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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

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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ABSTRACT

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

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

1 Introduction

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

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

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

58 phenylpyruvic acid is then reduced to PLA by a dehydrogenase (Vermeulen, Gánzle,
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.,

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

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

2 Materials and Methods

2.1 Materials

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

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

2.2 Cultures, media and growth conditions

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

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

2.3 Production of LAB cell-free supernatants

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

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

2.4 Evaluation of *in vitro* antifungal activity and determination of minimal inhibitory concentration (MIC_{90})

Pre-trials were carried out to determine the ideal fungal spore concentration to investigate the increase of antifungal performance in the respective cell-free supernatants. Thereby, a concentration of 10^6 spores/mL as applied in the assay described were chosen for the subsequent experiments (data not shown). To determine the *in vitro* antifungal activity, 1 mL of *Fusarium culmorum, Aspergillus niger* or *Penicillium expansum* spore solution (containing approximately 10^6 spores) was transferred into a 2 mL microcentrifuge tube and centrifuged at 3,000 *g* (10 min, 4°C). The supernatant was discarded and the spore pellet resuspended in 1.0 mL of diluted LAB cfs (10%, v/v, in unfermented MRS). Subsequently, 200 µL aliquots were pipetted into a 96-well microplate (Sarstedt AG and Co, Nurembrecht, Germany). To control for the effect of condensation, each supernatant was also inoculated with 1 mL of sterile synthetic nutrient-poor bouillon, instead of fungal spore solution (blank). The microplate was sealed with an optically clear seal for Q-PCR (Thermo Scientific, Waltham, USA) and incubated in a Multiskan FC microplate-reader (Thermo Scientific, Waltham, USA) for 5 days at 25°C. The optical density was recorded at 620 nm (OD620) every 2 h, with agitation in 4 s intervals. Values of each cfs 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 MIC_{90} 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 MIC_{90}. The
159 MIC_{90} 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**

163 **2.5.1 Determination of carboxylic antifungal compounds**

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 µm; Agilent, Cork,
175 Ireland), equipped with a guard column (50×7.7 mm, 8 µm; Agilent, Cork, Ireland)

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

Antifungal phenolic compounds were extracted, using a QuEChERS approach, as described by Peyer et al. (2016). Separation was carried out using a Gemini C_{18} column (150 x 2.0 mm, 5 µm; Phenomenex, Macclesfield, UK), equipped with a guard column (AF0-8497; Phenomenex, Macclesfield, UK). The mobile phase consisted of A) H_2O with 0.1% FA and B) ACN with 0.1% FA. To ensure separation from other compounds, a gradient flow was performed (0 – 5 min – 90% A, 30 min – 80% A, 35 min – 60% A, 45 min – 5% A, 45 – 70 min – 90% A), using a flow rate of 0.2 mL/min at a temperature of 30°C. The volume injected was 10 µL and detection carried out at a wavelength of 210 nm.

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

2.5.3 Colorimetric assay for reuterin quantification

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

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

2.6 Heat stability of antifungal fermentation products

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

2.7 Application of cfs in bread system and shelf life analysis

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

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

2.8 Statistical analysis

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

3 Results and Discussion

3.1 Effect of phenylalanine on antifungal activity and production of phenyllactic acid

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

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

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

Finally, after 48 h of fermentation, all cfs showed the best spore germination inhibition for the respective medium. The fermentation of unsupplemented MRS resulted in limited inhibition of fungal spore germination over 120 h of incubation. Compared to the unfermented medium, spore inhibition rates of 18.4±1.4%, 2.2±0.4% and 3.1±0.4% were found against *F. culmorum*, *A. niger* and *P. expansum*, respectively. In contrast, the addition of 1.0% Phe to the medium led to substantially improved antifungal activity (60.3±3.2%) over 120 h when challenged against *F. culmorum*. The most efficient medium composition, in terms of antifungal performance, was the addition of 1.5% Phe. This cfs resulted in 84.1±3.2% inhibition of spore germination against F. culmorum after 120 h. When challenged against the other fungal genera, spore germination inhibition was found to be less substantial, yet significantly increased compared to the unsupplemented medium. In contrast, the supplementation with higher amounts of Phe did not further improve the antifungal performance. The addition of less than 1.0% Phe showed no positive effect on the antifungal performance. In general, the quantities of PLA determined for each cfs correlate well with its respective antifungal activity. Thus, up to the addition of 1.5% Phe, the amounts of PLA increased in direct correlation with the level of Phe initially added. But no further increase could be achieved by supplementation with higher quantities of Phe. Furthermore, the concentration of PLA after 48 h of fermentation was always the highest for the respective medium, compared with shorter fermentation times. This also correlates with the findings of Oliveira et al. (2015) who concluded that PLA is produced by resting cells. The reason behind this is the promotion of amino acid metabolism under nitrogen poor conditions (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008), which yields the antifungal carboxylic 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 MIC_{90} 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. MIC_{90}
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 10^7
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±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 (unsupplemented 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

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

This 2-step fermented supernatant was further used to determine the MIC_{90} of reuterin, as no commercial reuterin standards are available. Values found were 4 mM against *F. culmorum* and 8 mM against *A. niger* and *P. expansum* (data not shown). These values are in good correlation with the findings of Chung, Axelsson, Lindgren, & Dobrogosz (1989). Furthermore, it highlights the great potential of reuterin as antimicrobial and antifungal agent.

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

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

3.3 Heat stability of PLA and reuterin

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

For the Pcfs, no significant reduction of inhibitory capacity against *F. culmorum* was found. Even after 120 h the heat treated supernatant resulted in 80±3% of fungal growth inhibition, compared to 84±3% for the not heat treated cfs (Figure 1A). Chromatographic analysis of the heat treated supernatant further revealed no substantial loss in PLA, compared to the unheated one (data not shown). Consequently, this cfs can be considered as heat stable with regards to the antifungal performance and the concentration of its lead antifungal substance, PLA. 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, Zurbriggen, &
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.

3.4 Effect of PLA and reuterin on bread microbial shelf life

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

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

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

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

4. CONCLUSIONS

In conclusion, this study demonstrates three possibilities for improving the efficiency of antifungal LAB *in vitro*, using *Lactobacillus reuteri* R29 as an example. Further understanding regarding production and stability of antifungal compounds was obtained. In particular, the key role of PLA for the antifungal performance of *L. reuteri* R29 became evident. However, from the MIC_{90} 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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Table 1: Concentration (ppm) of phenyllactic acid (PLA) and antifungal activity over 120 h (% of growth inhibition) of cell-free supernatants of *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]		
			P. expansum	*A. niger*	*F. culmorum*
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]

[#] Results shown are mean values ± confidence interval. Values in one column followed by the same lower case letter are not significantly different, values 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 *L. reuteri* R29	fermentation time [h]	reuterin [mM]	antifungal activity [% growth inhibition against 10^7 *F. culmorum* spores/mL]		
			P. expansum	*A. niger*	*F. culmorum*
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 *F. culmorum* spores/mL]		
			P. expansum	*A. niger*	*F. culmorum*

distilled water + 400mM glycerol	0 (control)	-[a]	-[a]	-[a]	-[a]
	2 (sample)	93.1±2.0	100±0	100±0	100±0

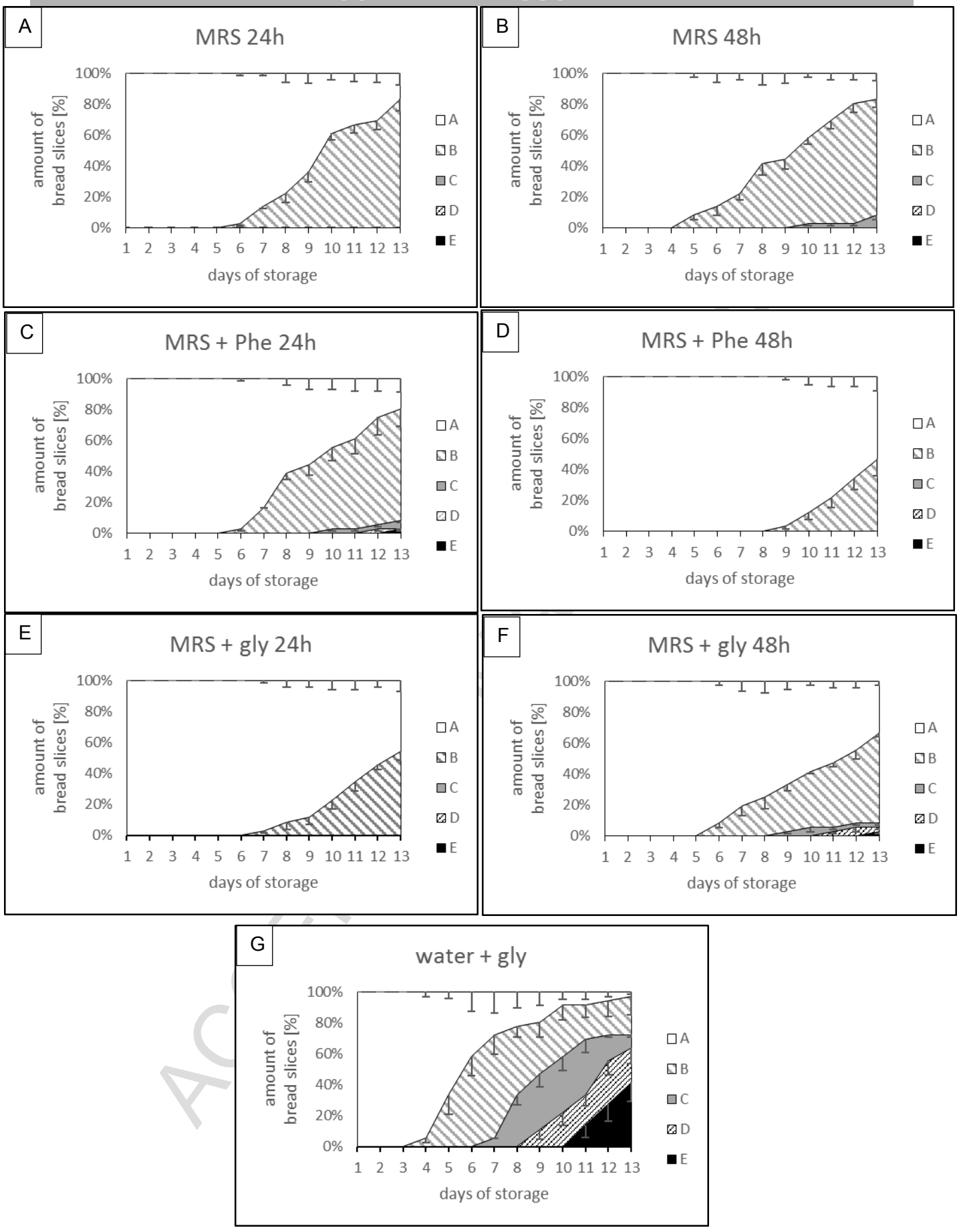
735 # Results shown are mean values ± confidence interval. Values in one column followed by the same
736 lower case letter are not significantly different, values without letter of significance are significantly
737 different from all other values in the same column ($p<0.05$)

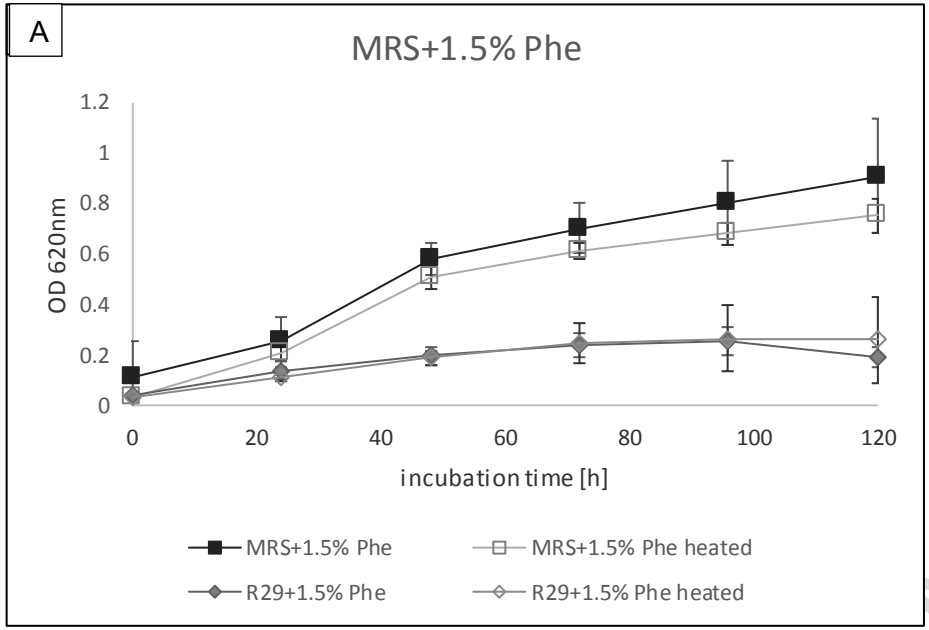
738

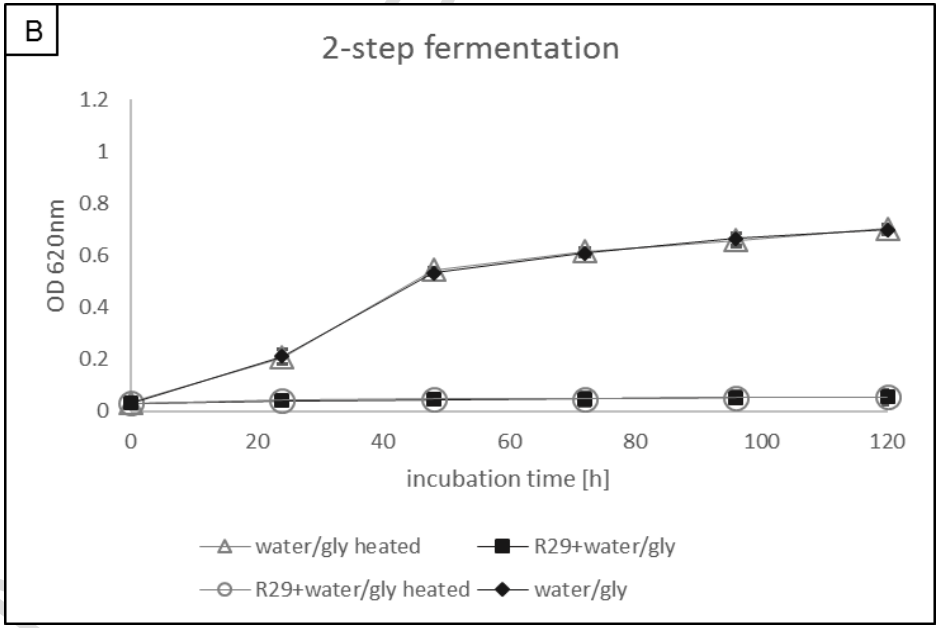
739 **Figure 1:** *In vitro* antifungal activity against *F. culmorum* in A) MRS + 1.5% Phe (48h
740 fermented), B) 2-step fermentation supernatant, C) impact of heat treatment on
741 reuterin content of the cell-free supernatants and D) MRS + 500mM glycerol; 1.5%
742 glucose (24h fermented).

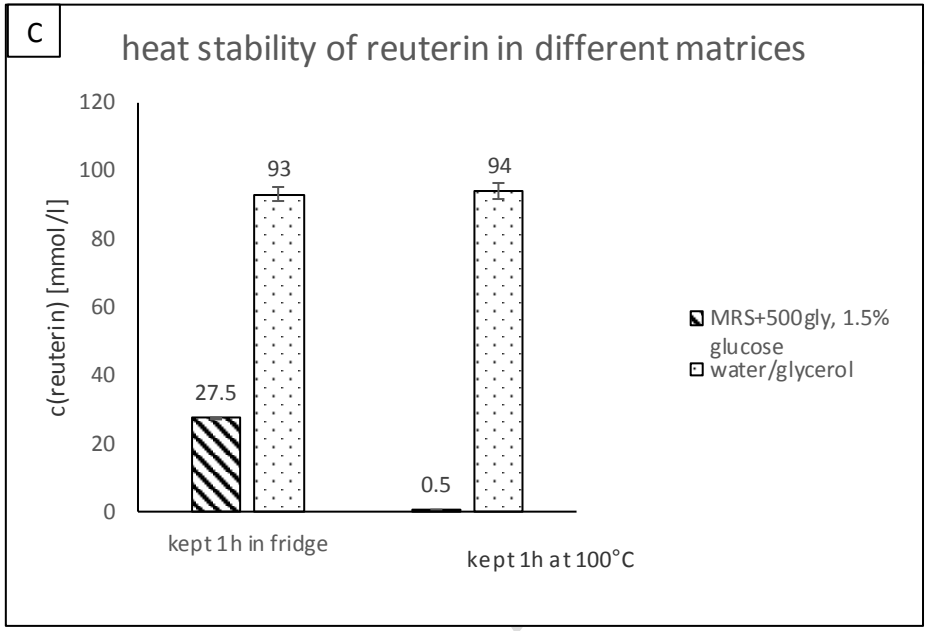
743 **Figure 2:** Shelf life of wheat bread against environmental mould during a 13-day
744 storage period is indicated as the percentage of the total surface area of each slice,
745 where fungal growth occurred. Mould-free slices (A), <10% mouldy (B), 10-24%
746 mouldy (C), 25-49% mouldy (D) and >50%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

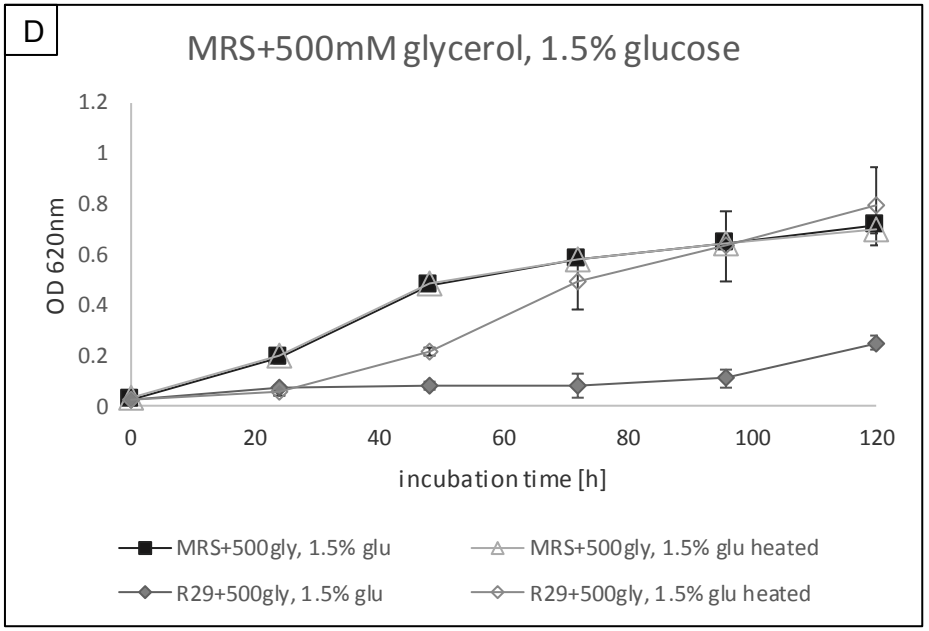
748




A
MRS+1.5% Phe
OD 620nm
incubation time [h]
MRS+1.5% Phe
MRS+1.5% Phe heated
R29+1.5% Phe
R29+1.5% Phe heated


B
2-step fermentation
OD 620nm
incubation time [h]
water/gly heated
R29+water/gly
R29+water/gly heated
water/gly


C
heat stability of reuterin in different matrices
c(reuterin) [mmol/l]
27.5
93
0.5
94
kept 1h in fridge
kept 1h at 100°C
MRS+500gly, 1.5% glucose
water/glycerol


D
MRS+500mM glycerol, 1.5% glucose
OD 620nm
incubation time [h]
MRS+500gly, 1.5% glu
MRS+500gly, 1.5% glu heated
R29+500gly, 1.5% glu
R29+500gly, 1.5% glu heated