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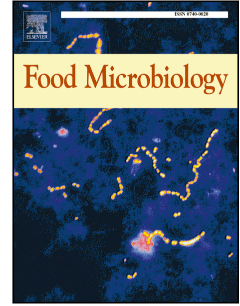

Application of qPCR for multicopper oxidase gene *(MCO)* in biogenic amines degradation by *Lactobacillus casei*

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1 **Application of qPCR for multicopper oxidase gene *(MCO)* in biogenic amines
2 degradation by *Lactobacillus casei***

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5 **Running title**

6 **Multicopper oxidase gene in *L. casei* - application of real-time PCR**

7

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28 **Abstract**

29 Degradation of undesirable biogenic amines (BAs) in foodstuffs by microorganisms is
30 considered one of the most effective ways of eliminating their toxicity. In this study, we
31 designed two sets of primers for the detection and quantification of the multicopper oxidase
32 gene (*MCO*), which encodes an enzyme involved in BAs degradation, and endogenous
33 (glyceraldehyde-3-phosphate dehydrogenase) gene (*GAPDH*) in *Lactobacillus casei* group by
34 real-time PCR (qPCR). We tested 15 *Lactobacillus* strains in the screening assays (thus, *MCO*
35 gene possessing assay (PCR) and monitoring of BAs degradation by HPLC-UV), in which
36 *Lactobacillus casei* CCDM 198 exhibited the best degradation abilities. For this strain, we
37 monitored the expression of the target gene *(MCO*) in time (qPCR), the effect of redox
38 treatments (cysteine, ascorbic acid) on the expression of the gene, and the ability to degrade
39 BAs not only in a modified MRS medium (MRS/2) but also in a real food sample (milk).
40 Moreover, decarboxylase activity (ability to form BAs) of this strain was excluded. According
41 to the results, CCDM 198 significantly ($P < 0.05$) reduced BAs (putrescine, histamine,
42 tyramine, cadaverine), up to 25% decline in 48 hours. The highest level of relative expression
43 of *MCO* (5.21 ± 0.14) was achieved in MRS/2 media with cysteine.

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50 **Keywords:**

51 Biogenic amines degradation, histamine, qPCR, primers, *Lactobacillus casei*

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53 **1. Introduction**

54 Biogenic amines (BAs) are low-molecular-weight nitrogen compounds that are formed in
55 foods and beverages during fermentation by bacterial species possessing the specific amino
56 acid decarboxylases. BAs could be toxic to human health in higher concentrations (Silla
57 Santos, 1996; Stratton et al., 1991). The most dangerous biogenic amine is histamine, which
58 is responsible for the majority of BAs-related food poisonings. Parente et al., 2001 pointed
59 out that levels of histamine greater than 100 mg.kg^{-1} can be health threatening; thus, its
60 quantity in foodstuffs must be monitored. European legislation (Commission Regulation (EC)
61 No 2073/2005, 2005) lays down food safety criteria for histamine in fishery products of up to
62 100 mg.kg^{-1} and for fishery products, which have undergone enzyme maturation treatment in
63 brine, of up to 200 mg.kg^{-1}. However, high levels of BAs may occur in all fermented
64 foodstuffs and beverages - the threat of their increased accumulation is mainly found in
65 cheeses, sausages and wine. Concentrations of BAs exceeding 1 g.kg^{-1} have been reported in
66 cheese, with histamine and tyramine being the most commonly present BAs (Alvarez and
67 Moreno-Arribas, 2014; Fernández et al., 2007).

68 Removing histamine and other BAs formed is very complicated because of their persistence
69 (Zaman et al., 2010). The strategies for diminishing BAs levels in foodstuffs are primarily
70 targeted at reducing their precursors (amino acids), reducing the growth of spoilage bacteria
71 and inoculating starter cultures without amino acid decarboxylases (Callejón et al., 2014).

72 Probably, the most effective solution is to use microorganisms, which can degrade amines
73 formed as a part of the starter or adjunct cultures. The ability to degrade biogenic amines in
74 culture media or foodstuffs is based on the fact that some microorganisms are capable of
75 producing degrading enzymes, such as amine oxidases and multicopper oxidases (MCO)
76 (Alvarez and Moreno-Arribas, 2014; Callejón et al., 2014). Amine oxidases are the large
77 group of enzymes catalysing the degradation of BAs to substances that can be utilised by

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78 microorganisms as a source of energy and growth. Several studies have described these
79 enzymes and deamination pathways (Sekiguchi et al., 2004; Wang et al., 2013; Yagodina et
80 al., 2002). Later, Callejón et al., 2014 described the degradation of BAs in wine by lactic acid
81 bacteria (LAB) possessing multicopper oxidases.

82 LAB play an essential role in the production of fermented dairy products, with *Lactococcus*
83 *lactis*, *Leuconostoc* sp., *Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *lactis*
84 and *Lactobacillus helveticus* being the species most commonly used as primary fermentation
85 starters (Ladero et al., 2015; Parente et al., 2017; Renes et al., 2019; Silva et al., 2020). The
86 development of the final organoleptic properties of fermented dairy products frequently
87 participate facultative, heterofermentative lactobacilli belonging to the species *Lactobacillus*
88 *casei/paracasei*, *Lactobacillus plantarum* or *Lactobacillus curvatus* (Ladero et al., 2015).
89 LAB are generally regarded as safe (GRAS) and thus are used in foodstuffs due to their
90 inhibitory properties against spoilage bacteria and foodborne pathogens (Özogul and Hamed,
91 2018). The *Lactobacillus casei* group has an important place among LAB, which includes
92 species: *L. casei, L. paracasei* and *L. rhamnosus*. These species are well-researched due to
93 their applicability in the food, biopharmaceutical and medical industries. Their health-
94 promoting capabilities have been documented in several studies suggesting their potential for
95 their use in the treatment, or prevention, of a variety of diseases (Hill et al., 2018).

96 A powerful tool for searching for strains with degradation abilities could be real-time PCR
97 (qPCR). This advanced technique offers the advantages of speed, sensitivity, simplicity and
98 the specific detection and quantification of target genes in one step (Landete et al., 2007). The
99 food industry is increasingly using qPCR for genes detection and quantification involved in
100 BAs production (Elsanhoty and Ramadan, 2016; Ladero et al., 2015, 2010; Postollec et al.,
101 2011). The situation is different for monitoring the expression of genes involved in BAs
102 degradation. Very few studies have described primers that allow the monitoring of the

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103 expression of the degrading genes (Eom et al., 2015; Herrero-Fresno et al., 2012). Some
104 authors have developed primers for the detection of the multicopper oxidase gene in LAB by
105 PCR (Callejón et al., 2014; Guarcello et al., 2016), which are not suitable for qPCR due to the
106 length of the PCR amplicons.

107 The purpose of this study is to design new primers for the specific detection of the
108 multicopper oxidase gene *(MCO)* in the *Lactobacillus casei* group, which catalyse the
109 degradation of common BAs present in fermented foodstuffs. Using new set of primers, we
110 identified 15 *Lactobacillus casei* strains capable of degrading BAs. Moreover, we examined
111 all tested strains for decarboxylase activity to eliminate the possibility that potential degraders
112 are also BAs producers. We also specify the degradation capacity of BAs of *L. casei* CCDM
113 198 in broth and milk. Strain CCDM 198 was isolated from aidam cheese and is used as a
114 starter culture, which is declared by the supplier (Laktoflora, Czech Republic). So far as we
115 know, no previous work tested degradation abilities of BAs of this strain. Furthermore, we
116 determine the influence of redox potential treatments cysteine and ascorbic acid on the BAs
117 degradation.

118

119 **2. Materials and methods**

120 The number of cells and the growth curve phase have a key effect on the degradation;
121 therefore, the growth curves of degrading strains and the effect of redox treatments or
122 cultivation media on bacterial growth were also monitored.

123 *2.1 Strains and cultivation conditions*

124 **Microorganisms:** The *L. casei* strains used in this study (CCDM 198, CCDM 145) were
125 obtained from the Laktoflora, the Culture Collection of Diary Microorganisms (CCDM),
126 Czech Republic. Thirteen strains of *L. paracasei* (S3_1 - S3_13) were isolated from

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127 sourdough of traditional Portuguese sourdough bread at the School of Agriculture at the
128 Polytechnic Institute of Beja, Portugal.
129 **Growth conditions for the production of BAs:** All 15 strains were first grown in tubes with
130 7 mL of MRS broth (HiMedia, Mumbai, India) at 37°C in 5% (v/v) CO_2 for 24 hours. Then
131 a 50 µL culture was inoculated into MRS broth (7 mL) to obtain the initial concentration of
132 bacteria (6.2±0.3 log CFU/mL). The medium was supplemented with amino acids (arginine,
133 ornithine, histidine, tyrosine, lysine and phenylalanine) at 0.2 $g.L^{-1}$ each, and hydrochloric
134 acid (0.1 M or 1 M HCl) was added to adjust a pH of the medium to 6.5±0.1. Incubation was
135 performed at 37°C in 5% (v/v) CO_2 for 48 hours.
136 **Growth conditions for the preliminary test of BAs degradation:** The inoculum was grown
137 in MRS broth (HiMedia, Mumbai, India) to achieve maximum cell count; however,
138 degradations were performed in a depleted/modified MRS (MRS/2; 50% of weight
139 Lactobacillus MRS broth) medium. The depleted medium provides less nutrients which may
140 support the use of BAs as an alternative source of carbon and nitrogen. All 15 strains were
141 first grown in tubes with 7 mL of MRS broth at 37°C in 5% (v/v) CO_2 for 24 hours. Then, the
142 50 µL culture was inoculated into 7 mL of MRS/2 broth to obtain initial concentration of
143 bacteria (6.2±0.3 log CFU/mL). The medium was supplemented with biogenic amines
144 (histamine, tyramine, putrescine, and cadaverine) at 0.2 $g.L^{-1}$ each, and hydrochloric acid (0.1
145 M or 1 M HCl) was added to adjust a pH of the medium to 6.5±0.1. Incubation was performed
146 at 37°C in 5% (v/v) CO_2 for 48 hours.
147 **Growth conditions for the degradation of BAs:** *L. casei* strains were first grown in tubes
148 with 7 mL of MRS broth (HiMedia, Mumbai, India) at 37°C in 5% (v/v) CO_2 for 24 hours and
149 a 200 µL culture was inoculated into MRS/2 broth (50 mL) to obtain the initial concentration
150 of bacteria (6.2±0.3 log CFU/mL). The medium was supplemented with biogenic amines
151 (histamine, tyramine, putrescine, and cadaverine) at 0.2 $g.L^{-1}$ each, and hydrochloric acid

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152 (0.1 M or 1 M HCl) was added to adjust a pH of the medium to 6.5±0.1. Incubation was
153 performed at 37°C in 5% (v/v) CO_2. The relative expression level and biogenic amine
154 degrading capability were determined after incubation for 0, 12, 24 and 48 hours.

155 **Effects of cysteine, ascorbic acid and milk on the growth of *L. casei* CCDM 198 and**
156 **biogenic amines degradation:** To support bacteria growth and/or biogenic amines
157 degradation, 1% (w/v) cysteine (concentration recommended by supplier Laktoflora) or 0.1%
158 (w/v) ascorbic acid (Demain et al., 1961) were added to a 50 mL MRS/2 medium
159 (composition described in growth conditions) before inoculation.

160 Growth and degradation of *L. casei* CCDM 198 were observed not only in the broth but
161 also in the real food - milk. UHT low-fat milk (50 mL) was supplemented with biogenic
162 amines (histamine, tyramine, putrescine, and cadaverine) at 0.2 $g.L^{-1}$ each, and hydrochloric
163 acid (0.1 M or 1 M HCl) was added to adjust a pH of the medium to 6.5±0.1. Then a 200 µL
164 culture (ca. 109 CFU) was inoculated into milk (50 mL) to obtain the initial concentration of
165 bacteria (6.2±0.3 log CFU/mL). Concentration of bacteria during growth cycle was monitored
166 by plate method. Incubation was performed at 37°C in 5% (v/v) CO_2 at 30°C and 37°C.

167 **Bacterial growth curves:** To indicate bacterial growth in MRS/2 with BAs and to determine
168 the effect of cysteine and ascorbic acid on the growth of *L. casei* CCDM 198, we added
169 200 µL of media to each well followed by inoculation to obtain the initial concentration of
170 bacteria (6.2±0.3 log CFU/mL). The covered microplates were incubated for 72 hours at
171 37°C. During the incubation period, optical density was measured at 550 nm in a Multimode
172 Microplate Reader (Tecan Infinite 200 PRO, Switzerland) at regular intervals.

173 The bacterial counts in milk with BAs were determined by the plate method. Cultured milk
174 samples were serially diluted with sterile phosphate buffer (1:9), and 100 µL of each sample
175 was loaded on the plate with MRS agar. Bacterial colonies were counted after 48 hours of
176 incubation at 37°C. Results were expressed as CFU per millilitre.

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177

178 *2.2 Primer design*

179 Specific gene primers were designed from conserved sequences of the multicopper oxidase
180 gene. In this study, endogenous gene primers were designed for the glyceraldehyde-3-
181 phosphate dehydrogenase *(GAPDH)* (Zhao et al., 2011). The sequences of the multicopper
182 oxidase gene *(MCO)* and endogenous gene *(GAPDH)* for ten different *Lactobacillus casei*
183 strains were obtained from the National Centre for Biotechnology Information ("NCBI",
184 2017). New sets of primers for target genes were designed based on the Primer Design
185 genefisher2 (Giegerich et al., 1996). Furthermore, the properties of sets of primers were
186 verified using the NCBI Primer-Blast tool. Primers in this study were synthesised by Merck
187 (Darmstadt, Germany).

188

189 *2.3 DNA extraction, Polymerase chain reactions (PCR)*

190 To verify the presence of target genes in 15 *Lactobacillus* strains, DNA was extracted from
191 bacterial cells. Genomic DNA was prepared from 1 mL of bacterial strains that were grown in
192 MRS/2 broth. Pellets of these strains were obtained by centrifuging at 3000 x *g* for 5 min.
193 DNA was extracted using the Genomic DNA from Tissue Kit (Macherey-Nagel, Germany)
194 according to the manufacturer´s instructions. The purity and concentration of the DNA were
195 measured using a Multimode Microplate Reader Infinite 200 PRO (Tecan, Switzerland).
196 PCR was performed by using a commercial mix, G2 Hot Start Green Master Mix (ROCHE,
197 Germany). The reaction volume, 25 μL, included 12.5 μL of the commercial mix (ROCHE,
198 Germany), 800 nmol.L^{-1} of a forward primer, 800 nmol.L^{-1} of a reverse primer and 10-100 ng
199 of template. Additionally, we prepared a negative control sample without a template. The
200 PCR conditions were as follows: initial denaturing at 95°C for 5 min, followed by 35 cycles

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201 each comprising 95°C for 30 sec of denaturing, 61°C for 30 sec of annealing, and 72°C for 30
202 sec of extension; the final extension was performed at 72°C for 10 min.
203 The PCR products were separated into 1% (w/v) agarose gel in a TAE buffer with ethidium
204 bromide by agarose electrophoresis run for 25 min at 90 V on a 1% gel. The GeneRuler
205 100 bp Plus DNA Ladder (Thermo Fisher Scientific, USA) was used as molecular weight
206 marker.
207 The partial nucleotide sequence of the amplified genes *MCO* and *GAPDH* were verified by
208 sequencing with our set of primers (Tab. 1). PCR products were purified using
209 NucleoMag® Tissue (Macherey-Nagel, Germany). The resulting sequences were compared
210 against NCBI database using the Basic Local Alignment Search Tool program (NCBI, 2009).
211
212 *2.4 Reverse transcriptase and qPCR*
213 In order to quantify *MCO* gene expression, it was necessary to isolate RNA from *L. casei*
214 strains (CCDM 198, CCDM 145) and transcribe it by reverse transcription into cDNA, which
215 serves as a template for qPCR. RNA isolation was done using the RNeasy PowerLyzer Tissue
216 & Cells Kit (QIAGEN, Germany) according to the manufacturer´s instructions. RNA
217 isolation from *L. casei* CCDM 198 cultivated in milk was also done using the RNeasy
218 PowerLyzer Tissue & Cells Kit, but with a small modification. After the collection of
219 samples, 1 mL of culture was centrifuged at 2000 x *g* for 5 min at 4°C. The upper layer of fat
220 was sterile removed, and a sample was frozen and thawed three times. Homogenisation was
221 performed with PowerLyzer Ceramic Bead Tubes. The procedure was finished according to
222 the enclosed instructions. First-strand cDNA was synthesized from 11 μL RNA using a
223 Transcriptor First Strand cDNA Synthesis Kit (ROCHE, Germany).
224 qPCR was performed by using thermocycler CFX 96 Real-Time (Bio-Rad, Hercules, CA,
225 USA) with the commercial kit FastStart Universal SYBR Green Master (ROCHE, Germany).

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226 The total reaction volume, 25 µL, included 12.5 µL of ROCHE mix, 250 nmol.L^{-1} of a
227 forward primer, 250 nmol.L^{-1} of a reverse primer and 1-2 µg of cDNA template. The qPCR
228 conditions were as follows: initial denaturing at 95°C for 3 min, followed by 45 cycles each
229 comprising 95°C for 30 sec of denaturing, 60°C for 30 sec of annealing, and 72°C for 1 min
230 of extension; final extension was performed at 72°C for 5 min. Data were normalized to
231 *GAPDH* expression. Reference control and nontemplate negative controls (using water
232 instead of cDNA) were included in every run for both genes.
233 The baseline and cycle threshold were automatically calculated using the C1000 Touch
234 Thermal Cycler equipped with a CFX 96 Touch™ System Software, version 2.1 (Bio-Rad,
235 CA, USA). The melt curve analysis was performed on the same device (CFX 96 Real-Time)
236 after the completion of qPCR. Obtained PCR products of the *MCO* and *GAPDH* had melting
237 temperatures of 76±0.5°C and 77±0.5°C, respectively.
238
239 *2.5 Determination of biogenic amine content*
240 The degrading capacity of the strains was tested in a modified nutrient broth (MRS/2) and
241 milk by HPLC/UV. Samples (3 mL) were collected in determined hours (0, 12, 24 and 48)
242 and centrifuged at 2000 x *g* for 10 minutes. Supernatant (600 µL) was diluted 1:1 (v/v) with
243 0.6 M perchloric acid (Acros, Belgium). Three independent extractions were performed on
244 each culture sample. Subsequently, mixtures were derivatised using dansyl chloride (Sigma-
245 Aldrich, Missouri, USA) with 1,7-heptanediamine (Fluka, Switzerland) as an internal
246 standard according to Dadáková et al., 2009.
247 BAs (histamine, tyramine, putrescine and cadaverine) were detected using high-performance
248 liquid chromatography, Dionex HPLC UltiMate 3000 (Thermo Fischer Scientific, Waltham,
249 Massachusetts, USA), following preceding derivatisation using dansyl chloride (Dadáková et
250 al., 2009). The chromatographic column used for separation was an Agilent Zorbax RRHD

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251 Eclipse Plus C18 with the dimensions of 50 x 3.0 mm, 1.8 µm (Agilent, Paolo Alto, USA).
252 Spectrophotometric detection was carried out at a wavelength of 254 nm and a column
253 temperature of 30°C. The flow rate was 0.453 $mL.min^{-1}$. The detection and separation of
254 biogenic amines were performed according to (Dadáková et al., 2009; Smělá et al., 2004).
255 Data were acquired and evaluated using Chromeleon™ 6.8 software (Thermo Fisher
256 Scientific, USA).
257
258 *2.6 Statistical evaluation*
259 Non-parametrical analyses of variance from the Kruskal-Wallis and Wilcoxon tests (Unistat®
260 6.5 software; Unistat, London, UK) were used to evaluate the results obtained (the
261 significance level was 0.05). To estimate of the dependence of threshold cycle on DNA
262 concentration regression line (linear least squares method) was used (Unistat® 6.5; software
263 Unistat, London, UK).
264
265 **3. Results and Discussion**
266 *3.1 Screening of LAB strains possessing the MCO gene*
267 The ability to degrade BAs depends not only on the species but also on the strains. Thus,
268 testing suitable strains using conventional techniques is unreliable or labour intensive and
269 time-consuming. For these reasons, molecular biology methods are increasingly being used in
270 the food industry (Postollec et al., 2011).
271 To allow rapid screening of strains possessing the *MCO* gene and to examine the expression
272 of this gene, we designed and tested gene-specific primers. Then we searched for a strain with
273 degrading properties using new primers.
274 Firstly, we screened *L. casei* and *L. paracasei* strains possessing the multicopper oxidase gene
275 by PCR. Secondly, we performed a preliminary test of the degradative ability of strains when

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276 we grew a culture in 7 mL MRS/2 with BAs for 48 hours. At the end of this test, we
277 monitored the decrease in the content of BAs by HPLC/UV. At the same time, we examined
278 whether or not strains with degradation abilities are producers of BAs. According to obtained
279 results (data not shown), we found that only two strains of *L. casei* (CCDM 198, CCDM 145)
280 are not BAs producers. We subsequently focused attention on the CCDM 198 strain, given
281 that it had significantly higher degradation capabilities than the strain CCDM 145. During the
282 preliminary tests, we also verified that the biogenic amines degradation ability of the CCDM
283 198 was four times higher in the depleted medium MRS/2 compared to MRS (data not
284 shown). Therefore, depleted medium was preferred in this work. Finally, based on the results,
285 we observed relative expression of the *MCO* gene within 48 hours for the selected CCDM
286 198 strain and used the CCDM 145 strain as a positive control for qPCR (*3.3 Expression of*
287 *Gene Encoding Multicopper Oxidase)*. We verified the ability of strains CCDM 198 and
288 CCDM 145 to degrade BAs in 50 mL of MRS/2 using HPLC/UV and experienced the effect
289 of redox treatments and milk on the BAs degradation of strain CCDM 198. We also observed
290 the growth of cells during the degradation process.
291
292 *3.2 Specific primer design*
293 In this study, we designed three sets of primers for the multicopper oxidase gene (*MCO*) in
294 *L. casei,* which is responsible for the degradation of BAs. Due to the normalisation of the
295 target gene with an endogenous standard, we designed and tested primers for the
296 glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH*). According to the PCR tests, we
297 chose the set of primers which do not form dimers or nonspecific products. The new sets of
298 primers anneal to the multicopper oxidase gene of *L. casei* and *L. paracasei* strains. The final
299 length of the PCR product for the detection of the multicopper oxidase gene (*MCO*) is 94 bp,
300 and for the detection of the endogenous gene *(GAPDH),* primers with a length of 137 bp were

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301 selected (Table 1). The sequencing followed by analysis in BLAST (NCBI, 2009) confirmed
302 that PCR products corresponded to the *MCO* and *GAPDH* partial nucleotide sequences,
303 respectively.
304
305 **Parameters of qPCR:** Amplification efficiency values with our sets of primers were in the
306 optimal range between 90% and 110% (Broeders et al., 2014), which corresponds to the slope
307 of the long-linear phase of the amplification reaction between -3.58 and -3.10 (Figure 1). In
308 addition, the linearity of the qPCR reaction used to determine the efficiency r^2 was ≥0.98 for
309 each target. Post-amplification melting-curve analysis (data not shown) confirmed that the
310 chosen sets of gene-specific primers do not form dimers or non-specific products. The Pfaffl
311 method was used to calculate the relative expression (Pfaffl, 2001).
312 **The verification of target genes by PCR and selecting strains to monitor the relative**
313 **expression of target genes during BAs degradation:** Before qPCR analysis, we checked for
314 the presence of the multicopper oxidase gene *MCO* and endogenous gene *GAPDH* in bacterial
315 strains using PCR. The presence of the multicopper oxidase gene in *L. casei* and *L. paracasei*
316 strains is shown in Figure 2. HPLC/UV showed that all strains possessing the multicopper
317 oxidase gene were able to degrade BAs. However, *L. paracasei* strains are also BAs
318 producers (for more details, see *Chapter 3.4*). Therefore, relative expression was only tested
319 in *L. casei* strains.
320
321 *3.3 Expression of Gene Encoding Multicopper Oxidase*
322 qPCR is a standard method for measuring gene expression. This quantitative analysis requires
323 no postprocessing; results are obtained quickly and, therefore, it could be used for the routine
324 detection of bacterial strains that have potential to degrade histamine and other BAs (Wong
325 and Medrano, 2005).

326 We performed qPCR analysis to study the expression of the target gene in the selected
327 bacterial strains. The degradation capacity of total BAs of strain CCDM 145 is only 6±0.46 %
328 after 48 hours (Figure 3); thus, this strain possesses the *MCO* gene, but its expression is low.
329 For this reason, the CCDM 145 strain was used as a positive control to calculate relative
330 expression. The relative expression levels are also shown in Figure 3.

331 Strain CCDM 198 exhibited the highest level of relative expression after 12 hours of
332 cultivation in modified MRS/2 media with cysteine (5.21±0.14). A somewhat lower level of
333 relative expression was achieved in MRS/2 broth (5.04±0.45); however, significantly lower
334 levels of relative expression were recorded in milk: 3.58±0.52 at 37°C and 2.53±0.35 at 30°C.
335 Therefore, the highest expression level of multicopper oxidase was recorded in the
336 exponential phase of the growth of cells. This was followed by a decline in relative expression
337 levels of all samples after 24 hours. At this time, the highest values (2.21±0.38) were reached
338 in MRS/2 broth. After 48 hours of cultivation, there was a further decrease in the relative
339 expression levels at values around 1.00. The ability to degrade BAs was confirmed by
340 HPLC/UV analysis.

341

342 *3.4 Detection of biogenic amine content*

343 High performance liquid chromatography is the most commonly used technique because of its
344 great versatility, efficiency, sensitivity and reproducibility, and is, therefore, the conventional
345 technique for analysing histamine in foods (Commission Regulation (EC) No 2073/2005,
346 2005; Marcobal et al., 2006).

347 **Selection of BAs-degrading LAB strains:** We performed a preliminary degradation test of
348 two strains of *L. casei* and 13 strains of *L. paracasei* bearing the multicopper oxidase gene to
349 quantify their BAs degrading ability using the HPLC/UV method.

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350 The results obtained showed that all strains tested can degrade BAs but with different
351 efficiency (data not shown). The highest decrease of BAs content was observed in the *L. casei*
352 CCDM 198 strain and was therefore chosen to observe expression during BAs degradation.
353 *L. casei* CCDM 198 showed approximately 25% degradation of BAs in a preliminary test
354 (data not shown). Other authors confirmed the excellent abilities of *L. casei* strains. García-
355 Ruiz et al., 2011 demonstrated great potential for histamine, tyramine and putrescine
356 degradation (54%, 55% and 65%, respectively) of the strain *L. casei* IFI-CA 52 strain in
357 culture media. Herrero-Fresno et al., 2012 isolated 17 *L. casei* strains with histamine and/or
358 tyramine degradation rate up to 40% in a cheese manufacturing model.
359 **Exclusion of BAs-producing LAB strains:** Since some lactobacilli are significant BAs
360 producers (Herrero-Fresno et al., 2012), we performed the test to exclude potential BAs
361 producers for industrial applications. *L. casei* (CCDM 198, CCDM 145) and 13 strains of *L.*
362 *paracasei* we tested for biogenic amine production in a medium with biogenic amines
363 precursors. Test results showed that *L. casei* (CCDM 198, CCDM 145) are not producers of
364 phenylethylamine, histamine, tyramine, cadaverine and putrescine. On the other hand, all
365 strains of *L. paracasei* are capable of producing one or more biogenic amine in amounts up to
366 30 mg.L^{-1} and are not suitable for industrial use (data not shown). Therefore, expression of the
367 target gene was not tested in *L. paracasei*, although all 13 strains contained the *MCO* gene
368 (Figure 2).
369 **Relationship between BAs (histamine, tyramine, cadaverine, putrescine) content and**
370 **relative gene expression:** The decrease of biogenic amines corresponds to the achieved
371 relative expression values (Figure 3). The highest difference in BAs content was after 12
372 hours of cultivation in broth, when the highest relative expression was also recorded; this was
373 in contrast to cultivation in milk, where the decrease of BAs content was lower than 3%. This

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374 was probably caused by the longer lag phase and low cell count after 12-hour cultivation
375 (Figure 4).
376 After 48 hours, the highest decrease in total BAs at 77.36±3.13% was achieved in MRS/2
377 with 1% cysteine. Only a little higher BAs content (78.78±4.17%) remained in MRS/2 broth.
378 Although a significantly higher number of cells was observed in cultivation at 30°C in milk,
379 low relative expression may have caused the highest biogenic amines content (85.02±1.83%)
380 at the end of cultivation.
381 **Effects of media on growth pattern of selected LAB strains:** Many studies have
382 demonstrated the positive effect of oxygen scavenging agents (cysteine and ascorbic acid) on
383 viability of probiotic bacteria (Dave and Shah, 1997; Demain et al., 1961; Rickes et al., 1949;
384 Shah, 2000). Oxygen reduces the growth of these bacteria, and the use of cysteine and
385 ascorbic acid may lower redox potential by scavenging oxygen, thus affecting their growth
386 (Shah, 2000). The positive effect of cysteine on the growth of cells of the CCDM 198 strain
387 was also observed in our test (Figure 4), but there was no marked effect on the increase of
388 multicopper oxidase expression level (Figure 3). This corresponds to the results recorded in
389 Figure 5. Lower amounts of histamine, tyramine and cadaverine were observed in samples
390 with 1% cysteine, but the difference was not significant ($P>0.05$) compared to MRS/2 broth.
391 Moreover, the highest degradation, thus the lowest content was observed for putrescine in
392 MRS/2 (75.18±3.95%). The final amount of putrescine in the 1% cysteine medium was
393 slightly higher (75.60±3.43%). On the other hand, the lowest degradation capacity was
394 recorded for tyramine, where the content of no sample falls below 80% (Figure 5). *L. casei*
395 CCDM 198 also significantly ($P < 0.05$) reduced histamine, the most dangerous BA, in
396 MRS/2 (76.86±4.21%) and in MRS/2 with 1% cysteine (76.35±2.87%) after 48 hours (Figure
397 5). Based on obtained results, the presence of ascorbic acid may not support the degradation

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398 of BAs because the content of all monitored BAs was higher in samples with 0.1% ascorbic
399 acid than in MRS/2 and MRS with cysteine (Figure 3 and 5).
400 **The effect of milk environment on content of histamine, tyramine, cadaverine and**
401 **putrescine:** After 48 hours, the content of all monitored BAs was 3-6% higher in milk than in
402 MRS/2 broth with exception of tyramine. A temperature of 37°C is more preferable for the
403 CCDM 198 strain because results showed lower histamine, cadaverine and putrescine content
404 at the end of cultivation than at growth temperature of 30°C (Figure 3 and 5). Although strain
405 CCDM 198 is isolated from the dairy product and therefore well adapted to the dairy
406 environment, our results are consistent with some studies showing that *L. casei* prefers MRS
407 medium (Avonts et al., 2004; Zuraw E A et al., 1960). Avonts et al., 2004 demonstrated that
408 *L. casei* strains were able to develop to high cell numbers in a milk medium, but fermentation
409 of milk was slow, and the production of bacteriocin was lower compared to the MRS
410 medium. We observed lower multicopper oxidase expression in this medium, which can also
411 be caused by amino acid imbalances in milk. Nevertheless, *L. casei* CCDM 198 proved to
412 significantly ($P<0.05$) decrease of histamine, tyramine, cadaverine and putrescine in milk
413 after 48 hours of cultivation.
414
415 *3.5 Growth of cells*
416 During degradations tests of *L. casei* CCDM 198, we monitored bacterial growth curves to
417 examine the effect of cysteine, ascorbic acid to the growth of bacterial cells. In order to follow
418 the growth curve at half-hour intervals, we used a spectrophotometric method for cultivation
419 in MRS/2 broth. The bacterial counts in milk were determined by the plate method at times of
420 collection of samples for qPCR and HPLC/UV. The findings of growth bacteria in both media
421 supplemented by BAs during 72 hours of cultivation are reported in Figure 4.

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422 **The growth of *L. casei* CCDM 198 and CCDM145 in MRS/2 broth:** The lag phase of both
423 strains was approx. 2 hours when OD_{550} was almost unchanged. Then OD_{550} rose rapidly, and
424 the exponential phase occurred between 4 and 17 hours of cultivation. After 22 hours, the
425 growth curves of both strains came into a stationary phase. While the OD_{550} of strain CCDM
426 198 slightly decreased at the beginning of the stationary phase, the OD_{550} of strain CCDM
427 145 maintained moderate growth until the end of the cultivation.
428 **The effect of cysteine and ascorbic acid on *L. casei CCDM 198* growth:** Since *L. casei* is
429 a facultative anaerobic bacterium, reducing agents cysteine and ascorbic acid were added to
430 promote cells growth. So far few authors reported the stimulatory effect of ascorbic acid on
431 the growth of *L. casei* with various recommended concentrations. Demain et al., 1961 tested
432 stimulating and toxic amounts for *Lactobacillus heterohiochi*. These results follow the study
433 Rickes et al., 1949, which dealt with the stimulating amount of ascorbic acid for *L. casei*
434 growth. In accordance with previous studies, two different concentrations of ascorbic acid
435 (0.1% and 0.01%) were chosen for our tests. Although the OD_{550} was slightly higher at 0.01%
436 compared to 0.1% concentration, the degradation of BAs decreased by approximately 10%
437 (data not shown). Thus, further tests were performed with 0.1% ascorbic acid. The addition of
438 0.1% (w/v) ascorbic acid significantly decreased OD_{550} throughout the growth curve, in
439 particular in the exponential phase. In contrast, the effect of 1% (w/v) cysteine in MRS/2
440 broth resulted in a slight increase of OD_{550} over the entire growth curve compared to growth
441 in MRS/2 itself. Strain *L. casei* CCDM 145 was used as a control sample for the relative
442 expression level; therefore, the effect of cysteine and ascorbic acid on the growth was not
443 tested.
444 **The effect of UHT low-fat milk on *L. casei* CCDM 198 growth:** Some strains are unable to
445 develop in unsupplemented milk because pure milk is generally low in free amino acid
446 content. Nevertheless, for a considerable number of different lactobacilli species is cow´s

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447 milk a naturally complex medium that supports their growth (Elli et al., 1999). The strain
448 CCDM 198 was originally isolated from a dairy product thus is well adapted to a milk
449 environment. In our test, the inoculum was grown in MRS; therefore, the lag phase in milk
450 was longer (approx. 2 hours), and the stationary phase came after 48 hours of cultivation
451 (Figure 4). Although the optimum cultivation temperature for CCDM 198 is 37°C,
452 significantly higher CFU values were achieved at 30°C ($2.6 \cdot 10^9$ CFU/mL).
453

454 **4. Conclusion**

455 In this work, we designed and tested new sets of primers for the detection of the multicopper
456 oxidase gene and endogenous gene for species *L. casei* and *L. paracasei.* We have proved that
457 the primers allow the detection and quantification of target genes by qPCR. Using this method
458 enables faster and easier searching for the strains capable of reducing histamine and tyramine,
459 the two abundant toxic BAs in foodstuffs and beverages. We also described a new way to
460 isolate RNA from curled milk.

461 In conclusion, we demonstrated that *L. casei* CCDM 198 used in dairy technology is not a
462 BAs producer and can significantly reduce histamine, tyramine, cadaverine, and putrescine in
463 milk. However, a noteworthy positive effect of cysteine and ascorbic acid on the degradation
464 of BAs has not been demonstrated. *L. casei* has been recognised as GRAS and was placed on
465 the QPS (qualified presumption of safety) list by the European Food Safety Authority (EFSA,
466 2016); therefore, nothing prevents the use of the CCDM 198 strain to reduce BAs in dairy
467 products.
468

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Table 1

Primer sequences for multicopper oxidase gene *(MCO)* catalysing BAs degradation and housekeeping glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) used in qPCR.

Primers	**Sequence 5´-3´**	**Amplicon length (bp)**
LCMCO4-L	GCGTGGTGACATCAAAATAGGG	94
LCMCO4-R	TGGGACTACCGGGCTGATTA	
LCGAPD4-L	GCACAGCGTGTTTCTGTTGT	137
LCGAPD4-R	TCGTTCCAGCCAAAGCTAGG	

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Figure captions

Figure 1

qPCR standard curves of multicopper oxidase gene (*MCO*) and endogenous gene (*GAPDH*). The templates were cDNA purified from bacterial cells grow in MRS/2 broth after 48 hours cultivation.

Figure 2

PCR testing of new primers. **a)** DNA fragments of multicopper oxidase gene (*MCO*) were amplified by primers LCMCO4-L and LCMCO4-R from *L. casei* strains: CCDM 198 (1) and CCDM 145 (2). **b)** DNA fragments of endogenous gene (*GAPDH*) were amplified by primers LCGAPD4-L and LCGAPD4-R from *L. casei* strains: CCDM 198 (1) and CCDM 145 (2). **c)** DNA fragments of multicopper oxidase gene (*MCO*) were amplified by primers LCMCO4-L and LCMCO4-R from 13 strains of *L. paracasei*.

Figure 3

Comparison of the relative expression levels of the multicopper oxidase gene in *L. casei* CCDM 198 performed by qPCR with the biogenic amines content in media determined by HPLC/UV during 48 hours of cultivation.

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Figure 4

Growth of the *L. casei* strains during BAs degradation in MRS/2 broth supplemented by histamine, tyramine and putrescine at 37°C, pH 6.5 ± 0.1 for 72 hours performed by optical density measurement and influence of 1% cysteine and 0.1% ascorbic acid on the growth. Compared to the growth of the *L. casei* CCDM 198 during BAs degradation in milk, which was determined by the colony counting method.

Figure 5

Content of histamine, tyramine, cadaverine and putrescine measured by HPLC/UV. Reaction was carried out in MRS/2 broth and milk inoculated by *L. casei* CCDM 198, pH 6.5±0.1 for 48 hours.

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Figure 1 (Pištěková et al.)

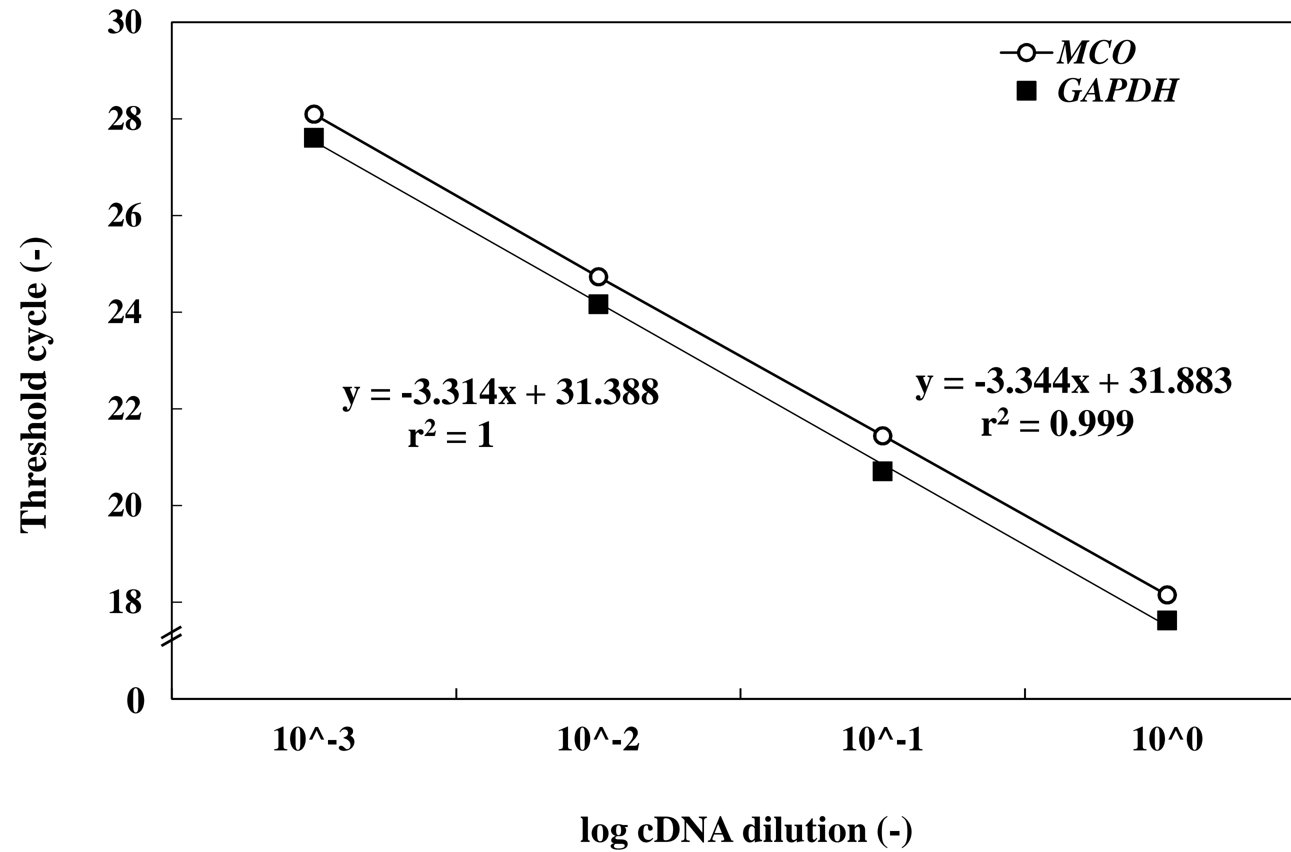

Figure 2 (Pištěková et al.)

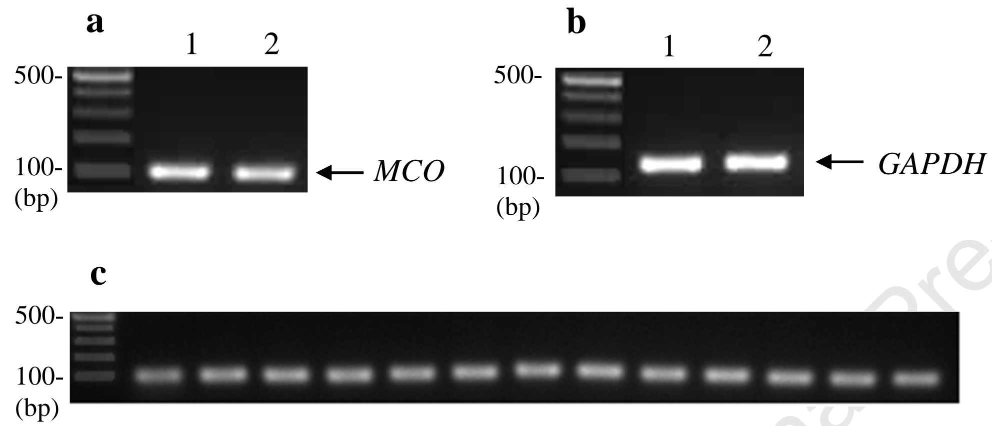

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Figure 3 (Pištěková et al.)

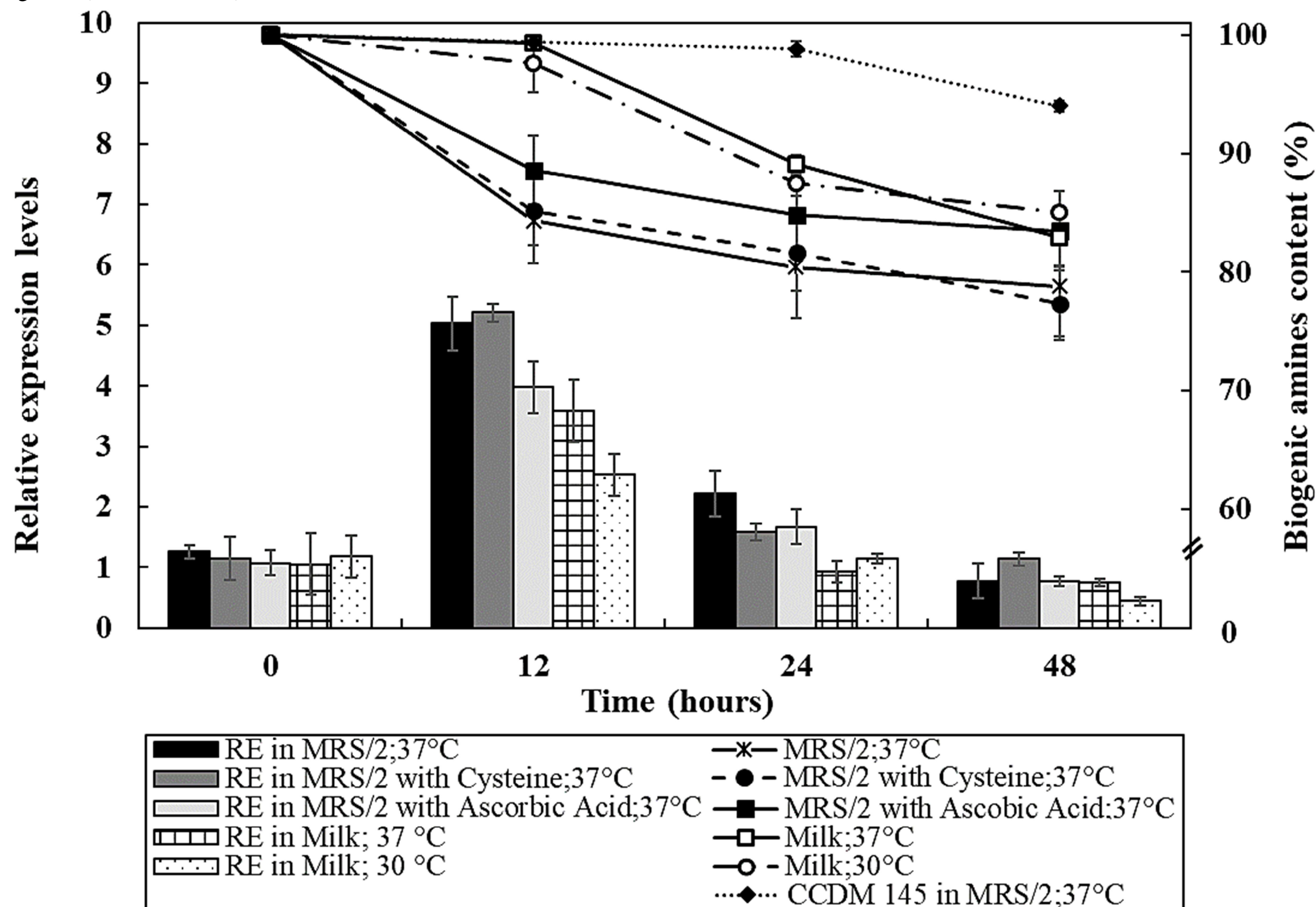


Figure 4 (Pištěková et al.)

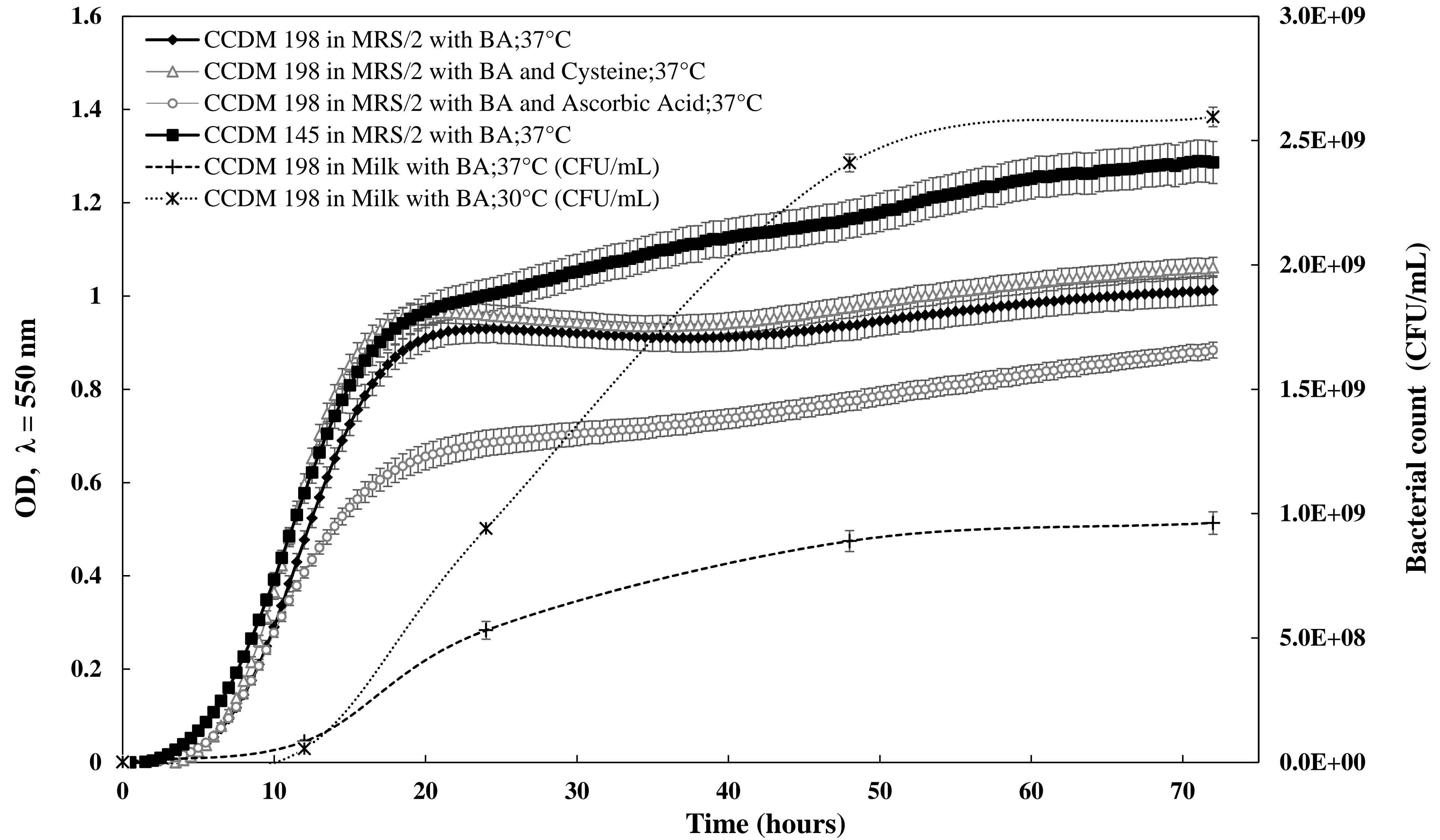
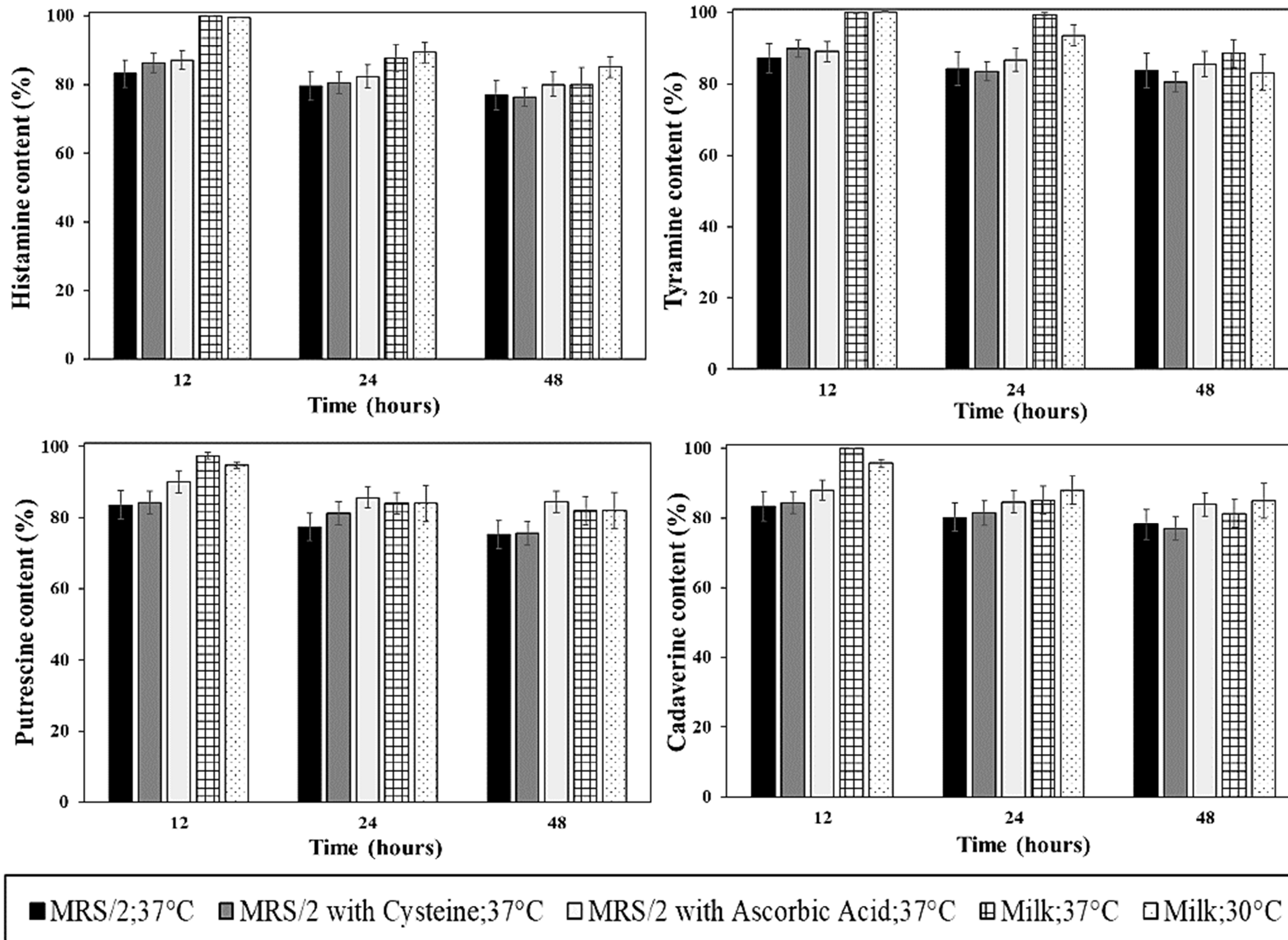


Figure 5 (Pištěková et al.)



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Highlights

- High levels of BAs in fermented foodstuffs constitute a health risk for consumers.
- qPCR is a key method for searching for suitable strains for starter or adjunct cultures.
- We designed primers for the multicopper oxidase gene expression analysis of *L. casei*.
- We monitored the degradation abilities of *L. casei* CCDM 198 used in the food industry.

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Conflict of interest

Declaration of interest: NONE.

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