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Reduction of histamine, putrescine and cadaverine by the bacteria Lacticaseibacittus casei depending on selected factors in the real condition of the dairy product*

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ABSTRACT

One way to effectively reduce the number of biogenic amines (BAs) in food is through enzymatic reduction using bacteria, such as lactic acid bacteria. This study focuses on the ability of the bacterial strain Lacticaseibacillus casei $CCDM$ 198 to reduce the number of three important BAs (histamine, putrescine and cadaverine) over time, depending on different conditions (temperature and pH) in vitro and for the real dairy product - skimmed milk. The obtained results show that the studied strain significantly ($P < 0.05$) affects the number of individual amines, and the content of all amines has a decreasing character compared to the initial relative content of BAs at time zero. Furthermore, a statistical dependence ($P < 0.05$) of the rate of amine degradation on the combination of investigated factors was demonstrated. The presence and the activity of multicopper oxidase enzyme was also detected in this bacterial strain. This is the first known publication demonstrating multicopper oxidase activity in Lacticaseibacillus casei $CCDM$ 198. Moreover, the studied strain is able to reduce the tested BAs in skimmed milk and would be a good candidate for degrading these toxic compounds in other dairy products, such as cheese. These findings could significantly enhance the food safety of dairy products.

Keywords: Biogenic amines degradation, histamine, putrescine, cadaverine, multicopper oxidase, lacticaseibacillus casei, dairy products

[*]*Interpretative summary: Biogenic amines (BAs) constitute a health risk for consumers. One strategy to reduce these unwanted substances is through enzymatic degradation using microorganisms. The aim of the current work was to describe and analyse the ability of the strain Lacticaseibacillus casei (previously Lactobacillus casei) CCDM 198 to reduce histamine, putrescine and cadaverine over time, depending on technological parameters (pH and incubation temperature) in vitro and in the real dairy product - skimmed milk. The aim of the study was also to detect the presence and the activity of the enzyme responsible for BAs degradation in L. casei CCDM 198.*

1. Introduction

Food quality and safety can be affected by the presence of biogenic amines (BAs) due to their physiological and toxicological effects. These compounds contribute to numerous outbreaks of foodborne illness worldwide and are of great importance to public health (**EFSA, 2017**). BAs are lowmolecular-weight nitrogenous organic substances formed in foodstuff, mainly by microbial enzymatic decarboxylation of the relevant amino acids (**Santos, 1996**). The toxicity rate of BAs is very difficult to establish because their effects do not depend on their presence alone but are also influenced by the presence of other compounds and the efficiency of the detoxifying mechanism in the intestinal tract (**Ladero et al., 2010; Omer et al., 2021**). Their accumulation depends on the presence of precursor amino acids, the presence of microorganisms with decarboxylase activity and suitable environmental conditions for their growth and the activity of the decarboxylases (**Ruiz-Capillas and Herrero, 2019**). There are many studies showing increased formation of BAs in various foods (**Fernández et al., 2006, 2007; Henríquez-Aedo et al., 2012; Lonvaud-Funel, 2001**). Due to the presence of BAs, non-fermented foods can be considered to be low risk compared to the fermented products (wine, beer, dairy products and fermented meat products), which often contain high levels of these compounds and are considered more hazardous. Excessive amines in unfermented foods are a sign of undesirable microbial activity, which can be caused, for example, by improper handling and storage temperature (**Sarkadi, 2019**). Hence their detection can be used as an indicator of quality and freshness and of unwanted contaminants that cause food spoilage (**Mietz and Karmas, 1978**). For example, the presence of histamine is regulated in fish (especially in the families Scombridae, Clupeidae, Engraulidae, Cor-yfenidae, Pomatomidae and Scombresosidae), with up to a maximum of 100 mg kg^{-1} in fresh fish and 200 mg kg^{-1} in fermented fish products, in the European Union, according to Commission Regulation EC No. 2073/2005 (**European Regulation, 2005**). On the other hand, formation of BAs in fermented foods is due to necessary microorganisms, e.g. lactic acid bacteria (LAB), that are used as a starter culture in production processes of foods (**Alvarez and Moreno-Arribas, 2014**).

For these reasons, the reduction of BAs has been the subject of research during recent years, and several strategies have been published. Traditional methods for delaying BA accumulation in individual foods include storage at low temperature (**Buňková et al., 2010**), use of food additives and preservatives (**Mah and Hwang, 2009**), and irradiation (**Kim et al., 2005**). However, these methods do not degrade already formed amines; they only delay the formation of amines in food, primarily through the inhibition of bacterial decarboxylase enzymes responsible for BA formation. Another promising strategy is the application of microorganisms with BA-degrading ability or their purified enzymes. Most studies have attributed these enzymatic activities exclusively to amino oxidases (**Yamada et al., 1965; 1967; Sekiguchi et al., 2004**). However, **Callejón et al.** (**2014**) were the first to identify that another enzyme, namely multicopper oxidase (MCO), is involved in the degradation of BAs in LAB. They demonstrated its presence in Lac-tiplantibacillus plantarum (previously Lactobacillus plantarum) and Ped-iococcus acidilactici isolated from wines. In later publications, they identified and characterized all these enzymes as laccases (EC No. 1.10.3.2; **Callejón et al., 2016, 2017**). MCO is enzyme that is widespread especially in fungi, higher plants and bacteria. Its catalytic center contains four reactive copper atoms that gives them their characteristic blue colour. MCO oxidizes a wide range of phenolic and nonphenolic aromatic substrates, while reducing molecular oxygen to water. Electrons from substrate are accepted at the center of a mononuclear copper and are transferred to a trinuclear copper center of MCO. The one molecule of oxygen is combined with the trinuclear copper center after four electrons transfer and is reduced to two molecules of water (**Riva 2006; Guan et al., 2018; Li and Lu, 2020**). Since then, several studies have been published demonstrating the presence of MCO and its amino degradability in LAB (**Guarcello et al., 2016; Li et al., 2020; Pistěková et al., 2020; Wang**

et al., 2022), and there is growing interest in laccases and microorganisms with these enzymes for potential application in food biotechnology. However, the use of MCO enzymes in the real food is still limited. Available studies show that bacterial MCO enzymes exhibit a broad substrate specificity and their optimal pH and temperature varies depending on individual substrates including BAs. Moreover, the ability of laccases to degrade the same BAs is different for individual microorganisms (**Callejón et al., 2016, 2017; Li et al., 2020; Olmeda et al., 2021; Ni et al., 2022; Tepkasikul et al., 2022; Wang et al., 2022**). For these reasons, it is necessary to experimentally analyse the BA-degrading ability of a particular LAB strain in a specific food matrix.

The purpose of the current work was to describe and analyse the ability of the strain Lacticaseibacillus casei (previously Lactobacillus casei) $CCDM$ 198 (Laktoflora, Czech Republic) to reduce content of three toxicologically important BAs: histamine, putrescine and cadaverine, in culture medium (in case of in vitro) and in skimmed milk. The influence of a combination of selected factors, such as pH and temperature, on BA-degrading ability of L. casei $CCDM$ 198 was evaluated. Finally, the presence and MCO activity was detected by electrophoresis and spectrophotometry respectively. Identifying the enzymes responsible for amine reduction and analysing their activity depending on different conditions allows for better designing of targeted applications for specific foods in the case of industrial use, and reduces the cost of implementing such an approach. Subsequent selection of suitable strains could effectively and safely reduce the presence of unwanted BAs in fermented foods, potentially without affecting the quality.

2. Material and methods

2.1. Bacteria strains and cultivation conditions

2.1.1. Microorganisms

The bacterial strain Lacticaseibacillus casei $CCDM$ 198 was obtained from Laktoflora (Czech Republic). The L. casei was cultivated anaerobically in the deMan Rogosa Sharpe (MRS) broth (Darmstadt, Germany) at 30 $\pm$ 1 °C for 24 h.

2.1.2. Growth conditions for the degradation of BAs in vitro and milk

A 24 hr-grown bacterial culture (5.9 $\pm$ 0.2 log CFU-ml^{-1}) was inoculated into the MRS broth (in the case of in vitro) and into milk (UHT-treated skimmed milk; Progolaktos, Czech Republic) supplemented with histamine, putrescine and cadaverine (initial concentrations of each BAs 0.2 g l^{-1}; Sigma-Aldrich, USA). The impact of selected external factors (a cultivation temperature 11 $\pm$ 1 °C, 23 $\pm$ 1 °C, 30 $\pm$ 1 °C and a pH of the media 5.4 $\pm$ 0.1, 6.2 $\pm$ 0.1, 7.0 $\pm$ 0.1) on the BA degradation ability of L. casei $CCDM$ 198 was also determined in vitro and in milk environment. The different cultivation and sampling times (interval 0-336 h, 0-96 h and 0-72 h) were chosen based on the growth curves of L. casei at the chosen temperatures in the previous experiment. The methodology used in this work is schematically summarized in **Fig. 1**.

2.2. Determination of biogenic amines

The content of individual BAs (histamine, putrescine and cadav-erine) was determined by high-performance liquid chromatography ($HPLC$). Before the detection, samples were diluted 1:1 (v/v) with

1.2 mol l^{-1} $HClO_4$ (MERCK, Darmstadt, Germany) and derived with dansylchloride (Sigma-Aldrich, USA), according to **Dadáková et al.** (**2009**), and then filtered (syringe filter with a porosity of 0.22 μm). The resulting solution was injected into the $HPLC$. The separation (Zorbax $RRHD$ Eclipse Plus C18 column, 50 × 3.0 mm, 1.8 μm, Agilent; Palo Alto, USA) and detection (diode array detector, $\lambda = 254$ nm) were employed according to **Smělá et al.** (**2004**). Data were acquired and evaluated using Agilent OpenLab Data Analysis.

2.3. Detection of multicopper oxidase

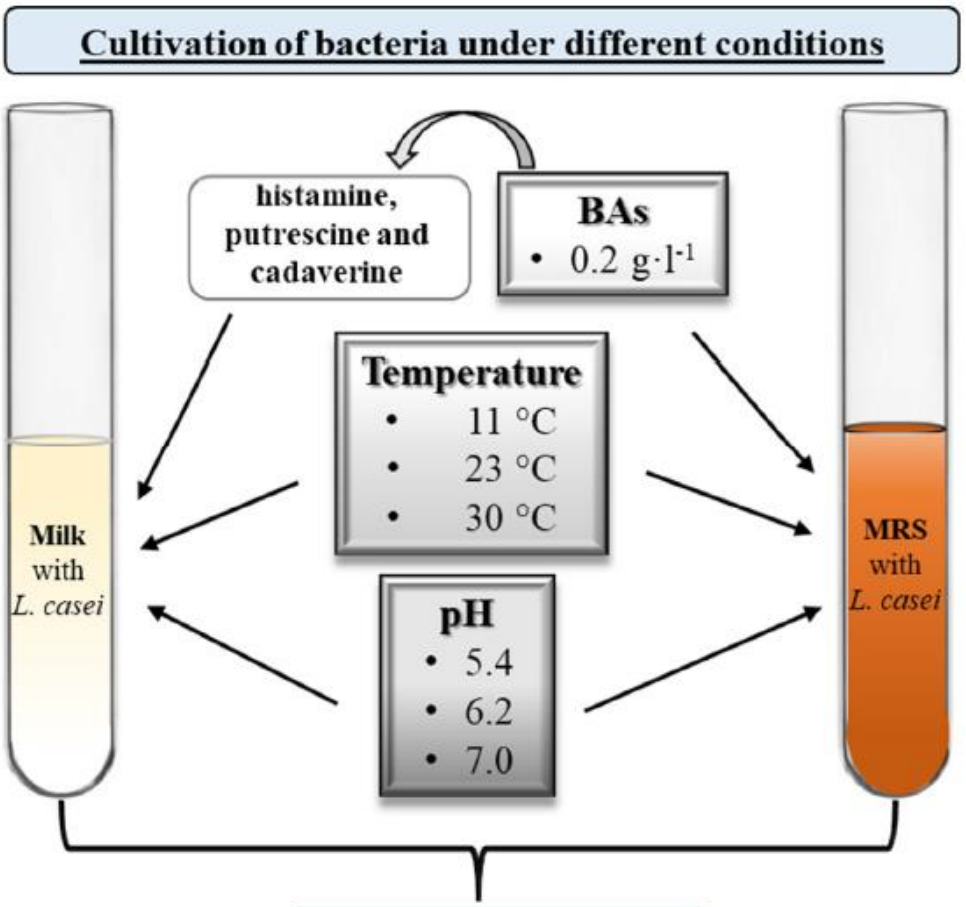
Cell-free extract and determination of presence and enzyme activity was performed, with slight modifications, according to **Callejón et al.** (**2014, 2017**).

2.3.1. Extraction

24-hr bacterial culture (an initial cell count of 5.9 $\pm$ 0.1 log CFU-ml^{-1}) was cultivated in the MRS broth supplemented with BAs for 24, 48 and 72 h at 30 $\pm$ 1 °C. After this time, samples were centrifuged (6500 rpm for 20 min). Cells were washed with 50 mmol l^{-1} sodium phosphate buffer (pH 7.4) and resuspended in the same buffer supplemented with 1 mmol l^{-1} phenylmethylsulfonyl fluoride (Sigma-Aldrich, USA). Cell lysis was performed with 1 g of 106 μM glass beads (Sigma-Aldrich, USA) for three cycles of 3 min each, with cooling on ice between cycles. After centrifugation (6500 rpm for 15 min), the supernatant was used to study the enzyme. The presence MCO enzyme was determined in a polyacrylamide gel and the activity was determined spectrophoto-metrically. Protein concentration was determined by the Bradford method (**Bradford, 1976**).

2.3.2. Determination the presence of the enzyme using in gel assays

The gel (8% polyacrylamide after native electrophoresis) was incubated in 0.1 mol l^{-1} sodium acetate buffer, pH 4.0, containing 10 mmol l^{-1} substrate 2,6-dimethoxyphenol (2,6-DMP; Sigma-Aldrich, USA) for 5 min and then for 10 min in the same buffer containing 1 mmol l^{-1} $CuSO_4$. The presence of MCO was demonstrated by the presence of a brown-orange coloured band.



Temperature	Cultivation time [hours]								
11 °C	0	48	96	144	192	240	288	336	
23 °C	0	12	24	30	36	48	72	96	
30 °C	0	8	12	24	28	32	36	48	72

*Detection of enzyme activity was monitored only in vitro and at selected times.

Fig. 1. Schematic representation of the methodology used in this study. Cultivation of the bacterium Lacticaseibacillus casei $CCDM$ 198 in vitro (the MRS medium) and in milk containing the BAs histamine, putrescine and cadaverine at initial concentrations of each BAs 0.2 g l^{-1}. Incubation was conducted at pH = 5.4, pH = 6.2 and pH = 7.0 and at temperatures of 11 °C, 23 °C and 30 °C. From the samples, the amount of BA was subsequently determined by $HPLC$, and the activity of the MCO enzyme was responsible for their degradation spectrophotometrically and in the gel.

2.3.3. Determination of enzyme activity using liquid spectrophotometrically assays

MCO activity was determined by monitoring the increase in absorbance at 420 nm during oxidation of the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS$; Sigma-Aldrich, USA) as a substrate in the reaction mixture (cell-free extract, 100 mmol l^{-1} acetate buffer, pH 4.0, and 5 mmol l^{-1} $ABTS$), with a multimode microplate reader (Tecan Infinite 200 PRO, Switzerland). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol $ABTS$ per minute. Enzyme activity was plotted as a percentage relative to maximum enzyme activity of 100% (% relative activity).

2.4. Statistical evaluation

The Kruskal-Wallis and Wilcoxon tests were used to evaluate the differences between BA occurrence in individual samples. The statistical program Unistat 6.5 (Unistat Ltd, London, UK) was used to process the data, and the significance level was 0.05.

3. Results

3.1. Determination of biogenic amines

In the present study, the development of the content of three BAs (histamine, putrescine and cadaverine) was determined using the bacterial strain L. casei $CCDM$ 198, depending on a combination of different technological factors (incubation temperature, cultivation time, pH of medium and type of medium). The incubation temperatures were 11 $\pm$ 1 °C, 23 $\pm$ 1 °C and 30 $\pm$ 1 °C. Depending on the selected temperature, samples were taken in the interval from 0 h to 336 h. Development of degradability was detected both in vitro (the MRS broth) and in a real food system (skimmed milk) at different pH levels (5.4 $\pm$ 0.1, 6.2 $\pm$ 0.1, 7.0 $\pm$ 0.1).

3.1.1. Influence of factors on histamine degradation

Fig. 2 shows the whole development of relative content of histamine (expressed as %) in vitro depending on the above-described tested factors. A decrease of histamine content ($P < 0.05$) was observed at all studied temperatures and pH values in vitro. A decrease ranged from 15% to almost 40% at the end of incubation. As shown, one of the most significant reduction ($P < 0.05$) was recorded for cultivation at a temperature of 11 °C in the MRS broth with pH 7.0 (**Fig. 2**C). The histamine level was reduced by more than 34% of the initial value after 48 h of cultivation, and during the cultivation there was a decrease that did not differ significantly ($P > 0.05$) from this value. Another noteworthy reduction ($P < 0.05$) in vitro was found during cultivation at pH 6.2 at a temperature of 30 °C. Histamine was reduced by more than 20% after 8 h of cultivation and at the end of the incubation, there was a further decrease of about 17% (**Fig. 2**B). Under the other tested conditions in vitro, the bacterial strain L. casei $CCDM$ 198 reduced histamine maximally in the range of 10%-20% at the end of the incubation.

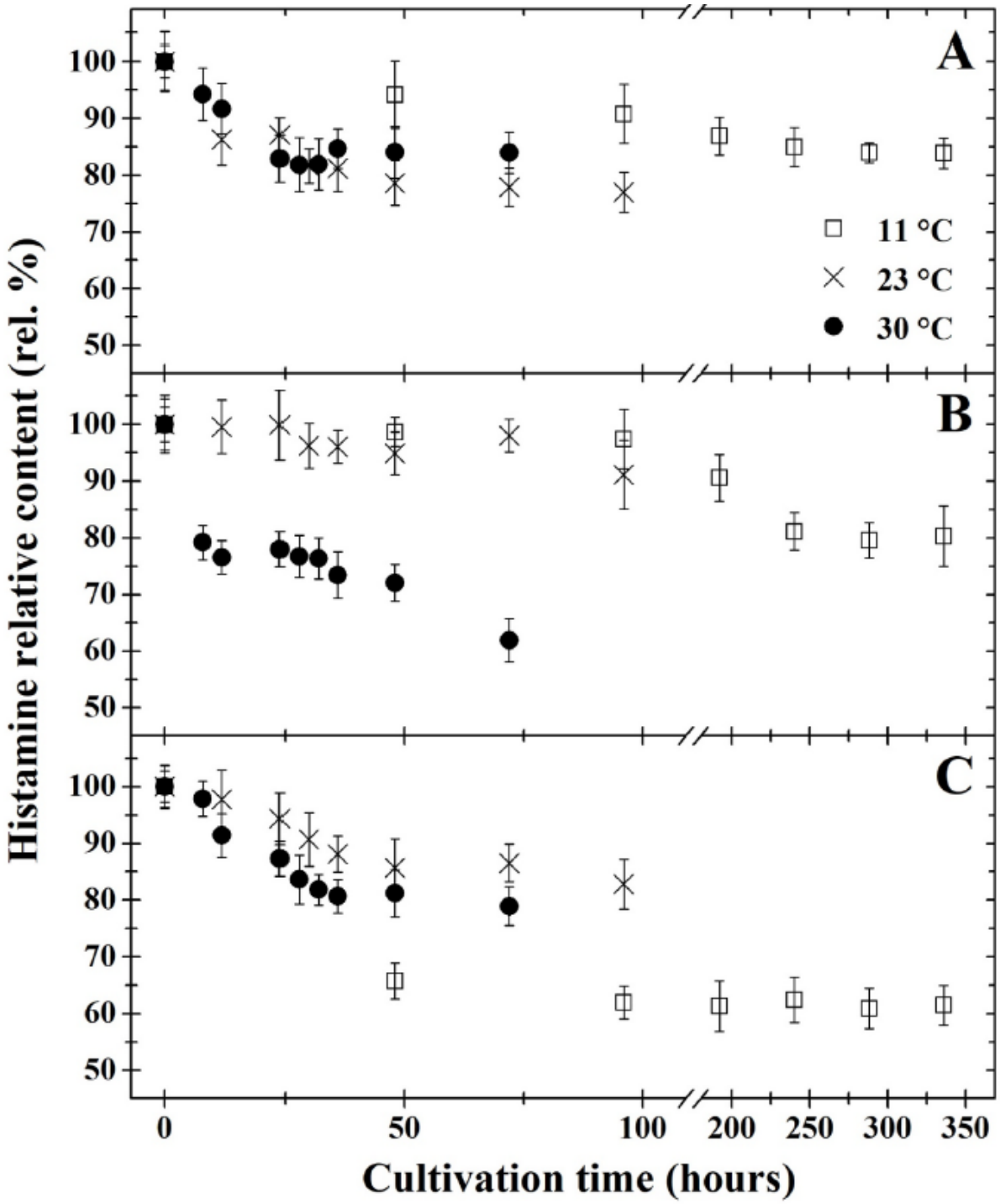


Fig. 2. The influence of Lacticaseibacillus casei $CCDM$ 198 on histamine content (rel.%) in vitro depending on cultivation time (hr), incubation temperatures and pH. The initial histamine relative content at the time zero was always 100%. (□) temperature 11 °C, (X) temperature 23 °C and (•) temperature 30 °C; (Part A) pH = 5.4, (Part B) pH = 6.2 and (Part C) pH = 7.0. The results are presented using the means and standard deviations (bars; $n = 6$).

Development of histamine relative content (rel. %) in milk is described in **Fig. 3**. The capacity of the tested strain $CCDM$ 198 to degrade histamine was also observed in milk under all studied factors. The reduction in histamine content ranged between 17% and 40% ($P < 0.05$) at the end of the incubation. As already mentioned above, a comparable histamine-reduction was also observed in vitro. However, the highest decrease of histamine in milk was achieved under different combinations of factors than

under in vitro conditions. The reduction of almost 40% was detected at the end of cultivation time in the samples of milk with pH 5.4 incubated at 11 ° C (**Fig. 3**A) and in the samples of milk with pH 6.2 incubated at 23 °C (**Fig. 3**B). Another noteworthy decrease of histamine content ($P < 0.05$) was observed in the samples of milk with pH 5.4 incubated at 23 °C (a reduction by 35%; **Fig. 3**A).

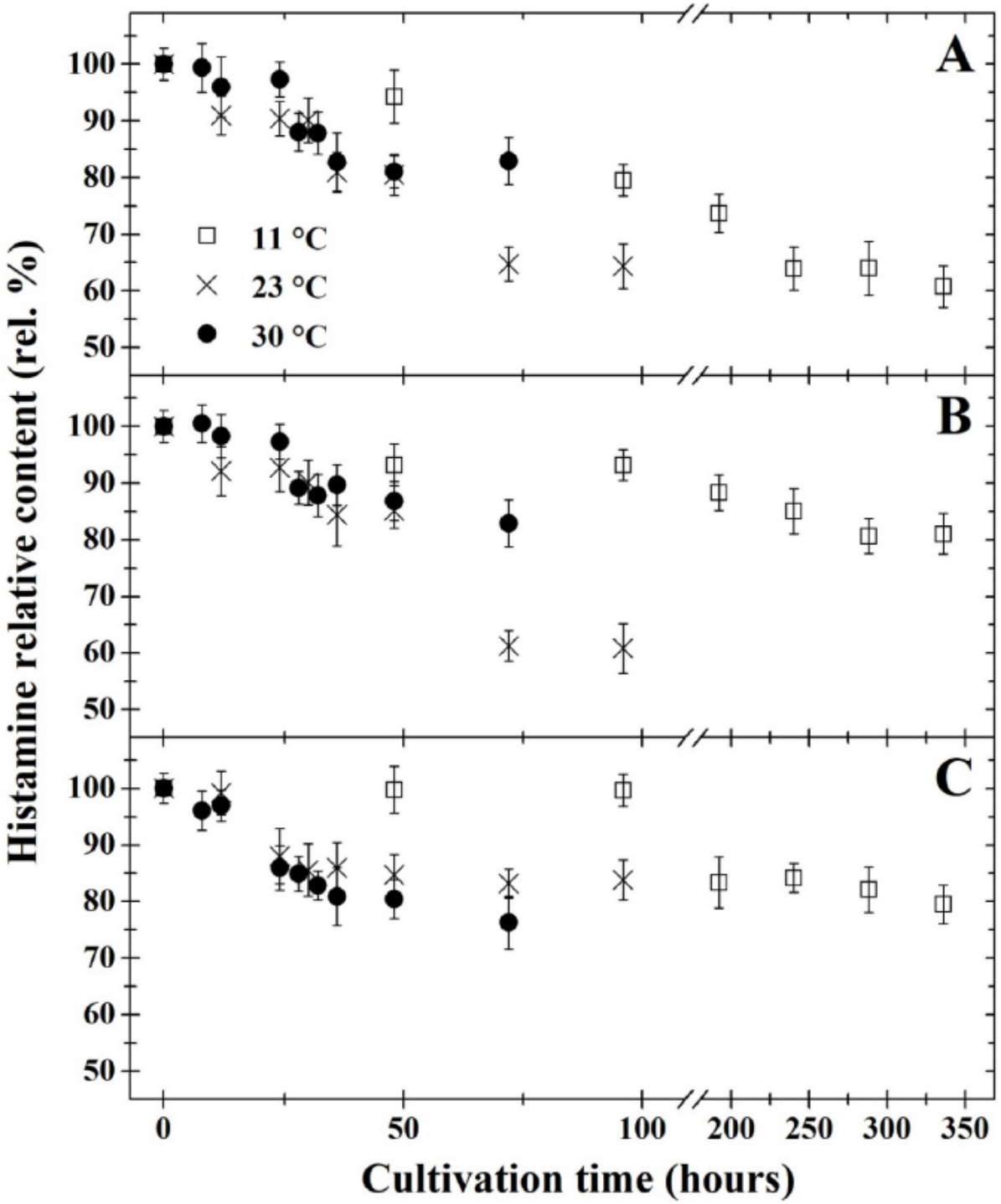


Fig. 3. The influence of Lacticaseibacillus casei $CCDM$ 198 on histamine content (rel.%) in milk depending on cultivation time (hr), incubation temperatures and pH. The initial histamine relative content at the time zero was always 100%. (□) temperature 11 °C, (×) temperature 23 °C and (•) temperature 30 °C; (Part A) pH = 5.4, (Part B) pH = 6.2 and (Part C) pH = 7.0. The results are presented using the means and standard deviations (bars; $n = 6$).

3.1.2. Influence of factors on putrescine degradation

The combination of tested factors had a significant effect ($P < 0.05$) on the reduction of putrescine by the strain $L.$ casei $CCDM$ 198 in vitro (**Fig. 4**; a reduction of putrescine ranging from 10% to 30% at the end of the incubation). The most suitable condition for the reduction of putrescine by the bacteria $L.$ casei $CCDM$ 198 in vitro was at 23 °C and pH 5.4 (a maximum decrease of 30%; **Fig. 4**A). On the contrary, at temperature 30 °C, the highest reduction was detected at a pH value of 7.0 (a maximum decrease of 25%; **Fig. 4**C). The least suitable temperature was 11 °C. $L.$ casei $CCDM$ 198 at all studied pH values of the medium did not reduce putrescine by more than 15% after 336 h of cultivation.

Higher or equal reduction of putrescine was achieved in milk (compared in vitro) under all tested conditions (**Fig. 5**). A putrescine degradation ranged from 25% to nearly 49% at the end of the incubation. The most suitable conditions for a putrescine reduction were in milk with a pH of 5.4 and 6.2. At these pH values, there was a reduction of more than 30% at a temperature of 30 °C and by more than 45% at a temperature of 23 °C and 11 °C (**Fig. 5**A and B). The least suitable conditions were recorded in milk with a pH of 7. A putrescine decrease did not exceed 27% at all tested temperature (**Fig. 5**C).

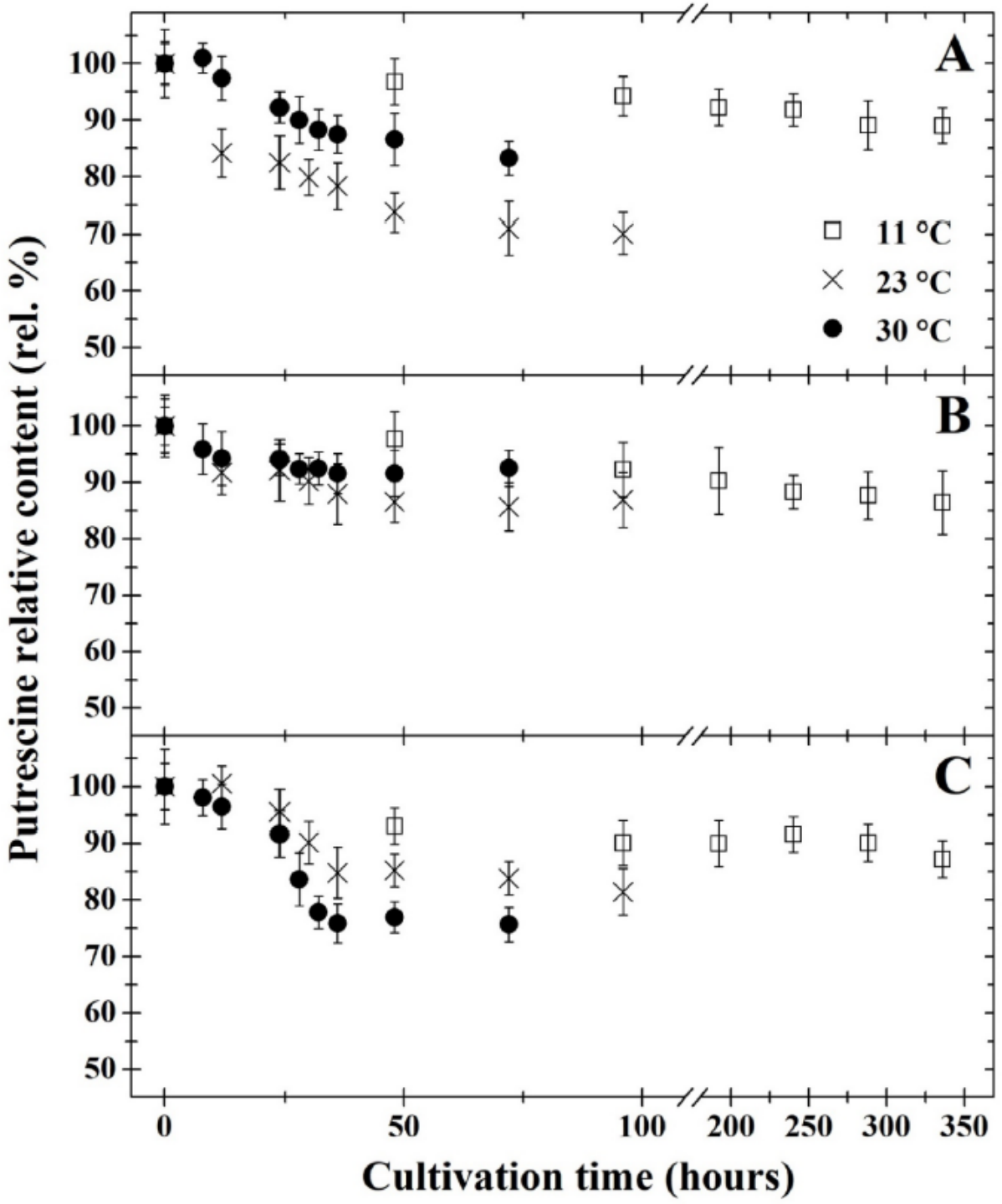


Fig. 4. The influence of Lacticaseibacillus casei $CCDM$ 198 on putrescine content (rel.%) in vitro depending on cultivation time (hr), incubation temperatures and pH. The initial histamine relative content at the time zero was always 100%. (□) temperature 11 °C, (×) temperature 23 °C and (•) temperature 30 °C; (Part A) pH = 5.4, (Part B) pH = 6.2 and (Part C) pH = 7.0. The results are presented using the means and standard deviations (bars; $n = 6$).

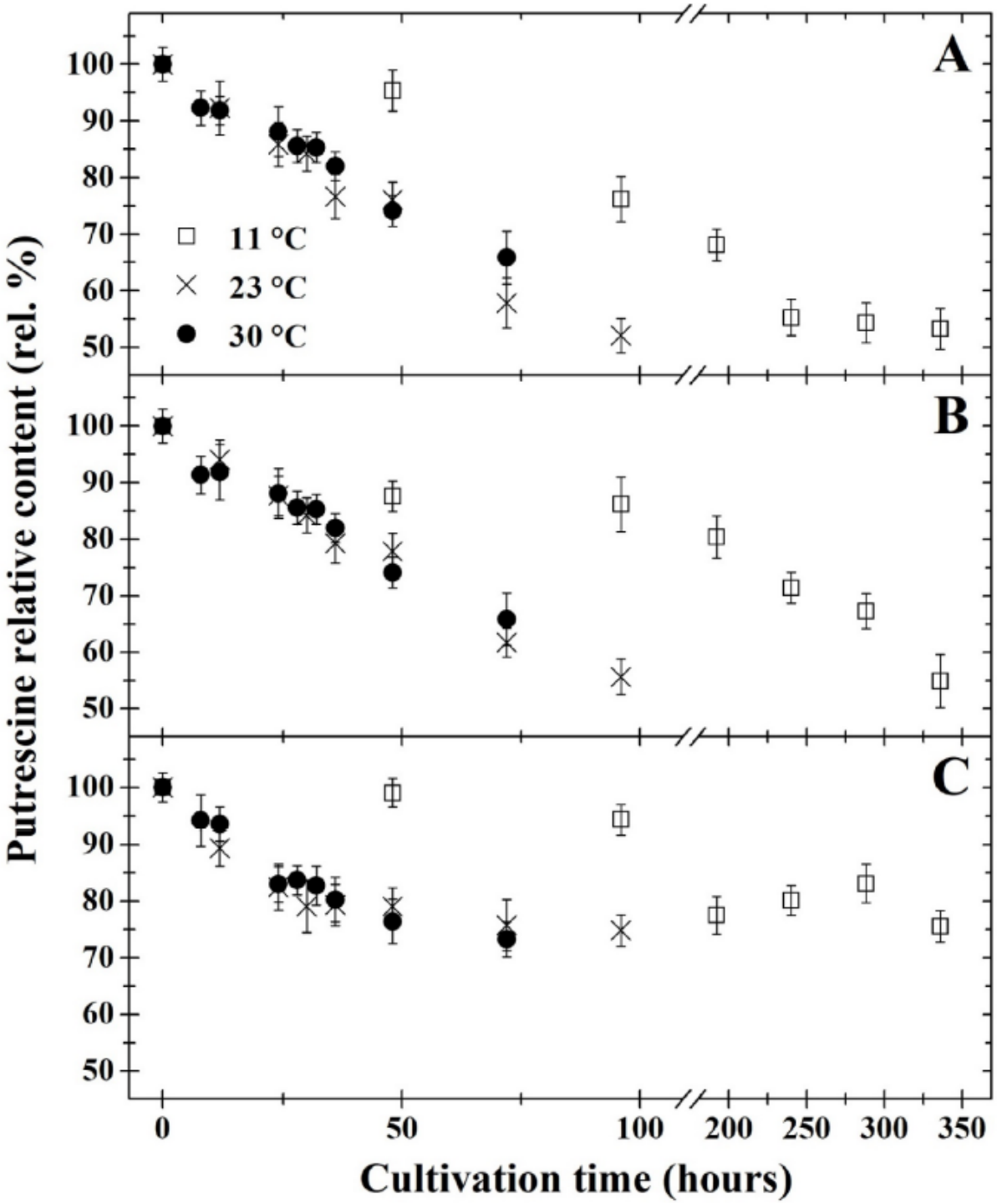


Fig. 5. The influence of Lacticaseibacillus casei $CCDM$ 198 on putrescine content (rel.%) in milk depending on cultivation time (hr), incubation temperatures and pH. The initial histamine relative content at the time zero was always 100%. (□) temperature 11 °C, (×) temperature 23 °C and (•) temperature 30 °C; (Part A) pH = 5.4, (Part B) pH = 6.2 and (Part C) pH = 7.0. The results are presented using the means and standard deviations (bars; $n = 6$).

3.1.3. Influence of factors on cadaverine degradation

The influence of studied conditions on the bacteria's ability to reduce cadaverine is summarized in **Figs. 6** and **7**. It is possible to see a decreasing trend of cadaverine content under all studied combination of factors. In vitro at a temperature of 30 °C, a significant reduction (a decrease by 30%, $P < 0.05$) was achieved only in the samples with adjusted pH of 7 (**Fig. 6**C), at other tested pH values the cadaverine

degradation did not exceed 10% during the cultivation time (**Fig. 6**A and B). The opposite trend was found at 23 °C. At this temperature, the highest decrease of cadaverine was detected at pH 5.4 (a decrease by 33%; **Fig. 6**A), whereas at the remaining two pH values, a maximum cadaverine reduction of 17% was detected (**Fig. 6**B and C). At a temperature of 11 °C, no significant difference ($P > 0.05$) between cadaverine content and pH values was noted. Under these conditions, a decrease of around 15% was measured after 336 h of cultivation.

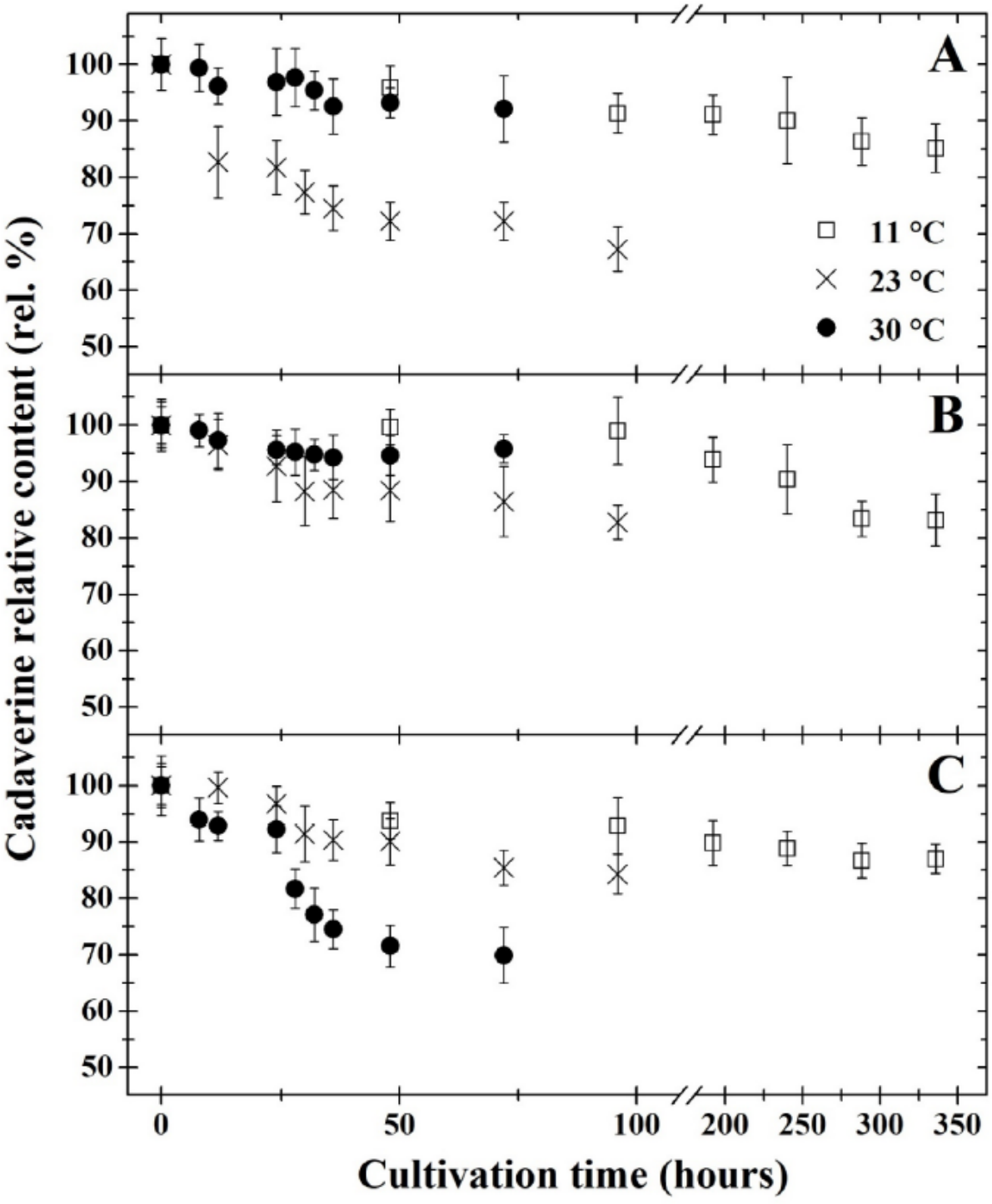


Fig. 6. The influence of Lacticaseibacillus casei $CCDM$ 198 on cadaverine content (rel.%) in vitro depending on cultivation time (hr), incubation temperatures and pH. The initial histamine relative content at the time zero was always 100%. (□) temperature 11 °C, (×) temperature 23 °C and (•) temperature 30 °C; (Part A) pH = 5.4, (Part B) pH = 6.2 and (Part C) pH = 7.0. The results are presented using the means and standard deviations (bars; $n = 6$).

On the contrary, a higher reduction was achieved in milk, ranging from 30% to 40% at the end of incubation under all studied conditions (**Fig. 7**). The most suitable combination of factors for $L.$ casei $CCMD$ 198 was milk with a pH of 7 and a temperature of 30 °C. After 72 h, the amount of cadaverine was reduced by approximately 40%, compared to the initial concentration of cadaverine at time zero (**Fig. 6**C).

3.2. Detection of multicopper oxidase

The ability to reduce BAs is related to the activity of MCO, a laccase subtype. To support the results obtained for $HPLC$, the research addressed the detection of this enzyme and its enzymatic activity. For these purposes, a cell-free extract was used that was obtained from the bacterial culture of $L.$ casei $CCDM$ 198 in the MRS broth during 0, 24, 48 and 72 h at 30 $\pm$ 1 °C. Enzyme detection was performed in gel after nondenaturing $PAGE$ and enzyme activity was determined spectrophotometrically.

3.2.1. Determining the presence of the enzyme in gel

The results from detection in nondenaturing $PAGE$ gel are shown in **Fig. 8**A. As evidenced by the gel description in **Fig. 8**A, a positive reaction was revealed by a brown-orange band caused by the oxidation of 2,6-DMP, a typical substrate for this enzyme. The presence of the enzyme in the studied strain was proven at all investigated times (0, 24, 48 and 72 h).

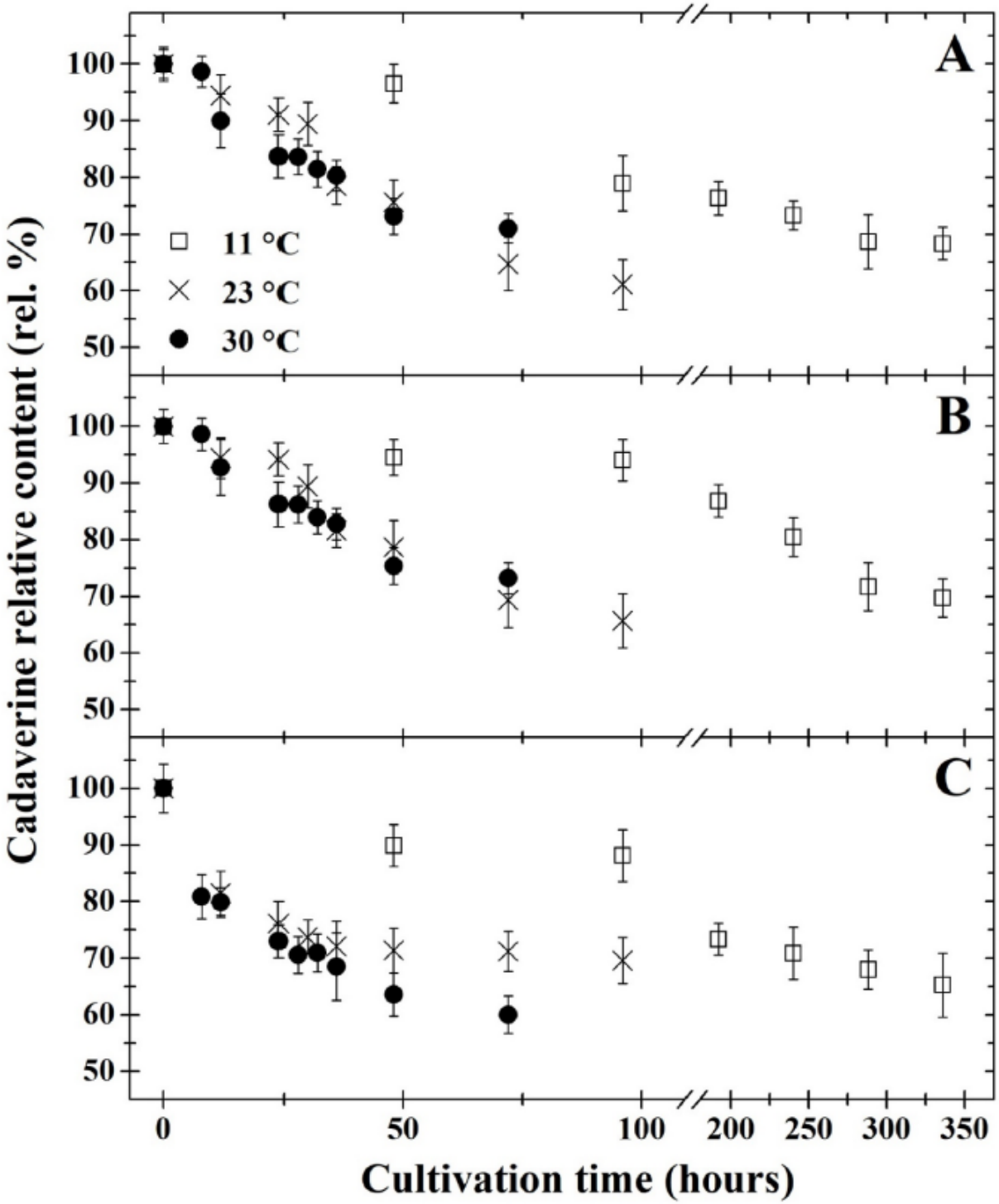


Fig. 7. The influence of Lacticaseibacillus casei $CCDM$ 198 on cadaverine content (rel.%) in milk depending on cultivation time (hr), incubation temperatures and pH. The initial histamine relative content at the time zero was always 100%. (□) temperature 11 °C, (×) temperature 23 °C and (•) temperature 30 °C; (Part A) pH = 5.4, (Part B) pH = 6.2 and (Part C) pH = 7.0. The results are presented using the means and standard deviations (bars; n = 6).

3.2.2. Determination of enzyme aktivity

The method chosen to examine MCO activity was the spectropho-tometric determination of the increase in absorbance at a wavelength of 420 nm during the oxidation of ABTS, also a typical substrate of MCO. The time course of the MCO enzyme activity after 0, 24, 48 and 72 h of incubation is shown

graphically in **Fig. 8**B. Enzyme activity (μmol-min^{-1}) over the time is plotted as a percentage relative to maximum value (% relative activity). Obtained results proved the presence and the activity of the MCO enzyme in the studied strain. Moreover, we detected the different activity of this enzyme over time. After 24 h of incubation, there was a significant increase in enzyme activity by more than 35% compared to the initial time. However, no further significant ($P < 0.05$) increase occurred during the rest of incubation time (time interval 24-72 h).

4. Discussion

A possible way to effectively reduce the number of BAs is enzymatic reduction using LAB, that are typically classified as Generally Regarded As Safe and features in the Qualified Presumption of Safety lists (**EFSA BIOHAZ Panel, 2020**).

From the preliminary research (unpublished data), it was found that the strain $L.$ casei $CCDM$ 198 could significantly degrade histamine under optimal growth conditions. In current study, we proved the presence and the activity of enzyme MCO in $L.$ casei $CCDM$ 198 by two methods. To the authors' knowledge, no previous work has proved the activity of this enzyme in this species. In addition, higher enzyme activity was also detected during incubation compared to time 0, corresponding to the higher relative gene expression in this strain (**Pištěková et al., 2020**). This finding aligns with other publications that describe enzyme activity or the presence of this enzyme gene in other LAB, e.g. Lacticaseibacillus paracasei, Latilactobacillus sakei (previously Lactobacillus sakei), Limosilactobacillus fermentum (previously Lactobacillus fermentum), Lactiplantibacillus paraplantarum (previously Lactobacillus paraplantarum), Lactiplantibacillus plantarum, Pediococcus acidilactici, Pediococcus pentosaceus and Staphylococcus xylosus (**Callejón et al., 2014; Guarcello et al., 2016; Li and Lu, 2020; Wang et al., 2022**).

Histamine is generally considered to be the most hazardous, and it is also the only amine regulated by legislation (EC No. 2073/2005; **European Regulation, 2005**). However, it is also highly important to control the level of other amines, such as putrescine and cadaverine. These secondary amines could synergistically enhance the negative effects of histamine and could further react with nitrite to form carcinogenic ni-trosamines (**Linares et al., 2011; Sarkadi, 2019**). Given the possible health risks described above, the ability of tested strain $CCDM$ 198 to degrade putrescine and cadaverine was also studied. The type of food matrix significantly affects the catalytic activity of enzyme MCO due to a presence of different natural competitive inhibitors and mediators in individual foods (**Wang et al., 2022**). Therefore, the BA-degradation ability of investigated strain $L.$ casei $CCDM$ 198 was determined not only in vitro conditions but also in skimmed milk. Milk was chosen because it is a starting material in the production of fermented dairy products that can contain large amounts of BAs, for example cheese (**Linares et al., 2011**).

The tested strain $L.$ casei $CCDM$ 198 significantly ($P < 0.05$) reduces histamine, putrescine and cadaverine in vitro but above all in a real food system - skimmed milk. The obtained results show that milk was a suitable culture medium for $L.$ casei $CCDM$ 198. Moreover, even a slightly higher reduction of tested BAs was detected in milk. The reduction of nearly 40% of histamine, almost 49% of putrescine and 40% of cadaverine was determined. In comparison, the content of histamine was reduced in vitro by more than 37%, putrescine by 30% and cadaverine by 33%. One possible explanation could be the fact that the strain $L.$ casei is an isolate from dairy product and this strain could be better adapted to this environment. Based on the available information from the literature, a rather opposite trend was expected (**García-Ruiz et al., 2011; Wang et al., 2022**). **García-Ruiz et al.** (**2011**) compared the degradation ability of $L.$ casei in a medium and in wine.

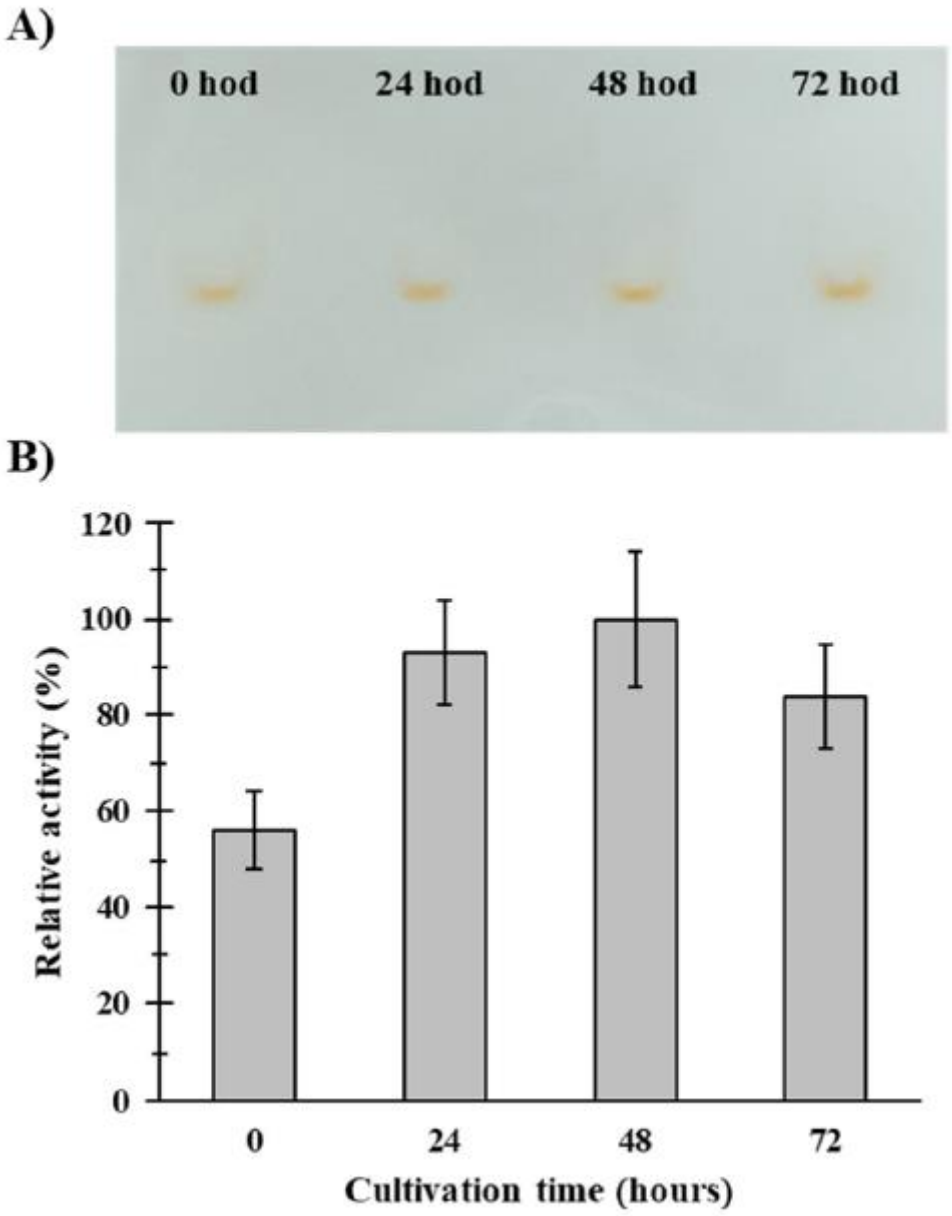


Fig. 8. Detection and change in the level of multicopper oxidase (MCO) enzyme relative activity in Lacticaseibacillus casei $CCDM$ 198. Part A demonstrates presence of MCO by gel staining from L. casei $CCDM$ 198 cell-free extracts after 0, 24, 48 and 72 h incubation in vitro with 2,6-DMP. The positive reaction revealed an orange-brown band. Part B describes spectrophotometric detection of MCO enzyme relative activity (% rel. activity) from cell-free extracts of Lacticaseibacillus casei $CCDM$ 198 using $ABTS$ substrate oxidation after 0, 24, 48 and 72 h incubation in vitro. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 pmol $ABTS$ per minute. Enzyme activity is plotted as a percentage relative to maximum value (% rel. activity) The values are means $\pm$ standard deviations of triplicate assays. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The L. casei IFI-CA 52 degraded histamine by 54%, tyramine by 55% and putrescine by 65% in the medium. Smaller decreases in concentration were observed in wine, with histamine decreasing by 16%, tyramine by 15% and putrescine by 8%. The possible real application of the investigated strain to dairy products is supported not only by these results but also by the fact that BA-degrading strains (including L. casei $CCDM$ 198) do not significantly affect the basic chemical parameters (pH values, dry matter content, salt content and fat content) or texture (hardness) of natural cheeses (**Adámek et al.,**

2021; Guarcello et al., 2016). Reducing ability was demonstrated for L. casei in a study by **Herrero-Fresno et al.** (**2012**). The authors of the paper identified 17 cheese isolates with the ability to differently reduce histamine and tyramine as L. casei. The degradation of amines in dairy products by microorganisms has been confirmed in other publications (**Adámek et al., 2021; Guarcello et al., 2016; Tittarelli et al., 2019**). LAB also reduce the presence of BAs, for example, in a wine (**Callejón et al., 2014**), a fish silage (**Dapkevicius et al., 2000**), a fermented soybean paste (**Lee et al., 2016**) and a sauerkraut (**Rabie et al., 2011**).

For real use in the food industry, it was also important to determine how a combination of technological factors, such as pH and temperature, could affect the degradation activity of this bacterial strain. The individual tested temperatures were selected to represent the most common production and storage temperatures. The temperature 30 °C is the optimal temperature for the studied strain, 11 ° C represents the cold storage temperature as well as the ripening temperature for many cheeses and other foods while 23 °C is room temperature at which foods are often stored incorrectly. The tested pH values are intended to cover a wide range of pH environments of both acidic and non-acidic foods. Moreover, pH 6.2 is the optimal pH for the strain L. casei. The reason for testing the effect of a combination of different multiple factors was to estimate the model system closer to real food samples, which also contain several factors/variables.

Tested temperatures and pH values and their combinations have a significant effect ($P < 0.05$) on the development of individual BAs. Based on the measured results, we were unable to find a common trend for the reduction of all tested amines. Moreover, a different ability of L. casei $CCDM$ 198 to reduce the number of individual amines was found depending on different combinations of factors. It seems, putrescine and cadaverine showed a similar trend but different optimal conditions for reduction were found for histamine. For example, at a temperature of 30 °C and a pH of 6.2 under in vitro conditions, histamine was reduced by more than 35% at the end of the cultivation time but under these some conditions the strain L. casei $CCDM$ 198 reduced the amount of putres-cine and cadaverine by less than 10%. In the case of pH 7, the most suitable temperature for the reduction of putrescine and cadaverine in vitro was 30 °C but it was a temperature of 11 °C for the reduction of histamine. This finding was very surprising, as we expected a similar degradation capacity towards all tested amines by L. casei $CCDM$ 198 under the same tested conditions. A possible explanation could be the structure of the individual amines that could influence the affinity of the enzyme for the substrate. Putrescine and cadaverine are short-chain aliphatic amines (therefore probably showed a similar trend in degradation), while histamine is an aromatic amine. Our theory would also explain the observed different optimal pH and temperature values for individual amines during degradation by L. casei $CCDM$ 198. The specific pH values and temperature could cause temporary conformational changes in the active site of the enzyme MCO, thereby making the binding of the specific substrate to the enzyme accessible or inaccessible. This hypothesis is confirmed, for example, by the publication of **Tepkasikul et al.** (**2022**). In this study authors investigated the reducing ability of Bacillus piscicola FBU1786 towards BAs and also concluded that BA degradation by the same bacterium is selective, at least based on the BA structure. Bacillus piscicola FBU1786 first removed short-chain aliphatic amines (cadaverine and putrescine), followed by aromatic amines, and then long-chain aliphatic amines (such as spermine and spermidine). Apart from this publication, our results are also consistent with other recent studies showing that the enzyme MCO within one studied bacterium could have a greater affinity for one substrate than another (including for individual BAs), and that individual substrates have different optimal pH and temperature. In addition, it also appears that laccases from individual microorganism may differ in optimal pH and temperature for the same substrate (**Callejón et al., 2016, 2017; Li et al., 2020; Olmeda et al., 2021; Ni et al., 2022; Tepkasikul et al., 2022; Wang et al., 2022**). **Callejón et al.** (**2016, 2017**) found a difference in substrate specificity for laccases. These differences

concern both typical substrates for laccases and individual BAs. Recombinant laccase from Lactiplantibacillus plantarum J16 (CECT 8944) had an optimum pH of 3.5 for $ABTS$ substrate and 7.0 for 2,6-DMP (**Callejón et al., 2016**). The optimal pH for recombinant laccase from Pediococcus acidilactici CECT 5930 (Lpa5930) was approximately 4.0 for $ABST$ (**Callejón et al., 2017**). Both enzymes showed highly similar catalytic ability for tyramine. The optimum temperature for tyramine degradation by laccase from Lactiplantibacillus plantarum was 28 °C. Degradation activity was also demonstrated at 4 °C (tyramine reduction by 44%). The optimum pH values were 4.0 and 9.5. The same optimal values were found for recombinant laccase from Pediococcus acidilactici (optimal pH values 4.0 and 9.5, and optimal temperature 28 °C). Recombinant laccase from Lactiplantibacillus plantarum oxidized all investigated amines (tyramine, histamine and putrescine) under optimal conditions for tyramine but putrescine and histamine were reduced to a lesser extent than tyramine (**Callejón et al., 2016**). In addition, the ability of the recombinant laccase enzyme from Pediococcus acidilactici under optimal conditions for tyra-mine to oxidize histamine and putrescine was not clearly demonstrated. Different results were obtained for recombinant laccase (rLac) from Bacillus velezensis TCCC 111904. The optimal temperature and pH for this enzyme were 80 °C and 5.5 in the case of the substrate $ABTS$ (**Li et al., 2020**). Authors of the work **Tepkasikul et al.** (**2022**) studied effects of environmental factors (including pH and cultivation temperature) on the degradation of histamine by laccase from Bacillus piscicola FBU1786. A histamine reduction was studied in a pH range of 4-10. The highest reduction was recorded in the pH range from 6 to 9, while no or little histamine reduction was found at pH 4, 5 and 10. At the investigated temperatures of 30, 37, and 45 °C, the bacterial strain FBU1786 completely reduced histamine. **Wang et al.** (**2022**) demonstrated a higher reduction of histamine and tyramine in the neutral environment than in the acidic environment by $rMCO$ which is a different pH for optimal degradation of tyramine by laccase from Lactiplantibacillus plantarum (**Callejón et al., 2016**). On the other hand, laccases from Pediococcus laccases 5930 and Pediococcus pentosaceus 4816 were unable to degrade histamine, putrescine or phenylethylamine. Both laccases (5930 and 4816) were able to oxidize tyramine but only in the presence of a mediator (**Olmeda et al., 2021**).

Due to the fact that there are still very few publications dealing with this issue and also the fact that there are significant differences in the optimal conditions for the degradation of amines in individual bacterial species, it is important to further explore this issue for greater understanding of this enzyme mechanism. Understanding the degradation mechanism could lead to the possible application of the MCO enzyme to a wider range of foods in which the presence of bacteria can be undesirable.

5. Conclusion

The findings in this study demonstrate a significant degradation capacity of histamine, putrescine and cadaverine in the strain L. casei $CCDM$ 198 in vitro and in milk by the enzyme MCO, a laccase subtype. The presence and the activity of the MCO enzyme was detected in L. casei $CCDM$ 198. To the current authors' knowledge, this is the first mention of the presence of MCO enzyme activity in this species. Additionally, the influence of technological factors on BA-degrading ability of the studied strain has been provided. The results of this study show the real potential for the application of the investigated strain in the fermented dairy products to reduce undesirable BAs. The use of appropriate degraders could be an effective strategy to minimize adverse effects on consumers, enhancing food safety.

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