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

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

Keywords:
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1. Introduction

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

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

Probably, the most effective solution is to use microorganisms, which can degrade amines formed as a part of the starter or adjunct cultures. The ability to degrade biogenic amines in culture media or foodstuffs is based on the fact that some microorganisms are capable of producing degrading enzymes, such as amine oxidases and multicopper oxidases (MCO) (Álvarez and Moreno-Arribas, 2014; Callejón et al., 2014). Amine oxidases are the large group of enzymes catalysing the degradation of BAs to substances that can be utilised by microorganisms as a source of energy and growth. Several studies have described these enzymes and deamination pathways (Sekiguchi et al., 2004; Wang et al., 2013; Yagodina et al., 2002). Later, Callejón et al. (2014) described the degradation of BAs in wine by lactic acid bacteria (LAB) possessing multicopper oxidases.

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

A powerful tool for searching for strains with degradation abilities could be real-time PCR (qPCR). This advanced technique offers the advantages of speed, sensitivity, simplicity and the specific detection and quantification oftarget genes in one step (Landete et al., 2007). The food industry is increasingly using qPCR for genes detection and quantification involved in BAs production (Elsanhoty and Ramadan, 2016; Ladero et al., 2015, 2010; Postollec et al., 2011). The situation is different for monitoring the expression of genes involved in BAs degradation. Very few studies have described primers that allow the monitoring of the expression of the degrading genes (Eom et al., 2015; Herrero-Fresno et al., 2012). Some authors have developed primers for the detection of the multicopper oxidase gene in LAB by PCR (Callejón et al., 2014; Guarcello et al., 2016), which are not suitable for qPCR due to the length of the PCR amplicons.

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

2. Materials and methods

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

2.1. Strains and cultivation conditions

Microorganisms: The *L. casei* strains used in this study (CCDM 198, CCDM 145) were obtained from the Laktoflora, the Culture Collection of Diary Microorganisms (CCDM), Czech Republic. Thirteen strains of *L. paracasei* (S3_1 - S3_13) were isolated from sourdough of traditional Portuguese sourdough bread at the School of Agriculture at the Polytechnic Institute of Beja, Portugal.

Growth conditions for the production of BAs: All 15 strains were first grown in tubes with 7 mL of MRS broth (HiMedia, Mumbai, India) at 37 °C in 5% (v/v) CO_2 for 24 h. Then a 50 µL culture was inoculated into MRS broth (7 mL) to obtain the initial concentration of bacteria (6.2 ± 0.3 log CFU/mL). The medium was supplemented with amino acids (arginine, ornithine, histidine, tyrosine, lysine and phenylalanine) at 0.2 $g.L^{-1}$ each, and hydrochloric acid (0.1 M or 1 M HCl) was added to adjust a pH of the medium to 6.5 ± 0.1. Incubation was performed at 37 °C in 5% (v/v) CO_2 for 48 h.

Growth conditions for the preliminary test of BAs degradation: The inoculum was grown in MRS broth (HiMedia, Mumbai, India) to achieve maximum cell count; however, degradations were performed in a depleted/modified MRS (MRS/2; 50% of weight Lactobacillus MRS broth) medium. The depleted medium provides less nutrients which may support the use of BAs as an alternative source of carbon and nitrogen. All 15 strains were first grown in tubes with 7 mL of MRS broth at 37 °C in 5% (v/v) CO_2 for 24 h. Then, the 50 µL culture was inoculated into 7 mL of MRS/2 broth to obtain initial concentration of bacteria (6.2 ± 0.3 log CFU/mL). The medium was supplemented with biogenic amines (histamine, tyramine, putrescine, and cadaverine) at 0.2 $g.L^{-1}$ each, and hydrochloric acid (0.1 M or 1 M HCl) was added to adjust a pH of the medium to 6.5 ± 0.1. Incubation was performed at 37 °C in 5% (v/v) CO_2 for 48 h.

Growth conditions for the degradation of BAs: *L. casei* strains were first grown in tubes with 7 mL of MRS broth (HiMedia, Mumbai, India) at 37 °C in 5% (v/v) CO_2 for 24 h and a 200 µL culture was inoculated into MRS/2 broth (50 mL) to obtain the initial concentration of bacteria (6.2 ± 0.3 log CFU/mL). The medium was supplemented with biogenic amines (histamine, tyramine, putrescine, and cadaverine) at 0.2 $g.L^{-1}$ each, and hydrochloric acid (0.1 M or 1 M HCl) was added to adjust a pH of the medium to 6.5 ± 0.1. Incubation was performed at 37 °C in 5% (v/v) CO_2. The relative expression level and biogenic amine degrading capability were determined after incubation for 0, 12, 24 and 48 h.

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

Growth and degradation of *L. casei* CCDM 198 were observed not only in the broth but also in the real food - milk. UHT low-fat milk (50 mL) was supplemented with biogenic amines (histamine, tyramine, putrescine, and cadaverine) at 0.2 $g.L^{-1}$ each, and hydrochloric 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 pL culture (ca. 109 CFU) was inoculated into milk (50 mL) to obtain the initial concentration of bacteria (6.2 ± 0.3 log CFU/mL). Concentration of bacteria during growth cycle was monitored by plate method. Incubation was performed at 37 °C in 5% (v/v) CO_2 at 30 °C and 37 °C.

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

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

2.2. Primer design

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

2.3. DNA extraction, polymerase chain reactions (PCR)

To verify the presence of target genes in 15 *Lactobacillus* strains, DNA was extracted from bacterial cells. Genomic DNA was prepared from 1 mL of bacterial strains that were grown in MRS/2 broth. Pellets of these strains were obtained by centrifuging at 3000X *g* for 5 min. DNA was extracted using the Genomic DNA from Tissue Kit (Macherey- Nagel, Germany) according to the manufacturer's instructions. The purity and concentration of the DNA were measured using a Multimode Microplate Reader Infinite 200 PRO (Tecan, Switzerland).

PCR was performed by using a commercial mix, G2 Hot Start Green Master Mix (ROCHE, Germany). The reaction volume, 25 pL, included 12.5 pL of the commercial mix (ROCHE, Germany), 800 $nmol.L^{-1}$ of a forward primer, 800 $nmol.L^{-1}$ of a reverse primer and 10-100 ng of template. Additionally, we prepared a negative control sample without a template. The PCR conditions were as follows: initial denaturing at 95 °C for 5 min, followed by 35 cycles each comprising 95 °C for 30 s of denaturing, 61 °C for 30 s of annealing, and 72 °C for 30 s of extension; the final extension was performed at 72 °C for 10 min.

The PCR products were separated into 1% (w/v) agarose gel in a TAE buffer with ethidium bromide by agarose electrophoresis run for 25 min at 90 V on a 1% gel. The GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific, USA) was used as molecular weight marker.

The partial nucleotide sequence of the amplified genes *MCO* and *GAPDH* were verified by sequencing with our set of primers (Table 1). PCR products were purified using NucleoMag® Tissue (Macherey-Nagel, Germany). The resulting sequences were compared against NCBI database using the Basic Local Alignment Search Tool program (NCBI, 2009).

2.4. Reverse transcriptase and qPCR

In order to quantify *MCO* gene expression, it was necessary to isolate RNA from *L. casei* strains (CCDM 198, CCDM 145) and transcribe it by reverse transcription into cDNA, which serves as a template for qPCR. RNA isolation was done using the RNeasy PowerLyzer Tissue & Cells Kit (QIAGEN, Germany) according to the manufacturer's instructions. RNA isolation from *L. casei* CCDM 198 cultivated in milk was also done using the RNeasy PowerLyzer Tissue & Cells Kit, but with a small modification. After the collection of samples, 1 mL of culture was centrifuged at 2000xg for 5 min at 4 °C. The upper layer of fat was sterile removed, and a sample was frozen and thawed three times. Homogenisation was performed with PowerLyzer Ceramic Bead Tubes. The procedure was finished according to the enclosed instructions. First-strand cDNA was synthesised from 11 µL

RNA using a Transcriptor First Strand cDNA Synthesis Kit (ROCHE, Germany).

qPCR was performed by using thermocycler CFX 96 Real-Time (Bio-

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	

Rad, Hercules, CA, USA) with the commercial kit FastStart Universal SYBR Green Master (ROCHE, Germany). The total reaction volume, 25 µL, included 12.5 µL of ROCHE mix, 250 nmol.L^{-1} of a forward primer, 250 nmol.L^{-1} of a reverse primer and 1-2 µg of cDNA template. The qPCR conditions were as follows: initial denaturing at 95 °C for 3 min, followed by 45 cycles each comprising 95 °C for 30 s of denaturing, 60 °C for 30 s of annealing, and 72 °C for 1 min of extension; final extension was performed at 72 °C for 5 min. Data were normalized to *GAPDH* expression. Reference control and nontemplate negative controls (using water instead of cDNA) were included in every run for both genes.

The baseline and cycle threshold were automatically calculated using the C1000 Touch Thermal Cycler equipped with a CFX 96 Touch™ System Software, version 2.1 (Bio-Rad, CA, USA). The melt curve analysis was performed on the same device (CFX 96 Real-Time) after the completion of qPCR. Obtained PCR products of the *MCO* and *GAPDH* had melting temperatures of 76 ± 0.5 °C and 77 ± 0.5 °C, respectively.

2.5. Determination of biogenic amine content

The degrading capacity of the strains was tested in a modified nutrient broth (MRS/2) and milk by HPLC/UV. Samples (3 mL) were collected in determined hours (0, 12, 24 and 48) and centrifuged at 2000xg for 10 min. Supernatant (600 µL) was diluted 1:1 (v/v) with 0.6 M perchloric acid (Acros, Belgium). Three independent extractions were performed on each culture sample. Subsequently, mixtures were derivatised using dansyl chloride (Sigma-Aldrich, Missouri, USA) with 1,7-heptanediamine (Fluka, Switzerland) as an internal standard according to Dadakova et al. (2009).

BAs (histamine, tyramine, putrescine and cadaverine) were detected using high-performance liquid chromatography, Dionex HPLC UltiMate 3000 (Thermo Fischer Scientific, Waltham, Massachusetts, USA), following preceding derivatisation using dansyl chloride (Dadakova et al., 2009). The chromatographic column used for separation was an Agilent Zorbax RRHD Eclipse Plus C18 with the dimensions of 50 x 3.0 mm, 1.8 pm (Agilent, Paolo Alto, USA). Spectrophotometric detection was carried out at a wavelength of 254 nm and a column temperature of 30 °C. The flow rate was 0.453 mL min^{-1}. The detection and separation of biogenic amines were performed according to (Dadakova et al., 2009; Smela et al., 2004). Data were acquired and evaluated using Chromeleon™ 6.8 software (Thermo Fisher Scientific, USA).

2.6. Statistical evaluation

Non-parametrical analyses of variance from the Kruskal-Wallis and Wilcoxon tests (Unistat® 6.5 software; Unistat, London, UK) were used to evaluate the results obtained (the significance level was 0.05). To estimate of the dependence of threshold cycle on DNA concentration regression line (linear least squares method) was used (Unistat® 6.5; software Unistat, London, UK).

3. Results and discussion

3.1. Screening of LAB strains possessing the MCO gene

The ability to degrade BAs depends not only on the species but also on the strains. Thus, testing suitable strains using conventional techniques is unreliable or labour intensive and time-consuming. For these reasons, molecular biology methods are increasingly being used in the food industry (Postollec et al., 2011).

To allow rapid screening of strains possessing the *MCO* gene and to examine the expression of this gene, we designed and tested gene-specific primers. Then we searched for a strain with degrading properties using new primers.

Firstly, we screened *L. casei* and *L. paracasei* strains possessing the multicopper oxidase gene by PCR. Secondly, we performed a preliminary test of the degradative ability of strains when we grew a culture in 7 mL MRS/2 with BAs for 48 h. At the end of this test, we monitored the decrease in the content of BAs by HPLC/UV. At the same time, we examined whether or not strains with degradation abilities are producers of BAs. According to obtained results (data not shown), we found that only two strains of *L. casei* (CCDM 198, CCDM 145) are not BAs producers. We subsequently focused attention on the CCDM 198 strain, given that it had significantly higher degradation capabilities than the strain CCDM 145. During the preliminary tests, we also verified that the biogenic amines degradation ability of the CCDM 198 was four times higher in the depleted medium MRS/2 compared to MRS (data not shown). Therefore, depleted medium was preferred in this work. Finally, based on the results, we observed relative expression of the *MCO* gene within 48 h for the selected CCDM 198 strain and used the CCDM 145 strain as a positive control for qPCR (*3.3 Expression of Gene Encoding Multicopper Oxidase)*. We verified the ability of strains CCDM 198 and CCDM 145 to degrade BAs in 50 mL of MRS/2 using HPLC/UV and experienced the effect of redox treatments and milk on the BAs degradation of strain CCDM 198. We also observed the growth of cells during the degradation process.

3.2. Specific primer design

In this study, we designed three sets of primers for the multicopper oxidase gene (*MCO*) in *L. casei,* which is responsible for the degradation of BAs. Due to the normalisation of the target gene with an endogenous standard, we designed and tested primers for the glyceraldehyde 3- phosphate dehydrogenase gene (*GAPDH*). According to the PCR tests, we chose the set of primers which do not form dimers or nonspecific products. The new sets of primers anneal to the multicopper oxidase gene of *L. casei* and *L. paracasei* strains. The final length of the PCR product for the detection of the multicopper oxidase gene (*MCO*) is 94 bp, and for the detection of the endogenous gene *(GAPDH),* primers with a length of 137 bp were selected (Table 1). The sequencing followed by analysis in BLAST (NCBI, 2009) confirmed that PCR products corresponded to the *MCO* and *GAPDH* partial nucleotide sequences, respectively.

Parameters of qPCR: Amplification efficiency values with our sets of primers were in the optimal range between 90% and 110% (Broeders et al.,

2014), which corresponds to the slope of the long-linear phase of the amplification reaction between -3.58 and -3.10 (Fig. 1). In addition, the linearity of the qPCR reaction used to determine the efficiency r^2 was ≥0.98 for each target. Post-amplification melting-curve analysis (data not shown) confirmed that the chosen sets of

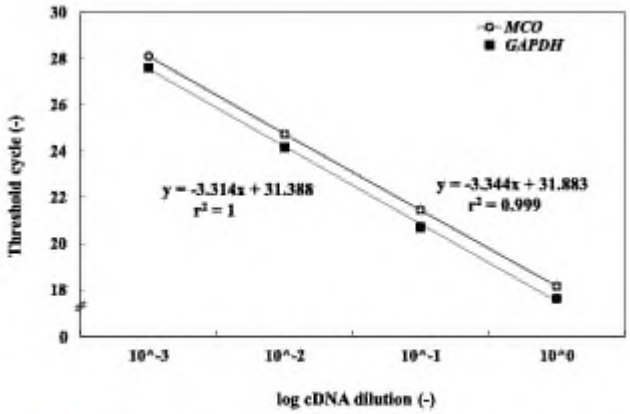

Fig. 1. qPCR standard curves of multicopper oxidase gene (*MCO*) and endogenous gene (*GAPDH*). The templates were cDNA purified from bacterial cells grow in MRS/2 broth after 48 h cultivation.

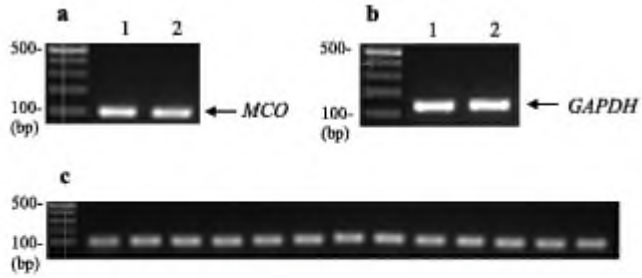

Fig. 2. PCR testing of new primers. **a)** DNA fragments of multicopper oxidase gene (*MCO*) were amplified by primers LCMCO4-L and LCMCO4-R from *L. casei* strains: CCDM 198 (1) and CCDM 145 (2). **b)** DNA fragments of endogenous gene (*GAPDH*) were amplified by primers LCGAPD4-L and LCGAPD4-R from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

gene-specific primers do not form dimers or non-specific products. The Pfaffl method was used to calculate the relative expression (Pfaffl, 2001).

The verification of target genes by PCR and selecting strains to monitor the relative expression of target genes during BAs degradation: Before qPCR analysis, we checked for the presence of the multicopper oxidase gene *MCO* and endogenous gene *GAPDH* in bacterial strains using PCR. The presence of the multicopper oxidase gene in *L. casei* and *L. paracasei* strains is shown in Fig. 2. HPLC/UV showed that all strains possessing the multicopper oxidase gene were able to degrade BAs. However, *L. paracasei* strains are also BAs producers (for more details, see *Chapter 3.4*). Therefore, relative expression was only tested in *L. casei* strains.

3.3. Expression of Gene Encoding Multicopper Oxidase

qPCR is a standard method for measuring gene expression. This quantitative analysis requires no postprocessing; results are obtained quickly and, therefore, it could be used for the routine detection of bacterial strains that have potential to degrade histamine and other BAs (Wong and Medrano, 2005).

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

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

3.4. Detection of biogenic amine content

High performance liquid chromatography is the most commonly used technique because of its great versatility, efficiency, sensitivity and reproducibility, and is, therefore, the conventional technique for

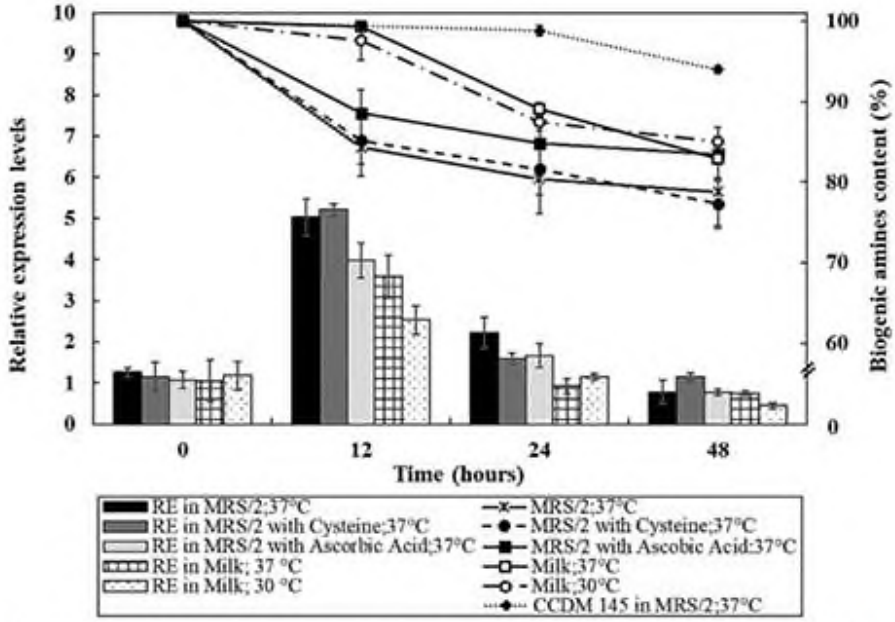

Fig. 3. Comparison of the relative expression levels of the multicopper oxidase gene in *L. casei* CCDM 198 performed by qPCR with the biogenic amines content in media determined by HPLC/UV during 48 h of cultivation.

analysing histamine in foods (Commission Regulation (EC) No 2073/ 2005, 2005; Marcobal et al., 2006).

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

The results obtained showed that all strains tested can degrade BAs but with different efficiency (data not shown). The highest decrease of BAs content was observed in the *L. casei* CCDM 198 strain and was therefore chosen to observe expression during BAs degradation.

L. casei CCDM 198 showed approximately 25% degradation of BAs in a preliminary test (data not shown). Other authors confirmed the excellent abilities of *L. casei* strains. Garcia-Ruiz et al. (2011) demonstrated great potential for histamine, tyramine and putrescine degradation (54%, 55% and 65%, respectively) of the strain *L. casei* IFI-CA 52 strain in culture media. Herrero-Fresno et al. (2012) isolated 17 *L. casei* strains with histamine and/or tyramine degradation rate up to 40% in a cheese manufacturing model.

Exclusion of BAs-producing LAB strains: Since some lactobacilli are significant BAs producers (Herrero-Fresno et al., 2012), we performed the test to exclude potential BAs producers for industrial applications. *L. casei* (CCDM 198, CCDM 145) and 13 strains of *L. para- casei* we tested for biogenic amine production in a medium with biogenic amines precursors. Test results showed that *L. casei* (CCDM 198, CCDM 145) are not producers of phenylethylamine, histamine, tyramine, cadaverine and putrescine. On the other hand, all strains of *L. paracasei* are capable of producing one or more biogenic amine in amounts up to 30 $mg.L^{-1}$ and are not suitable for industrial use (data not shown). Therefore, expression of the target gene was not tested in *L. paracasei*, although all 13 strains contained the *MCO* gene (Fig. 2).

Relationship between BAs (histamine, tyramine, cadaverine, putrescine) content and relative gene expression: The decrease of biogenic amines corresponds to the achieved relative expression values (Fig. 3). The highest difference in BAs content was after 12 h of cultivation in broth, when the highest relative expression was also recorded; this was in contrast to cultivation in milk, where the decrease of BAs content was lower than 3%. This was probably caused by the longer lag phase and low cell count after 12-hour cultivation (Fig. 4).

After 48 h, the highest decrease in total BAs at 77.36 ± 3.13% was achieved in MRS/2 with 1% cysteine. Only a little higher BAs content (78.78 ± 4.17%) remained in MRS/2 broth. Although a significantly higher number of cells was observed in cultivation at 30 °C in milk, low relative expression may have caused the highest biogenic amines content (85.02 ± 1.83%) at the end of cultivation.

Effects of media on growth pattern of selected LAB strains: Many studies have demonstrated the positive effect of oxygen scavenging agents (cysteine and ascorbic acid) on viability of probiotic bacteria (Dave and Shah, 1997; Demain et al., 1961; Rickes et al., 1949; Shah, 2000). Oxygen reduces the growth of these bacteria, and the use of cysteine and ascorbic acid may lower redox potential by scavenging oxygen, thus affecting their growth (Shah, 2000). The positive effect of cysteine on the growth of cells of the CCDM 198 strain was also observed in our test (Fig. 4), but there was no marked effect on the increase of multicopper oxidase expression level (Fig. 3). This corresponds to the results recorded in Fig. 5. Lower amounts of histamine, tyramine and cadaverine were observed in samples with 1% cysteine, but the difference was not significant ($P > 0.05$) compared to MRS/2 broth. Moreover, the highest degradation, thus the lowest content was observed for putrescine in MRS/2 (75.18 ± 3.95%). The final amount of putrescine in the 1% cysteine medium was slightly higher (75.60 ± 3.43%). On the other hand, the lowest degradation capacity was recorded for tyramine, where the content of no sample falls below 80% (Fig. 5). *L. casei* CCDM 198 also significantly ($P < 0.05$) reduced histamine, the most dangerous BA, in MRS/2 (76.86 ± 4.21%) and in MRS/2 with 1% cysteine (76.35 ± 2.87%) after 48 h (Fig. 5). Based on obtained results, the presence of ascorbic acid may not support the degradation of BAs because the content of all monitored BAs was higher in samples with 0.1% ascorbic acid than in MRS/2 and MRS with cysteine (Figs. 3 and 5).

The effect of milk environment on content of histamine, tyramine, cadaverine and putrescine: After 48 h, the content of all monitored BAs was 3-6% higher in milk than in MRS/2 broth with exception oftyramine. A temperature of37 °C is more preferable for the CCDM 198 strain because results showed lower histamine, cadaverine and putrescine content at the end of cultivation than at growth temperature of30 °C (Figs. 3 and 5). Although strain CCDM 198 is isolated from the dairy product and therefore well adapted to the dairy environment, our results are consistent with some studies showing that *L. casei* prefers MRS medium (Avonts et al., 2004; Zuraw et al., 1960). Avonts et al. (2004) demonstrated that *L. casei* strains were able to develop to high cell numbers in a milk medium, but fermentation of milk was slow, and the production of bacteriocin was lower compared to the MRS medium. We observed lower multicopper oxidase expression in this medium, which can also be caused by amino acid imbalances in milk. Nevertheless, *L. casei* CCDM 198 proved to significantly ($P < 0.05$) decrease of histamine, tyramine, cadaverine and putrescine in milk after 48 h of cultivation.

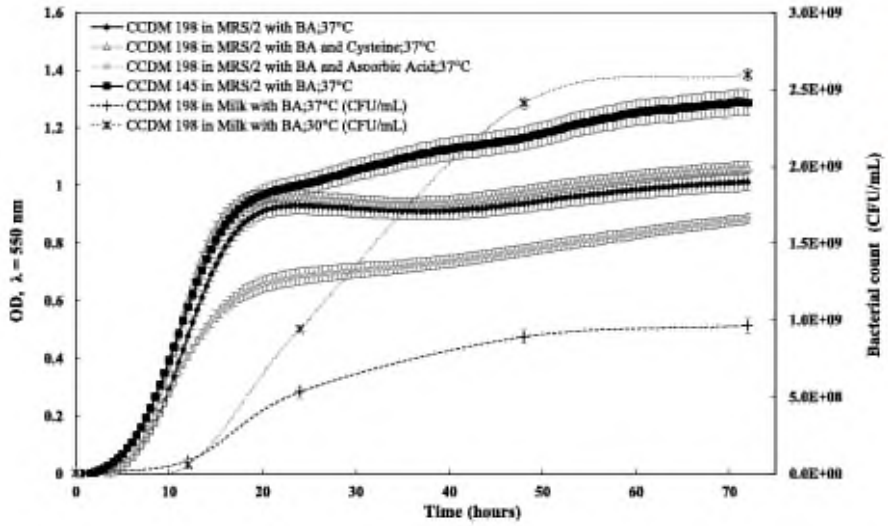

Fig. 4. Growth of the *L. casei* strains during BAs degradation in MRS/2 broth supplemented by histamine, tyramine and putrescine at 37 °C, pH 6.5 ± 0.1 for 72 h performed by optical density measurement and influence of 1% cysteine and 0.1% ascorbic acid on the growth. Compared to the growth of the *L. casei* CCDM 198 during BAs degradation in milk, which was determined by the colony counting method.

3.5. *Growth of cells*

During degradations tests of *L. casei* CCDM 198, we monitored bacterial growth curves to examine the effect of cysteine, ascorbic acid to the growth of bacterial cells. In order to follow the growth curve at half-hour intervals, we used a spectrophotometric method for cultivation in MRS/2 broth. The bacterial counts in milk were determined by the plate method at times of collection of samples for qPCR and HPLC/UV. The findings of growth bacteria in both media supplemented by BAs during 72 h of cultivation are reported in Fig. 4.

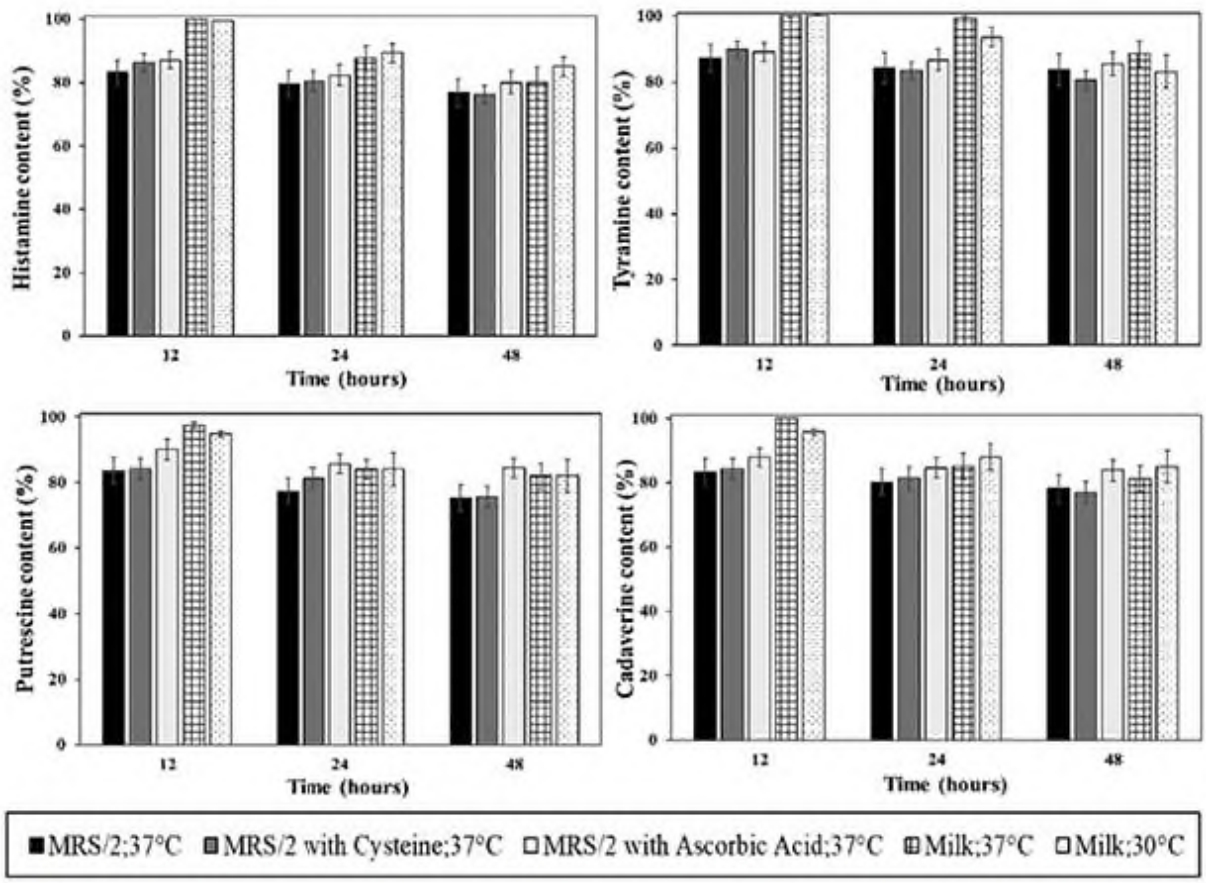

Fig. 5. Content of histamine, tyramine, cadaverine and putrescine measured by HPLC/UV. Reaction was carried out in MRS/2 broth and milk inoculated by *L. casei* CCDM 198, pH 6.5 ± 0.1 for 48 h.

The growth of *L. casei* CCDM 198 and CCDM145 in MRS/2 broth: The lag phase of both strains was approx. 2 h when OD_{550} was almost unchanged. Then OD_{550} rose rapidly, and the exponential phase occurred between 4 and 17 h of cultivation. After 22 h, the growth curves of both strains came into a stationary phase. While the OD_{550} of strain CCDM 198 slightly decreased at the beginning of the stationary phase, the OD_{550} of strain CCDM 145 maintained moderate growth until the end of the cultivation.

The effect of cysteine and ascorbic acid on *L. casei CCDM 198* growth: Since *L. casei* is a facultative anaerobic bacterium, reducing agents cysteine and ascorbic acid were added to promote cells growth. So far few authors reported the stimulatory effect of ascorbic acid on the growth of *L. casei* with various recommended concentrations. Demain et al. (1961) tested stimulating and toxic amounts for *Lactobacillus heterohiochi*. These results follow the study Rickes et al. (1949), which dealt with the stimulating amount of ascorbic acid for *L. casei* growth. In accordance with previous studies, two different concentrations of ascorbic acid (0.1% and 0.01%) were chosen for our tests. Although the OD_{550} was slightly higher at 0.01% compared to

0.1% concentration, the degradation of BAs decreased by approximately 10% (data not shown). Thus, further tests were performed with 0.1% ascorbic acid. The addition of 0.1% (w/v) ascorbic acid significantly decreased OD_{550} throughout the growth curve, in particular in the exponential phase. In contrast, the effect of 1% (w/v) cysteine in MRS/2 broth resulted in a slight increase of OD_{550} over the entire growth curve compared to growth in MRS/2 itself. Strain *L. casei* CCDM 145 was used as a control sample for the relative expression level; therefore, the effect of cysteine and ascorbic acid on the growth was not tested.

The effect of UHT low-fat milk on *L. casei* CCDM 198 growth: Some strains are unable to develop in unsupplemented milk because pure milk is generally low in free amino acid content. Nevertheless, for a considerable number of different lactobacilli species is cow's milk a naturally complex medium that supports their growth (Elli et al., 1999). The strain CCDM 198 was originally isolated from a dairy product thus is well adapted to a milk environment. In our test, the inoculum was grown in MRS; therefore, the lag phase in milk was longer (approx. 2 h), and the stationary phase came after 48 h of cultivation (Fig. 4). Although the optimum cultivation temperature for CCDM 198 is 37 °C, significantly higher CFU values were achieved at 30 °C ($2.6 \cdot 10^9$ CFU/ mL).

4. Conclusion

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

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

Declaration of competing interest

None.

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