cfs was relatively
259 poor. After 120 h no noteworthy inhibition was detected against either of the three
260 fungal isolates. The cfs obtained through fermentation of MRS+1.5% Phe and
261 MRS+2% Phe resulted after 120 h of incubation in significantly ($p < 0.05$) better
262 inhibition of fungal spore germination, compared to cfs produced from
263 unsupplemented MRS. But, it needs to be mentioned that the supernatants
264 presented stronger inhibition against *F. culmorum* than against *A. niger* and *P.*
265 *expansum*. Hence, it can be concluded that *F. culmorum* is the most sensitive of the
266 three fungal species towards PLA. Similarly, the concentration of PLA in the cfs was
267 found to be 3 times higher compared to the control. Addition of 0.5% or 1.0% Phe
268 was much less efficient in terms of PLA accumulation and antifungal activity.

269 Finally, after 48 h of fermentation, all cfs showed the best spore germination
270 inhibition for the respective medium. The fermentation of unsupplemented MRS
271 resulted in limited inhibition of fungal spore germination over 120 h of incubation.
272 Compared to the unfermented medium, spore inhibition rates of $18.4\pm 1.4\%$,
273 $2.2\pm 0.4\%$ and $3.1\pm 0.4\%$ were found against *F. culmorum*, *A. niger* and *P. expansum*,
274 respectively. In contrast, the addition of 1.0% Phe to the medium led to substantially
275 improved antifungal activity ($60.3\pm 3.2\%$) over 120 h when challenged against *F.*
276 *culmorum*. The most efficient medium composition, in terms of antifungal
277 performance, was the addition of 1.5% Phe. This cfs resulted in $84.1\pm 3.2\%$ inhibition
278 of spore germination against *F. culmorum* after 120 h. When challenged against the
279 other fungal genera, spore germination inhibition was found to be less substantial,
280 yet significantly increased compared to the unsupplemented medium. In contrast, the
281 supplementation with higher amounts of Phe did not further improve the antifungal
282 performance. The addition of less than 1.0% Phe showed no positive effect on the
283 antifungal performance. In general, the quantities of PLA determined for each cfs
284 correlate well with its respective antifungal activity. Thus, up to the addition of 1.5%
285 Phe, the amounts of PLA increased in direct correlation with the level of Phe initially
286 added. But no further increase could be achieved by supplementation with higher
287 quantities of Phe. Furthermore, the concentration of PLA after 48 h of fermentation
288 was always the highest for the respective medium, compared with shorter
289 fermentation times. This also correlates with the findings of Oliveira et al. (2015) who
290 concluded that PLA is produced by resting cells. The reason behind this is the
291 promotion of amino acid metabolism under nitrogen poor conditions (Hazelwood,
292 Daran, van Maris, Pronk, & Dickinson, 2008), which yields the antifungal carboxylic
293 acids, like PLA. Thus, after consuming the majority of nitrogen during the initial

294 growth phase, the resting bacteria have to survive in a relatively nitrogen poor
295 environment. This leads to increased catabolism of Phe and hence the high release
296 of PLA.

297 Furthermore, it was evident that the antifungal activity of cfs obtained from *L. reuteri*
298 R29 was clearly related to the amounts of PLA accumulated in the medium. For all
299 supernatants tested, a significant increase in PLA yield also led to improved
300 antifungal performance. These results are in correlation with previous findings of
301 Oliveira et al. (2015), who reported the antimicrobial activity of PLA and its role as
302 lead antifungal substance produced by *Lactobacillus reuteri* R29. Conversely, Axel et
303 al. (2016) found that it is not necessarily the strain with the highest production of
304 antifungal compounds that shows the best antifungal activity, demonstrating the key
305 role of the type of the antifungal compounds and their synergistic effects.
306 Nevertheless, the increased accumulation of antifungal active compounds by *L.*
307 *reuteri* R29 improves the overall antifungal performance of this particular strain
308 (Valerio, Di Biase, Lattanzio, & Lavermicocca, 2016). Thus, the increased
309 accumulation of PLA, as the lead antifungal compound of *L. reuteri* R29, led to
310 improved antifungal activity of the cfs. This clearly demonstrates the great potential
311 of microbial produced PLA as an antifungal substance.

312 It has to be mentioned however, that MIC₉₀ values of synthetic PLA in acidified MRS
313 against all three fungi tested are more than 10 times higher compared to the
314 amounts detected in the bacterial supernatants showing fungal inhibition. MIC₉₀
315 values determined are 6,000 ppm against *F. culmorum* and 15,000 ppm against *A.*
316 *niger* and *P. expansum* (data not shown). Considering the high concentration of 10⁷
317 fungal spores/mL these values are in good correlation with the findings of

318 Lavermicocca, Valerio, & Visconti (2003). After all, these findings highlight that PLA,
319 although being of major importance for antifungal performance, is not the only
320 component responsible. Instead the synergy of PLA with other bacterial metabolites
321 resulting in antifungal performance is evident.

322 In conclusion, the addition of Phe to the bacterial growth medium resulted in a
323 supernatant which exhibited improved antifungal activity *in vitro* against fungal
324 spores belonging to the genera of *Fusarium*, *Aspergillus* and *Penicillium*. This effect
325 was clearly related to the higher PLA accumulation during LAB fermentation. The
326 capacity to transfer this functionality to a food system remains unclear and will be
327 investigated further in sections 3.3 and 3.4. This would allow the exploitation of the
328 antifungal nature of LAB, particularly in systems that are unsuitable for direct
329 bacterial fermentation. Thus, these results highlight novel and promising
330 perspectives for the use of LAB as bio-preservatives.

331 As the 48 h fermentation of MRS broth supplemented with 1.5% phenylalanine was
332 found to be the best antifungal medium, it was carried forward for further
333 investigation, as discussed in sections 3.3 and 3.4. The medium and supernatant will
334 henceforth be referred to as PMRS and Pcfs, respectively.

335 **3.2 Effect of glycerol and sugar content on antifungal activity and reuterin** 336 **production**

337 Another promising antimicrobial compound with possible application in food systems
338 and produced by certain strains of *Lactobacillus reuteri*, is the multicomponent
339 system reuterin, with 3-hydroxy propionaldehyde (3-HPA) being the main active
340 component. However, to date no study has demonstrated the ability of *Lactobacillus*
341 *reuteri* R29 to produce reuterin via the bioconversion of glycerol. Various

342 combinations of glycerol and glucose in the growth medium were tested, in order to
343 achieve the highest possible reuterin yield and the best antifungal activity *in vitro*
344 against *F. culmorum*. Results for the different cfs, fermented for 0, 24 and 48 h,
345 respectively are summarised in Table 2.

346 Firstly, the results show that all the unfermented media (0 h fermentation) provide a
347 suitable substrate for spore germination of *F. culmorum*. The growth curve obtained
348 for each respective medium was used as reference, in order to evaluate the
349 antifungal activity of the respective fermentation cfs. Furthermore, concentrations of
350 antifungal carboxylic and phenolic acids were determined for all samples, showing
351 no substantial differences between any of the 24 and 48 h fermented samples were
352 found.

353 After 24 h of fermentation, the cfs obtained from unmodified MRS contained no
354 detectable amounts of reuterin, as MRS broth contains no glycerol. However, MRS
355 supplemented with 250 – 1000 mM glycerol and a glucose content of 1.0 – 1.5% led
356 to cfs containing various concentrations of reuterin. Values determined ranged
357 between 12.5 ± 0.1 mM (250 mM glycerol, 1.0% glucose) and 27.5 ± 0.6 mM (500 mM
358 glycerol, 1.5% glucose). Any further increase of glucose in the medium resulted in
359 significantly lower reuterin concentrations, as the data for the media containing 2%
360 glucose (Table 2) demonstrate. This suggests that 1.5% glucose in the initial
361 medium is the ideal amount required for *L. reuteri* R29 to accumulate reuterin. At this
362 level of glucose, the bacteria are able to grow to high cell density in the medium,
363 resulting in a high glycerol conversion to reuterin. However, this concentration is low
364 enough to avoid expression of the 3-HPA reductase responsible for the reductive
365 effect on antifungal activity by removing reuterin from the system through its

366 conversion to 1,3-propanediol. Comparison of reuterin contents for media containing
367 similar amounts of glycerol but varying glucose contents suggests that in presence of
368 sufficient amounts of glucose the 3-HPA reductase is expressed already during the
369 first 24 h of fermentation, preventing the accumulation of reuterin. The ideal amount
370 of added glycerol was found to be 500 mM, as it resulted in the highest reuterin
371 concentrations for each respective level of glucose in the medium. The amount of
372 reuterin produced by *L. reuteri* R29 correlates well with the *in vitro* antifungal activity
373 against fungal spores of *F. culmorum*, *A. niger* and *P. expansum*. As mentioned in
374 section 3.1 the cfs obtained from normal MRS broth showed, due to the high spore
375 concentration, very low levels of inhibition against the three fungi after 120 h of
376 incubation. In contrast, the supernatants containing reuterin presented substantially
377 better spore germination inhibition, demonstrating the antifungal potential of reuterin.
378 The cfs with the highest reuterin concentration (500 mM glycerol, 1.5% glucose)
379 showed total inhibition of *F. culmorum* against 10^7 spores/mL over 96 h of incubation
380 (data not shown), and, after 120 h still had $69\pm 2\%$ fungal inhibition. Similarly to the
381 Pcfs, inhibition of *Aspergillus* and *Penicillium* spores was found to be less substantial
382 but still significant compared to the control (un-supplemented MRS). In conclusion, it
383 is evident from these results that a higher reuterin concentration results in enhanced
384 antifungal performance of the cfs.

385 In contrast, after 48 h of fermentation, the amounts of reuterin and the antifungal
386 performance for each supernatant decreased substantially, compared to the
387 respective 24 h fermented medium. This may be due to the extremely high reactivity
388 of reuterin. As MRS broth is a complex medium, it provides plenty of potential
389 reaction opportunities for reuterin. Furthermore, if the fermentation time is too long
390 the reuterin acts as a self-inhibiting antimicrobial compound against the LAB in the

391 medium (Lüthi-Peng et al., 2002). Although *L. reuteri* R29 was shown to be able to
392 produce and accumulate reuterin, exact control of the fermentation parameters, such
393 as time and medium composition is crucial. As a consequence, cfs obtained after 48
394 h of fermentation showed no significant difference in antifungal performance
395 compared to the unsupplemented control.

396 Overall, the most efficient modification in terms of reuterin yield and *in vitro*
397 antifungal activity against *F. culmorum* was obtained by 24 h fermentation of MRS
398 supplemented with 500 mM glycerol and 1.5% glucose, instead of the conventional
399 2% glucose. This concentration appears to be the best compromise to allow
400 sufficient bacterial growth and at the same time inhibit the expression of 3-HPA
401 reductase. This correlates well with the findings of Lüthi-Peng et al. (2002).
402 Consequently, the modification of the bacterial growth medium to induce reuterin
403 production and accumulation was shown to be a promising method of improving the
404 suitability of *L. reuteri* R29 as a bio-preservative agent.

405 In addition, the possibility of a combined increase in PLA and reuterin accumulation
406 during LAB fermentation was attempted. However, this resulted in a total loss of
407 antifungal activity (data not shown) of the cfs, due to the high reactivity of reuterin
408 with the free amino-group of the Phe, needed as pre-cursor for PLA (Hazelwood et
409 al., 2008).

410 As reuterin has industrial relevance as pre-cursor for the production of acrolein,
411 several studies have been conducted to increase the reuterin production by LAB.
412 These studies solved the problem regarding the carbohydrate content by application
413 of a 2-step fermentation process. Although this process leads to high reuterin
414 accumulation, with the cfs of the first fermentation step, also antifungal compounds

415 produced by *L. reuteri* R29 are removed. Thus, it is uncertain if this process
416 ultimately leads to effective fungal inhibition when applied *in vitro*. Therefore, the 2-
417 step fermentation method of Doleyres et al. (2005) was adopted and tested for both,
418 yield of reuterin and *in vitro* antifungal activity. Here, a reuterin content of up to
419 93.1 ± 2.0 mM was achieved. Although this is much lower than the previously reported
420 176 mM (Doleyres et al., 2005) it equates to a more than 3 fold increase compared
421 to the yield in the 1 step fermentation procedure (27.5 ± 0.6 mM). This relatively low
422 level of reuterin, compared to Doleyres et al. (2005), is most likely due to the use of a
423 different strain of *Lactobacillus reuteri*. When the cfs was applied *in vitro* in the
424 antifungal assay, it resulted in a total inhibition of all fungi tested over 120 h of
425 incubation. Consequently, the significantly increased yield of reuterin in the 2-step
426 fermentation method fully compensated for the loss of carboxylic acids. In fact, the
427 cfs produced by this method had the highest *in vitro* antifungal activity of all the
428 treatments investigated in this study.

429 This 2-step fermented supernatant was further used to determine the MIC₉₀ of
430 reuterin, as no commercial reuterin standards are available. Values found were 4
431 mM against *F. culmorum* and 8 mM against *A. niger* and *P. expansum* (data not
432 shown). These values are in good correlation with the findings of Chung, Axelsson,
433 Lindgren, & Dobrogosz (1989). Furthermore, it highlights the great potential of
434 reuterin as antimicrobial and antifungal agent.

435 In conclusion, *Lactobacillus reuteri* R29 was shown to have the ability to metabolise
436 glycerol to reuterin. This can be achieved using a 1 or 2 step fermentation
437 procedure. Both approaches resulted in very high rates of fungal inhibition, when
438 challenged against 10^7 spores/mL over 120 h (Table 2). Thus, the modification of the

439 fermentation process of *L. reuteri* R29 towards the production of reuterin shows the
440 potential for bio-preservation. But the need to control parameters such as glucose
441 and glycerol content, and fermentation time carefully, to allow accumulation of
442 reuterin has to be kept in mind. For a successful application in a food system, the
443 high reactivity of reuterin might impose further challenges. This applies in particular,
444 if heat treatment is involved, as this would further increase the reactivity of reuterin.
445 Furthermore, there is the risk of acrolein formation due to thermal dehydration
446 (Gänzle, 2015).

447 **3.3 Heat stability of PLA and reuterin**

448 In order to get a more detailed picture with respect to the possible applications of the
449 various antifungal cell-free supernatants obtained in sections 3.1 and 3.2, three
450 selected media were subjected to heat treatment. In particular for use in food
451 systems, a certain heat resistance would broaden the range of possible applications
452 immensely. Therefore, the impact of heat treatment (1 h at 100°C) on PLA and
453 reuterin content, and on the antifungal performance *in vitro* was determined, using *F.*
454 *culmorum* as indication strain.

455 For the PcfS, no significant reduction of inhibitory capacity against *F. culmorum* was
456 found. Even after 120 h the heat treated supernatant resulted in 80±3% of fungal
457 growth inhibition, compared to 84±3% for the not heat treated cfs (Figure 1A).
458 Chromatographic analysis of the heat treated supernatant further revealed no
459 substantial loss in PLA, compared to the unheated one (data not shown).
460 Consequently, this cfs can be considered as heat stable with regards to the
461 antifungal performance and the concentration of its lead antifungal substance, PLA.
462 This result is in good correlation with the findings of Cortés-Zavaleta, López-Malo,

463 Hernández-Mendoza, & García (2014). Thus, the fermentation of Phe enriched
464 medium by *L. reuteri* R29 appears to be very promising for the production of
465 antifungal cfs and use as a bio-preservative in food systems.

466 For the reuterin-containing supernatants different results were obtained, depending
467 on the fermentation method. For the cfs produced with the 2-step fermentation
468 procedure, no substantial reduction in antifungal performance (Figure 1B) or reuterin
469 content (Figure 1C) was observed. However, heat treatment of the cfs produced by
470 the 1-step fermentation procedure resulted in a complete loss of antifungal activity
471 (Figure 1D). Likewise, the concentration of reuterin in the cfs decreased from
472 27.5 ± 0.6 mM to 0.5 ± 0.1 mM, due to the heating process (Figure 1C). The reason for
473 these differences is in the constituents of the respective supernatant, as heating
474 primarily increases the reactivity of reuterin (Vollenweider, Evers, Zurbruggen, &
475 Lacroix, 2010). The 2-step fermentation produces a cfs that consists only of water,
476 glycerol and reuterin, and thus no adequate reactive partner is present to sequester
477 reuterin. Hence, the antifungal activity of this cfs was not notably effected by the
478 heating process. On the other hand, the 1-step fermentation produces a supernatant,
479 containing all the ingredients of MRS broth and several metabolites produced by the
480 bacteria which are potential reactive partners for the reuterin. Consequently, the
481 increase in reactivity due to heating resulted in a substantial decrease in reuterin
482 content and hence severely reduced antifungal activity.

483 To examine this topic further and evaluate the antifungal activity *in situ*, the reuterin
484 enriched (1- and 2-step fermentation process) and the Pcfs were applied into a
485 model system for food production that involves heat treatment, the bread making
486 process.

487 **3.4 Effect of PLA and reuterin on bread microbial shelf life**

488 In order to investigate the antifungal activity *in situ*, it was attempted to transfer and
489 exploit the increased antifungal activity (*in vitro*) in the food processing chain, using
490 bread making as an example. Therefore, the water in the dough preparation was
491 replaced with the cell-free supernatants and the effect on microbial shelf life was
492 tested by challenge against environmental fungi. Thus, use of the *in vitro* fermented
493 cfs enabled comparison of the *in situ* antifungal performance of the different
494 supernatants without the influence of other ingredients on the bacterial growth.

495 As demonstrated in previous studies the application of LAB fermented media into a
496 cereal food matrix is possible (Le Lay et al., 2016; Peyer et al., 2016; Russo et al.,
497 2015; Saladino et al., 2016). Furthermore, the routinely use of LAB as starter
498 cultures for sourdough bread shows that the acidity has no substantial negative
499 impact on the product quality and taste (Pawlowska et al., 2012). Hence, application
500 of bacterial cfs, despite the strong acidic pH, as promising perspective for natural
501 extension of microbial shelf-life.

502 As shown in Figure 2A, the use of cfs obtained by fermentation of normal MRS (24
503 h) resulted in a microbial shelf life of 5 days. After 13 days of storage 83% of the
504 bread slices contained mould colonies (category B), whereas the remaining 17%
505 were still completely mould free. A very similar result was achieved for the same
506 medium, fermented for 48 h (Figure 2B). The microbial shelf life was determined to 4
507 days and after 13 days of storage 17% of the slices were still mould free. However,
508 the mould was found to spread slightly faster, than with the 24 h fermented
509 supernatant, as there were 6.5% of the slices in the “C” category (11 – 24% mouldy)
510 and the rest in “B”. This outcome is interesting, as the antifungal activity *in vitro* was

511 evident after 48 h of fermentation. However, as the breads were challenged against
512 environmental fungi and not just one specific strain, variations in the antifungal
513 performance are possible. In particular, as the antifungal performance is based on a
514 synergistic mechanism, the amount of organic acids produced cannot serve as sole
515 indicator for antifungal activity. Thus, it is possible that the mixture of antifungal
516 compounds present after 24 h is more effective against certain environmental fungi
517 than after 48 h. These samples, served as controls in order to evaluate if the
518 increased antifungal activity as evident in *in vitro* trials could also translated *in situ*.

519 In comparison, the Pcfs (24 h, Figure 2C) did not significantly increase the shelf life,
520 compared to the respective control (MRS/24 h). The microbial shelf life was
521 determined to be 5 days and after 13 days, 19% of the slices were still mould free. At
522 the same time, 6.5% of the slices were mouldy to more than 10%. These differences
523 to the 24 h fermented control are not statistically significant ($p < 0.05$). Thus, the 24 h
524 fermentation of Phe enriched MRS did not increase the antifungal performance *in*
525 *situ*. In contrast, after 48 h of fermentation, the Pcfs led to substantially increased
526 shelf life (Figure 2D) when compared to the respective control. The shelf life
527 increased from 4 to 8 days (100%), due to the use of Pcfs. In addition, the spread of
528 fungal outgrowth was found to be notably retarded and the number of mouldy slices
529 after 13 days of monitoring was, with 53%, significantly reduced also. This correlates
530 well with the results of the *in vitro* assays for the Pcfs against *F. culmorum*. These
531 results strengthen the conclusions previously made by Crowley et al. (2013), who
532 reported that LAB are, due to their antimicrobial acids, very promising candidates as
533 food bio-preservatives. In addition, this result demonstrates the broad antifungal
534 activity of PLA against various spoilage organisms *in situ*. Thus, the results obtained
535 in this study further prove the suitability of *L. reuteri* R29 as natural food

536 preservative, using bread making as an example of such an application. The use of
537 *L. reuteri* R29 as bio-preservative, in particular with emphasis on high PLA
538 production appears to be a promising alternative to conventional preservatives.

539 Despite showing less promising results *in vitro* after heat treatment, the reuterin
540 containing 1- and 2-step fermentations were also applied in the baking process. The
541 respective 24 h and 48 h 1-step fermented supernatants (Figures 2E and F,
542 respectively) were found to result in a shelf life increase of 1 day, compared to their
543 respective controls. Also the spread of the fungal outgrowth appeared to be slower.
544 However, these improvements were not statistically significant ($p < 0.05$). Due to the
545 high reactivity of reuterin, in particular at high temperatures as shown in section 3.3,
546 it is likely that the reuterin reacted with other dough constituents. Thus, when the
547 microbial challenge test was carried out, no noteworthy levels of active reuterin were
548 present. This indicates that the breads obtained from this supernatant should
549 perform similar to the control in terms of microbial shelf life, as the normally
550 produced antifungal acids were present in both control and 1-step fermentation.
551 Hence, the outcome of the shelf life test, where no significant difference to the
552 control was found, is in good correlation with the previous results, as discussed in
553 section 3.3.

554 Finally, the 2-step fermented cfs showed the least antifungal activity of all samples,
555 when applied to the baking process (Figure 2G). The shelf life decreased
556 substantially by 2 days, compared to the 24 h MRS control. The fungal spread was
557 also much faster than in any other sample, resulting in 42% of the bread slices being
558 covered to more than 50% by fungi (category "E"), after 13 days of monitoring. Thus,
559 the cfs obtained from the 2-step fermentation was found to be completely unsuitable

560 for *in situ* microbial preservation in bread. The explanation for this, again, is related
561 to the high reactivity of reuterin, in particular at high temperatures. Although this
562 supernatant showed heat stability when tested *in vitro*, the dough matrix is very
563 complex and thus provides plenty of reaction opportunities for the reuterin. In
564 particular, free thiol groups which are the main target in the inactivated microbes
565 present in the flour can interfere with the reuterins antimicrobial performance.
566 Despite the fact that levels of free thiol groups in a dough formulation as it was used
567 here are 10 – 100 times below the reuterin concentrations (Kohler, 2003; Reinbold,
568 Rychlik, Asam, Wieser, & Koehler, 2008) they are likely to compromise the antifungal
569 activity significantly (Engels et al., 2016). Furthermore, the production process of this
570 cfs eliminates the other metabolites produced by *L. reuteri* R29, such as antifungal
571 acids. As a consequence, by losing the reuterin due to reactions with other dough
572 constituents, no active antifungal compounds were present anymore. Hence, the
573 outcome of an even further decreased shelf life, compared to the control. The results
574 of this study clearly demonstrate that reuterin is not suitable as food preservative if it
575 is subjected to heating. However, it may still be suitable for unheated foods like
576 salami or cheese, for which it has already been successfully applied (Gomez-Torres
577 et al., 2014; Ortiz-Rivera et al., 2017).

578 4. CONCLUSIONS

579 In conclusion, this study demonstrates three possibilities for improving the efficiency
580 of antifungal LAB *in vitro*, using *Lactobacillus reuteri* R29 as an example. Further
581 understanding regarding production and stability of antifungal compounds was
582 obtained. In particular, the key role of PLA for the antifungal performance of *L. reuteri*
583 R29 became evident. However, from the MIC₉₀ values of synthetic PLA it also

584 became evident that microbial PLA just in synergy with other bacterial metabolites
585 can serve as efficient antifungal agent. The results achieved *in vitro*, could only partly
586 be transferred into the bread making process. Reuterin, due to its high reactivity, in
587 particular at high temperatures (Vollenweider et al., 2010), did not lead to
588 satisfactory results *in situ*. In contrast, the supplementation with Phe, to increase the
589 production of PLA, was found to be very efficient in both *in vitro* and *in situ*. Hence,
590 Phe supplemented fermentation media should be considered as promising options to
591 improve the antimicrobial performance of LAB during production of food, such as
592 bread or beverages. The proteolytic activity reported for *L. reuteri* R29 (Axel et al.,
593 2016) also proposes the possibility to achieve this antifungal effect upon
594 supplementation with Phe rich proteins. This work shows the potential for further
595 exploitation of LAB as bio-preservatives, particularly in environments unsuitable for
596 bacterial fermentation, for example, during grain storage. This demonstrates the
597 potential to enlarge the field of application for the antimicrobial properties of LAB.
598 Further research is required on the *in situ* production of PLA, including its stability
599 and influence on sensory parameters. In addition, application of such methodologies
600 investigated in this study in further food systems will serve to increase our
601 knowledge in this increasingly pertinent area.

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ACCEPTED MANUSCRIPT

726 **Table 1: Concentration (ppm) of phenyllactic acid (PLA) and antifungal activity over 120 h (% of growth inhibition) of cell-free supernatants of**
 727 ***Lactobacillus reuteri* R29 grown in MRS for 0, 24 or 48 h at 37°C in presence of various amounts of phenylalanine.#**

Medium	fermentation time [h]	PLA [ppm]	antifungal activity after 120 hours of incubation [% growth inhibition against 10 ⁷ spores/mL]		
			<i>P. expansum</i>	<i>A. niger</i>	<i>F. culmorum</i>
MRS	0	_a	_a	_a	_a
	24	38.4±1.3 ^b	1.2±0.2 ^b	0.2±0.1 ^a	1.5±0.2
	48	85.3 ±1.1	3.1±0.4 ^c	2.2±0.4 ^b	18.4±1.4 ^c
MRS + 0.5% Phe	0	_a	_a	_a	_a
	24	41.6±1.8 ^b	1.4±0.1 ^b	3.2±0.5 ^{bc}	3.4±2.2 ^{bc}
	48	103.2±2.3	3.6±0.3 ^c	3.7±1.4 ^c	14.1±2.8 ^c
MRS + 1.0% Phe	0	_a	_a	_a	_a
	24	57.5±1.5	2.2±0.3	2.8±1.4 ^b	6.1±2.5 ^c
	48	237.1±1.4	16.5±1.3	24.7±3.7	50.3±3.2
MRS + 1.5% Phe	0	_a	_a	_a	_a
	24	116.4±1.3 ^c	8.3±0.8	11.0±0.5	25.4±3.1 ^d
	48	361.2±2.4 ^d	32.7±0.2 ^d	63.5±2.1 ^d	84.1±3.2 ^e
MRS + 2.0% Phe	0	_a	_a	_a	_a
	24	113.2±1.8 ^c	14.1±2.5	16.0±1.1	27.1±2.1 ^d
	48	363.8±2.0 ^d	33.1±0.6 ^d	62.9±1.6 ^d	83.5±2.2 ^e

728 # Results shown are mean values ± confidence interval. Values in one column followed by the same lower case letter are not significantly different, values
 729 without letter of significance are significantly different from all other values in the same column (p<0.05)

730 **Table 2: Concentration of reuterin (mM) and antifungal activity (% growth inhibition) against**
 731 ***Penicillium expansum*, *Aspergillus niger* and *Fusarium culmorum* of cell-free supernatants of**
 732 ***Lactobacillus reuteri* R29 grown in various MRS formulations, containing 1.0 – 2.0% of glucose**
 733 **and 250 – 1000mM of glycerol, for 0, 24 and 48h (1-step fermentation process) and for the 2-**
 734 **step fermentation process (2h in water/glycerol).#**

Medium fermented by <i>L. reuteri</i> R29	fermentation time [h]	reuterin [mM]	antifungal activity [% growth inhibition against 10 ⁷ <i>F. culmorum</i> spores/mL]		
			<i>P. expansum</i>	<i>A. niger</i>	<i>F. culmorum</i>
Control (MRS)	0	–a	–a	–a	–a
	24	–a	0.2±0.1	0.5±0.1 ^b	0.3±0.2 ^a
	48	–a	3.1±0.7 ^b	8.2±0.4 ^c	18.4±1.4 ^b
MRS + 250mM glycerol; 1.0% glucose	0	–a	–a	–a	–a
	24	12.5±0.1	18.3±1.7 ^d	22.5±0.2 ^d	39.5±2.7
	48	–a	2.1±0.4 ^b	8.9±0.6 ^c	8.4±1.2 ^c
MRS + 250mM glycerol; 1.5% glucose	0	–a	–a	–a	–a
	24	20.5±1.1 ^b	17.0±1.2 ^d	24.5±1.3 ^d	55.8±3.1 ^d
	48	0.4±0.1	3.5±0.4 ^b	3.5±0.6 ^e	8.7±2.1 ^c
MRS + 250mM glycerol; 2% glucose	0	–a	–a	–a	–a
	24	0.9±0.2	4.5±0.2 ^e	11.1±1.9 ^f	15.7±2.6 ^e
	48	–a	2.2±0.3 ^b	1.8±0.5	14.6±0.1 ^e
MRS + 500mM glycerol; 1.0% glucose	0	–a	–a	–a	–a
	24	18.6±0.3	35.6±1.6	21.8±0.1 ^d	58.7±2.3 ^d
	48	0.2±0.1 ^a	2.4±0.2 ^b	3.3±0.3 ^e	8.8±0.6 ^c
MRS + 500mM glycerol; 1.5% glucose	0	–a	–a	–a	–a
	24	27.5±0.6 ^c	55.0±2.1	64.9±1.8	69.1±0.4
	48	1.2±0.3 ^d	3.1±1.1 ^b	4.5±0.4 ^e	17.9±2.1 ^{be}
MRS + 500mM glycerol; 2% glucose	0	–a	–a	–a	–a
	24	1.3±0.2 ^d	8.4±0.5	7.6±0.9	19.1±1.5 ^e
	48	–a	2.8±0.7 ^b	2.2±0.5 ^g	16.3±0.4 ^e
MRS + 1000mM glycerol; 1.0% glucose	0	–a	–a	–a	–a
	24	21.5±1.4 ^b	14.7±1.0	13.4±0.2 ^f	57.1±0.5 ^d
	48	0.3±0.1 ^a	–a	0.2±0.1 ^b	–a
MRS + 1000mM glycerol; 1.5% glucose	0	–a	–a	–a	–a
	24	26.6±0.3 ^c	26.5±1.1	28.5±1.5 ^d	52.9±2.4 ^d
	48	0.5±0.4 ^a	1.0±0.1 ^c	1.4±0.2 ^h	18.6±1.9 ^{be}
MRS + 1000mM glycerol; 2% glucose	0	–a	–a	–a	–a
	24	1.4±0.1 ^d	3.6±0.7 ^e	2.6±0.1 ^g	18.1±1.6 ^{be}
	48	–a	0.8±0.2 ^c	1.2±0.2 ^h	17.5±1.2 ^{be}
Medium (2-step fermentation process)	fermentation time [h]	reuterin [mM]	antifungal activity [% growth inhibition against 10 ⁷ <i>F. culmorum</i> spores/mL]		
			<i>P. expansum</i>	<i>A. niger</i>	<i>F. culmorum</i>

distilled water + 400mM glycerol	0 (control) 2 (sample)	_{-a} 93.1±2.0	_{-a} 100±0	_{-a} 100±0	_{-a} 100±0
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736 lower case letter are not significantly different, values without letter of significance are significantly
737 different from all other values in the same column (p<0.05)

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739 **Figure 1:** *In vitro* antifungal activity against *F. culmorum* in A) MRS + 1.5% Phe (48h
740 fermented), B) 2-step fermentation supernatant, C) impact of heat treatment on
741 reuterin content of the cell-free supernatants and D) MRS + 500mM glycerol; 1.5%
742 glucose (24h fermented).

743 **Figure 2:** Shelf life of wheat bread against environmental mould during a 13-day
744 storage period is indicated as the percentage of the total surface area of each slice,
745 where fungal growth occurred. Mould-free slices (A), <10% mouldy (B), 10-24%
746 mouldy (C), 25-49% mouldy (D) and >50% mouldy (E). Mean values are shown
747 (n=3); error bars indicate standard deviations.

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