



13

## ABSTRACT

14 Fungal spoilage limits the shelf life of fermented dairy products. To address the problem,  
15 this study explores the potential of lactic acid bacteria as antifungal adjunct cultures in  
16 dairy matrices. Strains of lactic acid bacteria (113) representing 19 species were screened  
17 for their activity against *Penicillium caseifulvum*, *Aspergillus clavatus* and *Mucor*  
18 *racemosus* in modified MRS medium, milk, and yogurt. Strains of *Lactiplantibacillus*  
19 *plantarum*, *Furfurilactobacillus milii*, and *Lentilactobacillus parabuchneri* inhibited the  
20 growth of mycelial fungi. The inhibitory effects of lactic acid bacteria against yeasts were  
21 also determined in yogurt with *Candida sake*, *Saccharomyces bayanus*, and *Torulaspora*  
22 *delbrueckii* as challenge strains. The inhibition of yeasts by lactic acid bacteria was strain-  
23 specific and unrelated to the activity towards mycelial fungi. Organic acids and hydroxy  
24 fatty acids were quantified by liquid chromatograph coupled with refractive index detector  
25 and tandem mass spectrometry, respectively. Principal component analysis indicated 10-  
26 OH 18:1 fatty acids and acetate are the main antifungal metabolites and explained over 50%  
27 of the antifungal activity. The correlation analysis of metabolites and mold-free shelf life  
28 of milk and yogurt confirmed the role of these compounds. The genomic study analysed  
29 genes related to the production of major antifungal metabolites and predicted the formation  
30 of 1,2-propanediol and acetate but not of hydroxy unsaturated fatty acids. The findings  
31 provide new perspectives on the selection of antifungal strains, the characterization of  
32 antifungal metabolites and the exploration of antifungal mechanisms among different  
33 species.

34 **KEYWORDS:** Antifungal, Dairy products, Lactic acid bacteria, Metabolites, Genome.

35

## 36 1 INTRODUCTION

37 Yeasts and molds are the major spoilage organisms of fermented dairy products that  
38 account for most of the dairy consumption in Canada in 2020 (Anonymous, n.d.). Yeasts  
39 and mycelial fungi grow in refrigerated fermented dairy products at low pH and at low aw.  
40 Fungal growth generates off-odors and changes the appearance of the products (Ledenbach  
41 and Marshall, 2009; Pitt and Hocking, 2009). Yeasts and molds including *Penicillium*  
42 *camemberti* and *Debaromyces hansenii* are also used in cheese manufacture as surface  
43 ripening cultures; however, in any product that is not ripened by surface cultures, the  
44 growth of these molds and yeasts constitutes spoilage (Lessard et al., 2012).

45 In food production, preventative control plans that include HACCP aim to reduce  
46 contamination, and the use of hurdle technologies aims to limit the growth of molds and  
47 yeasts after contamination has occurred. The latter includes post-packaging heat treatments,  
48 control of water activity, vacuum packaging, or the addition of preservatives to control  
49 fungal contaminants. However, most of these methods have limited use in fermented dairy  
50 products (Garnier et al., 2017; Snyder and Worobo, 2018). Lactic acid bacteria (LAB) with  
51 antifungal activity have been explored for control of fungal spoilage in dairy products.  
52 Screening of LAB for strains exhibiting antifungal activity has demonstrated that  
53 *Lactiplantibacillus plantarum*, *Lacticaseibacillus rhamnosus* and *Lacticaseibacillus casei*  
54 include strains with antifungal activity (Delavenne et al., 2012; Fernandez et al., 2017; Xu  
55 et al., 2021). However, these homofermentative lactobacilli do not represent the metabolic  
56 diversity of food-fermenting LAB (Gänzle, 2015) or the diversity of organisms that are  
57 used as starter cultures or adjunct cultures in fermented dairy products (Bourdichon et al.,

58 2019; Gänzle, 2015; Hutkins, 2019). Antifungal LAB exert inhibitory effects through the  
59 competition for nutrients (Hibbing et al., 2010) or through the production of antifungal  
60 metabolites (Siedler et al., 2019). Dairy products are nutrient rich matrices and readily  
61 support microbial growth but manganese depletion by *Lc. rhamnosus* and *Lc. paracasei*  
62 restricted growth of yeasts and fungi in yogurt (Siedler et al., 2020). Metabolites of LAB  
63 with antifungal activity include acetic acid, propionic acid, reuterin, diacetyl, cyclic  
64 dipeptides, and hydroxy fatty acids (Axel et al., 2017). Several of these compounds  
65 including acetic acid, propionic acid and diacetyl are also flavor volatiles and their flavor  
66 threshold concentration is lower than the minimum inhibitory concentration against fungi;  
67 i.e. concentrations that are active against fungi also beneficially or adversely impact the  
68 flavor of products (Siedler et al., 2019). Glycerol metabolism and reuterin production by  
69 *Lm. reuteri* inhibits clostridia that cause the late-blowing effect of cheeses (Gómez-Torres  
70 et al., 2014) but its efficacy against fungal spoilage of dairy products has not been evaluated.  
71 For other metabolites including cyclic dipeptides and hydroxy fatty acids, it remains  
72 unclear whether they accumulate to active concentrations in dairy fermentations.

73 Analysis of antifungal compounds in milk fermentates produced with *Lc. rhamnosus* and  
74 *Acidipropionibacterium jensenii* identified propionic, acetic and butyric acids as the most  
75 abundant antifungal compounds (Garnier et al., 2020). In addition, a 9-amino acid fragment  
76 from casein with antifungal activity was identified and its activity was validated *in vitro*  
77 (Garnier et al., 2020). Correlation of the antifungal effect of lactobacilli that were used as  
78 an adjunct culture in yogurt, cheese and sour cream with the concentration of metabolites  
79 identified acetic acid, diacetyl, phenylacetate and medium chain fatty acids as potential  
80 contributors to antifungal activity (Leyva Salas et al., 2019). The concentration of all of

81 these compounds in yogurt, sour cream or cheese, however, was considerably lower than  
82 their *in vitro* MICs (Leyva Salas et al., 2019). Collectively, these studies indicate that  
83 antifungal activity of LAB in dairy products is based on synergistic or additive activity of  
84 several compounds that are present in concentration below their MIC. The presence of  
85 long-chain hydroxy unsaturated fatty acids (HUFA), that are among the most relevant  
86 antifungal metabolites accumulating in cereal fermentations (Black et al., 2013; Quattrini  
87 et al., 2019), was not accounted for. Therefore, this study aims to screen a broad range of  
88 LAB with respect to their antifungal activity in laboratory media, in milk and yogurt.  
89 Antifungal compounds including organic acids and HUFA were quantified, and the  
90 accumulation of these antifungal metabolites was related to the genome sequences of  
91 antifungal strains.

## 92 **2 MATERIAL AND METHODS**

### 93 **2.1 Microbial strains and chemical reagents.**

94 The 113 strains of lactic acid bacteria that were used in this study and their origin are listed  
95 in Table 1. *Aspergillus clavatus* FUA 5005, *Penicillium caseicolum* PCa03 and *Mucor*  
96 *racemosus* MUR 01 were used as fungal challenge organisms; the spoilage yeasts used in  
97 this study include *Candida sake* CDS01, *Saccharomyces bayanus* SCPa01, and  
98 *Torulasporea delbrueckii* TOD01.

99 Lactic acid bacteria were cultivated in modified De Man, Rogosa Sharpe (mMRS) medium  
100 containing (w/v) 1% peptone, 0.5% beef extract, 0.5% yeast extract, 1% maltose  
101 monohydrate, 0.5% fructose, 0.5% glucose, 0.4% K<sub>2</sub>HPO<sub>4</sub>, 0.26% KH<sub>2</sub>PO<sub>4</sub>, 0.3% NH<sub>4</sub>Cl,  
102 0.1% Tween 80, 0.05% L-cysteine hydrochloride monohydrate, 0.02% MgSO<sub>4</sub>, 0.005%

103 MnSO<sub>4</sub>, and 1% malt extract; 1.5% agar was added to obtain solid media. Filamentous  
104 fungi were cultured in malt extract (ME) agar for 7 days. Yeasts was cultured in ME broth  
105 for 2 days, agitation, at 30 °C.

106 Yogurt was fermented in microplates by heating pasteurized milk with 3.25% milk fat  
107 (Dairyland, Canada) to 43°C in a water bath for 30 min. Then, 0.8 g of lyophilized  
108 *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* mix S129  
109 (Sacco Srl, Italy) and 82 g of pre-warmed pasteurized milk were mixed in a stomacher bag  
110 and homogenized with a stomacher for 2 min. One gram of this mixture was then mixed  
111 with another aliquot of 10.35 g of pre-warmed pasteurized milk and homogenized in an  
112 orbital shaker for 1 min, 200 rpm. One mL of this solution was diluted with 24 g of pre-  
113 warmed milk, mixing in an orbital shaker at 200 rpm for 1 min, and 200 µl per well of the  
114 inoculated milk were transferred to microtiter plates.

115 Large scale yogurt fermentation was performed with pasteurized skimmed milk (0% milk  
116 fat) or low-fat milk (2% milk fat) that was re-pasteurized at 90 °C for 10 min, followed by  
117 cooling at 4 °C overnight. The starter culture was prepared by mixing 1 g of *Streptococcus*  
118 *thermophilus* and *L. delbrueckii* subsp. *bulgaricus* Y350A (Sacco Srl, Italy) with 100 g of  
119 re-pasteurized skimmed milk with 0% milk fat. An aliquot of 0.6 mL of this mixture was  
120 then used to inoculate 500 mL of milk with 2% milk fat, followed by addition of 2.5 mL  
121 of E120 colorant (0.4 % in water sterilized by filtration). For experiments with unfermented  
122 milk, pasteurized milk was autoclaved at 121°C for 5 min and cooled down at 4 °C  
123 overnight before use.

124 Spores of filamentous fungi were separated from mycelia by filtering and centrifugation.  
125 The spore count in the spore suspension was determined microscopically with a  
126 haemocytometer (Magnusson and Schnürer, 2001; Zhang et al., 2010) and spore  
127 suspensions were diluted with saline (0.9% NaCl; 0.1% Tween 80), to a spore count of  $10^4$   
128 spores / mL.

129 Microbiological media were obtained from Fisher Scientific (Ottawa, ON, Canada), other  
130 chemicals were obtained from Sigma Aldrich (Oakville, ON, Canada); milk was obtained  
131 at a local supermarket.

## 132 **2.2 Screening of the antifungal activity of bacterial strains.**

133 Screening of lactic acid bacteria was carried out in three media, mMRS, milk, and yogurt.  
134 Subcultures (200  $\mu$ L) of each strain were made from one single colony and incubated in  
135 mMRS broth for two successive overnight incubations in 96-well plates at 30 °C.  
136 Microtiter plates containing 100  $\mu$ L of mMRS media, autoclaved milk or yogurt were  
137 inoculated with 15  $\mu$ L of the LAB cultures. After 2 d of incubation at 30 °C, the cultures  
138 were inoculated with diluted spore suspension to achieve 5 spores / microtiter plate well.  
139 The growth of filamentous fungi was observed visually. Antifungal activity of selected  
140 LAB strains was also confirmed in a 6 mL fermentation culture and all the parameters were  
141 scaled accordingly.

142 Yogurt fermentation was repeated with selected antifungal adjunct strains with a 43 °C  
143 fermentation temperature, followed by storage at 10 °C. For challenge tests with molds, an  
144 LAB inoculum of  $\sim 10^6$  cfu/mL was inoculated into 62.5 mL portions of yogurt in a small  
145 jar. The portioned and inoculated yogurt mixture were then fermented in 43 °C for about 8

146 h to reach a final pH of  $4.5 \pm 0.2$ . After fermentation, fungal spore suspension was added  
147 at 23 spores/62.5 mL and the yogurt was then stored at 10 °C. Fungal growth were observed  
148 daily visually.

149 For yeast challenge test, a LAB inoculum of  $\sim 5 \times 10^6$  cfu/mL was added into 25 mL yogurt  
150 portioned in 50 mL-falcon tubes. The fermentation was also performed at 43 °C for about  
151 8 h to reach final pH= $4.5 \pm 0.2$ . An aliquot of 5mL of the fermented yogurt was taken for  
152 pH measurement. To inoculate yeast, 50 cells /mL was then inoculated in the yogurt. The  
153 inoculated samples were vortexed and stored at 10 °C. Yeast growth was measured by  
154 performing cell counts on yeast extract glucose chloramphenicol (YGC) agar (5.0 g/L yeast  
155 extract, 20.0 g/L glucose, 0.1 g/L chloramphenicol, and 14.9 g/L agar), cultured for 3 days  
156 at 30 °C.

### 157 **2.3 Quantification of organic acids by LC-RI.**

158 mMRS, autoclaved milk or yogurt (500  $\mu$ L) were inoculated with 75  $\mu$ L of overnight  
159 cultures, fermented at 30 °C for 2 d and incubated at 25 °C for another 14 d to match  
160 conditions of the challenge assays. An equal portion (575  $\mu$ L) of 7 % (v/v) perchloric acid  
161 was added in the mixtures, incubated at 4 °C overnight, and solids were removed by  
162 centrifugation. The formation of organic acids and propanediol was quantified by HPLC  
163 with a refractive index (RI) detector (LC-RI).

164 Separation was performed on an Aminex HPX-87H column (Bio-Rad, Mississauga,  
165 Canada). The column was eluted with 5 mM H<sub>2</sub>SO<sub>4</sub> at 70 °C and a constant flow rate of  
166 0.4 mL/min. The concentrations of lactate, acetate, propanediol were measured using a  
167 calibration curve of external standards.



168 **2.4 LC-MS/MS-target analysis of hydroxy unsaturated fatty acids (HUFA).**

169 To identify the antifungal HUFA produced during fermentation, milk and yogurt samples  
170 were prepared as outlined above for the quantification of organic acids. The samples were  
171 extracted three times by combing 500 µL sample with 3 mL hexane-isopropanol solution  
172 (3:2, v/v) and phase separation was achieved by centrifugation. The organic supernatants  
173 were collected, evaporated under nitrogen and stored at -20 °C until use. HUFA were  
174 identified by Liquid Chromatography/Atmospheric Pressure Photo Ionization Tandem  
175 Mass Spectrometry (LC-APPI-MS/MS) according to (Liang et al., 2020b) with  
176 modifications. Specifically, the organic extracts were redissolved in 1 mL methanol and a  
177 200 µL aliquot was further diluted with 800 µL methanol before injection. The targeted  
178 compounds were identified using multiple reaction monitoring (MRM) mode and their  
179 retention times checked against HUFA standards (Liang et al., 2020b).

180 **2.5 *In silico* identification of genes encoding for production of antifungal**  
181 **metabolites.**

182 To relate the formation of antifungal metabolites to the genome sequences of antifungal  
183 strains, genomes of selected strains were sequenced and annotated. Genomic DNA was  
184 extracted using Wizard Genomic DNA Purification Kit (Promega, Madison, Wisconsin,  
185 USA). Briefly, cells from 5 mL of cultures of bacterial strains in mMRS were harvested by  
186 centrifugation, the cell pellet was washed with 5 mL saline (0.9% NaCl and 0.1% Tween  
187 20) and then washed with 5 mL EDTA solution (50 mM, pH 8). Subsequent steps were  
188 performed according to the instructions of the manufacturer.

189 The quantity and purity of DNA was examined by Nanodrop (Thermo Fisher, Waltham,  
190 MA, USA); the identity of the DNA was verified by High Resolution Melting (HRM)  
191 analysis and sequencing of the 16S rRNA genes. The gDNA samples were sequenced on  
192 the Illumina MiSeq2000 platform by service of Genome Quebec. The quality of reads was  
193 checked with Fastqc (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>),  
194 adapter sequences were removed with Trimmomatic (Bolger et al., 2014), reads were  
195 assembled in SPAdes (Bankevich et al., 2012) and the quality of assemblies was checked  
196 in QUAST (<http://bioinf.spbau.ru/quast>). Genomes were annotated with the RAST server  
197 (Aziz et al., 2008). Genes that relate to antifungal activity were identified with BLASTp  
198 with query sequences shown in Table 3 and cut-off values of 40% protein identity and 68%  
199 coverage.

## 200 **2.6 Statistical analysis.**

201 All experiments were conducted in biological triplicates. The strain screening data was  
202 analyzed by R 3.1.2 (R Core Team, 2014). Significant difference assessed at the level of  
203  $P < 0.05$  (5% probability of error). Principle component analysis carried out with  
204 MetaboAnalyst 5.0 (<http://www.metaboanalyst.ca>). Correlation analysis to relate the  
205 antifungal activity with the concentration of specific metabolites was carried out with the  
206 linear regression tool implemented in SigmaPlot 12.5 (Jandel Scientific, San Jose, CA,  
207 U.S.A.). Correlation coefficients of 0.75 or higher were interpreted as strong correlations.

## 208 **3 RESULTS**

### 209 **3.1 Screening of LAB for antifungal activity in mMRS, milk, and yogurt.**

210 To identify strains of LAB with antifungal activity, 113 LAB strains were screened in three  
211 different substrates with the three filamentous fungi *P. caseicolum*, *A. clavatus* and *M.*  
212 *racemosus* as indicator organisms. This experimental design allows to compare the  
213 antifungal activity of different adjunct cultures and of different challenge organisms  
214 growing in the same substrate. The screening results are summarized as a heat map in Fig.  
215 1. In all three substrates, fungal growth was observed in the negative control after 2 d while  
216 some LAB inhibited mold growth for up to 14 d (Fig. 1). When screened in mMRS, most  
217 strains of *Ln. parabuchneri*, *Lp. plantarum* and *Ff. milii* were active against two or more  
218 of the indicator strains and most *Lacticaseibacillus* spp. were active against *P. caseicolum*  
219 (Fig 1). The antifungal activity of *Lp. plantarum* TMW1.460 in milk and yogurt was  
220 consistently higher than the antifungal activity of *Lp. plantarum* TMW1.460 $\Delta$ *lah* which  
221 lacks 10-linoleate hydratase (Fig. 1). In contrast, only few strains exhibited strong  
222 antifungal activity in milk or yogurt. *A. clavatus* was overall the most sensitive indicator  
223 strain. Only few strains inhibited mold growth for 8 d or more in at least 6 out of the 9  
224 combinations of substrate and indicator strains; these strains were all assigned to the  
225 species *Lp. plantarum*, *Ff. milii* and *Ln. parabuchneri* (Fig 1).

### 226 **3.2 Anti-yeast activity of LAB in yogurt.**

227 To study the activity of LAB against yeasts, 14 of the antifungal strains (Fig. 1) were  
228 selected. *Lc. casei* and *Lc. paracasei* were additionally included because strains of this  
229 species are used commercially as adjunct cultures to improve the flavor of fermented dairy  
230 products (Stefanovic et al., 2017). The inhibitory activity was assessed in yogurt that was  
231 challenged with *Saccharomyces bayanus* SCPa01, *Candida sake* CDS01, and *Torulaspora*

232 *delbrueckii* TOD01. Growth of *S. bayanus* during storage is shown in Fig. 2. During 12 d  
233 of storage at 10°C, *Ln. parabuchneri* LPB02, *Lc. rhamnosus* FUA3185, and *Lc. paracasei*  
234 FUA3186 significantly inhibited the growth of *S. bayanus*, while *Ff. millii* or *Ff. rossiae*  
235 and *Lp. plantarum* species did not show inhibitory effects. None of the strains inhibited  
236 growth of *T. delbrueckii* and *C. sake* (Fig. S1 and S2).

237 Inhibition of *S. bayanus* and *C. sake* was additionally evaluated after fermentation at 30 °C  
238 and during storage at 25 °C (Fig 3 and Fig. S3). All adjunct cultures inhibited growth of *S.*  
239 *bayanus* on day 1, 2 and 3 while *Ff. millii* FUA3583, *Ln. parabuchneri* LPB02 and *Lp.*  
240 *plantarum* LP023 inhibited growth of *C. sake*. None of the antifungal adjunct strains  
241 inhibited or delayed yeast growth for more than 3 d (Fig. 3 and Fig. S3). Overall, these  
242 results indicate that inhibitory activity against yeasts is weaker than inhibitory activity  
243 against molds, and that anti-mold activity does not predict yeast inhibition.

### 244 **3.3 Exploration of the antifungal activity of *Lc. casei* and *Lc. paracasei*.**

245 Inhibition to *S. bayanus* by strains of the *Lc. casei* group (Fig. 2) contrasted the lack of  
246 inhibitory activity of the same strains against mycelial molds (Fig. 1). To further explore  
247 the findings, an exploratory test was carried out in a larger scale in tightly sealed jars with  
248 62.5 mL yogurt and 17.5 mL headspace using *P. caseicum* as indicator (Table S1). In a  
249 first experiment, *Lc. rhamnosus* FUA3185 and *Lc. paracasei* FUA3186 were tested and  
250 strains of *Lp. plantarum*, *Ff. rossiae*, *Ff. millii* and *Ln. parabuchneri* were used for  
251 comparison. The yogurt samples were stored at 10 °C for 15 d and mold growth was  
252 assessed visually. Both *Lc. rhamnosus* FUA3185 and *Lc. paracasei* FUA3186 as well as  
253 *Ln. parabuchneri* LPB02 and *Lp. plantarum* FUA3183 inhibited growth of *P. caseicum*

254 for 15 d. In a second experiment, additional strains of lacticaseibacilli were included, only  
255 *Ln. parabuchneri* LPB02 was used for comparison, and yogurt was incubated for 20 d  
256 (Table S1). All strains of *Lc. casei*, *Lc. paracasei* or *Lc. rhamnosus* inhibited fungal growth  
257 for 20 d while mycelial growth was visible on yogurt inoculated with *Ln. parabuchneri*  
258 LPB02 (Tab. S1).

### 259 **3.4 Identification of antifungal metabolites.**

260 To explore the active antifungal metabolites that were produced by the 14 selected strains  
261 of *Ln. parabuchneri*, *Lp. plantarum*, *Ff. rossiae*, and *Ff. milii* in milk and yogurt, organic  
262 acids and HUFA produced during fermentation and storage were quantified by LC-RI and  
263 LC-MS/MS, respectively. The quantification of fatty acids also included saturated and  
264 unsaturated fatty acids without hydroxylation (Table S2 and Table S3).

265 The multivariate dataset consisting of antifungal activity against several molds, the  
266 concentration of organic acids, 1,2 propanediol, and free fatty acids and HUFA was  
267 initially analysed by PCoA (Fig. 4). For the linear discriminant analysis (Fig. 4A and Fig.  
268 4B), strains were categorized as having low, moderate, and high antifungal activity.  
269 Principle component 1 and 2 explained 51% and 57.1% of the variance in milk and yogurt,  
270 respectively. The analysis did not separate the strains based on their antifungal activity,  
271 either because the categorization was inaccurate, or because too many metabolites without  
272 antifungal activity were included. The loading plot for data obtained in milk (Fig. 4C) and  
273 yogurt (Fig. 4D) demonstrated that HUFA, particularly 10-OH 18:1, and acetate were  
274 highly correlated to the mold-free shelf life while saturated OH-fatty acids or fatty acids  
275 without hydroxylation were not correlated to antifungal activity.

276 The contribution of HUFA and acetate to the antifungal activity was confirmed by linear  
277 correlation of the metabolite concentrations to the mold-free shelf life (Table 2). The  
278 metabolites 13-OH C18:1, 10-OH C18:1, lactate and acetate were included; in addition,  
279 we used 1,2 propanediol, which is a co-metabolite of the conversion of lactate to acetate  
280 by lenticulobacilli (Gänzle, 2015). In milk, the concentration of 10-OH 18:1 and of (10-  
281 OH 18:1 + 13-OH 18:1) were significantly ( $p < 0.05$ ) correlated to the mold-free shelf life;  
282 in particular, the sum of the concentrations of 10-OH 18:1 and 13-OH 18:1 was strongly  
283 correlated to inhibition of *A. clavatus* with a correlation coefficient of  $> 0.7$ . Lactate and  
284 acetate did not correlate to the mold-free shelf life of milk but 1,2 propanediol strongly  
285 correlated with inhibition of *A. clavatus*. The production of 1,2 propanediol in *Ln.*  
286 *parabuchneri* alone was strongly correlated to the inhibition of *A. clavatus* and *P.*  
287 *caseicum*, with correlation coefficients were 0.96. In yogurt, the concentrations of 10-OH  
288 18:1, lactate, acetate and 1,2 propanediol all significantly correlated to its mold-free storage  
289 life. Specifically, acetate was strongly correlated to the inhibition of all the three indicator  
290 molds, while 10-OH 18:1 was strongly correlated to the inhibition of *A. clavatus*. The  
291 production of 1,2 propanediol in *Ln. parabuchneri* alone was strongly correlated to the  
292 inhibition of *P. caseicum*. Therefore, HUFA and acetic acid were identified as major  
293 antifungal metabolites of *Ln. parabuchneri*, *Lp. plantarum*, *Ff. rossiae* and *Ff. miii*  
294 produced in milk and yogurt matrices. Additionally, 1,2 propanediol contributed to the  
295 antifungal activity of *Ln. parabuchneri* and one strain of *Ff. rossiae*.

### 296 **3.5 Comparative genomic study.**

297 To understand the differences in antifungal activity of lactobacilli at the genetic level, the  
298 selected strains were ranked based on their antifungal abilities and their genomes were  
299 analysed with respect to the presence of genes that encode metabolic functions that relate  
300 to antifungal activity. The selection of enzymes included 10-linoleate hydratases (10-Lah),  
301 13-linoleate hydratases (13-Lah), lactaldehyde dehydrogenase (Lact) and propanediol  
302 dehydratase (PduC), which are responsible for the first steps in the conversion of lactate to  
303 1,2 propanediol and 1,2 propanediol to propionate, respectively, by lentilactobacilli. In  
304 addition, manganese (Mn) transport enzymes (MntH1, MntH2, and MntH3) were included  
305 in the analysis as the antifungal activity of *Lc. rhamnosus* and *Lc. paracasei* in fermented  
306 milk products relates to manganese accumulation (Siedler et al., 2020).

307 The presence of these genes in relation to the antifungal activity of the corresponding  
308 strains is shown in Table 3. Irrespective of their antifungal activity, genomes of all the  
309 studied strains of *Lp. plantarum*, *Ff. rossiae*, *Ff. millii* and *Ln. parabuchneri* included genes  
310 coding for 10-Lah, MntH1 and MntH2. The presence of genes encoding for 10-Lah  
311 predicted the production of 10-OH C18:1 (Table S2 and S3). The presence of 13-OH 18:1  
312 in milk and yogurt samples (Table S2 and S3) was not predicted by the presence of genes  
313 encoding for 13-Lah; 13-OH 18:1 concentration were low in all samples and unrelated to  
314 antifungal activity. The presence of lactaldehyde dehydrogenase predicted the production  
315 of 1,2 propanediol by *Ln. parabuchneri* LPB02 but not by strains of *Ff. millii* FUA3583  
316 and *F. rossiae* FUA3124. The genes encoding for the conversion of lactate to 1,2  
317 propanediol and acetate were previously identified in *Ff. rossiae* and *F. millii* (De Angelis  
318 et al., 2014; Simpson et al., 2022) but the pathway has not been shown to functional in  
319 furfurilactobacilli. *Ff. millii* FUA3509 produced 1,2-propanediol during growth in milk and

320 yogurt but the differentiating genotypic and phenotypic properties relative to other  
321 furfurilactobacilli that do not convert lactate remain to be elucidated. Taken together,  
322 genomic analysis predicted the formation of some but not all the antifungal metabolites  
323 and thus had only limited predictive value for the overall antifungal activity.

#### 324 **4 DISCUSSION**

325 This study compared the antifungal activity of 113 LAB strains covering 18 different  
326 species in a high-throughput way by using microplate and identified specific strains of  
327 *Lactiplantibacillus plantarum*, *Furfurilactobacillus rossiae* and *Ff. milii*, and  
328 *Lentilactobacillus parabuchneri* based on their inhibitory effects against 3 molds. The  
329 screening test of 113 LAB strains against molds in three matrices documented the  
330 importance of the food matrix for the antifungal activity of LAB. MRS agar medium that  
331 contains acetate is reported to strongly affect the production and expression of antifungal  
332 metabolites (Le Lay et al., 2016). In our study, modified MRS without addition of acetate  
333 was used to avoid interference of acetic acid as a component of the medium. A total of 64  
334 strains exhibited antifungal activity after growth in mMRS while only 8 and 6 strains  
335 exhibited antifungal activity after growth in milk and yogurt, respectively. The choice of  
336 indicator molds also affects the antifungal performance of LAB. In our study,  
337 *P. caseicum*, *A. clavatus* and *M. racemosus* were chosen to represent fungal contaminants  
338 in dairy industry. Because *P. caseicum* produces a white mycelium and has high lipolytic  
339 and proteolytic activity, it is used in surface-ripening of cheese (Gripon, 1993). However,  
340 its growth on non-mold-ripened dairy products constitutes spoilage (Ansari and Häubl,  
341 2016). *Aspergillus clavatus* is a representative aflatoxin-forming *Aspergillus* species that



342 may grow during cheese ripening (Delgado et al., 2016). *Mucor racemosus* belongs to the  
343 phylum of *Mucoromycota* and is taxonomically distinct from the other two molds that are  
344 classified in the phylum *Ascomycota*. *Mucor racemosus* is of concern in cheese ripening  
345 and post-storage as it causes a fuzzy surface on soft cheeses (Bekada et al., 2008). The  
346 presence of *Mucor circinelloides* can cause quality deterioration after container bloating in  
347 yogurt and induce spoilage (Snyder et al., 2016).

348 The anti-yeast activity of the selected strains was not correlated to their antifungal activity  
349 against mycelial molds. The sensitivity of yeasts and molds is greatly influenced by  
350 environmental conditions, i.e., pH of the substrate and specific type of metabolites. When  
351 tested in the supernatant of *Lp. plantarum* MiLAB14 culture, the yeasts *K. marxianus*, *P.*  
352 *anomala*, and *R. mucilaginosa* were more sensitive to 3-OH C10 than the filamentous fungi  
353 *A. fumigatus*, *A. nidulans*, *P. roqueforti*, and *P. commune* with MICs between 10 to 50  
354 mg l<sup>-1</sup> and 25 to 100 mg l<sup>-1</sup>, respectively (Sjögren et al., 2003). Conversely, *A. niger* and  
355 *P. roqueforti* are susceptible to C18:1 and C18:2 HUFAs with hydroxylation at position 9,  
356 10, 12 and 13, with MICs ranging from 230 to 500 mg l<sup>-1</sup> while *Candida albicans*,  
357 *Saccharomyces cerevisiae*, *Candida valida*, and *Pichia membranaefaciens* tolerated the  
358 same compounds at concentrations exceeding 1 g l<sup>-1</sup> (Liang et al., 2020a). In our study,  
359 the concentration of 10-OH C18:1 ranged from 0.8-2.9 mg/L in milk (Table S2) and from  
360 0.7-1.9 mg/L in yogurt (Table S3) which is about 100 times lower than its MIC towards  
361 molds in mMRS media (Liang et al., 2020a). The *in vitro* MIC values were measured in  
362 mMRS media at a pH of around 6.0 while the pH of yogurt used in this study was about  
363 4.5 after fermentation; production of lactic and acetic acids during storage (Table S3)  
364 reduced the pH further. Because the pKa of acetic acid and HUFA is around 4.75, both

365 acids are predominantly undissociated in yogurt and the sensitivity of yeasts and molds to  
366 undissociated organic acids is higher when compared to dissociated organic acids. Acetic  
367 acid exhibited better inhibitory effects against a broad spectrum of fungi at pH 5 compared  
368 to pH 7 (Lind et al., 2005). The concentration of acetate in milk and yogurt ranged from 10  
369 to more than 100 mM, which is in the range of MICs of acetate towards molds and yeasts  
370 (4-120 mM) (Lind et al., 2005).

371 The discrepancy of the inhibitory activity of *Lc. casei* group against mycelium fungi in  
372 microtiter plates and small jars likely relates to the impact of oxygen to the growth of molds.  
373 Strains of the *Lc. casei* group are used as adjunct culture in cheese because they produce  
374 diacetyl and acetoin from pyruvate to provide a desirable butter aroma (Branen and Keenan,  
375 1971). In the present study, the microtiter plates were not hermetically sealed while the jars  
376 were tightly closed. In addition, the headspace in the air-tight jars accounted only for 1/5<sup>th</sup>  
377 of the volume, which greatly limited the availability of oxygen available to sustain mold  
378 growth. Limiting the availability of oxygen is often used in the control of mold spoilage to  
379 extend shelf life of dairy products (Foltynowicz and Rikhie, 2020; Haghghi-Manesh and  
380 Azizi, 2017; Ledenbach and Marshall, 2009). In addition, hermetically sealed jars with a  
381 small headspace to volume ratio trap of antifungal volatiles, especially diacetyl. Strains of  
382 the *L. casei* group that encode for the acetolactate synthase (*als*) utilize the citric acid to  
383 produce diacetyl (Lo et al., 2018). In yogurt, diacetyl was reported as one of the major anti-  
384 mold volatiles produced by *Lactobacillus paracasei* DGCC 2132 (Aunbjerg et al., 2015).  
385 Exposure of dairy molds to diacetyl induced intracellular oxidative stress, leading to cell  
386 death (Shi and Knøchel, 2021). The production of HUFA by strains of the *Lc. casei* was not  
387 explored in this study and lactic acid, the main catabolite of lacticaseibacilli (Díaz-Muñiz

388 et al., 2006) reduces the pH but has no antifungal activity. The antifungal activity of *Lc.*  
389 *paracasei* and *rhamnosus* in yogurt was also attributed to the depletion of manganese  
390 (Siedler et al., 2020).

391 Specific strains of *Lp. plantarum*, *Ff. mii* and *Ln. parabuchneri* were identified as the  
392 most antifungal strains against both yeasts and molds. *Lp. plantarum* is used as an adjunct  
393 culture in fermented dairy products to improve health benefits and extend shelf life (Behera  
394 et al., 2018). It is a homofermentative organism that converts hexoses to lactate (Gänzle,  
395 2015). It also converts linoleic acid to 10-hydroxy-12-octadecenoic acid or 13-hydroxy-9-  
396 octadecenoic acid by linoleate hydratases. Both hydroxy fatty acids have antifungal activity  
397 (Black et al., 2013; Chen et al., 2016; Liang et al., 2017). Acetate levels produced by strains  
398 of *Lp. plantarum* were all below 50 mM and the HUFA concentration was far below the  
399 MIC (Table S2 and S3), therefore, the antifungal mechanism of *Lp. plantarum* is likely  
400 based on combined activity of several metabolites.

401 *Ln. parabuchneri* is a heterofermentative species that converts lactate to acetate and  
402 1,2-propanediol. It occurs in Swiss cheese and contributes to eye formation, the production  
403 of ornithine, histidine and glutamate (Fröhlich-Wyder et al., 2015, 2013). The formation  
404 of 1,2-propanediol is best characterized for lentalactobacilli although the relevant enzymes,  
405 lactaldehyde dehydrogenase and propanediol dehydrogenase, are also present on other  
406 lactobacilli including loigolactobacilli, furfurilactobacilli, limosilactobacilli, and  
407 levilactobacilli (Zheng et al., 2015). The strong correlation of 1,2-propanediol production  
408 in *Ln. parabuchneri* to mold-free shelf life of yogurt indicates that acetate, the co-product  
409 of the metabolic pathway, contributes to mold inhibition.

410 *Ff. rossiae* has been used as a biopreservative in bakery products (Garofalo et al., 2012;  
411 Samapundo et al., 2016) but the antifungal activity of *Ff. rossiae* or the recently described  
412 *Ff. milii* (Simpson et al., 2022) has not been explored in dairy products. *Ff. rossiae* grows  
413 poorly in milk because it lacks an extracellular proteinase but has been used as an adjunct  
414 culture in dairy products (De Angelis et al., 2014). Comparable to *Lp. plantarum*, the  
415 concentration of acetate and HUFA produced by furfurilactobacilli in dairy products was  
416 below the respective MICs of the compounds, therefore, the antifungal activity of  
417 furfurilactobacilli is likely also attributable to the additive or synergistic effects of HUFA  
418 and acetate in conjunction with the low pH.

419 In conclusion, this study explored the antifungal activity of LAB in dairy products  
420 mimicking practical storage conditions which will provide more in-depth references for the  
421 application of antifungal LAB cultures and their metabolites. This characterization  
422 identified long chain HUFA as novel compounds contributing to antifungal activity of  
423 dairy starter cultures. Antifungal activity was produced mainly by lacticaseibacilli,  
424 lactiplantibacilli, furfurilactobacilli and lentilactobacilli but the mechanisms of activity  
425 differed between the strains of the four genera with diacetyl production and manganese  
426 depletion, formation of long-chain hydroxylated fatty acids and acetate formation as major  
427 contributors to antifungal activity. Genomic analyses only partly predicted the production  
428 of organic acids but not HUFA which limited the possibilities of explaining the antifungal  
429 mechanism from the genomic level. The antifungal activity of different LAB is dependent  
430 on synergistic or additive activity of multiple metabolites.

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633

634 **FIGURE LEGENDS**

635 **Figure 1.** Heat map depicting the antifungal effect of 113 strains of lactic acid bacteria  
636 against 3 different indicator organisms. Experiments were conducted with mMRS, milk, or  
637 yogurt as fermentation substrate. The indicator organisms and the fermentation substrate  
638 are shown on the y-axis; the LAB strains are shown on the x-axis and the time to visible  
639 mycelial growth is indicated as a heat map. mMRS media, milk and yogurt were fermented  
640 with lactic acid bacteria at 30 °C for 2 d prior to addition of the conidiospores of the  
641 indicator organisms and further incubation at 25 °C for 15 d. Shown are the averages of  
642 quadruplicate independent experiments.

643 **Figure 2.** Cell counts of *Saccharomyces bayanus* SCPa01 in yogurt fermented with *L.*  
644 *delbrueckii* and *S. thermophilus* and the following adjunct cultures: *Furfurilactobacillus*  
645 *milii* FUA3115 (▲), *Furfurilactobacillus rossiae* FUA3126 (Δ), *Lacticaseibacillus*  
646 *rhamnosus* FUA3185 (▼), *Lacticaseibacillus paracasei* FUA3186 (▽), *Lentilactobacillus*  
647 *parabuchneri* LPB02 (■), *Lactiplantibacillus plantarum* LP023 (◇), *Lactiplantibacillus*  
648 *plantarum* LP024 (◆), and *Lactiplantibacillus plantarum* LP048 (◇). A control was  
649 fermented without adjunct cultures (●). Yogurt was fermented for 8 h at 43 °C, challenged  
650 with the spoilage yeast and incubated at 10 °C. Data are shown as means ± of triplicate  
651 independent experiments. Above the x-axis, the symbols corresponding to those adjunct  
652 cultures that significantly (P<0.05) reduced the cell counts of *S. bayanus* compared to the  
653 control are shown.

654 **Figure 3.** Cell counts of *Saccharomyces bayanus* SCPa01 in yogurt fermented with *L.*  
655 *delbrueckii* and *S. thermophilus* and the following adjunct cultures: *Furfurilactobacillus*

656 *rossiae* FUA3115 (▲), *Furfurilactobacillus rossiae* FUA3126 (Δ), *Furfurilactobacillus*.  
657 *rossiae* FUA3583 (▲), *Lacticaseibacillus casei* FUA3311 (▼), *Lacticaseibacillus*  
658 *paracasei* FUA3413 (▼), *Lacticaseibacillus. paracasei* FUA3491 (▼), *Lacticaseibacillus*.  
659 *paracasei* LPC31 (▽), *Lentilactobacillus parabuchneri* LPB02 (■), *Lentilactobacillus*.  
660 *parabuchneri* FUA 3154 (■) *Lactiplantibacillus plantarum* LP023 (◇) and  
661 *Lactiplantibacillus plantarum* LP024 (◆). A control was fermented without adjunct  
662 cultures (●). Yogurt was fermented for 2 d at 30 °C, challenged with the spoilage yeast and  
663 incubated at 25 °C. Data are shown as means ± of triplicate independent experiments.  
664 Above the x-axis, the symbol for the control experiment is shown on those time points  
665 where cell counts of *S. bayanus* were significantly (P<0.05) higher in the control  
666 experiment compared to all samples with adjunct cultures.

667 **Figure 4.** Linear discriminant analysis of mold free storage life in milk (Panels A and C)  
668 and yogurt (Panels B and D) challenged with *A. clavatus*, *P. caseicolum* and *M. racemosus*.  
669 and concentration of fatty acids and bacterial metabolites. **Panels A and B:** PCoA with  
670 strains categorized as having least, moderate and most effective strains with a cutoff of <  
671 4 d (least), < 8 d (moderate) and < 10.2 d (most) for milk (Panel A) and < 4.5 d (least), <  
672 6.75 d (moderate) and < 10.2 d (most) for yogurt. Cut-offs were selected to obtain an  
673 approximately equal number of strains in each category. **Panels C and D:** Loading plots  
674 showing correlations of fatty acids and bacterial metabolites with the mold-free storage life.



**Table 1. Lactic acid bacteria used in the study**

Species	Strain	Source (reference)
<i>Ft. sanfranciscensis</i>	ATCC27651T	Sourdough (Kline and Sugihara, 1971)h
<i>Ff. rossiae</i>	FUA3124	Sourdough
<i>Ff. rossiae</i>	FUA3126	Sourdough
<i>Ff. rossiae</i>	FUA3162	Sourdough
<i>Ff. rossiae</i>	FUA3442	Sourdough
<i>Ff. rossiae</i>	FUA3514	Sourdough
<i>Ff. milii</i>	FUA3509 (=C5)	Sourdough (Simpson et al., 2022)
<i>Ff. milii</i>	FUA3115	Sourdough (Simpson et al., 2022)
<i>Ff. milii</i>	FUA3119	Sourdough (Simpson et al., 2022)
<i>Ff. milii</i>	FUA3430	Sourdough (Simpson et al., 2022)
<i>Ff. milii</i>	FUA3583	Mahewu (Pswarayi and Gänzle, 2019; Simpson et al., 2022)
<i>Lc. casei</i>	FUA3091	Kvass (Dlusskaya et al., 2008)
<i>Lc. casei</i>	FUA3311	Ting (Sekwati-Monang and Gänzle, 2011)
<i>Lc. casei</i>	FUA3326	Ting (Sekwati-Monang and Gänzle, 2011)
<i>Lc. casei</i>	FUA3402	Wheat bran
<i>Lc. rhamnosus</i>	FUA3185	Oat drink
<i>Lc. paracasei</i>	FUA3166	Ting (Sekwati-Monang and Gänzle, 2011)
<i>Lc. paracasei</i>	FUA3413	Fermented milk
<i>Lc. paracasei</i>	FUA3491	Sourdough
<i>Lc. paracasei</i>	LPC44	Sacco S.r.L., Italy
<i>Lc. paracasei</i>	LPC46	Sacco S.r.L., Italy
<i>Lc. paracasei</i>	LPC04	Sacco S.r.L., Italy
<i>Lc. paracasei</i>	LPC31	Sacco S.r.L., Italy
<i>Lc. paracasei</i>	LPC36	Sacco S.r.L., Italy
<i>Lc. paracasei</i>	FUA3186	Oat drink
<i>Lc. rhamnosus</i>	LRH01	Sacco S.r.L., Italy
<i>Lc. rhamnosus</i>	LRH03	Sacco S.r.L., Italy
<i>Lc. rhamnosus</i>	LRH05	Sacco S.r.L., Italy
<i>Lc. rhamnosus</i>	LRH08	Sacco S.r.L., Italy
<i>Lc. rhamnosus</i>	LRH14	Sacco S.r.L., Italy
<i>Lc. rhamnosus</i>	LRH16	Sacco S.r.L., Italy
<i>Lc. rhamnosus</i>	LRH42	Sacco S.r.L., Italy
<i>Lc. rhamnosus</i>	LRH43	Sacco S.r.L., Italy
<i>Lc. rhamnosus</i>	LRH64	Sacco S.r.L., Italy
<i>Lc. rhamnosus</i>	LRH65	Sacco S.r.L., Italy
<i>Lc. rhamnosus</i>	LRH66	Sacco S.r.L., Italy
<i>Lc. rhamnosus</i>	FUA3554	
<i>Lp. plantarum</i>	FUA3112	Sourdough
<i>Lp. plantarum</i>	FUA3073	Sausage
<i>Lp. plantarum</i>	FUA3171	Ting (Sekwati-Monang and Gänzle, 2011)
<i>Lp. plantarum</i>	FUA3246	Sourdough
<i>Lp. plantarum</i>	FUA3247	Sourdough
<i>Lp. plantarum</i>	FUA3309	Ting (Sekwati-Monang and Gänzle, 2011)
<i>Lp. plantarum</i>	FUA3319	Ting (Sekwati-Monang and Gänzle, 2011)
<i>Lp. plantarum</i>	FUA3567	Mahewu (Pswarayi and Gänzle, 2019)
<i>Lp. plantarum</i>	FUA3584	Mahewu (Pswarayi and Gänzle, 2019)
<i>Lp. plantarum</i>	FUA3590	Mahewu (Pswarayi and Gänzle, 2019)
<i>Lp. plantarum</i>	LP01	Sacco S.r.L., Italy
<i>Lp. plantarum</i>	LP03	Sacco S.r.L., Italy
<i>Lp. plantarum</i>	LP15	Sacco S.r.L., Italy
<i>Lp. plantarum</i>	LP16	Sacco S.r.L., Italy
<i>Lp. plantarum</i>	LP21	Sacco S.r.L., Italy
<i>Lp. plantarum</i>	LP23	Sacco S.r.L., Italy
<i>Lp. plantarum</i>	LP24	Sacco S.r.L., Italy
<i>Lp. plantarum</i>	LP37	Sacco S.r.L., Italy

<i>Lp. plantarum</i>	LP48	Sacco S.r.L., Italy
<i>Lp. plantarum</i>	LP51	Sacco S.r.L., Italy
<i>Lp. plantarum</i>	FUA3038	Sourdough
<i>Lp. plantarum</i>	TMW1.460	Spoiled beer (Ulmer et al., 2000)
<i>Lp. plantarum</i>	TMW1.701	Sourdough (Chen et al., 2016)
<i>Lp. plantarum</i>	FUA3160	Sourdough
<i>Lp. plantarum</i>	FUA3183	Fermented milk
<i>Lp. plantarum</i>	FUA3302	Sourdough
<i>Lp. plantarum</i>	FUA3428	Sourdough
<i>Lp. plantarum</i>	TMW1.460 <sup>Alah</sup>	Beer spoilage (Chen et al., 2016)
<i>L. acidophilus</i>	ATCC43121	
<i>L. acidophilus</i>	FUA3410	Fermented raw milk
<i>L. acidophilus</i>	FUA3052	Mouse intestine
<i>L. acidophilus</i>	FUA 3050	Mouse intestine
<i>L. helveticus</i>	FUA3191	
<i>Lt. curvatus</i>	A543	Biena, Québec, Canada
<i>Lt. curvatus</i>	A549	Biena, Québec, Canada
<i>Lt. curvatus</i>	A550	Biena, Québec, Canada
<i>Lt. curvatus</i>	A537	Biena, Québec, Canada
<i>Lt. curvatus</i>	A534	Biena, Québec, Canada
<i>Lt. sakei</i>	LSK23	Sacco S.r.L., Italy
<i>Ln. buchneri</i>	FUA3170	
<i>Ln. buchneri</i>	FUA3252	
<i>Ln. buchneri</i>	FUA3405	Cereal beverage
<i>Ln. buchneri</i>	FUA3406	Cereal beverage
<i>Ln. parabuchneri</i>	LPB02	Sacco S.r.L., Italy
<i>Ln. parabuchneri</i>	FUA3154	Cereal beverage
<i>Ln. parabuchneri</i>	FUA3169	Ting (Sekwati-Monang and Gänzle, 2011)
<i>Ln. parabuchneri</i>	FUA3315	Ting (Sekwati-Monang and Gänzle, 2011)
<i>Ln. parabuchneri</i>	FUA3407	Cereal beverage
<i>Ln. parabuchneri</i>	FUA3408	Cereal beverage
<i>Ln. parabuchneri</i>	A535	Biena, Québec, Canada
<i>Lu. lactis</i>	FUA3359	Striped skunk
<i>Lu. lactis</i>	FUA3352	Red panda
<i>Lu. mesenteroides</i>	FUA3184	Fermented milk
<i>Lu. mesenteroides</i>	FUA3193	
<i>Lu. mesenteroides</i>	FUA3353	Coatimundis
<i>Lu. mesenteroides</i>	FUA3425	Sourdough
<i>Lu. mesenteroides</i>	FUA3516	Sourdough
<i>Lu. mesenteroides</i>	FUA3519	Sourdough
<i>Lu. mesenteroides</i>	FUA3143	Kefir
<i>Lu. mesenteroides</i>	FUA3219	Kimchi
<i>Lv. hammesii</i>	DSM16381	Sourdough (Valcheva et al., 2005)
<i>Lm. equigenerosi</i>	FUA3355	Zebra feces
<i>Lm. fermentum</i>	FUA3165	Ting (Sekwati-Monang and Gänzle, 2011)
<i>Lm. fermentum</i>	FUA3398	Wheat bran
<i>Lm. fermentum</i>	FUA3591	Mahewu (Pswarayi and Gänzle, 2019)
<i>Lm. fermentum</i>	FUA3566	Mahewu (Pswarayi and Gänzle, 2019)
<i>Lm. fermentum</i>	FUA3570	Mahewu (Pswarayi and Gänzle, 2019)
<i>Lm. fermentum</i>	FUA3573	Mahewu (Pswarayi and Gänzle, 2019)
<i>Lm. fermentum</i>	FUA3588	Mahewu (Pswarayi and Gänzle, 2019)
<i>Lm. fermentum</i>	FUA3582	Mahewu (Pswarayi and Gänzle, 2019)
<i>Lm. fermentum</i>	FUA3589	Mahewu (Pswarayi and Gänzle, 2019)
<i>Lm. fermentum</i>	FUA3565	Mahewu (Pswarayi and Gänzle, 2019)
<i>Loigolactobacillus coryniformis</i>	A532	Biena, Québec, Canada
<i>Loigolactobacillus coryniformis</i>	A546	Biena, Québec, Canada
<i>Loigolactobacillus coryniformis</i>	A552	Biena, Québec, Canada
<i>Loigolactobacillus coryniformis</i>	A533	Biena, Québec, Canada

**Table 2.** Correlation between metabolites produced by *Ln. parabuchneri*, *Lp. plantarum* and *Ff. rossiae* and the mold-free storage life of milk or yogurt that was inoculated with *A. clavatus*, *P. caseicolum* or *M. racemosus* and stored at 10 °C. Shown are the correlation coefficients and the *P*-values

	<i>A. clavatus</i>	<i>P. caseicolum</i>	<i>M. racemosus</i>
<b>Milk</b> (correlation coefficient / <i>P</i> -value)			
Mono-OH 18:0	n.s.	n.s.	n.s.
10-OH 18:1	0.59 / 0.03	0.598 / 0.024	0.624 / 0.016
13-OH 18:1	n.s.	n.s.	n.s.
(10-OH 18:1+13-OH 18:1)	0.985 / <0.001	0.645 / 0.013	0.629 / 0.016
Lactate	n.s.	n.s.	n.s.
Acetate	n.s.	n.s.	n.s.
1,2, propanediol (lentilactobacilli only)	0.854 / < 0.001 (0.957 / 0.043)	n.s. (0.964 / 0.036)	n.s. (0.003 / 0.007)
<b>Yogurt</b> (correlation coefficient / <i>P</i> -value)			
Mono-OH 18:0	n.s.	n.s.	n.s.
10-OH 18:1	0.725 / 0.003	0.625 / 0.017	0.558 / 0.038
13-OH 18:1	n.s.	n.s.	n.s.
(10-OH 18:1+13-OH 18:1)	0.637 / 0.014	0.591 / 0.026	0.572 / 0.033
Lactate	0.538 / 0.047	0.646 / 0.013	n.s.
Acetate	0.757 / 0.003	0.807 / < 0.001	0.776 / 0.001
1,2, propanediol (lentilactobacilli only)	0.606 / 0.022 n.s.	n.s. 0.953 / 0.047	0.574 / 0.032 n.s.

**Table 3.** Presence of genes related to antifungal activity of lactobacilli in genome sequenced strains that were used in this study

Strain	Shelf life <sup>A</sup>	10-Lah	13-Lah	MntH1	MntH2	MntH3	Lact	PduC	Accession number
<i>Ln. parabuchneri</i> LPB02	7	Red	Red	Red	Red	Red	Red	Red	JAIWIZ000000000
<i>Lp. plantarum</i> LP24	6	Red	White	Red	Red	Red	White	White	JAIWJW000000000
<i>Lp. plantarum</i> LP23	5	Red	White	Red	Red	Red	White	White	JAIWJV000000000
<i>Lp. plantarum</i> TMW1.460	5	Red	Orange	Red	Red	Red	White	White	GCA_009864015.1
<i>Lp. plantarum</i> FUA3073	4	Red	White	Red	Red	Red	White	White	JAIWJU000000000
<i>Lp. plantarum</i> FUA3171	4	Red	Orange	Red	Red	Red	White	White	JAIWJT000000000
<i>Lp. plantarum</i> FUA3247	4	Red	White	Red	Red	Red	White	White	JAIWJS000000000
<i>Lp. plantarum</i> FUA3309	4	Red	White	Red	Red	Red	White	White	JAIWJR000000000
<i>Lp. plantarum</i> LP03	4	Red	White	Red	Red	Red	White	White	JAIWJQ000000000
<i>Lp. plantarum</i> FUA3302	4	Red	Orange	Red	Red	Red	White	White	JAIWJP000000000
<i>Lp. plantarum</i> LP51	3	Red	White	Red	Red	Red	White	White	JAIWJO000000000
<i>Lp. plantarum</i> FUA3112	2	Red	White	Red	Red	Red	White	White	JAIWJN000000000
<i>Lp. plantarum</i> FUA3319	2	Red	Orange	Red	Red	Red	White	White	JAIWJM000000000
<i>Lp. plantarum</i> FUA3038	2	Red	White	Red	Red	Red	White	White	JAIWJL000000000
<i>Lp. plantarum</i> FUA3428	2	Red	White	Red	Red	Red	White	White	JAIWJK000000000
<i>Lp. plantarum</i> LP48	0	Red	White	Red	Red	Red	White	White	JAIWJJ000000000
<i>Lp. plantarum</i> FUA3183	0	Red	White	Red	Red	Red	White	White	JAIWJI000000000
<i>F. milii</i> FUA3583	4	Yellow	Orange	Red	Red	White	Orange	White	GCA_009863985.1
<i>F. milii</i> FUA3115	4	Yellow	White	Red	Red	White	White	White	JAIWJH000000000
<i>F. milii</i> FUA3119	3	Yellow	White	Red	Red	White	White	White	JAIWJG000000000
<i>F. milii</i> FUA3430	3	Yellow	White	Red	Red	White	White	White	JAIWJF000000000
<i>F. rossiae</i> FUA 3124	2	Yellow	Orange	Red	Red	White	Orange	Yellow	JAIWJE000000000

A: impact on mold free shelf life. Show is the number of replicates of a total of 9 experiments that extended the mold-free shelf life to 8 days or more. The 9 experiments represent the permutation of three challenge strains and 3 fermentation substrates shown in figure 1; each of these experiments was done in 4 independent replicates.

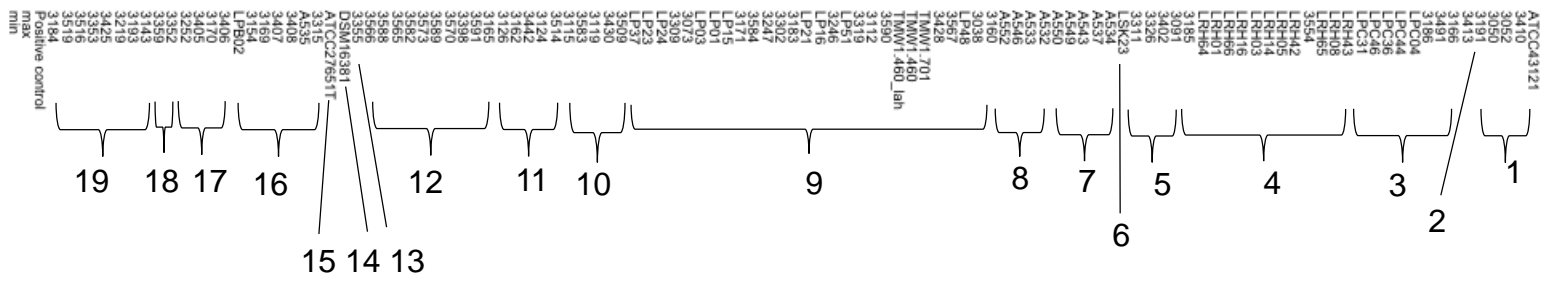
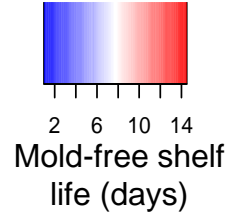
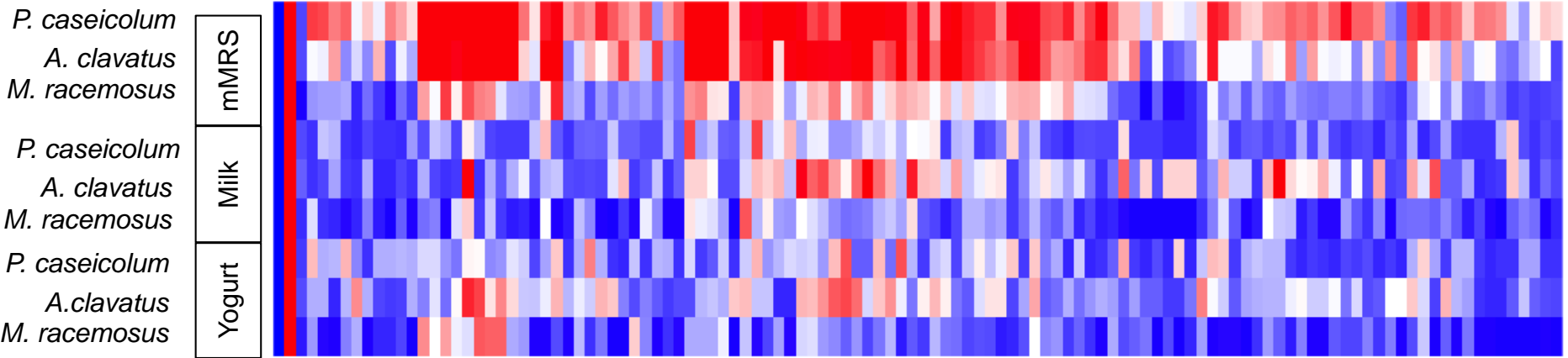
Protein names and accession numbers of query sequences: 10Lah, linoleate-10-hydratase *Lp. plantarum* (AOZ57083.1); 13Lah; linoleate-13-hydratase *Lactobacillus acidophilus* (AHW98239.1); MtnH1, Mn<sup>2+</sup> Nramp family transporter *Lp. plantarum* (AAO15439.1); MtnH2, Mn<sup>2+</sup> Nramp family transporter *Lp. plantarum* (AAO15440.1); MtnH3; Mn<sup>2+</sup> / Fe<sup>2+</sup> Nramp family transporter *Lp. plantarum* (EFK28456.1); Lact, lactaldehyde dehydrogenase *Lt. buchneri* (KRK67102.1); PduC, propanediol dehydratase medium subunit *Lt. buchneri* (WP\_153152761.1).

Red: >80% amino acid identity and >80% coverage.

Orange: >55% amino acid identity and >73% coverage.

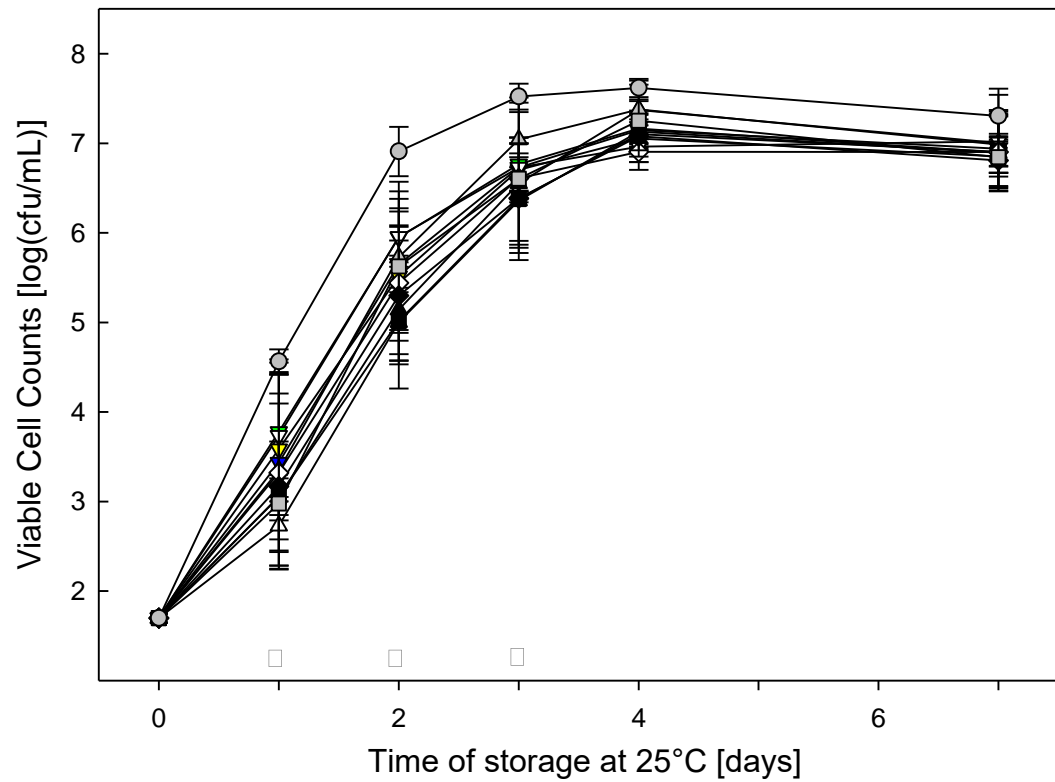
Yellow: >50% amino acid identity and > 68% coverage.

White: no Blast hit with <=50% amino acid identity.



1: *Lactobacillus acidophilus*; 2: *L. helveticus* 3: *Lc. paracasei*; 4: *Lc. rhamnosus* ; 5: *Lc. casei*; 6: *Lt. sakei*; 7: *Lt. curvatus*; 8: *Lg. coryniformis*; 9: *Lp. plantarum*; 10: *Ff. millii*; 11: *Ff. rossiae*; 12: *Lm. fermentum*; 13: *Lm. equigenosus*; 14: *Lv. hammesii*; 15: *Ft. sanfranciscensis*; 16: *Ln. parabuchneri*; 17: *Ln. buchneri*; 18: *Lu. lactis*; 19: *Lu. mesenteroides*.









**Online supplementary material to**

**Antifungal cultures and metabolites of lactic acid bacteria for use in dairy fermentations**

Nuanyi Liang#, Zheng Zhao#, Jonathan M. Curtis, and Michael G. Gänzle\*

**Figure S1.** Cell counts of *Torulaspora delbrueckii* TOD01 at 10 °C in yogurt fermented at 43°C with *L. delbrueckii* and *S. thermophilus* and adjunct cultures

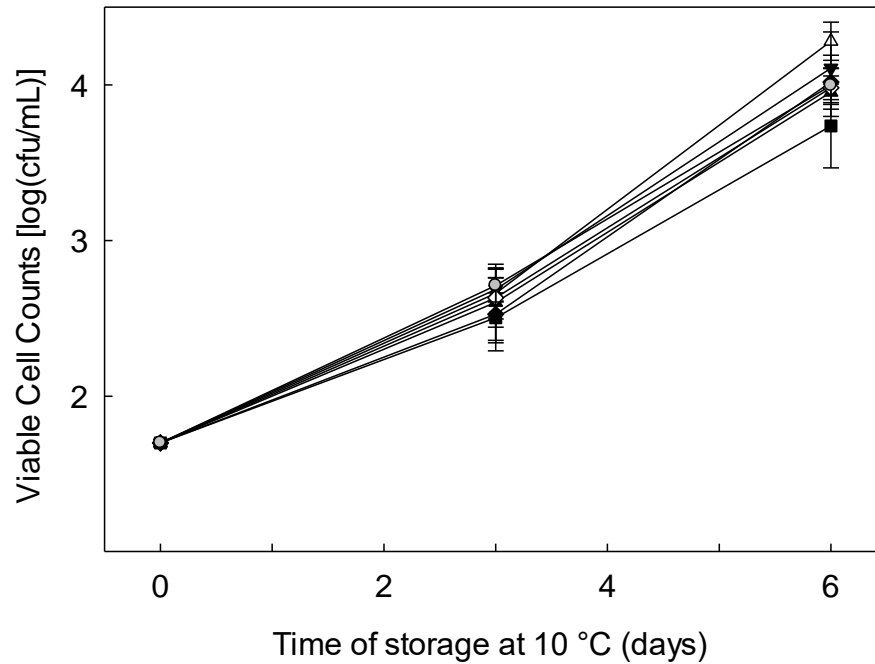
**Figure S2.** Cell counts of *Candida sake* CDS01 at 10 °C in yogurt fermented at 43°C with *L. delbrueckii* and *S. thermophilus* and adjunct cultures

**Figure S3.** Cell counts of *Candida sake* CDS01 at 25 °C in yogurt fermented at 30°C with *L. delbrueckii* and *S. thermophilus* and adjunct cultures

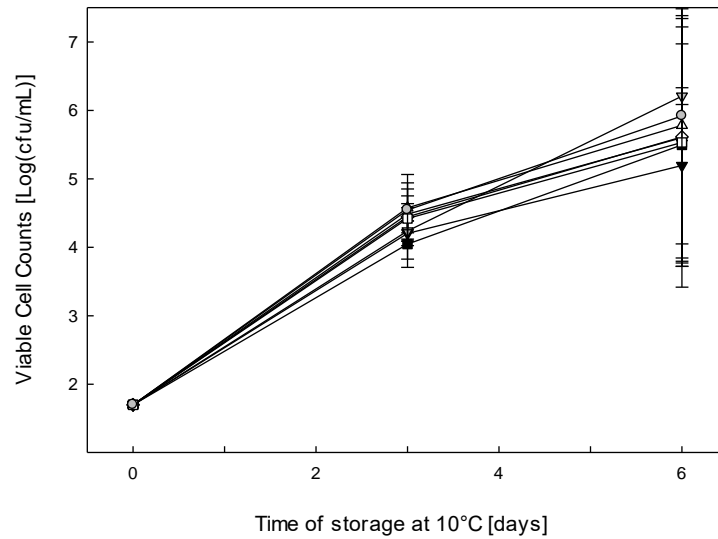
**Table S1.** Inhibitory effect of *Lc. (para)casei* strains in yogurt challenged by *P. caseicolum*

**Table S2.** Concentration of organic acids and fatty acids in milk

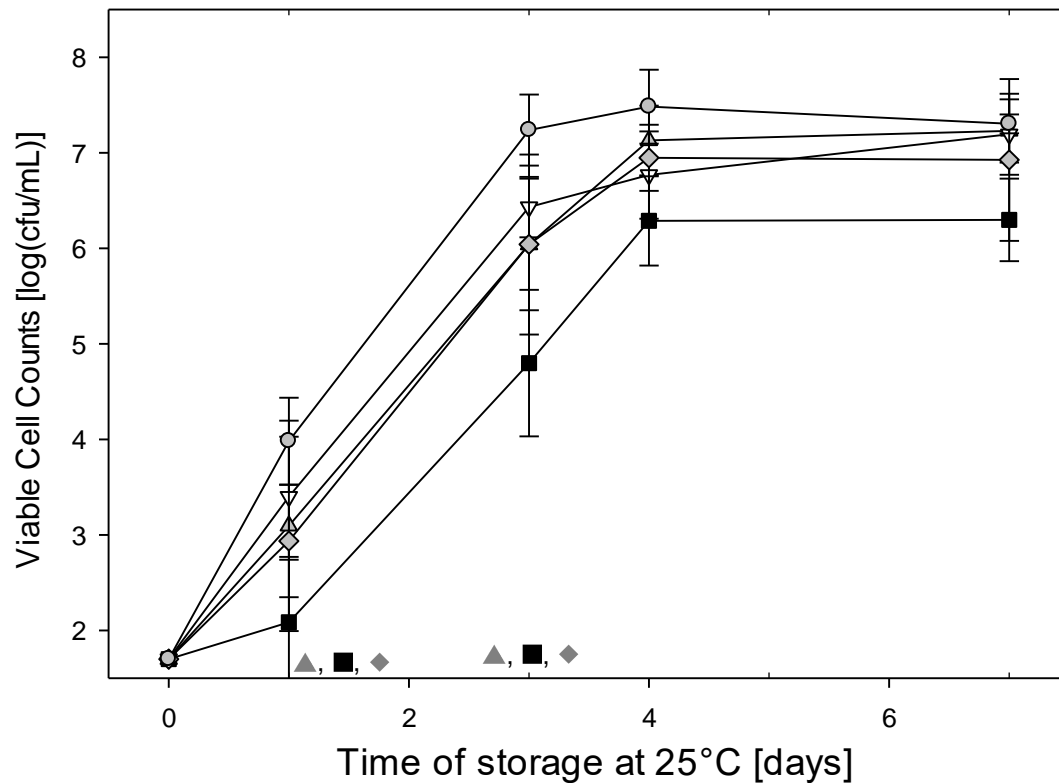
**Table S3.** Concentration of organic acids and fatty acids in yogurt



**Figure S1.** Cell counts of *Torulaspora delbrueckii* TOD01 in yogurt fermented with *L. delbrueckii* and *S. thermophilus* and the following adjunct cultures: *Furfurilactobacillus mii* FUA3115 (▲), *Furfurilactobacillus rossiae* FUA3126 (Δ), *Lacticaseibacillus rhamnosus* FUA3185 (▼), *Lentilactobacillus parabuchneri* LPB02 (■), *Lactiplantibacillus plantarum* LP023 (◇), *Lactiplantibacillus plantarum* LP024 (◆). A control was fermented without adjunct cultures (●). Yogurt was fermented for 8 h at 43 °C, challenged with the spoilage yeast and incubated at 10 °C. Data are shown as means  $\pm$  of triplicate independent experiments.



**Figure S2.** Cell counts of *Candida sake* CDS01 in yogurt fermented with *L. delbrueckii* and *S. thermophilus* and the following adjunct cultures: *Furfurilactobacillus miii* FUA3115 (▲), *Furfurilactobacillus rossiae* FUA3126 (Δ), *Lacticaseibacillus rhamnosus* FUA3185 (▼), *Lacticaseibacillus paracasei* FUA3186 (▽), *Lentilactobacillus parabuchneri* LPB02 (■), *Lactiplantibacillus plantarum* LP023 (◇), *Lactiplantibacillus plantarum* LP024 (◆). A control was fermented without adjunct cultures (●). Yogurt was fermented for 8 h at 43 °C, challenged with the spoilage yeast and incubated at 10 °C. Data are shown as means  $\pm$  of triplicate independent experiments.



**Figure S3.** Cell counts of *Candida sake* CDS01 in yogurt fermented with *L. delbrueckii* and *S. thermophilus* and the following adjunct cultures: *Furfurilactobacillus miii* FUA3583 (▲), *Lacticaseibacillus paracasei* LPC31 (▼), *Lentilactobacillus parabuchneri* LPB02 (■), or *Lactiplantibacillus plantarum* LP023 (◇). A control was fermented without adjunct cultures (●). Yogurt was fermented for 2 d at 30 °C, challenged with the spoilage yeast and incubated at 25 °C. Data are shown as means  $\pm$  of triplicate independent experiments. Above the x-axis, the symbols corresponding to those adjunct cultures that significantly ( $P < 0.05$ ) reduced the cell counts of *C. sake* compared to the control are shown.

**Table S1.** Comparison of the inhibitory effect of adjunct cultures on microtiterplates and in yogurt challenged by *P. caseicolum*

		mold-free shelf life in microplate <sup>A</sup> (d)	moldy scale in small jar <sup>B</sup>
			<b>D15</b>
<i>Ff. milli</i>	FUA3115	6.1	+++
<i>Ff. rossiae</i>	FUA3126	6.4	+++
<i>Lp. plantarum</i>	FUA3183	5.8	-
	LP23	6.3	++
	LP24	4.9	++
	LP48	6.1	++
<i>Ln. parabuchneri</i>	LPB02	7.5	-
<i>Lc. rhamnosus</i>	FUA3185	5.6	-
<i>Lc. paracasei</i>	FUA3186	5.6	-
Control		2.5	+++
			<b>D20</b>
<i>Ln. parabuchneri</i>	LPB02	7.5	+++
<i>Lc. rhamnosus</i>	FUA3185	5.6	-
<i>Lc. casei</i>	FUA3186	5.6	-
	FUA3311	8.5	-
	FUA3413	2.3	-
	FUA3491	2.5	-
<i>Lc. paracasei</i>	LPC46	8.3	+
	LPC31	5.4	+
Control		2.5	+++

A: Data was derived from Figure 1.

B: Results were based on two individual tests; -: no visible fungal growth; +: visible white mycelium but no conidiospores; ++: yogurt has dark spots on surface indicating conidiospores formation; +++: yogurt surface is covered with mycelium forming conidiospores.

1 **Table S2. Summary of the production of organic acids and fatty acids in milk.** Milk (575  $\mu$ L) was fermented for 2 d at 30 °C and  
 2 incubated for 14 d prior to sampling at 25 °C to match the conditions of the challenge assays. The negative control represents  
 3 uninoculated milk that was incubated at the same conditions.

strains	mold-free span	C18-2	C17-0	C14-0	C16-0	C18-0	C18-1	C18-3	C14-1	C16-1	mono-OH C18-0	10-OH C18-1	13-OH C18-1	Lactate	Acetate	1,2 propanediol
		(mg/L)										(mM)				
Neg. ctrl.	2.9	3.3	1.4	6.4	191.0	106.0	21.8	0.7	0.7	2.5	0.9	0.0	0.0	0.0	0.0	0.0
							<i>F. rossiae</i> and <i>F. mii</i>									
FUA3124	3.9	3.5	1.8	10.3	166.0	88.8	17.1	0.6	1.2	3.4	13.4	1.0	0.1	23.9	46.7	0.0
FUA3509	6.5	2.1	1.9	11.1	203.0	114.3	16.9	0.4	1.2	2.3	13.8	1.3	0.5	30.2	48.1	6.6
FUA3119	7.3	2.8	1.6	9.0	148.3	74.6	14.3	0.3	1.0	2.7	11.1	1.9	0.0	42.9	52.8	0.0
FUA3115	7.9	3.1	1.9	10.6	174.3	96.3	16.8	0.4	1.1	3.2	13.1	2.2	0.0	39.3	51.3	0.0
FUA3126	9.3	3.5	1.6	10.8	159.7	88.7	17.3	0.4	1.4	3.4	14.0	2.3	0.0	37.5	53.4	0.0
							<i>Ln. buchneri</i> and <i>Ln. parabuchneri</i>									
FUA3154	3.5	2.3	1.7	10.8	170.3	86.5	14.6	0.0	0.9	2.0	14.3	2.1	0.0	113.1	52.6	8.4
FUA3315	3.5	4.2	1.7	10.9	199.0	92.6	35.2	0.0	1.4	5.7	10.8	0.8	0.0	49.5	10.2	1.5
FUA3405	4.3	2.3	1.8	10.1	210.0	114.0	13.4	0.0	1.1	1.8	11.9	2.2	0.0	95.0	88.7	23.2
LPB02	10.2	2.3	2.0	11.1	172.5	88.1	9.4	0.0	1.0	2.1	7.3	2.9	0.0	236.4	139.5	77.7
							<i>Lp. plantarum</i>									
LP48	3.9	3.6	1.5	10.4	176.0	94.0	21.9	0.0	1.2	3.5	5.5	0.9	0.1	280.9	29.5	0.0
FUA3183	6.4	2.8	1.5	6.8	169.7	93.1	17.4	0.2	1.0	2.4	5.3	2.0	0.0	323.8	48.3	0.0
LP23	8.1	3.2	1.4	8.8	175.8	91.2	18.3	0.0	0.9	3.3	7.6	2.1	0.0	251.4	49.5	0.0
LP24	8.1	3.1	2.0	8.0	208.0	114.1	19.6	0.2	1.1	3.3	7.7	2.2	0.0	262.7	48.0	0.0

4 Shown are averages of three replicates

5 **Table S3. Summary of the production of organic acids and fatty acids in yogurt.** Yogurt (575 µL) was fermented for 2 d at 30 °C  
6 and incubated for 14 d prior to sampling at 25 °C to match the conditions of the challenge assays. The negative control represents  
7 yoghurt that was incubated without adjunct cultures at the same conditions.

strains	mold-free span	C18-2	C17-0	C14-0	C16-0	C18-0	C18-1	C18-3	C14-1	C16-1	mono-OH C18-0	10-OH C18-1	13-OH C18-1	Lactate	Acetate	1,2 propanediol
		mg / L										(mM)				
Neg. ctrl.	2.4	6.9	2.4	11.9	200.0	98.2	33.1	0.3	0.7	4.5	0.8	0.1	0.0	146.7	0.0	0.0
<i>F. rossiae</i> and <i>F. mii</i>																
FUA3509	3.3	2.3	2.2	9.2	211.0	103.3	12.4	0.0	0.5	2.2	9.2	1.0	0.4	161.6	60.9	6.5
FUA3119	4.5	2.9	1.7	9.6	192.7	97.2	12.3	0.2	0.5	2.5	8.8	1.3	0.0	181.9	60.3	0.0
FUA3126	4.9	3.3	2.2	10.1	211.0	114.4	14.0	0.0	0.5	2.7	9.7	1.2	0.0	200.9	38.1	0.0
FUA3124	6.1	3.4	2.1	10.7	217.3	112.9	16.0	0.2	0.7	3.0	10.5	0.8	0.4	177.9	56.3	0.0
FUA3115	6.9	3.4	1.8	9.4	190.3	99.0	14.6	0.2	0.6	2.8	9.8	1.3	0.0	171.9	61.8	0.0
<i>Ln. buchneri</i> and <i>Ln. parabuchneri</i> .																
FUA3315	3.3	4.1	1.9	11.6	202.7	96.5	34.4	0.0	1.2	4.6	9.3	0.7	0.0	160.5	14.9	1.5
FUA3405	6.8	2.7	1.9	11.2	221.7	107.1	15.7	0.0	0.6	1.7	9.3	1.6	0.1	172.1	107.8	50.7
LPB02	8.9	2.6	2.0	10.8	219.0	117.7	11.2	0.0	0.4	1.7	3.4	1.8	0.0	264.3	135.0	82.5
FUA3154	10.2	2.3	1.9	10.6	184.3	90.6	14.0	0.0	0.8	1.4	11.1	1.4	0.1	229.1	111.8	56.0
<i>Lp. plantarum</i>																
LP48	4.5	3.3	1.9	10.3	184.7	94.8	21.1	0.0	0.9	3.3	3.7	0.9	0.2	320.5	36.0	0.0
LP24	6.5	3.3	2.1	9.8	220.0	110.4	18.3	0.0	0.7	2.8	8.4	1.8	0.1	268.9	46.4	0.0
FUA3183	6.8	3.3	2.2	8.4	218.0	118.7	21.0	0.0	1.0	2.7	4.5	1.9	0.0	336.4	47.4	0.0
LP23	7.2	3.0	1.8	8.2	217.7	114.0	17.3	0.0	0.7	3.0	6.1	1.8	0.0	270.5	46.5	0.0

8 Shown are averages of three replicates

