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ROLE OF THE MICROBIAL COMMUNITY IN PRODUCTION OF BIOGENIC AMINES IN FERMENTED FOODS AND CHARACTERISATION OF A TYRAMINE-REDUCING *LACTOBACILLUS PLANTARUM* STRAIN

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presented by

JANINE ANDEREGG

MSc ETH in Food Science

born September 9, 1980

citizen of Koppigen (BE)

accepted on the recommendation of

Prof. Dr. Leo Meile, examiner

Prof. Dr. Christophe Lacroix, co-examiner

Dr. Michael Beer, co-examiner

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dedicated to my parents

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Abbreviations

ADC	Agmatine deaminase
AGDI	Agmatine deaminase cluster
ANOVA	Analyse of variance
ATP	Adenosine triphosphate
BA	Biogenic amine
BHI	Brain-heart infusion
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BP	Baird-Parker
Cad	Cadaverine
CE	Capillary electrophoresis
cfu	Colony-forming units
CK	Carbamate kinase
CNS	Coagulase-negative staphylococci
CO_2	Carbon dioxide
CPS	Coagulase-positive staphylococci
DNA	Deoxyribonucleic acid
DAO	Diamine oxidase
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
FFA	Free fatty acid
FSVO	Food Safety and Veterinary Office
G + C	Guanine + Cytosine
GABA	Gamma-aminobutyric acid
GC	Gas chromatography
GRAS	Generally Regarded As Safe
H^+	Hydrogen ions
HDC	Histidine decarboxylase cluster
HGT	Horizontal gene transfer
HHP	High hydrostatic pressure
His	Histamine

H_2O_2	Hydrogen peroxide
HPH	High-pressure homogenisation
HPLC	High-performance liquid chromatography
IC-PAD	Ion exclusion chromatography with pulsed amperometric detection
KEGG	Kyoto Encyclopedia of Genes and Genomes
KFS	Kenner Fecal *Streptococcus*
LAB	Lactic acid bacteria
LDC	Lysine decarboxylase cluster
LOAEL	lowest observed adverse effect level
MAO	Monoamine oxidase
MAP	Modified atmosphere packaging
mCDM	Minimal chemically defined medium
MRS	De Man-Rogosa-Sharpe
Na^+	Sodium ions
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
NH_3	Ammonium
NOAEL	Non-observed adverse effect level
NSLAB	Non-starter lactic acid bacteria
OD	Optical density
ODC	Ornithine decarboxylase cluster
PCR	Polymerase chain reaction
Phe	Phenylethylamine
PTC	Putrescine carbamoyltransferase
Put	Putrescine
QPS	Qualified Presumption of Safety
RNA	Ribonucleic acid
rRNA	ribosomal ribonucleic acid
SFF	Schweizer Fleisch-Fachverband – Swiss Meat Association
Spd	Spermidine
Spm	Spermine
TDC	Tyrosine decarboxylase cluster
Trp	Tryptamine
Tyr	Tyramine

Summary

Biogenic amines (BA) are organic bases, which can occur in high concentrations in fermented food products and can cause several adverse health reactions for the consumer. BAs are formed by decarboxylation of free amino acids by food-resident amino acid-decarboxylating bacteria, which can be part of the starter culture or may be introduced by contamination during food processing and storage. While cadaverine and putrescine tend to have an indirect toxic effect on human health by increasing the toxic effect of histamine and tyramine through competitive inhibition of detoxifying diamine oxidase enzymes, histamine and tyramine have a direct toxic effect. An intake of a higher concentration of histamine or tyramine can lead to serious health effects such as abdominal cramps, cardiac failure, diarrhoea, headache and migraine, hypertensive crises, intracranial haemorrhage, pounding heart and palpitations, pulmonary oedema and even death. While to date there is a legislation on the maximum histamine concentration in fish, despite the frequent occurrence in high concentrations and the range of toxic effects, there is no legal regulation of the maximum concentration of tyramine in fermented foods. Reasons are inter alia the lack of knowledge about tyramine production conditions as well as the main responsible tyramine-producing microorganisms in different fermented food products. Therefore, the aim of this doctoral thesis was to improve the knowledge about tyramine-producing microorganisms and on their mechanism and factors involved in tyramine production as well as to investigate a *Lactobacillus plantarum* strain for the ability to reduce tyramine accumulation in fermented food.

In a first part of the thesis, 62 salami-type fermented sausages from the Swiss market were investigated on tyrosine-decarboxylating bacterial strains and the content of cadaverine, histamine, putrescine and tyramine. BA concentrations were measured by ion-exclusion chromatography with pulsed amperometric detection (IC-PAD) and viable plate counts of enterococci, lactobacilli/lactococci and staphylococci were measured. Bacterial strains were geno- and phenotypically examined on their ability to produce tyramine. Positive strains were identified for the species by multiplex polymerase chain reaction (PCR) or 16S rRNA gene sequencing. In the analysed samples, all four BAs had higher concentrations in industrially-produced sausages compared to artisanally-produced ones. Tyramine was the major amine detected in 46 of 62 sausages, with a maximum of 785.22 mg kg^{-1}, and enterococci (74%), as well as coagulase-negative staphylococci (61%), mainly the meat starter culture *S. xylosus*,

could be identified as the main tyramine producers. Putrescine was found in 20 of 62 sausages, with a maximum observed concentration of 707.77 mg kg^{-1}. These two BAs showed a significant correlation ($P = 0.0407$) for their concentrations. Cadaverine and histamine were detected in nine and eight samples respectively, and both were found in significantly higher levels ($P = 0.019$) and ($P = 0.036$) in industrially-produced sausages. Interestingly, based on the quantitative tyramine content, five groups of fermented sausages were identified. Group 1 included products with a very high tyramine level (> 700 mg kg^{-1}), group 2 with a high level (400–700 mg kg^{-1}), group 3 with a moderate level (200–400 mg kg^{-1}), group 4 with a low level (< 200 mg kg^{-1}) and group 5 with a tyramine level below the detection limit (0.05 mg kg^{-1}). Samples with a tyramine level higher than 200 mg kg^{-1} could be considered as products of less quality because consumption of such samples could be unhealthy for sensitive individual consumers.

Among fermented products, tyramine appears with the highest frequency in cheese. Therefore, 274 cheese samples were investigated on tyrosine-decarboxylating bacterial strains and their content of the four BAs cadaverine, histamine, putrescine and tyramine. Tyramine was with a concentration up to 984.38 mg kg^{-1} by far the most abundant BA followed by cadaverine with a concentration up to 872.09 mg kg^{-1}. Moreover, the concentration of cadaverine significantly correlates with the concentration of tyramine. Furthermore, with a prevalence of 78% enterococci were by far the major tyramine producers in cheese samples and showed a significant correlation to tyramine. Besides, this study determined that the factors of milk treatment, milk origin as well as ripening time play an essential role in BA formation and accumulation in cheese. The highest concentration of all four BAs measured was found in semi-hard cheese made from cow's raw milk.

Sodium chloride is often used in fermented food products to control the growth of pathogens by reducing water activity, although it might have an enhancing effect on BA production and accumulation. To investigate the influence of sodium chloride in BA production and accumulation, eight tyramine-producing bacterial strains, representing Gram-positive species, were investigated on their tyramine production in a cheese-like micro-cheese model containing 2.5 mM tyrosine and different sodium chloride concentrations (0%, 1.5%, 3%, and 4.5%). It could be demonstrated that an increase in sodium

chloride concentration resulted in an increase of tyramine concentration in all eight tested tyramine-producing bacterial strains. In the tested *Staphylococcus xylosus* strain, for example, an increase in tyramine concentration of 871% was observed with measured tyramine concentrations of 67.91 mg L^{-1} and 569.17 mg L^{-1} when the sodium chloride concentration was increased from 0% to 1.5%. However, different tyramine-producing bacterial strains reacted differently to an increase in sodium chloride concentration. While the tyramine-producing *Enterococcus faecalis* strain showed high and relatively stable tyramine concentrations between 598.52 mg L^{-1} and 716.10 mg L^{-1} in all four tested sodium chloride concentrations, the strains *Enterococcus faecium*, *Enterococcus durans, Staphylococcus equorum*, *Lactococcus lactis* and *Lactobacillus parabuchneri* showed higher tyramine concentrations with increasing sodium chloride concentrations.

The last study in this thesis examined the *Lactobacillus plantarum* JA-1199 strain - harbouring a multicopper oxidase and a histidinol-phosphate transaminase gene - for its capacity to reduce tyramine accumulation in a micro-cheese model. Therefore, eight tyramine-producing bacterial strains were incubated in combination with the *Lb. plantarum* JA-1199 strain in a micro-cheese model containing 2.5 mM tyrosine and different sodium chloride concentrations (0%, 1.5%, 3%, and 4.5%). At the end of their ripening period, all micro-cheese models were analysed for their tyramine concentration. The result of the study showed a significant reduction of tyramine accumulation of up to 99% when *Staphylococcus simulans* or *Staphylococcus xylosus* were incubated in combination with the *Lb. plantarum* JA-1199 strain. Although the capacity of *Lb. plantarum* JA-1199 to reduce tyramine accumulation decreased with an increase of sodium chloride concentration and showed a maximum of reduction of tyramine accumulation of 9.23% at a sodium chloride concentration of 4.5%, the tested *Lb. plantarum* JA-1199 strain was able to reduce tyramine accumulation at any sodium chloride concentration without affecting the growth of the tyramine producing or other microorganisms such as starter cultures.

In conclusion, this doctoral thesis has given insight into the complex interaction of fermented food and tyramine concentration with tyramine-producing microorganisms. It was shown that high concentrations of tyramine in fermented sausages or cheeses are caused by enterococci alone or in combination with

coagulase-negative staphylococci and that significant correlations are existing between tyramine and other biogenic amines. The approach with a high number of cheeses and fermented sausages samples from Switzerland supports previous studies, which reported punctually the ability of different bacterial strains to produce different biogenic amines. Furthermore, the knowledge of the influence of different factors affecting tyramine production and potential mechanisms could be extended. It was shown that different environmental and physicochemical factors increase the tyramine concentration in fermented foods. Moreover, this doctoral thesis demonstrated reduction of tyramine accumulation in a complex food-like matrix with potential for application as a feasible strategy for decreasing tyramine concentration and therefore increase the safety level of fermented food products. These findings have established prerequisites for the application of the *Lb. plantarum* JA-1199 strain to prevent/reduce tyramine accumulation in fermented food products. However, further studies are needed to demonstrate the efficacy of *Lb. plantarum* JA-1199 in different fermented food products without negatively affecting the respective food matrix.

Zusammenfassung

Biogene Amine (BA) sind organische Basen, welche in hohen Konzentrationen in fermentierten Lebensmitteln vorkommen und beim Konsumenten mehrere negative Auswirkungen auf die Gesundheit verursachen können. BAs entstehen durch die Decarboxylierung freier Aminosäuren mittels lebensmittelbeständiger Bakterien, welche Decarboxylierungseigenschaften besitzen. Diese Bakterien können entweder Teil der Starterkultur sein, oder durch Kontamination bei der Lebensmittelverarbeitung und -lagerung in das Lebensmittel eingebracht werden. Während die BAs Kadaverin und Putrescin tendenziell eher eine indirekte toxische Wirkung auf die menschliche Gesundheit haben, indem sie die toxische Wirkung von Histamin und Tyramin durch konkurrierende Hemmung der entgiftenden Diaminooxidase-Enzyme erhöhen, haben Histamin und Tyramin eine direkte toxische Wirkung. Die Einnahme einer höheren Menge an Histamin oder Tyramin kann zu schwerwiegenden gesundheitlichen Folgen wie Bauchkrämpfe, Herzversagen, Durchfall, Kopfschmerzen und Migräne, hypertensiven Krisen, intrakraniellen Blutungen, erhöhten Herzschlägen und Herzklopfen, Lungenödemen und sogar zum Tod führen. Während eine Gesetzgebung über die Maximalkonzentration von Histamin in Fischen existiert, gibt es trotz des in hohen Konzentrationen und häufigen Auftretens und dessen Bandbreite der toxischen Wirkungen keine gesetzliche Grundlage zur Regelung der Maximalkonzentration von Tyramin in fermentierten Lebensmitteln. Gründe dafür sind unter anderem ein mangelndes Wissen über die Produktionsbedingungen von Tyramin und deren produzierenden Mikroorganismen in verschiedenen fermentierten Lebensmitteln. Ziel dieser Doktorarbeit war es deshalb, das Wissen über die Tyramin-produzierenden Mikroorganismen und deren Mechanismen und beeinflussenden Faktoren, welche an der Tyramin-Produktion beteiligt sind, zu verbessern sowie einen *Lactobacillus plantarum*-Stamm und dessen Fähigkeit, Tyramin-Akkumulation in fermentierten Lebensmitteln zu reduzieren, zu untersuchen.

In einem ersten Teil der Arbeit wurden 62 vom Schweizer Markt stammende fermentierte Würste des Typs Salami auf Tyrosin-decarboxylierende Bakterienstämme sowie auf den Gehalt der vier BAs Kadaverin, Histamin, Putrescin und Tyramin untersucht. Die Konzentrationen der BAs wurden mittels Ionenaustauschchromatographie mit amperometrischer Puls-Detektion (IC-PAD) gemessen und die Konzentrationen von Enterokokken, Laktobazillen/Laktokokken und Staphylokokken wurde mittels

dem Ausstrichverfahren auf semiselektivem Agar-Medien bestimmt. Bakterienstämme wurden geno- und phänotypisch auf ihre Fähigkeit zur Tyramin-Produktion untersucht, und positive Stämme wurden durch eine Multiplex-Polymerase-Kettenreaktion (PCR) oder mittels 16S rRNS-Gensequenzierung auf ihre Spezies identifiziert. Aller vier untersuchten BAs kamen in industriell-hergestellten Würsten des Typs Salami in einer höheren Konzentration vor als in artisanal-hergestellten Würsten. Tyramin war das am häufigsten vorkommende BA und wurde mit einer Maximalkonzentration von 785,22 mg kg^{-1} in 46 von 62 Wurstproben nachgewiesen. Als Hauptproduzenten von Tyramin konnten Enterokokken (74%) und Koagulase-negativen Staphylokokken (61%), hauptsächlich die Fleischstarterkultur *Staphylococcus xylosus*, identifiziert werden. Putrescin wurde mit einer maximalen Konzentration von 707,77 mg kg^{-1} in 20 von 62 Würsten gefunden und zwischen Putrescin und Tyramin wurde eine signifikante positive Korrelation ($P = 0.0407$) beobachtet. Kadaverin und Histamin wurden hingegen nur in neun beziehungsweise acht Proben nachgewiesen, allerdings in einer signifikant höheren Konzentration ($P = 0.019$) und ($P = 0.036$) in industriell-hergestellten Würsten im Vergleich zu artisanal-produzierten. Interessanterweise konnten auf der Grundlage des quantitativen Tyramin-Gehaltes fünf Gruppen von fermentierten Würsten identifiziert werden. Gruppe 1 umfasst Produkte mit einem sehr hohen Tyramin-Gehalt (> 700 mg kg^{-1}), Gruppe 2 Würste mit einem hohen Tyramin-Gehalt (400–700 mg kg^{-1}), Gruppe 3 Würste mit einem mässigen Gehalt an Tyramin (200–400 mg kg^{-1}), Gruppe 4 mit einem niedrigen Tyramin-Gehalt (< 200 mg kg^{-1}) und Gruppe 5 beinhaltet Würste mit einem Tyramin-Gehalt unterhalb der Nachweisgrenze (0.05 mg kg^{-1}). Proben welche der Gruppen 1, 2 und 3 angehören und somit einen Tyramin-Gehalt höher als 200 mg kg^{-1} aufweisen, können als Produkte geringerer Qualität angesehen werden, da der Verzehr solcher Würste bei empfindlichen Konsumenten gesundheitliche Probleme verursachen können.

Da Käse als fermentiertes Lebensmittel optimale Bedingungen zur Bildung von BAs aufweist und im Käse vor allem das BA Tyramin in Höchstkonzentrationen vorkommt, wurden 274 Käseproben auf den Gehalt der vier BAs Kadaverin, Histamin, Putrescin und Tyramin sowie auf das Vorkommen von Tyrosin-decarboxylierenden Bakterien untersucht. Tyramin war mit einer gemessenen Konzentration von bis zu 984,38 mg kg^{-1} mit Abstand das am häufigsten vorkommende BA, gefolgt von Kadaverin,

welches mit einer Konzentration von bis zu 872,09 mg kg^{-1} vorkam. Darüber hinaus wurde zwischen diesen beiden gemessenen BAs eine signifikante Korrelation beobachtet. Die geno- und phänotypische Untersuchung der Tyrosin-decarboxylierenden Bakterienisolate zeigten ganz klar, dass Enterokokken, mit einer Prävalenz von 78% die hauptverantwortlichen Tyramin-Produzententen im Käse sind. Des Weiteren konnte mittels einer statistischen Untersuchung eine signifikante positive Korrelation zwischen Enterokokken und der Tyramin-Konzentration im Käse nachgewiesen werden. Ebenfalls konnte in diesem Versuch bestätigt werden, dass Faktoren wie Milchbehandlung, der tierische Ursprung der Milch sowie die Reifungszeit des Käses wichtige Rollen bei der Bildung und Akkumulation von BAs spielen. Käse aus Rohmilch oder Halbhartkäse wiesen im Vergleich zu Käse aus thermisierter oder pasteurisierter Milch oder im Vergleich zu Weichkäse höhere Konzentrationen an BAs auf.

Natriumchlorid wird, obwohl es einen Einfluss auf die BA-Produktion und -Akkumulation haben kann, in fermentierten Lebensmitteln häufig dazu verwendet, das Wachstum von Krankheitserregern zu kontrollieren, indem es die Wasseraktivität im Lebensmittel reduziert. Um den genauen Einfluss von Natriumchlorid auf die BA-Produktion und -Akkumulation zu untersuchen, wurden acht Gram-positive Tyramin-produzierende Mikroorganismen, in einem Mikrokäse Modell, welches 2,5 mM Tyrosin und verschiedene Natriumchlorid-Konzentrationen (0%, 1,5%, 3% und 4,5%) enthält, untersucht. Es konnte gezeigt werden, dass eine Erhöhung der Natriumchlorid-Konzentration bei allen acht getesteten Bakterien eine Erhöhung der Tyramin-Produktion zur Folge hat. Beim Tyramin-produzierenden *Staphylococcus xylosus*-Stamm zum Beispiel, konnte eine Erhöhung der Tyramin-Konzentration von 871% mit gemessenen Tyramin-Konzentrationen von 67,91 mgL^{-1} und 569,17 mg L^{-1} bei einer Erhöhung der Natriumchlorid-Konzentration von 0 auf 1.5% beobachtet werden. Es zeigte sich jedoch, dass verschiedene Tyramin-produzierende Bakterienstämme unterschiedlich stark auf eine Erhöhung der Natriumchlorid-Konzentration reagieren. Während der Tyramin-produzierende *Enterococcus faecalis*-Stamm in allen vier Natriumchlorid-Konzentrationen hohe und relativ stabile Tyramin-Konzentrationen zwischen 598,52 mg L^{-1} und 716,10 mg L^{-1} aufwies, wiesen die Stämme *Enterococcus faecium*, *Enterococcus durans*, *Staphylococcus equorum*, *Lactococcus lactis* sowie

Lactobbacillus parabuchneri höhere Tyramin-Konzentrationen bei steigender Natriumchlorid-Konzentration auf.

Die letzte Studie in dieser Arbeit untersuchte den *Lactobacillus plantarum* JA-1199-Stamm - der entsprechend seiner Genomsequenz ein Gen für eine Multikupfer-Oxidase sowie ein Gen für eine Histidinol-Phosphat-Transaminase kodiert - auf seine Fähigkeit, die Tyramin-Akkumulation in einem Mikrokäse-Modell zu reduzieren. Hierfür wurden acht Tyramin-produzierende Bakterien in Kombination mit dem *Lb. plantarum* JA-1199-Stamm in einem Mikrokäse-Modell, welches 2,5 mM Tyrosin und vier verschiedenen Natriumchlorid-Konzentrationen (0%, 1,5%, 3% und 4,5%) enthält, inkubiert. Am Ende der Reifungszeit wurden alle untersuchten Mikrokäse-Modelle auf deren Tyramin-Gehalt analysiert. Die Ergebnisse der Studie zeigten eine deutliche Reduktion der Tyramin-Konzentration von bis zu 99% bei einer Inkubation von *Staphylococcus simulans* oder *Staphylococcus xylosus* in Kombination mit dem *Lactobacillus plantarum* JA-1199-Stamm. Obwohl die Kapazität von *Lactobacillus plantarum* JA-1199 zur Reduktion der Tyramin-Akkumulation bei einer Erhöhung der Natriumchlorid-Konzentration abnahm, und bei einer Natriumchlorid-Konzentration von 4.5% eine maximale Reduktion der Tyramin-Akkumulation von 9.23% zeigte, konnte der *Lactobacillus plantarum* JA-1199-Stamm die Tyramin-Akkumulation bei jeder Natriumchlorid-Konzentration verringern ohne das Wachstum der Tyramin-produzierenden Mikroorganismen oder der Starterkulturen zu beeinträchtigen.

Zusammenfassend erlaub diese Doktorarbeit einen Einblick in die komplexe Wechselwirkung zwischen fermentierten Lebensmitteln und deren Tyramin-Konzentrationen mit den dazugehörigen Tyramin-produzierenden Mikroorganismen. Es konnte gezeigt werden dass, abhängig vom jeweiligen Lebensmittel, hohe Tyramin-Konzentrationen entweder durch Enterokokken alleine oder in Kombination mit Koagulase-negativen Staphylokokken verursacht werden, und dass signifikante Korrelationen zwischen Tyramin und anderen BAs bestehen. Der Ansatz mit einer hohen Anzahl von Lebensmittelproben unterstützt frühere Studien, die über die Fähigkeit verschiedener Bakterienstämme, welche verschiedene BA produzieren, berichteten. Darüber hinaus konnte das Wissen über den Einfluss verschiedener Faktoren, die die Tyramin-Produktion beeinflussen, sowie das Verständnis der

involvierten Mechanismen bei der Tyramin-Produktion, erweitert werden. Es konnte gezeigt werden, dass verschiedene umweltbedingte und physikalisch-chemische Faktoren die Tyramin-Konzentration in fermentierten Lebensmitten beeinflussen. Des Weiteren zeigte diese Doktorarbeit eine praktikable Strategie zur Verringerung der Tyramin-Konzentration und damit zur Erhöhung des Sicherheitsniveaus fermentierter Lebensmittel auf, indem die Tyramin-Akkumulation in einer komplexen lebensmittelähnlichen Matrix reduziert wurde. All diese Erkenntnisse legen den Grundstein für die Anwendung des *Lactobacillus plantarum* JA-1199-Stammes zur Verhinderung bzw. Reduktion der Tyramin-Akkumulation in fermentierten Lebensmitteln. Dennoch sind zukünftige *in vivo* Studien erforderlich, um die Wirksamkeit des *Lactobacillus plantarum* JA-1199-Stammes in verschiedenen fermentierten Lebensmitteln zu bestätigen, ohne dass dabei die jeweilige Matrix des untersuchten Lebensmittels negativ beeinträchtigt wird.

Chapter 1

General Introduction

1. Biogenic amines

1.1 Characteristics and classification

Biogenic amines (BA), formed by decarboxylation of free amino acids, are basic, organic, nitrogenous compounds with biological activity. According to their chemical structure, they can be classified as aliphatic di-, tri-, and polyamines (agmatine, cadaverine, putrescine, spermidine, and spermine), aliphatic volatile amines (ethanolamine, ethylamine, isopentylamine, and methylamine) or as aromatic and heterocyclic amines (histamine, phenylethylamine, tryptamine, tyramine and serotonin). Furthermore, according to their number of amine groups they can be grouped in monoamine (ethanolamine, ethylamine, isopentylamine, methylamine, and tyramine), diamine (cadaverine, histamine, putrescine, tryptamine and serotonin) or as polyamine (agmatine, spermidine, and spermine) (Erdag et al., 2019; Linares et al., 2011; Spano et al., 2010).

1.2 Physiological role

In eukaryotic cells, BAs display essential physiological roles in organisms, and they are the most common active compounds that are precursors for the synthesis of alkaloids (Lauwers et al., 1975), aromatic compounds (Alves et al., 2017; He et al., 2017), hormones (Poljaroen et al., 2011), nucleotides (Ferrendelli, 1984), and proteins (Seitter et al., 2011). Putrescine and spermidine, for example, are needed for critical biological functions like protein synthesis and modulating DNA and RNA, while other BAs play an essential role as neurotransmitters (Gevrekci, 2017; Tabor and Tabor, 1985). In prokaryotic cells, the decarboxylation pathways of free amino acids are activated for a few reasons. One of those is a cellular defence mechanism to restore the external pH as a counter-reaction to an acidic environment. It has been shown that to withstand intracellular acidification, decarboxylation of free amino acids is coupled to an electrogenic amino acid/amine antiporter. Activation of transcription of the decarboxylase genes is induced by a low pH, thereby improving cell performances under acidic conditions (Marcobal et al., 2012; Molenaar et al., 1993; Pereira et al., 2009; Perez et al., 2015; Pessione et al., 2010; Romano et al., 2012). Furthermore, decarboxylation of free amino acids can support primary metabolism under critical environmental conditions by the additional production of ATP. This support

is achieved by generating a proton driving force by transferring a positive net charge outside the cell. This mechanism can be particularly important for microorganisms such as LAB that are lacking a respiratory chain (Figure 1.1) (Hernández-Jover et al., 1997; Konings, 2006; Pereira et al., 2009; Perez et al., 2015; Vido et al., 2004).

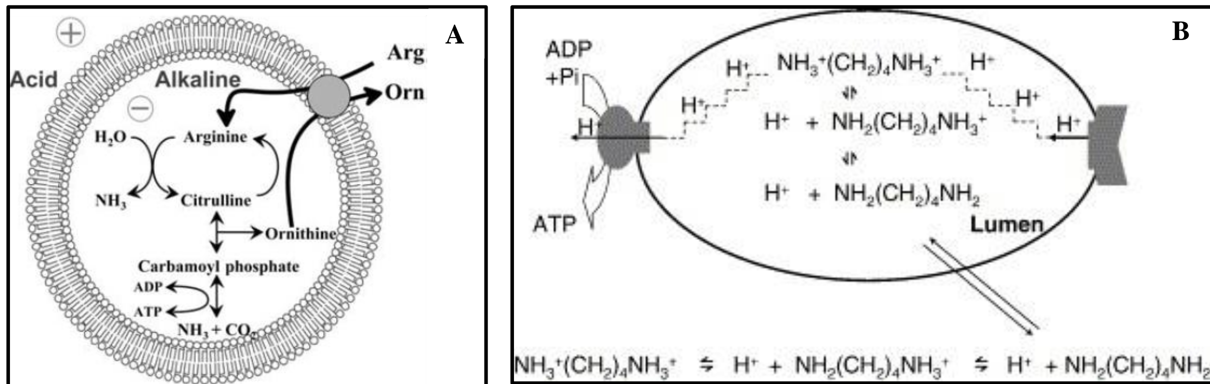

Figure 1.1: Arginine deaminase pathway in lactic acid bacteria (A) and oversimplified pathway of fully, semi- and un-charged putrescine (B). Adapted from Ioannidis et al., (2006); Konings, (2006); Pessione et al., (2010).

2. Production sources of biogenic amines

BAs can be produced naturally by animals, plants, and microorganisms. Currently, there are 11 BAs commonly existing in animals (Farooqui, 2013; Kamhi and Traniello, 2013), plants (Baciak et al., 2016; Farooqui, 2013) and foods (Ladero et al., 2016b; Linares et al., 2011; Naila et al., 2010; Spano et al., 2010; Suzzi and Gardini, 2003). They are namely cadaverine, dopamine, histamine, octopamine, phenylethylamine, putrescine, serotonin, spermidine, spermine, tyramine, and tryptamine, but there are existing many more (Figure 1.2) (Restuccia et al., 2019). In terms of origin or synthesis, spermidine and spermine, and sometimes also cadaverine and putrescine, are classified as polyamines when they are endogenous and formed naturally by animals, plants or microorganisms and as biogenic amines when they are formed exogenously by decarboxylation of free amino acids by means of the action of decarboxylase enzymes, which are mainly of microbial origin (Charalampos, 2019; Ruiz-Capillas and Herrero, 2019).

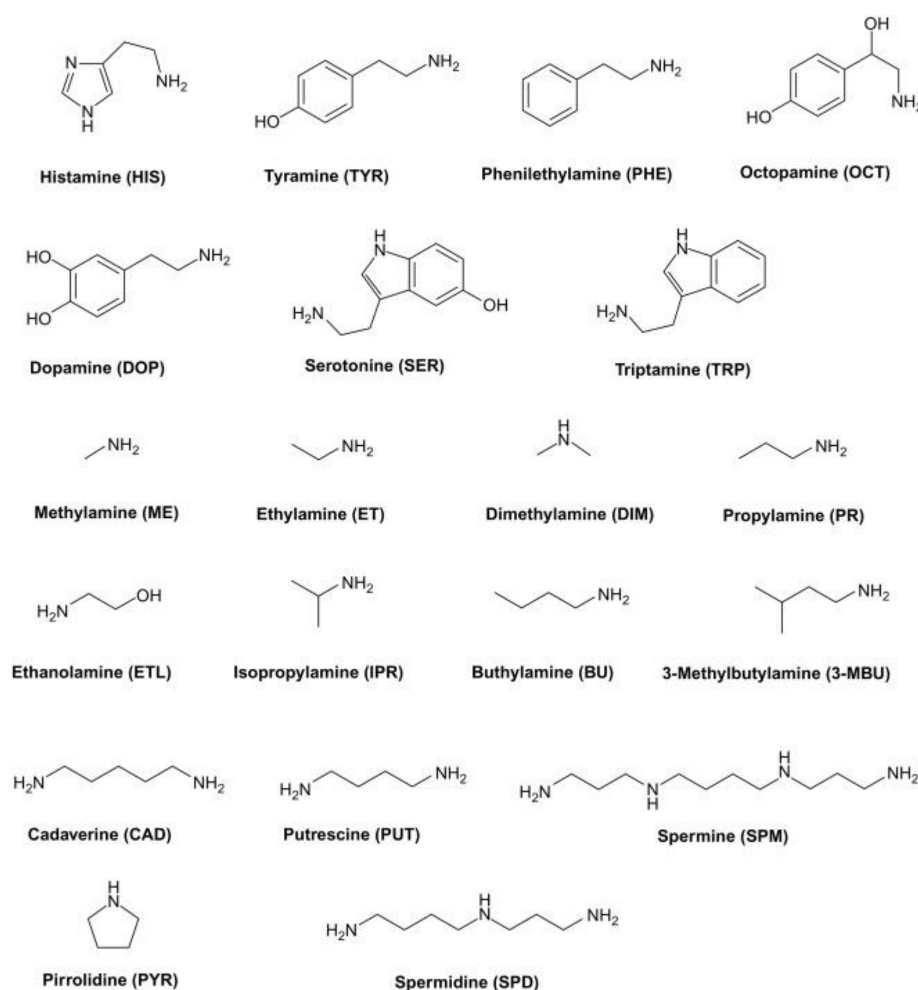

Figure 1.2: Chemical structures, names and abbreviations of different biogenic amines (Restuccia et al., 2019).

2.1 Biogenic amines in animals

Endogenous BAs are vital neuroactive molecules, which play an essential role in physiological functions in the central nervous system of invertebrates and vertebrates and can modulate multiple physiological and behavioural processes, such as aggression, circadian rhythms and locomotion, as well as emotions, in their function as neurohormones, neuromodulators, and neurotransmitters. Furthermore, they are essential in the regulation of gene expression, cell growth and differentiation, gastric secretions, immune response and inflammatory processes (Beaulieu and Gainetdinov, 2011; Farooqui, 2012; Nichols and Nichols, 2008; Ruiz-Capillas and Herrero, 2019). Histamine, serotonin and tyramine, for example, control blood pressure and are involved in nervous system functions, while cadaverine, putrescine, spermidine and spermine are important in the synthesis of DNA, RNA and proteins, and are involved in cell growth, proliferation and signalling (Pereira et al., 2017).

In insects, the stimulation of specific membrane proteins, which leads to subsequent changes of intracellular concentrations of secondary messengers, is mediated via the action of BAs like dopamine, octopamine, serotonin, and tyramine (Blenau and Baumann, 2001).

The BA octopamine, which is a derivate from tyramine, plays a vital role in the regulation and maintenance of energetic homeostasis in honeybees. An increase of appetite corresponds with an increase in octopamine levels in the brain of forage honeybees and lead to a decline in glucose, trehalose and fructose haemolymph sugar level (Mayack et al., 2019). Furthermore, octopamine promotes the flight for foraging behaviour. While young worker bees with a high level of tyramine and a low level of octopamine in the brain tend to stay in the hive, old worker bees with a high level of octopamine and a low level of tyramine go out of the hive to forage. The change of levels of tyramine and octopamine occur during ageing; tyramine levels decrease whereas octopamine levels increase contrary (Fussnecker et al., 2006; Sasaki and Nagao, 2002; Wagener-Hulme et al., 1999).

The BAs dopamine, octopamine, serotonin, and tyramine modulate the behaviours like colony foundation, nestmate recognition, predatory aggression, repertoire expansion, reproductive dominance, sub-caste-related division of labour, temporal polytheism, and worker behavioural development in ants (Kamhi and Traniello, 2013). The levels of BAs, for example, the concentration of serotonin, in the brain

of worker ants, are associated with age-related task performance and regulate task specialisation. The level of serotonin increases with the age of the ant, and therefore, major workers show significant differences compared with minors (Giraldo et al., 2013; Smith et al., 2013).

In humans, the BAs including catecholamines (epinephrine, dopamine and norepinephrine) and indolamine (serotonin) are classical neurotransmitters, which together with their amino acid precursors tyrosine and tryptophan, respectively, and metabolites participate in a signal transmission throughout the brain and body. They play an important role in the regulatory processes related to emotions, cognition and motor activity (Konieczna et al., 2016). Imbalances at the level of BAs are associated with several diseases of the nervous system including neurodegenerative disorders, e.g. Alzheimer's and depression as well as neuroendocrine tumours (Ippolito, 2006; Liu et al., 2011; Marc et al., 2011; Weismann et al., 2015). For example, in Parkinson's disease, reduced levels of both dopamine and its metabolite are observed (Guimarães et al., 2019). Furthermore, enhanced levels of serotonin, its metabolite and two precursors in body fluids have been linked to the neuroendocrine tumours (Yazar et al., 2005).

The endogenous monoamine serotonin, which is widely distributed in both the Central Nervous System and the periphery as well as in non-neuronal tissues such as blood, endocrine and gastrointestinal, is involved in numerous behavioural and physiological disorders, such as autism, anxiety, mania, major depression, modulation of pain, obesity and schizophrenia (Alfaro-Rodríguez et al., 2017). Moreover, a persistent deficit in cortical potassium-stimulated extracellular norepinephrine, dopamine, and serotonin, and reductions in striatal extracellular dopamine function leads to lasting cognitive and fine motor dysfunctions, including attention deficit hyperactivity disorder-like impairments in attention, impulse control, and cognitive flexibility (Bouchard et al., 2007; Lasley et al., 2019).

The synthesis and release of the BA γ-aminobutyric acid (GABA), a classical neurotransmitter, through the immune system is another important factor in humans. GABA has several effects on immune cells and can affect a variety of functional properties of the cells like activation or suppression of cytokine secretion, phagocytic activity and chemotaxis, migration of the cells or even the modification of cell proliferation. The ability to learn novel motor skills is a central part of our daily lives and can provide a model for rehabilitation after a stroke. During motor learning, for example, a significant reduction in

GABA concentration, as well as a strong correlation between the primary motor cortex and GABA and with the degree of subsequent learning, can be observed, such that greater inhibition is associated with poorer subsequent learning. This means that higher levels of cortical inhibition may present a barrier that must be overcome in order to achieve an increase in the primary motor cortex excitability, and hence encoding of a new motor skill (Kolasinski et al., 2019). Moreover, GABA also has an effect in autoimmune diseases like multiple sclerosis, rheumatoid arthritis, and type 1 diabetes and may also modulate the immune response to infections (Jin et al., 2013; Tian et al., 2011b, 2011a). Dysfunction of the type A GABA receptor for example, which is responsible together with its neurotransmitter GABA for fast inhibitory neurotransmission, results in neurological disorders and mental illnesses including epilepsy, anxiety and insomnia (Zhu et al., 2018). In addition, the gut microbiota affects many important host functions, including the immune response and the nervous system and *Bacteroides* spp. for example, can produce large quantities of GABA. In patients with major depressive disorder, a disease associated with an altered GABA-mediated response, the relative abundance levels of faecal *Bacteroides* spp. are negatively correlated with brain signatures associated with depression (Strandwitz et al., 2019).

2.2 Biogenic amines in plants and fungi

Ornithine decarboxylase is a key enzyme in the BA biosynthetic pathway, which is associated by its production of putrescine, an essential type of BA that is highly involved in response to abiotic stress in plants (Choi et al., 2014). The accumulation of putrescine, for example, improve the tolerance to dehydration and freezing stress in *Arabidopsis* plants (Alet et al., 2011). In addition, putrescine plays a role in the protection against osmotic stress and salt in the fungi *Ustilago maydis* (Valdés-Santiago et al., 2010).

Moreover, BAs such as putrescine, spermidine and spermine are associated with regulation of the responses to biotic and abiotic stress, and cellular growth and division and affect fruits in the stimulation of ripening and senescence in angiosperms (Bregoli et al., 2005). While polyamines and bioactive amines correlate with antioxidant activities and histamine and serotonin act as protective agents, dopamine and norepinephrine are related to the enzymatic darkening of fruits (Adão and Glória, 2005; Lima et al., 2008).

To prevent the effects of prolonged chemical stress and the accompanying concentration increases by fertilisers, pesticides, copper, zinc, agricultural and industrial wastes, aquatic and wheat plants produce osmolytes such as antioxidant enzymes, free amino acids, organic acids and soluble sugars, and they accumulate potassium ions (Adomas et al., 2013; Parvaiz Ahmad, 2012; Taie et al., 2019; Yang et al., 2010). BAs and their metabolism subsequently regulate the ion transport, the level of free radicals, and the photosynthetic apparatus and play therefore a significant role in the adaption mechanisms of plants under chemical stress (Baciak et al., 2016).

2.3 Biogenic amines in food

BAs are present in a wide range of food and are mainly exogenously-produced by the presence of microorganisms through decarboxylation of the corresponding amino acids. However, their levels are different depending on the type of food products (Table 1.1). The concentration of BAs is also influenced by the environment and the availability of different microorganisms in food (ten Brink et al., 1990). Low concentrations of BAs exist as endogenous components in fresh food, but the occurrence of BAs in high concentrations can be found primarily in fermented food products such as dairy products, meat and meat products, fish and fish products, fruit and vegetable products like soy sauce and sauerkraut, and alcoholic beverages (Spano et al., 2010). In general, the longer the food production cycle, storage time, trade, processing, and handling, the higher the content of BAs as each phase at each step can contribute to the accumulation of BAs either by technological treatments or spoilage phenomena (Naila et al., 2010). In fermented food products, it is not always clear to which extent BAs are produced by decarboxylase enzymes from the food or by the decarboxylating activity of resident or starter microorganisms (Ekici and Omer, 2018; Vodolazov et al., 2018).

Table 1.1: Content of biogenic amines [mg kg^{-1} or mg L^{-1}] in different food products. Data compiled from Borges et al., (2019); Doeun et al., (2017); Mohedano et al., (2015); Özdestan, (2014); Poveda, (2019).

Products	Biogenic amines [mg kg^{-1} or mg L^{-1}]							
	Cad	His	Phe	Put	Spd	Spm	Trp	Tyr
Dairy products								
Leerdamer cheese	0.01	0.02	0.01	0.07	0.95	0.65	0.04	n.d.
Parmesan cheese	1.98	40.64	0.20	0.83	0.83	0.82	0.07	3.75
Semi-hard Italian cheese	15.56	28.55	9.51	75.87	7.71	4.46	11.85	29.89
Semi-rippened cheese	33.49	24.38	25.75	22.60	24.35	23.40	28.14	32.92
Blue cheese	2101.40	376.60	39.70	257.20	71.60	18.90	128.80	1585.40
Roquefort blue cheese	8.90	9.90	7.70	18.30	4.60	18.10	n.d.	n.d.
Austrian cheese	1268.00	509.00	n.d.	840.00	n.i.	n.i.	161.00	4597.00
Spanish cheese	774.51	337.90	67.86	105.78	n.i.	n.i.	72.10	2519.98
Italian cheese	2127.60	761.40	232.40	986.00	n.i.	n.i.	n.i.	1771.30
Yogurt	0.38	n.d.	0.00	0.03	0.47	0.17	n.d.	n.d.
Fermented meat products								
Soppresata	60.80	21.90	3.40	98.80	40.00	35.50	n.i.	178.00
Salsiccia	6.70	n.d.	n.d.	19.70	18.80	2.80	n.i.	76.70
Salami	6.54	8.54	3.20	61.57	3.11	14.09	1.63	77.14
Turkish Sucuk	199.00	136.00	20.30	364.00	10.70	16.40	82.30	676.00
Saucisson (industrial)	103.00	71.00	4.00	279.00	5.10	91.00	3.90	220.00
Saucisson (traditional)	71.30	15.30	1.30	223.00	4.30	83.70	n.i.	164.30
Meetwurst	6.00	21.00	3.00	77.00	6.00	29.00	18.00	72.00
Belgian sausage	2.50	4.10	0.90	15.10	n.i.	n.i.	n.i.	36.80
Finish sausage	50.00	54.00	13.00	79.00	4.00	31.00	14.00	88.00
Russian sausage	10.00	89.00	11.00	93.00	5.00	33.00	22.00	110.00
Danish sausage	180.00	9.00	2.00	130.00	7.00	37.00	27.00	54.00
Egyptian sausage	19.20	5.25	33.25	38.62	2.30	1.75	12.70	19.25
Greek sausage	689.90	514.50	25.20	505.30	10.23	36.74	49.80	509.90
Fish and fish products								
Pacific mackerel	n.d.	2.70	2.80	9.80	35.20	3.80	n.d.	40.30
Pacific herring	59.50	9.10	9.50	43.90	3.00	3.20	13.30	23.30
Pacific saury	52.02	9.13	0.10	3.65	0.18	0.55	4.26	21.27
Canned tuna	0.07	0.33	0.03	0.35	7.60	11.25	0.02	0.06
Fish sauce	685.50	574.70	n.i.	308.20	9.90	3.70	30.50	117.30
Shrimp paste	80.00	382.00	30.00	40.00	36.00	43.00	67.00	3.70
Fish paste	58.00	263.00	n.d.	12.00	15.00	60.00	70.00	8.80
Fruits and vegetables								
Dessert bananas	n.i.	86.00	n.d.	271.00	181.00	148.00	n.i.	100.00

Products	Biogenic amines [mg kg^{-1} or mg L^{-1}]							
	Cad	His	Phe	Put	Spd	Spm	Trp	Tyr
Cooking bananas	n.i.	74.00	n.d.	314.00	190.00	144.00	n.i.	103.00
Fermented cabbage	21.50	37.01	0.73	108.90	10.98	1.20	0.18	60.66
Fermented cabbage juice	59.40	83.70	50.80	366.00	96.90	n.d	99.60	73.00
Korean Doenjang	323.60	279.50	870.50	429.20	880.40	972.90	280.80	661.60
Korean Natto	36.80	34.40	51.50	43.10	478.10	80.10	40.70	300.20
Chinese Soy sauce	550.00	592.00	n.i.	n.i.	486.00	145.00	n.i.	673.00
South Korean Soy sauce	n.d.	157.00	68.50	52.30	25.00	n.d.	n.d.	172.00
Korean Miso	1.30	24.40	11.70	14.09	28.30	2.80	9.70	66.70
Chinese Sufu	85.80	196.90	36.30	316.90	4.00	6.90	104.10	446.60
Alcoholic beverages								
Turkish Boza	1.50	7.90	5.00	5.60	3.50	4.00	3.30	65.00
Chinese Beer	n.i.	4.62	0.42	8.23	2.55	3.96	1.73	7.15
Portuguese Beer	1.38	0.34	0.15	12.77	n.i.	n.i.	n.i.	5.92
Spanish craft beer	1.30	5.70	0.93	8.90	4.00	n.i.	0.50	6.50
Chinese Rice wine	29.90	22.40	n.i.	58.90	n.i.	n.i.	n.i.	37.60
French Cider	3.00	5.00	n.i.	1.00	n.i.	n.i.	n.i.	14.00
Spanish Cider	34.00	16.00	n.i.	34.00	n.i.	n.i.	n.i.	7.00
Italian Wine	1.11	7.21	1.28	15.99	0.70	n.i.	n.i.	11.94
Spanish Wine	7.00	25.20	7.30	83.20	n.i.	n.i.	n.i.	20.00
South Korean Wine	0.59	2.84	1.17	3.12	0.80	n.i.	0.42	2.54
Chilean Wine	2.45	10.49	2.41	25.99	6.39	2.62	n.i.	7.24
Chinese Wine	13.00	10.45	4.58	19.00	3.82	0.75	n.d.	19.10
Red wine	0.40	3.67	0.61	8.50	2.08	0.07	0.03	1.93
Cab Sauvign. (2009 Maipo)	5.21	8.39	n.d.	19.81	4.21	0.67	n.i.	2.61
Merlot (2009 Maipo)	1.93	6.83	n.d.	10.03	4.60	1.94	n.i.	2.51
Carménère (2009 Maipo)	3.85	9.21	0.17	20.09	5.41	0.99	n.i.	3.82
Rose wine	0.90	0.58	0.16	3.28	0.09	n.d.	n.i.	0.76
White wine	0.79	0.18	n.d.	2.24	n.d.	n.d.	n.i.	0.41
Sauv Blanc (2009 Maipo)	0.62	1.36	0.72	5.87	0.46	0.12	n.i.	n.d.
Chocolate and coffee								
Chocolate	0.75	0.26	2.67	0.80	7.40	1.95	3.30	65.00
Turkish ground coffee	75.08	n.d.	22.82	15.20	n.d.	n.d.	37.85	99.58
Turkish brewed coffee	8.01	n.d.	4.99	1.55	n.d.	n.d.	20.18	19.70

Cad: cadaverine; His: histamine; Phe: phenylethylamine; Put: putrescine; Spd: spermidine; Spm: spermine; Trp: tryptamine; Tyr: tyramine; n.d.: not detected; n.i.: not investigated

In the following sections, 2.3.1–2.3.7 (2. – Biogenic amines in food), single groups of BA containing food products are further described to get an idea on their origin and conditions leading to high contents in food products. The microbiological background is explained in details in further section 4 biogenic amine-producing microorganisms.

2.3.1 Dairy products

Dairy products are essential components of the human diet in developed countries with relatively high consumption (Gerosa and Skoet, 2012). Through the process of milk processing by means of fermentation and the microorganism composition in the milk, dairy products, especially cheese, can accumulate high levels of BAs such as cadaverine, histamine, phenylethylamine, putrescine, tryptamine and tyramine by decarboxylation of free amino acids (Linares et al., 2011).

Milk, Yogurt and Kefir: Fermented milk products are prepared from milk by a specific microbiota and using several additives to induce some changes, e.g. in flavour, taste or texture. Fermented milk products have been developed as a mean of preserving milk against microbial spoilage, and generated products are known throughout the world (Oberman and Libudzisz, 1998). While the concentration of different BAs between 0.086 and 0.18 mg kg^{-1} in fresh milk were described as shallow, yoghurt already showed a slightly higher BA concentration between 0.5 and 13 mg kg^{-1} (Bodmer et al., 1999; Novella-Rodríguez et al., 2004, 2000). These concentrations imply that microorganisms involved in the fermentation process might produce BAs. Similarly, an increase of concentration of BAs compared to milk can be observed in kefir, a fermented milk product, often consumed in Middle Asian countries, Russia and Caucasia (Özdestan and Üren, 2010). Lactic acid bacteria (LAB) are among the most important bacteria involved in the dairy industry and non-starter LAB as well as starter LAB, which have been proposed as bioprotective cultures in the food industry, are able to produce BAs (Gardini et al., 2012; Ladero et al., 2015).

Cheese: Cheese represents a significant part of the world's food, and there is an extensive list of cheese varieties; however, the production is mostly based on comparable technologies. The main classification of cheeses relies on milk species, milk treatment and cheese moisture content. Cheese manufacturing is one of the major industries worldwide to enhance shelf life from milk. In cheese manufacturing milk protein (casein) and fat are concentrated, and the milk sugar (lactose) is fermented into lactic acid by acidification, dehydration and sodium chloride addition (Kindstedt, 2018; Stanley, 1998). The process of cheese making provides ideal conditions for the formation of BAs by microorganisms. During cheese ripening, proteolysis is a principal activity, which covers a series of biochemical events like breakdown of casein to peptides and free amino acids (Ardö et al., 2017). The proteolysis of the milk casein ensures the availability of free amino acids as substrates for decarboxylation by microorganisms. These microorganisms are already actively present during the manufacturing of cheeses as either part of the starter cultures or introduced by contamination before, during or after milk processing (Bover-Cid et al., 2001; Linares et al., 2012; Torriani et al., 2016). Therefore, the main BAs so far prominent in cheese, namely histamine, phenylethylamine, putrescine and tyramine, can reach concentrations up to 2000 mg kg^{-1} in Italian Pecorino cheeses for example (Fernández et al., 2007a; Mohedano et al., 2015).

2.3.2 Fermented meat products

Like dairy products, meat and meat products are an important component of the diet in developed countries. Meat fermentation has a long tradition to enhance the shelf life of meat. Fermented meat products are made from comminute meat and fat, mixed with sodium chloride, curing agents, spices and sugars and using a specific microbiota and filling into the casings. After fermentation, the meat products possess microbial stability and desired and organoleptic properties (Lücke, 1998). Fully ripened, they represent one of the foods in which significant concentrations of BAs in total up to 1000 mg kg^{-1} can be found (Papavergou et al., 2012), as a consequence of the use of poor quality raw materials, starter cultures or contaminants equipped with decarboxylating activities, sometimes paired with inappropriate conditions during processing and storage (Bover-Cid et al., 2006, 2000; Latorre-Moratalla et al., 2010; Mohedano et al., 2015; Roig-Sagués et al., 1999; Stadnik and Dolatowski, 2010; Suzzi and Gardini,

2003). Fermented meat products constitute the considerably higher concentration of BAs compare to fresh meat because of the increase of the nonprotein nitrogen fraction, e.g. free amino acids, the main precursor of BAs, during fermentation (Suzzi and Gardini, 2003). While cadaverine, histamine, putrescine and tyramine are the most prevalent BAs in fermented meat products, spermidine and spermine are the main BAs in fresh meat (Hernández-Jover et al., 1997; Jairath et al., 2015; Ruiz-Capillas and Jiménez-Colmenero, 2004).

2.3.3 Seafood and seafood products

Seafood is a specific type of food, which may contain several biological, chemical and physical hazards, such as pathogenic bacteria and viruses, biotoxins, metal inclusion and different BAs (Visciano et al., 2012). Cadaverine, putrescine, tyramine and especially histamine are the most common BAs in seafood (Lehane and Olley, 2000). Fishes, of the Scombridae family, are rich in free histidine, a precursor of histamine, in their muscles and histidine can be decarboxylated to histamine by decarboxylating bacteria any time after harvesting by improper handling mainly by storage duration and temperature (Auerswald et al., 2006; Kang et al., 2019; Prester, 2011). In mackerel and tuna, for example, histamine can reach very high levels of 3420 mg kg^{-1} and 4500 mg kg^{-1}, respectively by improper storage at ambient temperatures (Du et al., 2002; Kim et al., 2002). An abundant concentration of histamine has also been detected in Asian fish sauce, a liquid manufactured by spontaneous fermentation of various fishes, following by the addition of sodium chloride. Therein, high levels of histamine up to 700 mg kg^{-1} (Stute et al., 2002), but also high concentrations of tyramine, putrescine and cadaverine assisting the fermentation process. These can be explained because of the insoluble fish protein, which can be converted into a soluble form during fermentation, caused simultaneously by enzymes from fish and from microorganisms (Yongsawatdigul et al., 2004). This leads to two main products, namely free amino acids and polypeptides (Beddows, 1998). Furthermore, because of the reasons mentioned above, significant concentrations of BAs can also be found in products such as dried fish (Huang et al., 2010), fish paste (Naila et al., 2011) and shrimp paste (Tsai et al., 2006).

2.3.4 Fruits and vegetables

The dietary intake of fresh fruits, vegetables and their products like juices and sauces represent the dietary habits, lifestyle and eating culture of each country and the harvesting process, storage conditions and the processing part of the fruits and vegetables varies between different countries. Fresh fruits and vegetables contain BAs as endogenous compounds, and due to uncontrolled microbial enzymatic activity, they can be accumulated after harvesting and reach concentrations up to 300 mg kg^{-1} (Borges et al., 2019). The most frequently detected BAs in fruits and vegetables are cadaverine, histamine, putrescine, spermidine, spermine and tyramine. Fruits and fruit juice in which BAs have been detected are apples, apple juice, apricot, banana, black currant juice, cherry juice, grapefruit juice, grapes (red and white), litchi, mango, olives, orange, peach, pear, pineapples and pineapple juice, pomelo and red currant juice (Jastrzebska et al., 2015; Palermo et al., 2013). Preservation of vegetables by fermentation is a traditional technique since ancient times. Regarding the vegetables, sauerkraut, a product made with shredded white cabbage and followed by fermentation, can contain a high concentration of BAs up to 110 mg kg^{-1} (Mayr and Schieberle, 2012), which are generated in the fermentation process (Majcherczyk and Surówka, 2019).

Soybean products are a heterogeneous group of food products including Doenjang, Miso, Natto, soya bean, soya sauce, soybean paste, soymilk, soy sprouts, tamari, and tofu, all mainly consumed in Asia, but also in America and Europe (Papageorgiou et al., 2018a). As a result of fermentation, soybean products such as Doenjang, Miso, Natto, soya sauce tamari, can contain concentrations of BA’s up to 900 mg kg^{-1} (Mohedano et al., 2015); however, the content of BAs varies in different fermented soybean products. In Sufu, the most dominant BA is putrescine, followed by histamine and tryptamine (Guan et al., 2013) and Doenjang showed a BA profile with the dominant BA of spermine, followed by spermidine and phenylethylamine (Kim et al., 2011; Shukla et al., 2010), while in Miso and Natto, spermidine is the most dominant BA (Stratta and Badino, 2012). In different products of soy sauce, histamine is a dominant BA (Kirschbaum et al., 2000; Yongmei et al., 2009).

2.3.5 Alcoholic beverages

Alcoholic beverage is a main term, including beers, brandy, ciders, gin, liqueurs, rum, whiskey, and wine. Alcoholic beverages, which contains different BAs, may cause adverse health effects, despite containing health-promoting components such as high contents of phenolic compounds, occasionally present in wine (Preti et al., 2016). Until the date of the use of pure cultures for fermentation, alcoholic fermentation was natural or spontaneous evens that originated from the microbiota associated with the raw materials. However, despite the use of pure culture, natural microbiota still plays an important role and can promote the formation of BAs during fermentation (Fleet, 1998). The most common and most crucial BAs found in alcoholic beverages are cadaverine, histamine, putrescine, and tyramine. The presence of BAs in alcoholic beverages may indicate a deterioration of beverages through defective manufacture and undesired microbial activity (Gustaw and Wasko, 2017).

Beer: Beer is a low alcoholic beverage and is produced mainly by mixing hops, malted barley and selected yeast strains followed by the fermentation process. In beer, two categories of BAs can be found. On the one hand, they are endogenous BAs who are natural constitutes from either malt or hop such as agmatine, phenylethylamine, putrescine, spermidine and spermine and on the other hand, they are exogenous BAs linked to microbial contamination during brewing such as cadaverine, histamine and tyramine (Papageorgiou et al., 2018b). The level of BAs varies in different beers and can reach a concentration up to 34 mg L^{-1} (Gustaw and Wasko, 2017).

Boza: Boza is a traditional fermented beverage in Balkan countries, made from millet maize, wheat or rice, and it is often available as an unpasteurised cereal-based fermented beverage. BAs which were found in Boza are putrescine, spermidine and tyramine while putrescine and spermidine are natural endogenous BAs and tyramine a product from microbial decarboxylation in a concentration up to 65 mg L^{-1} (Yeğin and Üren, 2008). LAB and yeast, which can be found at high concentrations and diversity (Osimani et al., 2015) might be the primary suspicious BA producers.

Rice wine: Chinese rice wine is a traditional and most popular fermented alcoholic beverage made from cereals by the use of glutinous rice as a starchy substrate and wheat Qu as a source of amylolytic and proteolytic enzymes. The brewing is to some extent a spontaneous microbiological process for, which

indigenous bacteria are responsible and, which produce putrescine as a major BA in concentrations up to around 60 mg L^{-1} (Zhong et al., 2012).

Ciders: Cider, is a popular fermented alcoholic beverage in different countries all over the world and is made by fermenting pressed apple juice or crushed apples and sometimes also mixed with pears. Both alcoholic and malolactic fermentation occurs spontaneously with the participation of indigenous yeasts and LAB of the musts and is not microbiologically stabilised before bottling. The unstable microbiota and the fermenting process can lead to the formation of the essential BAs cadaverine, histamine, phenylethylamine, putrescine and tyramine up to a level of 34 mg L^{-1} in cider (Garai et al., 2006; Gustaw and Wasko, 2017).

Wine: Wine is an alcoholic beverage produced by the alcoholic fermentation of grapes primarily by means of yeasts. The main biogenic amines, which were reported in wine, are histamine, putrescine and tyramine, but there are more than 15 BAs that have been identified in wine, and their total concentration has been reported from a few mg L^{-1} up to 50 mg L^{-1} (Maintz and Novak, 2007; Papageorgiou et al., 2018a). Several factors are influencing the type of BAs, e.g. differences in the wine-making process, fermentation time and storage conditions, raw material quality, agricultural practise and climate conditions. Moreover, grapes themselves already contain the endogenous polyamines histamine and tyramine but also polyamines and volatile amines (Costantini et al., 2019; Gustaw and Wasko, 2017). Compared to red wine, white wine contains a lower concentration of BAs because malolactic fermentation driven by LAB does not occur in the fermentation process and therefore microorganisms involved in that are suspected to be BA producers (Henríquez-Aedo et al., 2012; Smit and du Toit, 2013).

2.3.6 Vinegar

Vinegar is typically produced by a two-stage fermentation process ending with an approximately 5% solution of acetic acid. In the first step, yeasts converting sugar into ethanol and in the second step, acetic acid bacteria oxidase the ethanol to acetic acid (De Roos and De Vuyst, 2018). The fermentation can occur spontaneously, while yeasts perform the alcoholic fermentation and the acetic acid bacteria carry out the acetic acid fermentation, also called oxidative fermentation (Pinho et al., 2001). Both Yeasts and acetic acid bacteria are mostly part of the plant products as natural microbiota (Adams, 1998). However, the vinegar production process in the first step is not well controllable, and therefore, undefined microorganisms can proliferate and produce BAs. At least, acetic acid bacteria involved in the second step were so far not reported as BA producers when tested *in vitro* (José M. Landete et al., 2007). However, in a survey of 26 Spanish vinegar, putrescine could be detected in most of them, and the total content of BAs was never below 24 mg L^{-1} and exceeded in five samples more than 500 mg L^{-1}. Tyramine was detected in only one sample at a remarkable value of 216 $mg^{-1,}$ but overall the concentration of BAs are much lower than in vine (Ordóñez et al., 2013).

2.3.7 Chocolate, Coffee and Tea

Although from quite different types of plants, these three commodities have several things in common such as the presence of caffeine and similar compounds as well as the development of the flavours, which starts immediately after harvesting all along with the processing due to the action of endogenous enzymes on carbohydrates, polyphenols and proteins (Cevallos-Cevallos et al., 2018; Fowler et al., 1998).

Chocolate: Chocolate products are the most popular snacks and desserts around the world. They are made from fermented cocoa beans from the *Theobroma* trees, and the main ingredients for all milk chocolate types are cocoa butter, cocoa liquor, milk powder and sugar (ICCO, 2016; Konstantas et al., 2018). During fermentation of the cocoa beans, the main ingredients of chocolate, BAs can be produced and in the final product, spermidine spermine and tyramine can be detected in a concentration up to 7.4 mg kg^{-1} as the most abundant BAs. Since the endogenous BAs spermidine and spermine are involved

in cell growth, renewal and metabolism, their presence in the cocoa beans can be expected. However, although cocoa beans undergo a quite long spontaneous fermentation process, driven mainly by yeast, LAB and acetic acid bacteria (De Roos and De Vuyst, 2018) the concentration of BAs are low compared to other fermented food products (Mayr and Schieberle, 2012).

Coffee: Coffee is one of the most common brewed beverages across the world produced by the spontaneous fermentation of *Coffea* beans and its subsequent roasting of coffee cherries. Coffee consists, by its endogenous substances and by the fermentation process, of caffeine, carbohydrates, chlorogenic acid, fibres, free amino acids, lipids, minerals, organic acids, and trigonelline (Papageorgiou et al., 2018a; Restuccia et al., 2015). During ontogeny of *Coffea*, the endogenous BAs putrescine, spermidine and spermine are predominant, and their concentration continuously increases during fruit development (Restuccia et al., 2019). Due to the fermentation process of the coffee cherries, BAs can accumulate, but with the roasting process, the content of BAs decrease again (Amorim et al., 1977; Casal et al., 2004). Cadaverine, putrescine, serotonin and tyramine are the main BAs occurs in coffee and can reach a level of 107 mg kg^{-1} at which the level of BAs in brewed coffee is lower than in ground coffee (Özdestan, 2014).

Tea: Tea is available and consumed worldwide in a large concentration. The primary tea production is black tea, but there are also other types such as white tea, green tea, red tea and oolong tea, for example. Black and red tea are produced by fermentation and drying of *Camellia* spp. leaves whereby the main differences among the types result from differences in the degree of fermentation (Fowler et al., 1998; Restuccia et al., 2019). In plants, putrescine, spermidine and spermine act as growth factors, and therefore it is not surprising that in dried tea leaves, these three endogenous BAs can be detected. In addition, cadaverine, ethylamine, methylamine, tryptamine and tyramine can be detected up to a total concentration of 3300 mg kg^{-1} (Restuccia et al., 2019; Zhang et al., 2014). In tea infusions and beverages, the sum of all these BAs, as well as serotonin, can be found up to a concentration of 431 mg L^{-1} (Brückner et al., 2012), with the following trend regarding BA concentration organic ≤ instant ≤ decaffeinated ≤ black ≤ conventional teas (Restuccia et al., 2019).

3. Biochemistry, biosynthesis pathway, enzymes and transporters of biogenic amines in fermented foods

The biosynthesis of BAs depends on the activity of at least two key enzymes. First, the biosynthesis contains a decarboxylase enzyme that catalyses the decarboxylation of the free amino acid as a substrate to the corresponding BA and secondly, a transporter that is responsible for the translocation of free amino acids into the cell and the excretion of the generated BA from it (Figure 1.3). The synthesis of BAs occurs in one or two steps, and usually, the two enzymes are encoded by two contiguous genes (Linares et al., 2011; Marcobal et al., 2012).

Figure 1.3: Biosynthesis pathway of the four BAs cadaverine, histamine, putrescine and tyramine via the enzymes lysine decarboxylase (CadA), histidine decarboxylase (HdcA), arginine decarboxylase (ADC), agmatine deiminase (AGDI), arginine deaminase (ADI), ornithine decarboxylase (ODC), carbamate kinase (CK), putrescine carbamoyltransferase (PTC) and tyrosine decarboxylase (TdcA). Adapted from D. M. Linares et al., (2011).

Cadaverine: Cadaverine is often found in fermented food products such as wine, cheese, cider, fermented sausages as well as fish and fish products and are mostly associated with Gram-negative bacteria such as *Escherichia coli*, *Salmonella enterica* and *Vibrio vulnificus* where cadaverine play a role in acid stress resistance (Geornaras et al., 1995; Haneburger et al., 2012; Kang et al., 2007). In Gram-negative bacteria, the cadaverine biosynthesis occurs via lysine decarboxylase (CadA; EC 4.1.1.18) converting lysine into cadaverine, and CO_2 (Figure 1.3, Figure 1.4) and the system consists in addition to CadA a lysine-cadaverine antiporter CadB (Ladero et al., 2016b; Mohedano et al., 2015). To date, in Gram-positive bacteria, lysine decarboxylase activity has been poorly characterised.

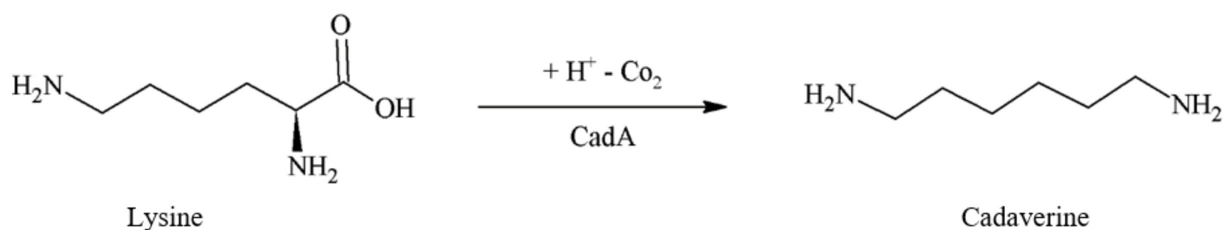

Figure 1.4: Cadaverine biosynthesis by the action of the lysine decarboxylase (CadA) by decarboxylation of lysine. Hydrogen (H^+) proton is consumed, and a carbon dioxide (CO^2) is produced. Adapted from V. Ladero et al., (2016).

Histamine: Histamine is one of the major BA that occurs in fermented food products. The production of histamine occurs via histidine decarboxylase (HdcA; EC 4.1.1.22) in bacteria and eukaryotes (Figure 1.3, Figure 1.5). There exist two classes of histidine decarboxylases, the one, which is produced by Gram-positive bacteria, needs a covalently bound pyruvoyl moiety as the prosthetic group, and the other one produced by eukaryotic cells and by Gram-negative bacteria requires pyridoxal phosphate as a cofactor (van Poelje and Snell, 1990). In addition, a histidine/histamine antiporter (HdcP) is needed to drive the exchange between histidine into the cell and histamine out of the cell (Cruz Martín et al., 2005; Trip et al., 2012).

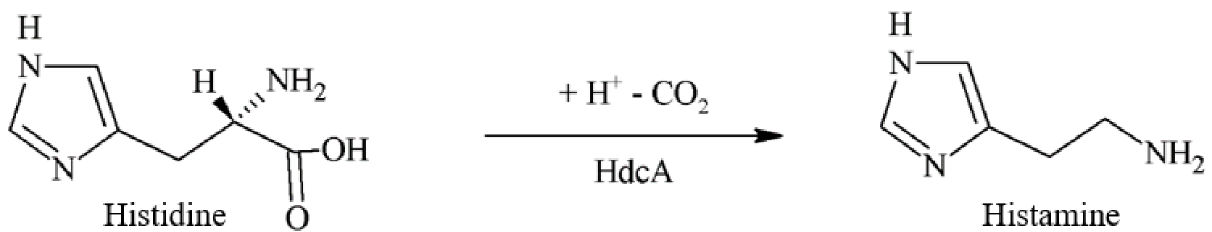

Figure 1.5: Histamine biosynthesis by the action of the histidine decarboxylase (HdcA) by decarboxylation of histamine. Hydrogen (H^+) proton is consumed, and a carbon dioxide (CO^2) is produced. Adapted from V. Ladero et al., (2016).

Putrescine: Putrescine is a common BA found in fermented food products, which can be produced by two different pathways via decarboxylation or deamination. One pathway is the ornithine decarboxylation pathway where arginine is first deaminated to form ornithine, which is afterwards decarboxylated by ornithine decarboxylase (ODC; EC 4.1.1.17) to form putrescine. The second pathway is the agmatine deamination (AGDI) pathway where arginine is first decarboxylated by arginine decarboxylase (ADC; EC 4.1.1.19) to generate agmatine followed by its deamination to putrescine (Figure 1.3, Figure 1.6) (Linares et al., 2013). The ornithine decarboxylase pathway is common in Gram-negative bacteria such as *Enterobacteriaceae* and *Pseudomonas* spp. or LAB deriving from wine, while the agmatine deaminase pathway is common in LAB found in fermented food products others than wine (M. Coton et al., 2010; Romano et al., 2014, 2012). In the agmatine deaminase pathway, four proteins are needed namely a deaminase enzyme (AgDI), an agmatine/putrescine antiporter (AgmP), a putrescine carbamoyltransferase (PTC), as well as a carbamate kinase (CK) (Barbieri et al., 2019; Linares et al., 2013).

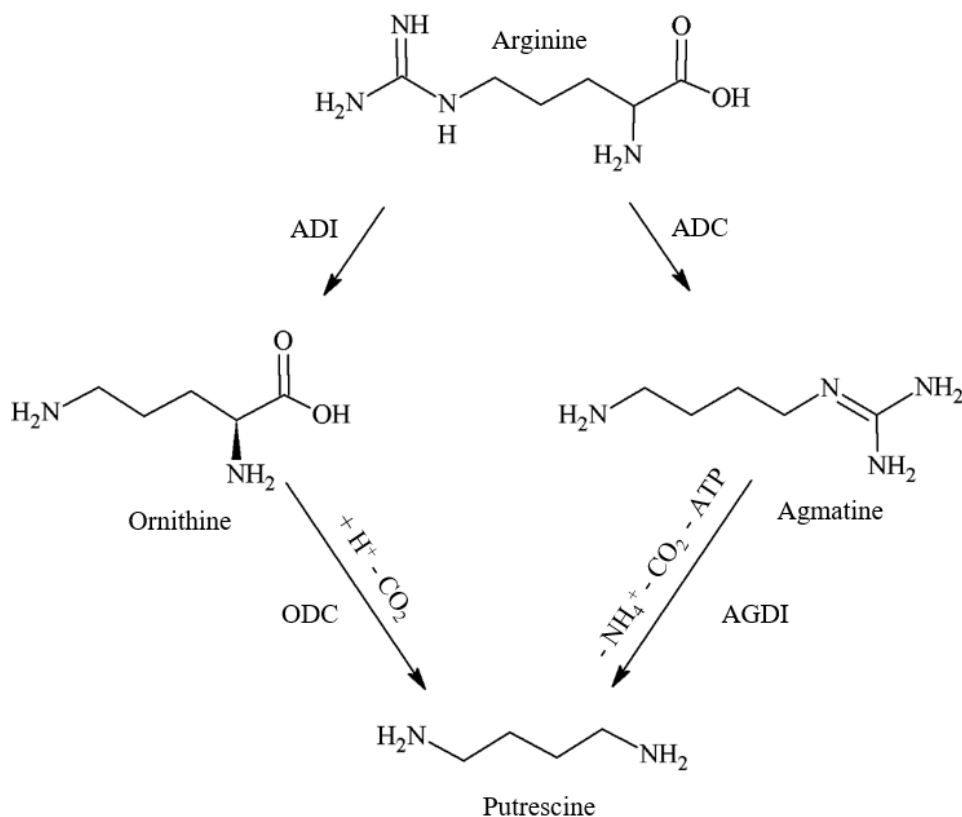

Figure 1.6: Putrescine biosynthesis via two different pathways – the ornithine decarboxylase (ODC) by the production of carbon dioxide (CO^2) and consummation of a hydrogen proton (H^+) and the agmatine deaminase (AGDI) pathway to form putrescine by the production of a carbon dioxide (CO^2), two ammonium ions (NH_4^+) and one adenosine triphosphate (ATP). Adapted from V. Ladero et al., (2016).

Tyramine: Tyramine is one of the most abundant BA in fermented foods and is produces by decarboxylation of tyrosine via the action of tyrosine decarboxylase (TdcA; EC 4.1.1.25) (Figure 1.3, Figure 1.7). TdcA together with the antiporter (TyrP), which encodes the tyrosine-tyramine antiporter, are the two key enzymes to enrich tyramine concentration (Coton et al., 2011; Linares et al., 2016, 2011; Marcobal et al., 2012). It is known, that tyrosine decarboxylase in different bacterial strains, can also act on the phenylalanine to generate 2-phenylethylamine, but at much lower efficacy compared to tyramine, and it is accumulated when tyrosine is almost depleted (Barbieri et al., 2019).

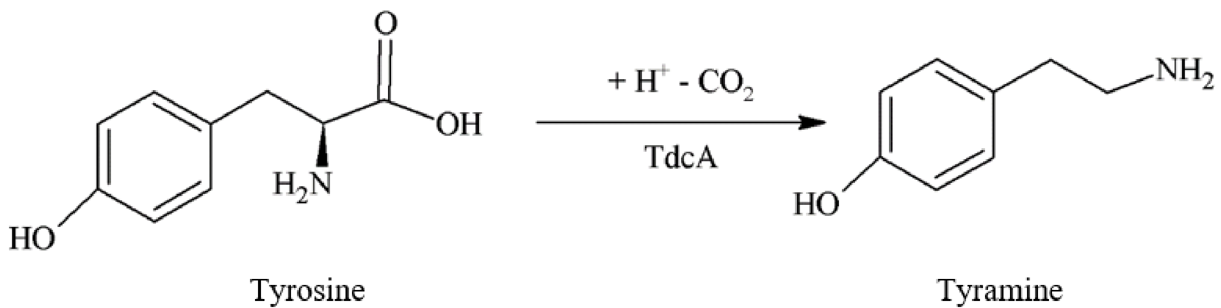

Figure 1.7: Tyramine biosynthesis by the action of the tyrosine decarboxylase (TdcA) by decarboxylation of tyramine. Hydrogen (H^+) proton is consumed, and a carbon dioxide (CO^2) is produced. Adapted from (Ladero et al., 2016b).

4. Biogenic amine-producing microorganisms

Fermented foods pose the risk of contamination with biogenic amines. Several microorganisms are able to produce BAs (Table 1.2) and in addition yeasts and moulds are also capable of producing BAs but in fewer concentrations (Coton et al., 2012; Gardini et al., 2006; Kang et al., 2019; José M. Landete et al., 2007; Linares et al., 2011; Qi et al., 2014). Although, most of LAB are "Generally Regarded As Safe" (GRAS) recognised by the US Food and Drug Administration (FDA) or have "Qualified Presumption of Safety" (QPS) status recognised by the European Food Safety Authority (EFSA), LAB are considered as main responsible bacteria for BA production in fermented foods. In contrast, the production of diamines is usually attributed to Gram-negative spoilage bacteria belonging to *Enterobacteriaceae* and *Pseudomonas* spp. (Linares et al., 2011; Spano et al., 2010; Suzzi and Gardini, 2003).

Table 1.2: Biogenic amines, precursors, decarboxylase enzymes and their producers in fermented food products. Data compiled from Barbieri et al., (2019); Benkerroum, (2016); Buňková et al., (2010a); Comas-Basté et al., (2019); Pircher et al., (2007).

Biogenic amine	**Amino acid precursor**	**Decarboxylase enzyme or pathway in bacteria**	**Biogenic amine-producing bacteria**
Cadaverine	Lysine	Lysine decarboxylase (PotE) (CadA)	**Gram-positive bacteria**: *Enterococcus faecium, E. faecalis, Lactobacillus brevis, Lb. casei, Lb. curvatus, Lb. paracasei, Leuconostoc mesenteroides, Pediococcus* spp., *Staphylococcus carnosus, St. xylosus, Streptococcus thermophilus, Tetragenococcus halophilus* **Gram-negative bacteria**: *Aeromonas* sp., *Enterobacteriaceae, Pseudomonaceae, Salmonella enterica, Vibrio vulnificus*
Histamine	Histidine	Histidine decarboxylase (HdcA)	**Gram-positive bacteria**: *Clostridium* sp., *E. faecium, E. faecalis, Lb. bavaricus, Lb. brevis, Lb. buchneri, Lb. casei, Lb. curvatus, Lb. helveticus, Lb. hilgardii, Lb. mali, Lb. parabuchneri, Lb. paracasei, Lb. paracollinoides, Lb. plantarum, Lb. reuteri, Lb. rhamnosus, Lb. rossiae, Lb. sakei, Lb. vaginalis, Leuc. mesenteroides, Oenococcus oeni, Pediococcus parvulus, P. damnosus, S. thermophilus, Tetragenococcus* spp., *Weissella cibaria, W. confusa, W. paramesenteroides* **Gram-negative bacteria**: *Aeromonas* spp., *Citobacter freundii, Enterobacter cloacae, Ent. aerogenes, E. coli, Hafnia alvei, Klebsiella pneumonia, Klebsiella oxycata, Lb. sakei, Morganella morganii, M. psychrotolerans, Proteus vulgaris, P. mirabilis, Pseudomonas fluorescens, Ps. putida, Pleisomonas shigelloides, Photobacterium phosphoreum, Phot. psychrotolerans, Serratia fonticola, Ser. liquefaciens*
Putrescine	Arginine	Ornithine decarboxylase (ODC)	**Gram-positive bacteria**: *E. casseliflavus, E. durans, E. faecalis, E. faecium, E. hirae, Lb. acidophilus, Lb. brevis, Lb. collinoides, Lb. sakei, Lb. curvatus, Lb. buchneri, Lb. plantarum, Lb. paracasei, Lb. mali, Lb. rhamnosus, Lb. rossiae, Lb. homohiochii, Lc. lactis, O. oeni, P. parvolus, S. thermophilus, S. mutans, T. halophilus* **Gram-negative bacteria**: *Enterobacteriaceae, Pseudomonaceae*
	Arginine	Agmatine deaminase (AGDI)	**Gram-positive bacteria**: *Carnobacterium divergens, C. maltaromaticum E. durans, E. faecalis, E. faecium, E. hirae, E. mundtii, Lb. brevis, Lb. curvatus, Lb. plantarum, Lc. Lactis, Leuc. mesenteroides, St. carnosus, St. xylosus S. mutans, S. thermophilus, O. oeni, P. parvulus, P. pentosaceus, W. halotolerans*
Tyramine	Tyrosine	Tyrosine decarboxylase (TdcA)	**Gram-positive bacteria**: *C. divergens, C. maltaromaticum, E. casseliflavus, E. durans, E. faecalis, E. faecium, E. hirae, E. mundtii, Lb. acidophilus, Lb. alimentarius, Lb. bavaricus, Lb. bifermentans, Lb. brevis, Lb. buchneri, Lb. bulgaricus, Lb. casei, Lb. curvatus, Lb. delbrueckii* subsp. *bulgaricus, Lb. divergens, Lb. hilgardii, Lb. homohiochii, Lb. johnsoni, Lb. plantarum, Lb. paracasei, Lb. reuteri, Lb. sakei, Lc. lactis, Leuc. mesenteroides, St. carnosus, St. xylosus, S. thermophilus, S. macedonicus, Sporolactobacillus* sp., *W. cibaria, W. confusa, W. paramesenteroides, W. viridescens, T. halophilus,* **Gram-negative bacteria**: *Cit. braakii, Cit. freundii, Ent. gergoviae, E. coli, Hafnia alvei, Pseudomonas putida, Raoultella ornithinolytica, Ser. liquefaciens*

4.1 Main biogenic amine-producing bacteria in fermented foods

Although many microorganisms found in fermented foods can produce BAs, some distinct bacterial groups are the main responsible for their high concentrations. These bacteria include the Gram-positive LAB enterococci, lactobacilli and lactococci, as well as staphylococci and the Gram-negative group of *Enterobacteriaceae* (Bover-Cid and Holzapfel, 1999; Durlu-Özkaya et al., 2001; Linares et al., 2011; Maifreni et al., 2013; Martuscelli et al., 2000; Özogul and Hamed, 2018; Rahmdel et al., 2018; Zhang et al., 2018). Decarboxylation of BAs occurs for several physiological reasons. The decarboxylation system is coupled with an electrogenic antiporter and decarboxylation of amino acids protons are consumed in the cytosol. These protons are incorporated in BAs and secreted with them, which helps to maintain the internal pH (Fernández et al., 2007b; Gardini et al., 2001; Lund et al., 2014; Schelp et al., 2001; Van de Guchte et al., 2002). Moreover, the primary metabolism in harsh environmental conditions can be supported by means of the transfer of the net positive charge outside the cell because it leads to cell membrane energisation and brings supplementary energy (Konings, 2006; Pereira et al., 2009; Perez et al., 2015; Soksawatmaekhin et al., 2004).

***Enterobacteriaceae*:** The family of *Enterobacteriaceae* contains over 60 genera and more than 250 species of Gram-negative, facultatively anaerobic, none-spore forming rod-shaped bacteria that includes harmless and pathogenic organisms (Adeolu et al., 2016; Munson and Carroll, 2019). Some of these bacteria, when present at elevated titers, such as *Citrobacter* spp. *Enterobacter* spp., *Escherichia coli*. *Hafnia* spp., *Klebsiella* spp., *Morganella* spp., *Proteus* spp., and *Serratia* spp. are identified as common contaminants in fermented food products and can synthesise detectable concentrations of cadaverine, histamine or putrescine (Durlu-Özkaya et al., 2001; Maifreni et al., 2013; Marino et al., 2000; Zhang et al., 2018). In cheese and fermented meat products, *Enterobacteriaceae* species such as *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter cloaceae*, *Hafnia alvei*, *Klebsiella* spp. *Proteus* spp., and *Serratia* spp. can produce cadaverine and putrescine and both species *Hafnia alvei* and *Serratia liquefaciens* or even able to produce small concentrations of histamine (Marino et al., 2000; Pircher et al., 2007; Wunderlichová et al., 2014). In fish and fish products, histamine occurs mainly due to the growth of naturally occurring *Enterobacteriaceae* such as *Enterobacter aerogens*, *Morganella morganii*, *Raoultella planticola*, *Raoultella ornithinolytica* and *Photobacterium damselae* and can reach

a high concentration (Björnsdóttir-Butler et al., 2010; Kang et al., 2019; Kristin et al., 2009; Visciano et al., 2012).

Enterococci: The genus of enterococci has neither a GRAS nor QPS status because most of the species frequently harbour a series of antibiotic resistance and virulence factors, and they are associated with several infections. Due to their adaptability and ability to grow in harsh conditions such as high sodium chloride content and low pH, they are often involved in fermentation processes of traditional cheeses and fermented sausages (Foulquié Moreno et al., 2006). Nevertheless, the occurrence of BAs in fermented foods is often associated with the presence of enterococci. Usually, enterococci are not part of supplied industrial starter cultures but may be introduced into the food by contamination before, during or after processing (Bover-Cid et al., 2001; Giraffa, 2003; Linares et al., 2012; Torriani et al., 2016). In dairy products, especially in cheese, the most common *Enterococcus* spp. are *E. faecalis*, *E. faecium* and *E. durans* and were mostly identified as tyramine and sometimes also as putrescine producers (Bonetta et al., 2016; Fernández et al., 2007a; Galgano et al., 2001; Ladero et al., 2012a; Linares et al., 2011). In meat, the presence of enterococci and their ability to produce BAs is a relevant food-related-issue, despite their role recognized in the development of sensory properties in fermented sausages (Bover-Cid et al., 2001; Coloretti et al., 2008; Hugas et al., 2003). As part of the natural microbiota of raw meat and many fermented meat products, *E. durans*, *E. faecium* and *E. faecalis* are the predominant species that can accumulate high levels of BAs, especially cadaverine, putrescine and tyramine (Garriga and Aymerich, 2014; Landeta et al., 2013b; Latorre-Moratalla et al., 2017; Suzzi and Gardini, 2003; Zgomba Maksimovic et al., 2018). In wine, *E. faecium* is, together with some *Lactobacillus* spp., the main responsible bacterium for tyramine accumulation and in tofu and other soybean food, *E. faecium* and *E. faecalis* has been recognised as responsible tyramine producers (Capozzi et al., 2011; M. Coton et al., 2010; Jeon et al., 2018; Pérez-Martín et al., 2014; Takebe et al., 2016).

Lactobacilli: In fermented food products, different *Lactobacillus* spp. are reported to be strong BA producers (Spano et al., 2010). In cheese, the non-starter lactic acid bacteria (NSLAB) strains *Lb. brevis*, *Lb. curvatus* and *Lb. paracasei* were identified as tyramine producers, and in yoghurt, the ability to produce tyramine by a *Lb. plantarum* strain was demonstrated. Furthermore, it has been reported that *Lb. brevis* and *Lb. curvatus* are even able to produce putrescine and tyramine (Buňková et al., 2010b; Komprda et al., 2008; Ladero et al., 2015; Pachlová et al., 2018; Yılmaz and Gökmen, 2017). Although Gram-negative bacteria are often linked to histamine production in cheese, *Lactobacillus* spp. such as *Lb. buchneri*, *Lb. curvatus*, *Lb. helveticus*, and *Lb. parabuchneri* are able to produce histamine even at refrigerate temperature and can be responsible for the accumulation of BAs in cheese (Burdychova and Komprda, 2007; Diaz et al., 2018; Ladero et al., 2008; Linares et al., 2014; Wüthrich et al., 2017). In fermented meat products, strains of *Lb. acidophilus*, *Lb. brevis*, *Lb. bulgaricus*, *Lb. casei*, *Lb. plantarum*, and *Lb. paracasei* were identified as tyramine and histamine producers (Komprda et al., 2010; Suzzi and Gardini, 2003), and *Lb. curvatus* as tyramine and putrescine producers (Li et al., 2018). In wine, several native *Lactobacillus* spp., together with *Oenococcus oeni* and *Pediococcus* spp. strains are responsible for BA production mainly during malolactic fermentation by the preactivity of *Lb. brevis* and *Lb. hilgardii* (Ancín-Azpilicueta et al., 2008; Gardini et al., 2005; Lerm et al., 2010; Marcobal et al., 2006d). While *Lb. hilgardii* strains seem to be able to produce histamine, 2-phenylethylamine and putrescine (Lucas et al., 2005), *Lb. brevis* is the main responsible bacterium for tyramine production in wine (Costantini et al., 2013; Moreno-Arribas and Lonvaud-Funel, 1999) as well as in beer (Lorencová et al., 2012).

Lactococci: *Lactococci* such as *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *lactis* and *L. lactis* subsp. *lactis* biovar *diacetylactis* are among the most important LAB used in the dairy industry as starter cultures (Fox and McSweeney, 2017). Although *Lactococcus* spp. own the GRAS and QPS status, some *L. lactis* spp. have been reported to be able to produce putrescine from agmatine via the AGDI pathway (Ladero et al., 2015, 2012a, 2011a). Also, strain combinations of *L. lactis* subsp. *cremoris* were reported to produce both putrescine and tyramine in a 90 g cheese-model exceeding 500 mg kg^{-1} (Flasarová et al., 2016).

Staphylococci: Coagulase-negative staphylococci (CNS) are often part of the surface of smeared cheeses, e.g. *Staphylococcus xylosus* (Hammer et al., 2019) but it is still unclear whether they produce therein BAs. In fermented meat products, they are used as starter cultures and are responsible for the red colour development due to their nitrate reductase activity during ripening and to modulate the aroma through the conversion of amino acids and free fatty acids (FFA) (Laranjo et al., 2017b, 2017a; Olesen and Stahnke, 2004). Nevertheless, despite the use as starter cultures, it is known that *S. carnosus* and *S. xylosus* are able to produce histamine (Martuscelli et al., 2000). Furthermore, it was found that the coagulase-negative *S. carnosus*, *S. equorum*, and *S. xylosus* strains are able to produce tyramine, albeit only in moderate concentrations of 24 mg L^{-1} up to 65 mg L^{-1} (Alfaia et al., 2018).

Streptococci: Within the genus *Streptococcus*, *S. thermophilus* is the only representative with QPS-status and is therefore often used as a starter culture in the dairy industry (Uriot et al., 2017). It has been reported, that *S. thermophilus* strains are able to produce histamine, putrescine and tyramine in dairy products such as yoghurt and cheese (Calles-Enríquez et al., 2010; La Gioia et al., 2011; Yılmaz and Gökmen, 2017). However, selected *S. thermophilus* strains distributed to date by the industry are well-checked and selected only when they fail to produce BAs (Eric Johansen, Chr. Hansen Cie. personal communication).

4.2 Genes involved in the biosynthesis of biogenic amines

In general, the expression of at least two genes is mandatory to synthesise BAs in bacteria, namely one coding for the amino acid decarboxylase and another coding for the transporter, which is involved in the exchange of the amino acid into the cell and the biogenic amine out of the cell. These two genes are located on a cluster that often includes further genes (Linares et al., 2011; Marcobal et al., 2012). To date, the ability to produce BAs are mostly strain-dependent rather than species-specific, and the presence of the decarboxylase genes involved the occurrence of horizontal gene transfer (HGT) between strains as a part of adaption to a specific environment and of survival (Coton and Coton, 2009; Lucas et al., 2005; Marcobal et al., 2006b).

LDC Cluster: The lysine decarboxylase cluster (LDC) is responsible for the synthesis of cadaverine in Gram-negative bacteria and has been extensively characterised in *E. coli* (Meng and Bennett, 1992). The LDC cluster of Gram-negative bacteria is composed of four adjacent genes: the *cadA* gene encodes the lysine decarboxylase (CadA), followed downstream by the gene *cadB*, which encodes the lysine/cadaverine antiporter (CadB), a gene called *cadC*, which encodes a regulatory protein (CadC), and by the fourth gene called *lysU* that encodes an aminoacyl-tRNA synthetase like protein (Figure 1.8) (Benkerroum, 2016; Mohedano et al., 2015). It suggests that the ability of Gram-negative bacteria to produce cadaverine is a species-specific trait that during gene evolution has become part of a "pathoadaptive" mutation to provide an advantage for survival and pathogenesis within the host (Torres, 2009).

HDC cluster: Histamine is one of the most abundant BA in fermented beverages and foods, and the histidine decarboxylase catalyses the synthesis of histamine by decarboxylation of histidine (Figure 1.5). Histidine decarboxylase can be found in Gram-negative and Gram-positive bacteria, though the enzymes were differentiated into pyruvoyl-dependent HDC (*hdcA*) for Gram-positive bacteria and into pyruvoyl-phosphate-dependent HDC for Gram-negative bacteria. Most of the characterised Gram-positive histamine-producing bacteria contain a HDC cluster composed of four genes. Upstream of the *hdcA* gene that encodes the histidine decarboxylase (HdcA), the gene *hdcP* is located that encodes the histidine/histamine antiporter. Downstream of the *hdcA* gene the *hdcB* gene is located, which encodes

HdcB that is involved in the conversion of the HdcA pro-enzyme to the active decarboxylase. The fourth gene is located downstream of the *hdcB* gene and is called *hisS* and encodes a protein similar to histidyl t-RNA synthetase (Ladero et al., 2016b; José M. Landete et al., 2008; Mohedano et al., 2015; Trip et al., 2011). In *Streptococcus* spp. the aminoacyl-tRNA synthetase gene *hisS* is missing, and the gene order is *hdcA–hdcP–hdcB* similar to that of *Staphylococcus* spp. where the *hdcB* gene is absent (Figure 1.8) (Benkerroum, 2016; Calles-Enríquez et al., 2010; Mohedano et al., 2015). In most of the histamine-producing bacteria, the HDC cluster is located in the chromosome. However, there are existing strains of *O. oeni*, *Lb. hilgardii* and *T. muriaticus*, especially those isolated from alcoholic beverages, in which the HTC cluster is found on an unstable plasmid. This suggests DNA acquirement by HGT. Furthermore, HGT is also supported by the fact, that the HDC cluster and its organisation, which is located on the chromosome in histamine-producing S. *thermophilus* strains, is unique among the LAB and more similar to the HDC cluster in *Staphylococcus* spp. and *Clostridium* spp. (Calles-Enríquez et al., 2010; José María Landete et al., 2008; Lucas et al., 2005, 2008).

ODC cluster: Putrescine can be synthesised via two pathways, namely via the ornithine decarboxylase (ODC) and the arginine deamination pathway (AGDI) (Figure 1.6). The ODC cluster contains only two genes: the ornithine decarboxylase gene *speF* and the antiporter gene called *potE* (Figure 1.8). This cluster, most probably acquired by horizontal gene transfer, is described for *Lactobacillus* spp. and *Oenococcus oeni*, all isolated from wine (Ladero et al., 2016b; Marcobal et al., 2006a; Romano et al., 2014).

AGDI cluster: The other route to synthesise putrescine is via decarboxylation of arginine to agmatine, followed by its deamination to form putrescine (Figure 1.6). This pathway is the main responsible one to accumulate putrescine in fermented food products, except in wine, and is used from different bacteria species (Ladero et al., 2012b, 2011b; Linares et al., 2012). For *Lactococcus* spp., the AGDI cluster contains five genes, which are involved in putrescine synthesis (Figure 1.8). Downstream of the *aguA* gene encoding the agmatine deaminase (AGDI), we can find the carbamate kinase gene *aguC* that encodes a carbamate kinase and upstream the *aguD* gene encoding the arginine/putrescine antiporter AguP. Further upstream, the gene *aguB* encoding a putrescine carbamoyl-transferase is located preceded

by gene *aguR* encoding a transmembrane transcription activator. *Enterococcus* spp. contain the same AGDI cluster arrangement as *Lactococcus* spp. except that the *aguR* gene is oriented in the opposite direction on the genome (Benkerroum, 2016; Mohedano et al., 2015). *Lactobacillus* and *Listeria* spp. contain a second *aguA* gene called *aguA2* and the order *aguB–aguD–aguA1–aguC–aguA2–aguR* is slightly different to that in *Enterococcus* and *Lactococcus* spp. (Chen et al., 2013; Cheng et al., 2013; Lucas et al., 2007; Romano et al., 2014). In *E. faecalis*, the production of putrescine seems to be a species-level trait, whereas, in *E. faecium,* only some strains of human origin can produce putrescine (Ladero et al., 2011a). This suggests HGT events of the AGDI cluster in contrast to the situation in *Lactococcus* spp. where putrescine production seems not to be mediated by such gene transfer events, although putrescine production in *L. lactis* is strain-dependent (Ladero et al., 2011a). It seems that the ability to produce putrescine via AGDI pathway may have been a species-specific trait that was lost during adaption to the milk environment via a mechanism of reductive genome evolution (Ladero et al., 2011a). Moreover, in certain *Lactobacillus* spp. the AGDI, ODC and TDC clusters are linked on a region of the chromosome as a genomic island involved in acid stress resistance and energy production. This cluster region can be transferred between different LAB strains and species (Coton and Coton, 2009; Lucas et al., 2007; Romano et al., 2014).

TDC cluster: Tyramine is one of the most abundant BA in fermented foods and is synthesised via tyrosine decarboxylation by means of the TDC cluster (Figure 1.8). There are four genes in the TDC cluster, which are involved in tyramine production. Upstream of the *tdcA* gene that encodes the tyrosine decarboxylase enzyme (TDC) the *tyrS* gene is located, which encodes an aminoacyl-tRNA synthetase. Downstream of the *tdcA* gene, *tyrP* encodes the tyrosine-tyramine antiporter and upstream of the *tyrP* gene the sodium/hydrogen Na^{+}/H^{+} gene (sometimes-called *nhaC*) is located, but its function in tyramine biosynthesis is unknown to date (Ladero et al., 2016b; Mohedano et al., 2015). For the first time, a TDC cluster was described in *E. faecalis* JH2-2, and similar clusters were annotated in the genomes of other bacteria (Bargossi et al., 2015a; Fernández et al., 2004; Gatto et al., 2016; Ladero et al., 2013). The distribution, the high sequence similarity as well as the similar organisation of the different TDC clusters in different bacteria suggest a horizontal gene transfer from a common source (Fernández et al., 2004;

Ladero et al., 2012b). However, in different strains, different transcriptional organisations of the TDC cluster can be observed (Lucas et al., 2003; Perez et al., 2015).

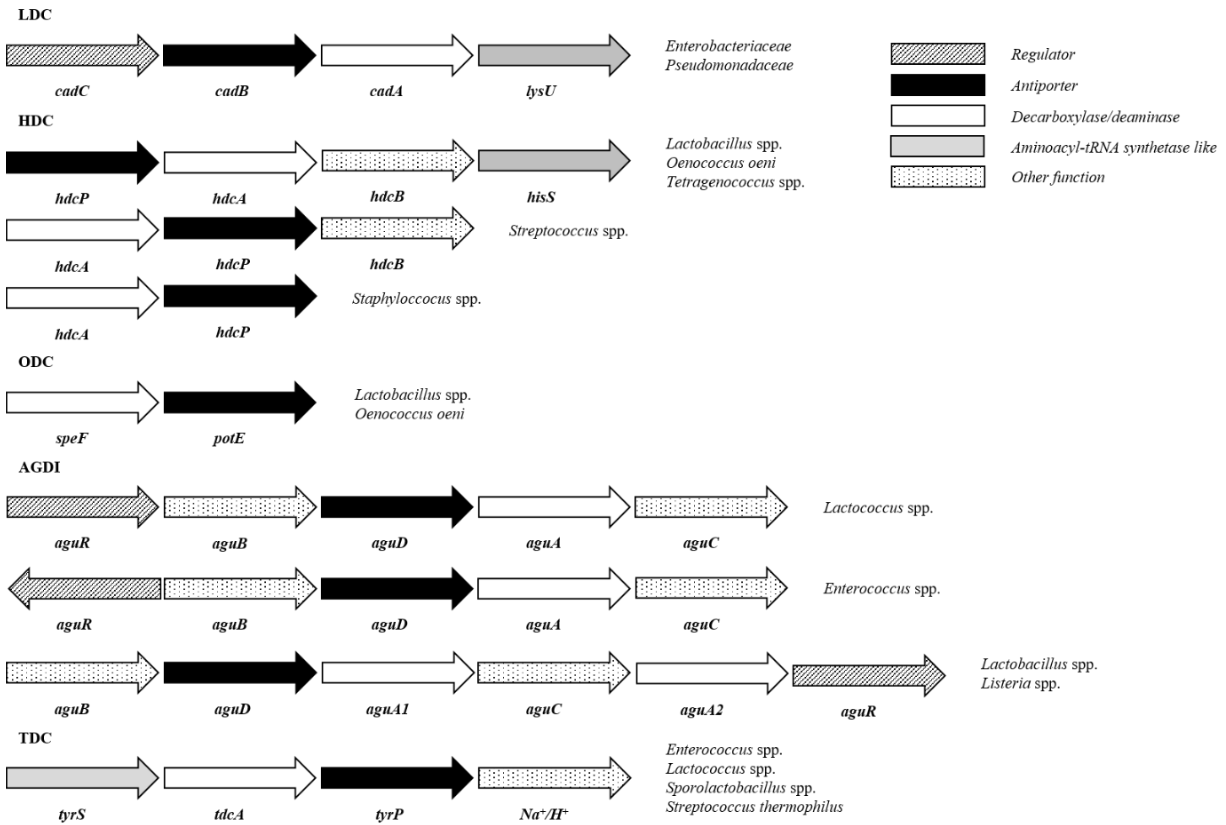

Figure 1.8: Organisation of the gene clusters of different BA-producing bacteria involved in the production of the four BA cadaverine (LDC cluster), histamine (HDC clusters), putrescine (AGDI clusters) and tyramine (TDC cluster). Adapted from Benkerroum, (2016); V. Ladero et al., (2016); Mohedano et al., (2015).

5. Toxicity and effects of biogenic amines on human health

Many BAs are essential for physiological functions and can be formed endogenously of regular metabolic activity in animals, humans, plants and microorganisms. Although they are naturally-produced endogenous, the consumption of foods containing high concentrations of BAs can have toxicological consequences. Under normal conditions during the food intake process, low concentration of exogenous BAs ingested with food are metabolised in the human gut to physiologically less active degradation products. There are two main types of amine oxidases, which are responsible for detoxification namely the monoamine oxidases (MAO) that deaminate BAs with one amino group and diamine oxidase (DAO) that deaminate those with two amino groups (Maśliński, 1975; Youdim et al., 2006). However, an excessive oral intake of BAs can provoke toxicological effects in sensitive individuals with a less effective detoxifying system because of genetic disposition or as a consequence of pharmacological treatment, alcohol intake or tobacco smoking (Berlin and Anthenelli, 2001; Bodmer et al., 1999; Flockhart, 2012; Herrero-Fresno et al., 2012; Hutchins et al., 2005; Ladero et al., 2016b; Novella-Rodríguez et al., 2004; Papageorgiou et al., 2018a). Not to be ignored is the ability of some BAs to potentiate the toxic effects of other BAs (Maintz and Novak, 2007).

Cadaverine: No human dose-response data are available concerning cadaverine, and to date, only one animal study in rats reported a non-observed adverse effect level (NOAEL) of 180 mg kg^{-1} body weight per day (Til et al., 1997). Although the pharmacological activities of cadaverine are lower than that of histamine and tyramine, high consumption can also lead to unhealthy effects such as bradycardia, dilatation of the vascular system, hypotension, increased cardiac output, and lockjaw and paresis of the extremities (EFSA, 2011). In addition, cadaverine inhibits via competition the detoxifying enzymes, which are involved in the oxidative catabolism of histamine and therefore potentiate the toxicity of histamine (Al Bulushi et al., 2009; Hui and Taylor, 1985; Lyons et al., 1983) Furthermore, cadaverine can react with nitrite to produce nitrosopiperidine, which is known as a carcinogen (del Rio et al., 2019; Ladero et al., 2010a; Linares et al., 2011; Warthesen et al., 1975).

Histamine: Scombroid poisoning or more general histamine poisoning describes a specific adverse reaction that can occur after the ingestion of food rich in histamine, whereby scombroid poisoning refers to the adverse reaction after the intake of histamine rich scombroids such as mackerel and tuna (Ladero et al., 2010a). Histamine poisoning is one of the significant toxicological effects of BAs and scombroid poisoning, is the most common cause of ichythyotoxicosis worldwide. Occasionally, cheeses, particularly Swiss cheese, have also been implicated in histamine poisoning (Feng et al., 2016; Taylor et al., 1989). In *in vitro* human intestinal epithelium cells, there exists a tested NOAEL of 3 mM and a lowest observed adverse effect level (LOAEL) of 4 mM, respectively for histamine. The symptoms which are associated with a scombroid poisoning are similar to an allergic reaction, with headache, migraine, abdominal cramps, nausea, flatulence, vomiting, diarrhoea, hypotension, palpitations, bronchospasms, respiratory distress, flushing, rash and urticarial (del Rio et al., 2017; Ladero et al., 2010a; Linares et al., 2016; Ruiz-Capillas and Herrero, 2019; Stratta and Badino, 2012). DAO is the main enzyme that is involved in histamine catabolism and detoxification, and healthy persons can ingest food with a concentration up to 70 mg kg^{-1} with no adverse effects. However, higher concentrations of histamine can cause serious reactions up to severe intoxication (Hungerford, 2010; Rauscher-Gabernig et al., 2009; Wöhrl et al., 2004). These adverse severe health effects can be enhanced by the joint appearance of histamine and other BAs like cadaverine and putrescine since they impair its catabolism by competitive inhibition of DAO (Hui and Taylor, 1985; Lehane and Olley, 2000; Maintz and Novak, 2007).

Putrescine: As well as for cadaverine, no human dose-response data are existing for putrescine but only an animal study in rats with a NOAEL of 180 mg kg^{-1} body weight per day (Til et al., 1997). Putrescine has, compared to histamine or tyramine, on its own a low toxicological activity even though putrescine can increase cardiac output, which can lead to hypotension or tachycardia (Ladero et al., 2010a; Wunderlichová et al., 2014). However, exogenous putrescine is involved in many destructive processes in the human body. For example, putrescine reacts with nitrite to nitrosopyrrolidine, which is known as carcinogenic (Ladero et al., 2016b, 2010a; Shalaby, 1996). In addition, putrescine is a key factor in carcinoma development caused by microorganisms such as *Helicobacter pylori* (Alam et al., 1994). Another carcinogenic effect of putrescine is the proliferation of neoplasms in the gastrointestinal tract and in the promotion of malignancy as well as in the involvement in the oncogenic process of developing colorectal adenocarcinomas together with spermidine and spermine (Gerner and Meyskens, 2004; Kim et al., 2017; Milovic and Turchanowa, 2003; Pegg et al., 1995; Vargas et al., 2012). Furthermore, putrescine, as well as cadaverine, potentiate the toxic effect of histamine by competitively inhibiting the detoxifying DAO enzymes involved in the degradation of histamine (Al Bulushi et al., 2009; Hui and Taylor, 1985).

Tyramine: Because tyramine is commonly present in high concentrations in cheese, tyramine toxicity has been referred as the "cheese reaction" though the high concentration of tyramine and therefore also intoxication have been observed in other fermented food products (Benkerroum, 2016; Blackwell, 1963; Linares et al., 2011; Pegg et al., 1995; Ruiz-Capillas and Jiménez-Colmenero, 2004; Stadnik and Dolatowski, 2010; Suzzi and Gardini, 2003). MAO degrades exogenous tyramine in the human intestine but if the average physiological level is exceeded tyramine can trigger a range of toxic effects such as cardiac failure, intracranial haemorrhage, hypertensive crises, pounding heart and palpitations, pulmonary oedema and even death. Furthermore, tyramine also causes lacrimation and salivation, increases respiration and blood sugar levels and dilates the pupils (Broadley, 2010; Finberg and Gillman, 2011; Shalaby, 1996). In addition, tyramine has been associated with neurological disorders, such as depression, Parkinson’s disease, Reyes’ syndrome and schizophrenia (Berry, 2008; Premont et al., 2001). Moreover, tyramine can react with dietary nitrites to form 3-diazotyramine that is known as an

inducer of oral cancer in rats and therefore, tyramine toxicity is related to an increased risk of carcinogenesis (Fujita et al., 1987; Shalaby, 1996). Furthermore, tyramine has the ability to affect the adherence of various bacteria to the intestinal epithelial cells, such as an increased attachment of enteropathogenic *E. coli* O157:H7 (De Palencia et al., 2011; Lyte, 2004). While in *in vitro* human intestinal epithelial cells a NOAEL of 1.8 mM and a LOAEL of 2.0 mM for tyramine exists, there is still insufficient information about the NOAEL in humans. However, in healthy individuals, which were exposed to a concentration of 600 mg tyramine per meal, no adverse health effects were observed (EFSA, 2011; Linares et al., 2016). Nonetheless, in sensitive individuals with a less effective detoxifying system the NOAEL decrease drastically to 50 or even 6 mg tyramine per meal (EFSA, 2011; Galgano et al., 2001; Ladero et al., 2010a; Novella-Rodríguez et al., 2004). Considering that many fermented foods contain a high concentration of tyramine, sensitive individuals are exposed to a not inconsiderable risk of tyramine intoxication.

6. Factors affecting biogenic amine formation and accumulation in food

The formation of BAs in fermented food products requires the presence of substrate amino acids, the presence of bacteria with decarboxylase and/or deaminase activities, and favourable environmental conditions. The final concentration of BAs in fermented food products depends on the gene expression activity, on the conditions for BA-producing bacterial growth as well as on environmental and technological factors.

6.1 Environmental factors

6.1.1 Hygienic condition of raw materials, processing and storage

BAs are already naturally present as endogenous BAs in fresh milk (Novella-Rodríguez et al., 2004) and grapes (Sass-Kiss et al., 2000), as well as in fruits and plants in general (Palermo et al., 2013), raw meat (Hernández-Jover et al., 1997), and fresh fish (Kang et al., 2019), before being eventually fermented. One of the major factors for the potential formation of additional exogenous BAs in fermented food products is the quality of the raw materials used, which may contain both microorganisms with decarboxylase and/or deaminase activities as well as the free amino acids acting as a substrate for the production of BAs (ten Brink et al., 1990). There are several microbial groups, which are identified to possess decarboxylase and/or deaminase activity. These bacteria are actively present during the production of fermented food products as either part of the starter cultures or may be introduced into the food by contamination before, during or after processing due to deficient hygiene conditions (Barbieri et al., 2019; Bover-Cid et al., 2001; Linares et al., 2012; Loizzo et al., 2016; Shalaby, 1996; Torriani et al., 2016).

6.1.2 Physicochemical factors

Physicochemical factors such as pH, sodium chloride and sugar concentrations, and temperature as well as humidity are essential factors in the production of fermented food products but also in the

accumulation of BAs. They can influence the growth of BA-producing bacteria, their gene expression, as well as the activity of their enzymes and transporters.

pH: The production of fermented food products require a low pH, and this acidity is also a preventive factor against microbial spoilage. However, since BA-producers in fermented foods are often LAB, they are able to grow under acidic conditions and moreover, produce BAs, because acidic pH induces the transcription of decarboxylase gene cluster to improve the fitness of the cell subjected to acidic stress (Marcobal et al., 2012; Pereira et al., 2009; Perez et al., 2015; Pessione et al., 2009; Romano et al., 2012). It has been shown that *L. lactis* subsp. *cremoris* strains are able to survive at pH values as low as pH 3 by the production of histamine, while the wild type of *L. lactis* subsp. *cremoris*, which is not able to decarboxylate histidine, is rapidly killed (Trip et al., 2012). In the production of putrescine via the AGDI pathway, ammonium ions are the main product, which indicates that a low pH and an acidic stress-induced resistance mechanism play an essential role in putrescine production (Chen et al., 2013; Griswold et al., 2006). However, the most extensive studies to investigate the influence of pH on BA production were done with tyramine. It was shown that the activity of the TDC cluster gene expression was the highest at a pH of about 5, while at pH 4 an extremely weak and at a neutral pH no activity was observed (Bargossi et al., 2015b; Linares et al., 2009; Zhang and Ni, 2014). Although an acidic environment promotes accumulation of BAs, it is very difficult to change parameters in the production of fermented foods, as an acidic pH contributes to ensuring the microbiological safety by inhibiting the growth of most pathogens and spoilage microorganisms, and affects the flavour of the fermented food product.

Sodium chloride concentration: Traditionally, the main role of sodium chloride is to control the growth of pathogens during the fermentation and ripening process by reducing water activity values to prevent spoilage and food poisoning. However, sodium chloride also has an effect on decarboxylase activity and therefore, on BA accumulation in fermented food products. A higher concentration of sodium chloride increase the concentration of BAs formed by enhanced activity of the enzymes and the expression of the genes, which are involved in BA production (Ladero et al., 2016b). A sodium chloride concentration of < 5 g 100 g^{-1} in milk, for example, does not affect the production of histamine, but a higher sodium

chloride concentration up-regulates the expression of the *hdcA* gene in *S. thermophilus* (Tabanelli et al., 2012). This suggests a potential role of the *hdcA* gene in osmoprotection and as a part of complex metabolic responses in the presence of stress conditions (Pessione et al., 2009; Rossi et al., 2011). Sodium ions (Na^+) are involved in the regulation of intracellular pH and are essential in sodium/proton antiporter systems by exchange with H^+ ions that are removed from the cell. Since the TDC cluster in tyramine-producing bacteria contains a Na^+/H^+ transporter coding gene Na^+ play an essential role in the tyrosine decarboxylation pathway and may explain the higher tyramine production at a higher sodium chloride concentration (Buáková et al., 2011; Buňková et al., 2012; Pereira et al., 2009).

Temperature and humidity: The production of BAs is generally favoured by temperatures close to the optimum growth values, promoting cell metabolism and proliferation (Marcobal et al., 2012, 2006c). Decreasing the temperature can reduce the production of endogenous BAs in raw materials for fermented foods. Therefore, a continuous cold chain during storage and commercialisation is a primary tool to reduce BA accumulation. However, in fermented food products, the range of temperature within is strict by controlled for the production of the different fermented food products. The temperature of fermentation and ripening controls the microbial activity of the desired microbiota (Andiç et al., 2010; Buňková et al., 2010b; Calles-Enríquez et al., 2010; Gardini et al., 2016; Knope et al., 2014; Komprda et al., 2012). Mostly, however, optimal temperatures and humidity, which are necessary for the fermentation process are also optimal for the activity of decarboxylase enzymes and therefore for the production and accumulation of BAs (Bargossi et al., 2015a; Beresford and Williams, 2004; Marcobal et al., 2012, 2006c).

6.2 Technological factors

6.2.1 Maturation period

The maturation process is a major factor in BA formation in fermented food products. In cheese, for example, the accumulation of BAs are affected by ripening and proteolysis. While starter LAB contribute to the protein break down (Lane and Fox, 1996; Lynch et al., 1997), the non-starter LAB are responsible for the peptidolysis and the release of free amino acids (Ardö et al., 2017; Muehlenkamp-Ulate and Warthesen, 1999). During ripening time, the proteolysis rate increases, which leads to an accumulation of free amino acids that serve as the substrate of decarboxylation activities and ultimately in BA accumulation if decarboxylase-positive microorganisms are available (Fernández et al., 2007a). Thus, due to higher proteolysis rate, long-ripened cheeses may have higher levels of BAs compared to short-ripened ones (Arlorio et al., 2003; Buňková et al., 2010b; Ladero et al., 2010c). In dry-fermented sausages BA production, proteolysis, and lipolysis are taking place during both fermentation and ripening, and the proteins change as a consequence of the action of microbial and endogenous proteolytic enzymes (Ordóñez et al., 1999). The increase of acidity, dehydration and action of sodium chloride are responsible factors for the denaturation of proteins and therefore for the proteolysis. During fermentation and drying, the non-protein nitrogen fraction increases and includes the presence of free amino acids, which acts as precursors of BAs (Gardini et al., 2016; Halász et al., 1994). Also in wine, an increase of BA concentrations during the maturation, mainly during the malolactic fermentation period, which is considered to one of the most crucial factors for BA production in wine, was observed (Henríquez-Aedo et al., 2016; Landete et al., 2005).

7. Methods to prevent biogenic amine accumulation

Fermented food products are favourable products for BA accumulation, and the most effective ways to maintain low concentrations of BAs in fermented food products are to reduce the initial microbial load and growth of decarboxylase-positive bacteria, maintain a low temperature during production, transport and storage, and maintain a neutral pH in the product. However, in fermented food products, the range of temperature and pH is tightly controlled and cannot be changed without impacting product quality and safety. Therefore, additional technological factors are needed to prevent BA accumulation in fermented food products.

7.1 Additives and antimicrobial substances

The use of preservatives and additives in food can inhibit the formation of BAs by acting on the microbial population rather than affecting the decarboxylase efficiency directly. There are various antimicrobial additives tested for the reduction of BAs in different food products. In pickled cabbage, for example, citric acid can lower the level of BAs during fermentation and garlic extract in fermented anchovy can reduce the concentration of BAs up to 8% (Bozkurt and Erkmen, 2004; Mah et al., 2009). Furthermore, In fermented sausages, nitrate and nitrite salts are often used to control hazardous bacteria and to develop colour and taste during fermentation but also to reduce BAs (Bozkurt and Erkmen, 2004; Gençcelep et al., 2014). Another method to reduce BAs in fermented sausages is the addition of sugar. The use of sugar, mainly glucose, sucrose and lactose, can reduce BAs content in fermented sausages enhancing growth and stronger acidification resulting from glucose fermentation by the LAB (Bover-Cid et al., 2008; González-Fernández et al., 2003). Moreover, in fermented fish, the action of an aminobiogenic *Enterobacter aerogenes* strain could be inhibited by the use of sorbate and benzoate, sometimes in combination with clove (Gençcelep et al., 2014; Lapa-Guimarães et al., 2011; Wendakoon and Sakaguchi, 1993). In alcoholic beverages, sulphur compounds play a key role in the reduction of BAs by inhibiting cell metabolism of decarboxylating bacteria (José M. Landete et al., 2008). A novel technology to reduce BAs in fermented food products is the use of specifically targeted bacteriophages to reduce the population of BA-producing bacteria. It could be shown that the use of the bacteriophage

Q69, which infects specifically the tyramine-producing *E. faecalis*, reduced tyramine in dairy products under different experimental conditions (Ladero et al., 2016a).

7.2 Hydrostatic pressure

High hydrostatic pressure (HHP) or high-pressure homogenisation (HPH) is a technological factor that can be used to inhibit the formation of BAs by modifying the microbiota of treated fermented foods both qualitatively and quantitatively (Georget et al., 2015; Naila et al., 2010). In general, the efficacy of BA reduction depends on the intensity of the pressure applied, but it has been shown, that HHP and HPH can reduce the concentration of BAs in fermented cheese and meat products (Calzada et al., 2013; Halász et al., 1994; Lanciotti et al., 2007; Ruiz-Capillas et al., 2007).

7.3 Irradiation

Food irradiation affects microbial growth and therefore increase the safety and shelf life of fermented food products. It has been reported that gamma-irradiation reduce BAs contents in food such as pepperoni, blue jack mackerel, Bonito, soybean paste, sausages, chicken, beef and pork as well as in cheese, due to the delay in the initial growth of adventitious microorganisms (Kim et al., 2005a, 2005b, 2004; Mbarki et al., 2008; Mendes et al., 2000; Min et al., 2007; Rabie et al., 2011, 2010).

7.4 Oxidising microorganisms able to metabolise biogenic amines

Microorganisms can produce amino oxidase enzymes, such as laccases (EC 1.10.3.2), ascorbate oxidases (EC 1.10.3.3), nitrite reductases (EC 1.7.2.1) as well as ferroxidases (EC 1.16.3.1), which are responsible for detoxification of BAs. In the presence of oxygen, these enzymes can convert BAs firstly by deamination with the production of ammonium (NH_3), hydrogen peroxide (H_2O_2) and aldehydes. Afterwards, the aldehydes formed are further reduced to the corresponding acids and transferred to the central metabolism of the cells. Therefore, in a poor nitrogen medium, this metabolic pathway can be used as a source of NH_3 (Cooper, 1997). These enzymatic activities have been evidenced *in vitro* in

several microorganisms such as *Brevibacterium linens* strains, *Bacillus* sp., *Lactobacillus* spp., *Pediococcus* and *Oenococcus* spp. as well as in *Staphylococcus* spp. (Capozzi et al., 2012; Fadda et al., 2001; García-Ruiz et al., 2011; Guarcello et al., 2016; Herrero-Fresno et al., 2012; Leuschner and Hammes, 1998; Zaman et al., 2011, 2010).

7.5 Packaging

Carbon dioxide in modified atmosphere packaging (MAP) plays an essential role for food preservation in the extension of shelf life and BA reduction by inhibiting microbial growth (Curiel et al., 2011; Özogul and Özogul, 2006). With MAP, the content of BAs in various fermented foods could be reduced such as in sardine, barramundi filets, Indian mackerel as well as in meat and chicken (Chouliara et al., 2007; Özogul and Özogul, 2006; Rodrigues et al., 2016; Yassoralipour et al., 2012; Yew et al., 2014; Zhang et al., 2015). Nevertheless, nowadays, the use of oxygen scavengers which eliminate oxygen in the packaging and the product are most used in active food packaging technology (Álvarez, 2000).

7.6 Smoking

Smoking is often used in fermented meat and fish products to yield products with aseptic characteristics, thus decreasing the level of BAs in the product by inhibiting the growth of active decarboxylase bacteria (Martuscelli et al., 2009).

7.7 Thermal treatments

The application of thermal treatments to raw materials such as milk before fermentation can contribute to the elimination of the wild decarboxylase-positive microbiota (Gardini et al., 2016). Compared to pasteurisation, thermisation is a mild thermal process with continuous heat treatment with a maximum temperature of 68°C followed by cooling. In pasteurisation, on the other hand, the milk is typically heated at 72–74°C for 15–30 seconds. During thermisation, almost all of the raw milk's properties are

unchanged and the bacterial microbiota, especially the psychotropic microbiota, is reduced considerably. In pasteurisation, on the other hand, all pathogenic microorganisms that may be present in the raw milk are inactivated. Therefore, thermal treatment increases safety and has the ability to reduce BAs in cheese (Doeun et al., 2017; Gardini et al., 2016; Ladero et al., 2016b; Panthi et al., 2017).

Use of starter culture: One of the major tools to counteract BA accumulation in fermented foods is the addition of selected starter cultures. For the use of starter cultures for the reduction of BA accumulation, two conditions are needed namely they should be characterised by the absence of any decarboxylating activity, and secondly, they should be able to inhibit the growth performances and aminobiogenic potential of food-resident-decarboxylating bacteria (Ayhan, Kolsarici, & Özkan, 1999; Sara Bover-Cid, Hugas, Izquierdo-Pulido, & Vidal-Carou, 2000). The use of selected starter cultures is applied in several fermented food products, and the European Food Safety Agency (EFSA) recommends to use strains originating from each specific fermented product, so-called autochthonous strains, with suitable technological profiles as starter cultures (EFSA, 2011; García-Ruiz et al., 2011; Gardini et al., 2002; Nieto-Arribas et al., 2009; Zaman et al., 2011).

8. Detection methods of biogenic amines

The detection of BAs in food is essential for two reasons. Firstly, BAs can be used as food quality markers and secondly, because BAs can have adverse health effects and possess a toxic potential. There existing two categories of BA detection methods. One is based on the detection of amino acid decarboxylase microorganisms, and the other one is based on the detection of accumulated BAs.

8.1 Detection based on amino acid decarboxylase microorganisms

Microorganisms can be tested geno- and phenotypically on their ability to produce BAs. The genotypic detection of the BA-producing microorganisms is based on polymerase chain reaction (PCR) and can detect potential BA-producing bacterial strains at any step of the food processing process. The multiplex PCR is a method for the simultaneous detection of bacteria that produce more than one BA based on the design of specific primers for each BA decarboxylase gene (De Las Rivas et al., 2005). The phenotypic identification of BA-producing microorganisms is carried out through a pH-induced medium containing an indicator such as bromocresol purple. This method is based on the change of the indicator colour of the medium by increasing the pH. The production of BAs results in an alkalised cytoplasm relative to the medium and therefore increase the cytoplasmic pH (Bover-Cid and Holzapfel, 1999; De Palencia et al., 2011; Wolken et al., 2006).

8.2 Detection based on biogenic amines

There existing several reasons why the identification of BAs in food is not simple, and they are one of the most significant challenges in food analysis. First of all, most kind of food products consisting of a very complex food matrix. Furthermore, BAs are compounds which are strongly polar and therefore higher soluble in water than in organic solvents. Moreover, the range of the variable concentration of BAs is broad, and several BAs can co-occur in the food. Therefore, the analytical methods based generally on amine extraction and derivatisation, followed by separation and quantification (Önal, 2007; Papageorgiou et al., 2018a).

8.2.1 Capillary electrophoresis

Although capillary electrophoresis (CE) shows a low sensitivity, it is the second most commonly performed method for the determination of BAs in food because CE is rapid, simple, cost-effective and reliable (Wei et al., 1990). Therefore, several approaches are used to enhance the sensitivity such as the application of selected buffer systems without derivatisation, the application of derivatisation processes or the application of specific kits (Kvasnička and Voldřich, 2006; Papageorgiou et al., 2018a).

8.2.2 Gas chromatography

Gas chromatography (GC) has been specified for the detection of BAs in fermented beverages and is not commonly used in other fermented food products. To increase the volatile properties and to decrease the polarities of BAs, derivatisation of the analytes must be done and depending on the detection technique used for final determination, a different type of derivatising reagents can be used (Papageorgiou et al., 2018b; Płotka-Wasylka et al., 2016).

8.2.3 Liquid chromatography

The most common analytical method to determine BAs in fermented food products is high-pressure liquid chromatography (HPLC) with Reversed-Phase separation using C18 columns. To prepare the samples for the final separation, an application of solvent extraction and derivatisation process to concentrate the analytes and remove compounds that may interfere with the analysis (Huang, 2016). There are existing several derivatisation reagents; however, dansyl chloride has been the major reagent since that its derivates can be detected using diode array detectors, fluorescence detectors and mass spectroscopy (Ahmad et al., 2020; Learey et al., 2018). Although ortho-phthalaldehyde derivates are less stable than dansyl chloride ones, it can be used at room temperature and without using a preliminary separation or clean-up. Therefore, it is also frequently used as a derivatisation reagent (Latorre-Moratalla et al., 2009).

9. Legislation concerning biogenic amine content in food

Although BAs are present in many different fermented food products and beverages and they can occur in high concentrations and cause severe health effects, a standard regulation limiting the concentrations of BAs in food is to date still lacking except for histamine in fish. The FDA has set a stringent limit of 50 mg kg^{-1} for histamine in fish products, whereas the EFSA considers a critical level of histamine in fish to a range between 200 and 100 mg kg^{-1} according to whether the products have undergone enzymatic maturation treatment in brine or not (EFSA, 2005; EU Commission, 2007; United States Department of Health and Human Services (HHS) Food and Drug Administration (FDA) and Applied Nutrition Center for Food Safety (CFSAN), 2019). It is difficult to quantify the potential risk of BAs because often the poisoning by BAs is underestimated or generally not reported to the health authorities. This could be either because symptoms are often misdiagnosed or because mild symptoms do not require a medical report (Russo et al., 2010). In addition, due to the intra- and inter-individual variations of sensitivity to BAs and the concomitant interference of inhibitors of the detoxification pathways such as antidepressant treatments, smoking or alcohol, it is difficult to establish a critical threshold of BAs in fermented food products (Spano et al., 2010).

10. Background and objectives of the thesis

BAs are compounds with biological activity. They are formed by decarboxylation of free amino acids by means of food-resident amino acid-decarboxylating bacteria, from the starter culture or introduced by contamination, during food processing and storage. Especially in fermented food products, such as dairy products, fish and fish products, fruit and vegetable products, meat and meat products, and alcoholic beverages high concentrations of BAs can be found because of the advantageous properties for bacterial activities. Despite the ability to degrade a low concentration of exogenous BAs in the human intestine, an excessive oral intake of BAs can lead to severe toxicological health effects in humans.

Tyramine is one of the most important BAs that can occur in high concentrations in various fermented food products. An intake of exogenous tyramine at a higher concentration than can be degraded by MAO in the human intestine can lead to serious health effects such as cardiac failure, intracranial haemorrhage, hypertensive crises, pounding heart and palpitations, pulmonary oedema and even death. Although tyramine is one of the most toxic BAs in fermented food products, there is still no legislation limiting the concentration of tyramine. Furthermore, there is still a lack of knowledge about tyramine production conditions as well as about the main responsible tyramine producers in different fermented food products. Former studies have demonstrated, that *L. brevis* in cheese, *L. curvatus* and *Carnobacterium* spp. in sausages and *S. thermophilus* in dairy products have the physiological capacity to produce tyramine during food processing and storage, but recent findings give evidence that *Enterococcus* spp. contribute to a high tyramine formation. Therefore, it was hypothesised for this study that *Enterococcus* spp. are the main tyramine producers in fermented food products, especially in cheeses and sausages for the Swiss market. Moreover, since it is known that some microorganisms can produce oxidases to degrade BAs, we raised the question if selected microorganisms are able to degrade tyramine accumulation in fermented food products without changing the food matrix.

10.1 General objective

The general objective of this study was to reduce tyramine accumulation in fermented food products and to increase the knowledge about tyramine-producing microorganisms and their producing-mechanisms.

10.2 Specific objectives

Accordingly, the specific objectives of this thesis were the following:

1. To identify tyramine-producing microorganisms in fermented cheese and sausage samples on the Swiss market.
2. To improve the mechanistic knowledge on tyramine production of microorganisms in fermented food products.
3. To screen *in vitro,* antagonistic *Lactobacillus* spp. strains against tyramine producers to decrease tyramine concentration in fermented food products.
4. To deliver data, which enable Swiss Food Safety authorities to propose measures and recommendations for tyramine reduction in fermented food.

Chapter 2

Detection of Biogenic Amines and Tyramine-Producing Bacteria in Fermented Sausages from Switzerland

Janine Anderegg, Moritz Fischer, Jovana Dürig, Alfonso Die, Christophe Lacroix and Leo Meile*

Laboratory of Food Biotechnology, Institute of Food, Nutrition and Health, ETH Zurich, Zurich, Switzerland

*Corresponding author

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Abstract

Fermented food products can cause human illness because of the unhealthy effect of biogenic amines (BA) that accumulate by decarboxylation of free amino acids. Salami-type fermented sausages can contain BAs, but it is still unclear which bacteria and which environmental factors contribute to BA production. Therefore, 62 sausages purchased on the Swiss market were investigated on their decarboxylating bacterial strains and the content of the BAs cadaverine, histamine, putrescine and tyramine. Based on the size and number of employees of the meat plants, sausages were distinct into two groups: artisanally- and industrially-produced ones. All four BAs had higher concentrations in industrially-produced sausages compared to artisanally-produced ones. Tyramine was the major amine detected in 46 of 62 sausages, with a maximum concentration of 785.22 mg kg^{-1} and enterococci, as well as coagulase-negative staphylococci, mainly the meat starter culture *S. xylosus*, could be identified as the main tyramine producers. Putrescine was found in 20 of 62 samples, with a maximum concentration of 707.77 mg kg^{-1}. These two BAs showed a significant correlation ($P = 0.0407$) for their concentrations. Cadaverine and histamine were detected in nine or eight samples respectively, and both were found in significantly higher levels ($P = 0.019$) and ($P = 0.036$) in industrially-produced sausages. Based on the quantitative tyramine content, five groups of fermented sausages were identified. Group 1 included products with a very high tyramine level (> 700 mg kg^{-1}), group 2 with a high level (400–700 mg kg^{-1}), group 3 with a moderate level (200–400 mg kg^{-1}), group 4 with a low level (< 200 mg kg^{-1}) and group 5 with a tyramine level below the detection limit (0.05 mg kg^{-1}). Samples with a tyramine level higher than 200 mg kg^{-1} could be considered as products of less quality because consumption of such samples could be unhealthy for sensitive individual consumers.

Introduction

Fermentation is a traditional meat preservation technique that provides relatively stable products with typical sensorial characteristics. A combination of lactic acid bacteria (LAB), mainly lactobacilli and coagulase-negative staphylococci (CNS), contribute as starter cultures to the acidification and flavour development, respectively (Cocolin et al., 2011). These starters can either be resident in the meat or intentionally added, especially in industrial production, to quickly reduce pH at the beginning of the ripening process (Ravyts et al., 2010). Although the type and manufacture of fermented sausages differ depending on the region, industrial manufacturing processes tend to standardised procedures. This trend is sometimes contrary to consumer expectations, who appreciate artisanally-produced sausages because of their unique sensory properties (Latorre-Moratalla et al., 2008). Traditional artisanal sausages are produced in small-scale plants by spontaneous fermentation or sometimes combined with selected starter cultures. It is assumed that artisanal producers may have technical or even financial difficulties to meet the requirements of hygiene and food safety standards which were established for industrial processes (Latorre-Moratalla et al., 2008). Regardless, the meat fermentation is generally used to prevent the growth of pathogenic and other unwanted bacteria. Enterococci represent one such group of bacteria, often regarded as contaminants that originate from meat or the environment and are able to proliferate under fermentation conditions. They are suspected of contributing to enhanced biogenic amine (BA) levels due to their proteinase activities in such a protein-rich environment (Suzzi and Gardini, 2003).

BAs are formed by microbial decarboxylation of amino acids. According to their chemical structure, BAs can be classified as aromatic (tyramine and phenylethylamine), heterocyclic (histamine and tryptamine), or as aliphatic (cadaverine, putrescine, spermine and spermidine), and belong to a group of basic nitrogenous organic compounds (Linares et al., 2011). The most well-known BAs are tyramine, histamine, cadaverine and putrescine derived from decarboxylation of the free precursor amino acids tyrosine, histidine, lysine and ornithine, respectively (Fernández et al., 2007b; Herrero-Fresno et al., 2012). BAs can be found in fermented food products including dairy products (Linares et al., 2011), fermented meat and fish (Suzzi and Gardini, 2003), as well as fermented fruits (Moreno-Arribas, 2002) and vegetables (Lee et al., 2019), and can thereby pose a risk of food intoxication (Suzzi and Gardini,

2003). Histamine and tyramine can cause severe allergic reactions; increase blood pressure, cardiac output, respiration, and blood glucose levels; and release norepinephrine (Broadley, 2010; EFSA, 2011). Compare to histamine and tyramine, cadaverine and putrescine have a much lower toxicological impact, but they can potentiate the toxic effect of other amines (Combarros-Fuertes et al., 2015).

Tyramine in fermented sausages is produced by a variety of LAB including enterococci, lactobacilli and lactococci, but also by some *Staphylococcus* spp. (Linares et al., 2012; Suzzi and Gardini, 2003; Torriani et al., 2016) that are either part of the starter cultures or introduced by contamination before, during or after meat processing (Bover-Cid et al., 2001). The key enzyme, a tyrosine decarboxylase (TdcA, EC.4.1.1.25), and a transporter protein for tyrosine/tyramine interchange are involved in tyramine enrichment (Coton et al., 2011; Linares et al., 2011; Marcobal et al., 2012). Corresponding genes are typically clustered in an operon (named TDC or TYR) on the chromosome or plasmids in tyramine producer strains (Fernández et al., 2004; Mohedano et al., 2015). One of the genes (named *tdcA* or *tdc*) encodes the tyrosine decarboxylase, while the other one (*tyrP*) encodes the tyrosine-tyramine antiporter. Also, sometimes a gene responsible for the regulation of tyrosine decarboxylase is part of the operon (Linares et al., 2011; Marcobal et al., 2012). This genetic setup and the fact that the production of tyramine (or the production of BAs in general) is strain-specific and not species-specific supports the hypothesis of horizontal gene transfer (Bonnin-Jusserand et al., 2012; Linares et al., 2011; Marcobal et al., 2012; Spano et al., 2010). Tyrosine decarboxylase has only been characterised extensively in few LAB species, including *Enterococcus faecalis, Enterococcus faecium*, *Lactobacillus brevis* and *Lactobacillus curvatus* (Linares et al., 2011) where knock-out mutants in the TDC operon were constructed (Suzzi and Gardini, 2003). A key factor for the production of tyramine is an acidic pH in media at pH 5 (Fernández et al., 2007b; Gardini et al., 2001; Moreno-Arribas, 2002).

Several studies reported the presence of high levels of BAs, particularly tyramine in fermented sausages from retail markets (Martuscelli et al., 2000; Suzzi and Gardini, 2003). Therefore, in artisanally-fermented sausages, the natural background microbiota of the meat used as raw material might be involved in tyramine production in addition to the used starter microorganisms which might be carriers of the TDC operon (Latorre-Moratalla et al., 2012, 2008; Martuscelli et al., 2000; Suzzi and Gardini,

2003). Nevertheless, levels of tyramine differ even between the products from the same manufacturer (Marcobal et al., 2012). Therefore, it seems that the quality of raw materials is one of several factors affecting tyramine formation in fermented dry sausages (Suzzi and Gardini, 2003). Peptides containing tyrosine or free tyrosine must be available for TdcA-positive microorganisms to produce tyramine (Marcobal et al., 2012). Additionally, other factors such as ingredients and additives, such as sugar, curing agents (nitrite/nitrate mixtures) and spices, processing conditions and parameters as well as technological ripening conditions like temperature and relative humidity might influence the formation of tyramine (Latorre-Moratalla et al., 2012).

The factors affecting tyramine production and the extent of its concentration in industrially- and artisanally-produced salami-type fermented products are not well described. Therefore, this study aimed to investigate BA accumulation in artisanal or industrial sausages purchased in supermarkets from Switzerland as a model country for both procedures. In particular, the correlation between tyramine concentration in different artisanal and industrial fermented sausages and the different factors that influence the concentration of tyramine were analysed, including pH, concentrations of enterococci, lactobacilli, lactococci and staphylococci, and presence of other BAs, cadaverine, histamine and putrescine. The BA contents in the final sausages were assessed to determine their potential health risk.

Material and Methods

Sausages samples and bacterial isolation

Sixty-two fermented salami-type sausage samples were obtained from local Swiss meat processors or bought in Swiss supermarkets (Table 2.1). The sausages used were made from pork, venison, sheep, chamois, horse, roe deer, goat, beef or wild boar meat and additives like sugar, spices, garlic and wild garlic were added sometimes. Sausages were produced either in small artisanal meat plants with less than 15 employees or in larger industrial meat plants, respectively.

After removal of the sausage casing, 10 g of each sample was homogenised for 10 min in 90 mL 0.9% NaCl containing 0.1% peptone (Merck, Darmstadt, Germany) at pH 7 using a Stomacher Bag Mixer (Interscience, Saint Nom, France). From the homogenised samples, 100 mL each were used for pH measuring, and dilutions were plated on three semi-selective agar-media: KF *Streptococcus* (KFS) (BD Difco, Allschwil, Switzerland), Baird-Parker (BP) (Biolife, Milano, Italy), and De Man-Rogosa-Sharpe with Tween 80 (MRS) (Biolife, Milano Italy), followed by incubation for 48 h at 43°C (KFS) or 37°C (BP and MRS). KFS- and BP-agar plates were incubated under aerobic conditions, whereas MRS plates were incubated anaerobically in 2.5 L culture jars with AnaeroGen AN 25 sachets (Oxoid, Basingstoke, UK). From each sample, three colonies per different solid medium were randomly picked, grown overnight in MRS broth for lactobacilli/lactococci or BHI broth for enterococci and staphylococci, respectively.

Identification of tyrosine-decarboxylating bacteria

Tyrosine decarboxylase activity of bacterial isolates was tested using the screening plate method (Joosten and Northolt, 1989) with slight modifications (Bover-Cid and Holzapfel, 1999). Strains were streaked on pH-induced medium agar-plates containing tyrosine and bromocresol purple as an indicator of pH increase and were incubated for 2 days at 37°C under aerobic conditions for *Enterococcus* spp. and *Staphylococcus* spp., and under anaerobic conditions for *Lactobacillus/Lactococcus* spp.

The presence of the *tdcA* gene in bacterial isolates was tested in DNA from single colonies after using the Goldenberger B extraction method (Goldenberger et al., 1995). The PCR assay was based on the primer pair DEC3 (5'-CCGCCAGCAGAATATGGAAYRTANCCCAT-3') and DEC5 (5'-CGTTGTT GGTGTTGTTGGCACNACNGARGARG-3') (Torriani et al., 2016), which were tested in previous studies in different *Enterococcus* spp., *Lactobacillus* spp. and in *Staphylococcus epidermidis* strains. For amplification of *tdcA* fragments, the PCR mixture (20 µL) was composed of 10 µL PCR Master Mix (2X) (Thermo Scientific, Reinach, Switzerland), 1 µM concentrations of each primer, 1 µL of extracted DNA and Milli-Q water. PCR analysis was conducted in a Biometra Professional Basic Gradient thermocycler (Biometra GmbH, Göttingen, Germany), under the following conditions: initial denaturation at 94°C for 5 min; 5 cycles of 94°C for 30 s, 47°C for 90 s, and 72°C for 90 s; 30 cycles of 90°C for 30 s, 50°C for 60 s, and 72°C for 60 s; and a final extension at 72°C for 7 min. The expected size of the amplicon was about 350 bp, and PCR products were visualised after electrophoresis in a 2% agarose gel.

Identification of bacterial isolates

For identification of *Enterococcus* spp., a multiplex PCR was used (Jackson et al., 2004) with primers to generate amplicons of 733 bp for the genus *Enterococcus*, 295 bp for *Enterococcus durans*, 360 bp for *Enterococcus faecalis*, and 215 bp for *Enterococcus faecium*. The PCR reaction mixture was performed as described above and PCR was conducted under the following conditions: initial denaturation at 95°C for 4 min; 30 cycles of 95°C for 30 s, 53°C for 60 s, and 72°C for 60 s; and a final extension at 72°C for 7 min.

All other strains were identified by 16S rRNA gene sequencing. The target DNA was amplified using the primers bak4 (5'-AGGAGGTGATCCARCCGCA-3') and bak11w (5'-AGTTTGATCMTGGCT CAG-3'), with an expected amplicon size of approximately 1500 bp (Dasen et al., 1998). The PCR mixture was prepared as described above and performed with an initial denaturation step at 95°C for 5 min followed by 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 2 min; and a final extension at 72°C for 7 min. PCR amplicons were purified by the Promega Wizard® SV Gel, and PCR Clean-Up

System (Promega, Fitchburg, USA) and DNA were analysed by Sanger sequencing at GATC (Konstanz, Germany).

Determination of biogenic amines

For the quantitative determination of BAs, ion exclusion chromatography with pulsed amperometric detection (IC-PAD) was used (Dionex, 2007). The Dionex method was modified by changing the eluent gradient: 5–10 mM MSA from 0–6 min, 10–20 mM from 6–16 min, and 20–45 mM from 16–20 min. The IC-PAD apparatus used was Dionex™ ICS-5000+ Hybrid HPIC™ system and IonPac CG18, 2 x 50 mm were used as a precolumn and IonPac CS18, 2 x 250 mm as separation column, respectively (Thermo Fisher Scientific, Switzerland). After removing of the sausage casing, 5 g of each sample was homogenised for 10 min in 22.5 mL 0.2 M perchloric acid (Sigma-Aldrich, Buchs, Switzerland) and 22.5 mL acetonitrile (Sigma-Aldrich, Buchs, Switzerland) in a Stomacher Bag Mixer (Interscience, Saint Nom, France). After homogenisation, the sample was incubated for 30 min at room temperature. Afterwards, 10 mL of the homogenate was transferred to a new tube and centrifuged for 10 min at 6,000 x g at 4°C. A portion (1.5 mL) of the supernatant was transferred into a new tube and centrifuged again for 10 min at 10,000 x g at 4°C. The supernatant was then stored at -20°C and used in a 1:50 dilution for determination of BA concentrations.

Statistical analysis

All samples were analysed in duplicate. Statistical analysis of cell counts and concentrations of BAs were performed using a one-way unpaired Mann-Whitney Rank Sum Test ($P < 0.05$) in the statistic software SigmaPlot 13 (Systat Software Inc., San Jose, California, USA). Linear regression with interaction was performed using the statistic software R (R Core Team) using the lme4, sjplot, sjmisc and ggplot2 packages. Means of technical replications were first calculated prior to comparison. As previously described, samples with cell counts and concentrations of BAs below the detection limit were replaced with artificial values of 99 and 9999 for cell count of staphylococci and enterococci and lactococci/lactobacilli, respectively and 0.049 for the concentration of BAs to allow statistical analysis (Garriga et al., 2002; Meier et al., 2018).

Results

Biogenic amine content in sausages

A number of 62 randomly purchased artisanally-(art.) and industrially (ind.)-produced salami-type fermented sausages were analysed for BA content. The total concentration of the four measured BAs was significantly higher ($P = 0.003$) in industrially-produced sausages with an average concentration of 140.67 mg kg^{-1} compared to artisanally produced ones with an average of 50.18 mg kg^{-1}. Moreover, only one of 36 artisanally-produced sausages contained all four BAs whereas in industrially-produced ones five of 26 samples containing all four BAs (Table 2.1).

Tyramine was by far the most abundant BA, with an average value of 145.94 mg kg^{-1} in artisanal and 183.50 mg kg^{-1} in industrial fermented sausages, and was found in 46 of 62 samples (Figure 2.1). Among artisanally- and industrially-produced sausages, based on the tyramine content, five groups each could be classified. Group 1 included products with a very high tyramine level (> 700 mg kg^{-1}). This group contained 6.5% (1 art., 3 ind.) of the products examined. Group 2 was characterised by a high level of tyramine (400–700 mg kg^{-1}) and contained 6.5% of the products (2 art., 2 ind.). Group 3, which was characterised by a moderate level of tyramine (200–400 mg kg^{-1}), included 14 samples (7 art., 7 ind.) and represented 22.5% of the products. Up to 38.7% of the analysed products were included in group 4 and presented a low level of tyramine with a concentration below 200 mg kg^{-1} while 25.8% (11 art., 5 ind.) contained in group 5 (below detection limit) (Table 2.1).

Putrescine was the second most abundant BA with corresponding average values of 48 mg kg^{-1} artisanal and 81.57 mg kg^{-1} for industrial sausages, respectively and was found in nine artisanally and 11 industrially-produced sausages (Figure 2.1). Putrescine was found in a concentration between 27.37 and 503.72 mg kg^{-1} in artisanal sausages and between 2.03 and 707.77 mg kg^{-1} in industrial sausages. In 19 of 20 samples, putrescine always occurred together with tyramine and samples with high concentrations of putrescine also tested tyramine in higher concentrations (> 300 mg kg^{-1}) (Table 2.1).

For the other BAs average values in artisanal and industrial sausages were 2.65 mg $kg^{-1}$and 56.63 mg kg^{-1} for cadaverine, respectively and 4.11 mg $kg^{-1}$and 30.79 mg $kg^{-1}$for histamine, respectively

(Table 2.1) and were with a *P*-value of 0.019 for cadaverine and 0.036 for histamine significant higher in industrially-produced sausages (Figure 2.1).

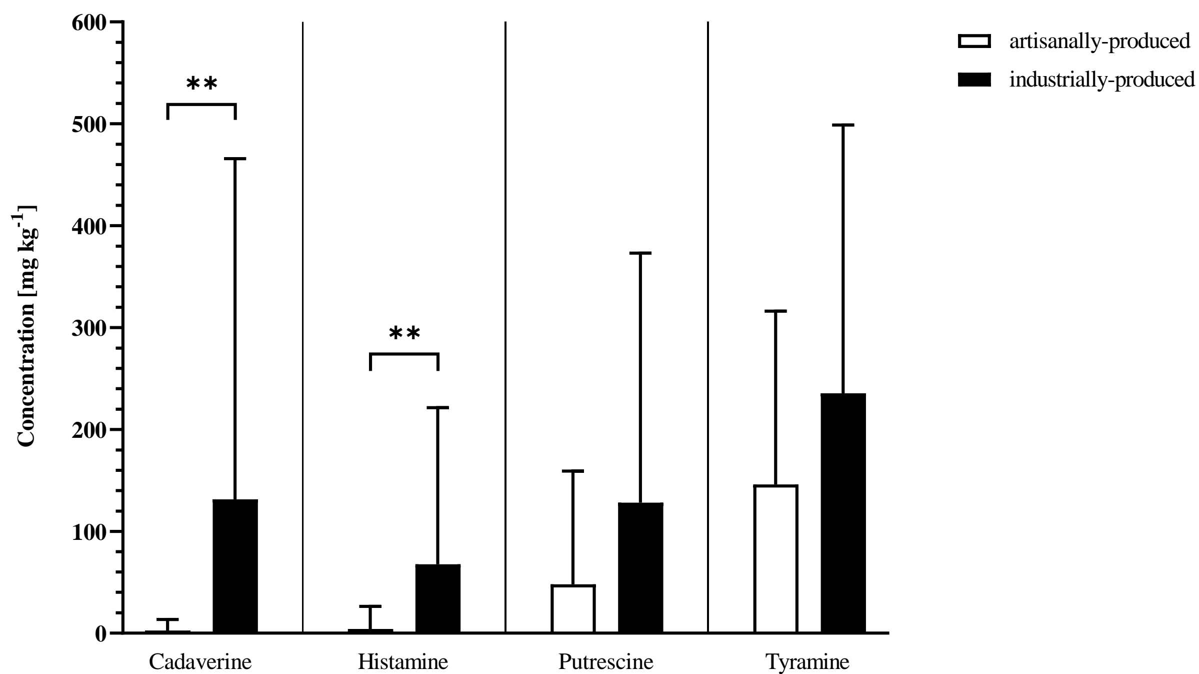

Figure 2.1: Concentration [mg kg^{-1}] of biogenic amines of 36 artisanally-produced salami-type fermented sausages and 26 industrially-produced salami-type fermented sausages. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 2.1: Group number, sample number (No.), meat origin, production method, pH, biogenic amine concentration and cell count of CNS (BP agar), CPS (BP agar), enterococci (KFS agar) and lactobacilli/lactococci (MRS agar) of the 62 fermented salami-type sausage samples bought in Switzerland.

Group No.	No. [c]	Animal	Production method	pH	Biogenic Amine [mg kg^{-1}] [a]				Cell count log$_{10}$ [cfu g^{-1}] [b]			
					TYR	CAD	HIS	PUT	CNS	CPS	enterococci	lactococci/ lactobacilli
1	**1**	Pork/Beef	Art.	5.6	710.32	ND	ND	ND	7.67	ND	2.48	10.11
2	**2**	Sheep	Art.	5.4	474.43	49.41	ND	294.22	5.45	4.26	6.55	8.63
2	**3**	Pork	Art.	4.5	428.66	ND	ND	ND	5.36	ND	4.46	6.75
3	**4**	Pork/Beef	Art.	5.4	360.99	ND	ND	503.72	7.36	ND	3.24	8.48
3	**5**	Pork	Art.	5.1	343.58	ND	ND	309.74	4.41	ND	ND	7.31
3	**6**	Pork/Beef	Art.	5.5	341.90	ND	ND	185.18	7.18	ND	ND	8.74
3	**7**	Goat	Art.	5.8	303.61	ND	132.40	146.40	7.34	ND	5.01	8.24
3	**8**	Pork	Art.	5.1	298.40	ND	ND	ND	3.68	ND	2.48	8.45
3	**9**	Roe Deer	Art.	4.7	281.30	ND	ND	ND	6.24	ND	3.77	6.75
3	**10**	Pork	Art.	5.6	234.26	44.30	13.94	27.37	3.48	ND	ND	7.40
4	**11**	Deer	Art.	5.0	158.35	ND	ND	100.49	3.81	ND	ND	7.46
4	**12**	Pork	Art.	6.1	153.53	ND	ND	ND	8.21	ND	3.04	7.00
4	**13**	Pork	Art.	5.4	150.34	ND	ND	ND	4.43	ND	4.32	7.42
4	**14**	Chamois	Art.	4.7	144.73	ND	ND	ND	6.08	ND	2.52	7.71
4	**15**	Pork	Art.	4.9	144.53	ND	ND	ND	6.30	ND	ND	8.21
4	**16**	Beef	Art.	6.2	140.84	ND	ND	ND	7.83	ND	2.60	8.41
4	**17**	Beef	Art.	5.2	133.75	ND	ND	80.40	5.94	ND	ND	8.49
4	**18**	Pork/Beef	Art.	5.2	125.52	ND	ND	ND	7.44	ND	2.48	8.73
4	**19**	Pork	Art.	4.9	101.23	ND	ND	ND	6.76	ND	6.08	ND
4	**20**	Pork	Art.	6.4	58.60	ND	ND	ND	7.11	ND	5.40	9.03
4	**21**	Pork	Art.	5.0	52.88	ND	ND	ND	5.49	ND	ND	7.55
4	**22**	Pork	Art.	5.0	33.60	ND	ND	ND	5.02	ND	ND	7.79

Group No.	No. [c]	Animal	Production method	pH	Biogenic Amine [mg kg^{-1}] [a]				Cell count log_{10} [cfu g^{-1}] [b]			
					TYR	CAD	HIS	PUT	CNS	CPS	enterococci	lactococci/ lactobacilli
4	**23**	Pork	Art.	5.1	32.17	ND	ND	ND	4.89	ND	4.04	8.82
4	**24**	Wild Boar	Art.	6.8	26.01	ND	ND	ND	10.00	ND	2.00	7.29
4	**25**	Pork	Art.	5.9	19.87	ND	ND	ND	3.95	ND	ND	ND
5	**26**	Pork	Art.	6.1	ND	ND	ND	79.20	ND	ND	ND	5.00
5	**27**	Pork	Art.	4.5	ND	ND	ND	ND	6.28	ND	ND	6.56
5	**28**	Pork	Art.	6.3	ND	ND	ND	ND	7.03	ND	ND	8.32
5	**29**	Deer	Art.	4.8	ND	ND	ND	ND	2.41	ND	ND	8.44
5	**30**	Pork	Art.	5.6	ND	ND	ND	ND	3.13	ND	2.70	7.67
5	**31**	Pork	Art.	4.8	ND	ND	ND	ND	5.95	ND	2.48	8.83
5	**32**	Horse	Art.	4.5	ND	ND	ND	ND	4.24	ND	5.03	7.68
5	**33**	Pork	Art.	4.8	ND	ND	ND	ND	6.76	ND	5.25	9.90
5	**34**	Pork	Art.	4.9	ND	ND	ND	ND	5.51	ND	2.30	8.64
5	**35**	Pork	Art.	5.1	ND	ND	ND	ND	10.00	ND	3.04	9.43
5	**36**	Pork	Art.	4.8	ND	ND	ND	ND	6.92	ND	ND	7.76
1	**37**	Roe Deer	Ind.	5.2	785.22	1266.77	463.77	687.41	4.23	ND	4.96	8.96
1	**38**	Venison	Ind.	5.4	750.23	ND	ND	ND	10.00	ND	2.30	8.09
1	**39**	Pork	Ind.	6.5	708.50	ND	ND	ND	10.00	ND	3.08	8.91
2	**40**	Pork	Ind.	4.7	656.05	336.97	467.90	707.77	2.90	ND	3.58	8.44
2	**41**	Chamois	Ind.	5.4	616.22	964.36	449.96	630.74	3.39	ND	4.33	8.61
3	**42**	Pork/Beef	Ind.	4.8	365.06	ND	ND	ND	6.76	ND	ND	8.41
3	**43**	Pork	Ind.	5.2	364.31	771.69	228.71	431.98	3.43	ND	ND	9.24
3	**44**	Pork	Ind.	5.0	315.33	ND	140.56	567.26	ND	5.47	4.55	8.74
3	**45***	Venison	Ind.	5.2	314.81	27.49	ND	65.14	6.53	ND	ND	8.85
3	**46**	Pork	Ind.	6.3	267.54	ND	ND	ND	10.00	ND	2.00	8.26

Group No.	No. [c]	Animal	Production method	pH	Biogenic Amine [mg kg^{-1}] [a]				Cell count $\log_{10}$ [cfu g^{-1}] [b]			
					TYR	CAD	HIS	PUT	CNS	CPS	enterococci	lactococci/ lactobacilli
3	**47***	Pork	Ind.	5.4	264.58	8.28	ND	22.06	6.72	ND	ND	8.87
3	**48***	Pork	Ind.	5.3	201.73	39.19	9.22	207.25	6.39	ND	ND	8.64
4	**49**	Pork	Ind.	5.9	126.40	ND	ND	2.03	7.07	6.48	3.48	8.76
4	**50**	Pork	Ind.	6.6	76.36	ND	ND	ND	10.00	ND	2.60	9.20
4	**51**	Pork	Ind.	6.1	63.81	ND	ND	3.21	9.00	9.00	2.95	8.67
4	**52**	Pork	Ind.	5.5	57.43	ND	ND	ND	5.95	6.34	ND	8.86
4	**53**	Pork	Ind.	5.8	49.91	ND	ND	ND	6.66	ND	ND	7.63
4	**54**	Pork	Ind.	6.3	44.60	ND	ND	3.89	7.08	ND	ND	9.14
4	**55**	Pork	Ind.	6.1	37.63	ND	ND	ND	5.08	5.15	ND	9.90
4	**56**	Sheep	Ind.	4.8	32.78	ND	ND	ND	3.69	ND	4.23	8.62
4	**57**	Pork	Ind.	5.6	24.57	ND	ND	ND	3.84	ND	ND	6.28
5	**58**	Venison	Ind.	5.4	ND	ND	ND	ND	6.23	ND	ND	7.06
5	**59**	Pork	Ind.	4.8	ND	ND	ND	ND	6.03	ND	2.95	8.54
5	**60**	Pork	Ind.	5.9	ND	ND	ND	ND	7.40	ND	3.61	8.25
5	**61**	Pork	Ind.	7.0	ND	ND	ND	ND	4.97	ND	ND	9.14
5	**62**	Pork	Ind.	5.9	ND	ND	ND	ND	7.34	ND	3.69	8.26

[a] ND, not detectable. Detection limit of biogenic amines is 0.05 mg kg^{-1}

[b] ND, not detectable. Detection limit of cell count for staphylococci and enterococci is 100 cfu g^{-1} (2 $\log_{10}$ cfu g^{-1}) and for lactococci and lactobacilli 10000 cfu g^{-1} (4 $\log_{10}$ cfu g^{-1})

[c] No. 1–36 artisanally-produced salami-type fermented sausage samples and No. 37–62 industrially-produced salami-type fermented sausage samples

* No. 49, 50 and 51 produced in Italy, all others in Switzerland

TYR: tyramine; CAD: cadaverine; HIS: histamine; PUT: putrescine

CNS: coagulase-negative staphylococci; CPS: coagulase-positive staphylococci

Microbial analysis of sausages

Microbial analysis was performed to determine whether there is a putative correlation of different bacterial counts as *tdcA* carriers and tyramine concentration taking in consideration of the pH after maturation. Moreover, a potential dependence of tyramine content on artisanal or industrial type manufacturing was taken into account.

Counts of CNS were between 2.41 to 10.00 log_{10} cfu g^{-1} and 2.90 to 10.00 log_{10} cfu g^{-1} in artisanally-produced (n=36), and industrially-produced (n=26) fermented salami-type sausages, respectively and were not significantly different irrespective of artisanally or industrially-produced ($P > 0.05$) (Table 2.1, Figure 2.2).

Only one sample (Table 2.1) of artisanally-produced sausage contained coagulase-positive staphylococci (CPS), with a level of 4.26 log_{10} cfu g^{-1}. Counts of CPS in industrially-produced sausage were found in five of 26 samples (Table 2.1) and were in a range of 5.15 to 9 log_{10} cfu g^{-1}. CPS in industrial sausages were significantly higher compared to those in artisanal sausages ($P = 0.028$) (Figure 2.2).

Enterococci were detected in 22 of 36 artisanally-produced sausages samples at levels between 2.0 to 6.5 log_{10} cfu $g^{-1}$and were not significantly different ($P > 0.05$) from the counts found in industrially produced ones (2.00 to 4.96 log_{10} cfu g^{-1}, found in 14 of 26 samples) (Table 2.1, Figure 2.2).

Lactococci/lactobacilli were detected in 60 of 62 samples. Counts of lactococci/lactobacilli in artisanally-produced sausages were between 5.00 to 10.11 log_{10} cfu g^{-1}, and were significantly higher ($P = 0.006$) in industrially-produced sausages, with counts of lactococci/lactobacilli of 6.28 to 9.90 log_{10} cfu g^{-1} (Table 2.1, Figure 2.2).

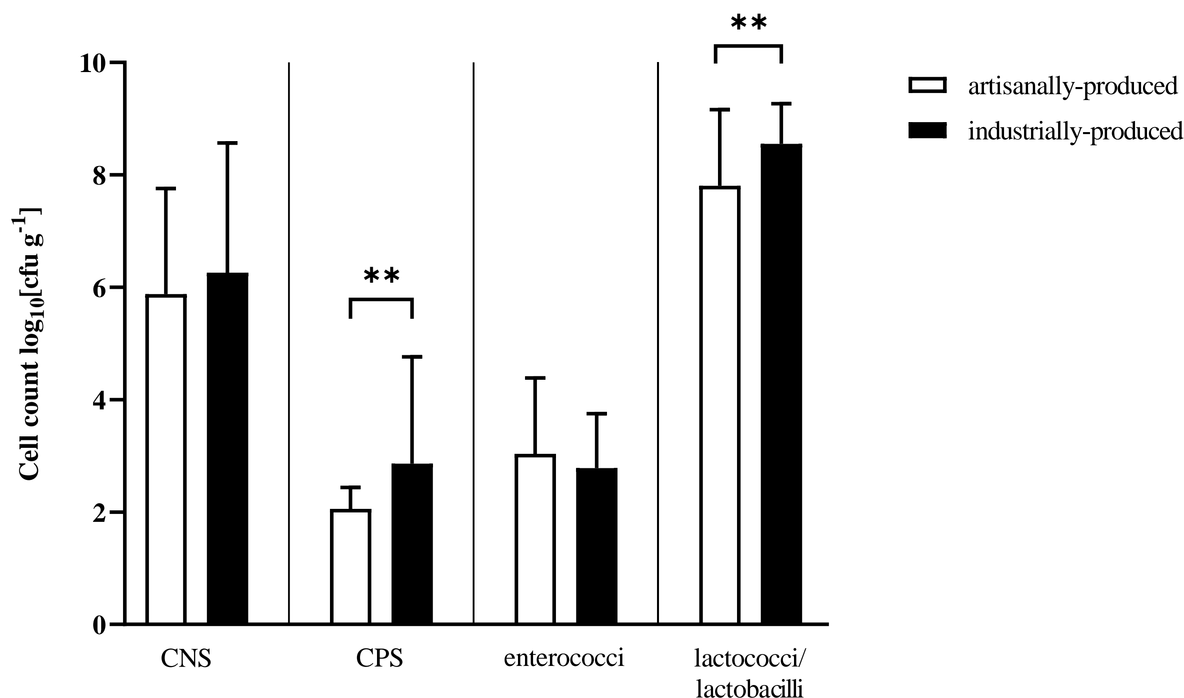

Figure 2.2: Cell count [$\log_{10}$ cfu g^{-1}]of coagulase-negative staphylococci (CNS), coagulase-positive staphylococci (CPS), enterococci and lactococci/lactobacilli of 36 artisanally-produced salami-type fermented sausages and 26 industrially-produced salami-type fermented sausages. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Identification of decarboxylating strains

A total of 329 bacterial isolates were selected from solid media. Of these isolates, 108 were CNS, 18 were CPS, 73 were enterococci, and 130 were lactococci or lactobacilli. Of these, 66 CNS isolates (61.1%) and 54 isolates (74.0%) of enterococci were PCR-positive for the 350-bp *tdcA* amplicon. For all *tdcA* amplicon-positive isolates, decarboxylating activity on the tyrosine-enriched solid medium was confirmed by colour change. Of the 130 analysed lactococci/lactobacilli, only one isolate originating from artisanal production showed a *tdcA*-specific amplification and decarboxylation activity. All CPS isolates were negative for the *tdcA* amplicon, and no decarboxylation activity was found on the tyrosine-enriched medium. Higher frequencies of decarboxylating CNS isolates of 15% (38 of 66) and decarboxylating enterococci of 11% (30 of 54) in artisanally-produced fermented salami-type sausages compared to industrially-produced ones were observed.

16S rRNA gene sequencing analysis of decarboxylase-positive isolates in the analysed sausages revealed that the three most abundant decarboxylating *Staphylococcus* spp. were *S. xylosus* with 45 of

66 (68.2%), *S. carnosus* with 16 of 66 (24.2%) and *S. saprophyticus* with five (7.6%) isolates. For *Enterococcus* spp. the most abundant decarboxylating species were *E. faecalis*, with 44 of 54 (81.5%) followed by *E. faecium* with 10 of 54 (18.5%). The sequenced lactococci/lactobacilli decarboxylating isolate was revealed as *Lactobacillus sakei*.

Factors influencing tyramine accumulation in salami type fermented sausages

The production of BAs can be influenced either directly or indirectly through interaction with the resident microbiota and pH in the meat. Likewise, different microorganisms are able to produce more than one BA; thus, different BAs may occur together in high concentrations. A significant higher ($P = 0.035$) pH value in the final ripened and stored sausages produced by the industrial manufacturing process compared to the pH value after maturation of artisanally-produced sausages could be observed (Table 2.1). Furthermore, the concentration of putrescine was significantly correlated ($P = 0.0407$) with tyramine concentration (Figure 2.3), whereas cadaverine and histamine did not demonstrate a correlation ($P > 0.05$). No significant correlation ($P > 0.05$) between the four tested bacterial groups, and tyramine was observed. Furthermore, the common interaction of enterococci and CNS and its influence on tyramine was also not significant ($P > 0.05$).

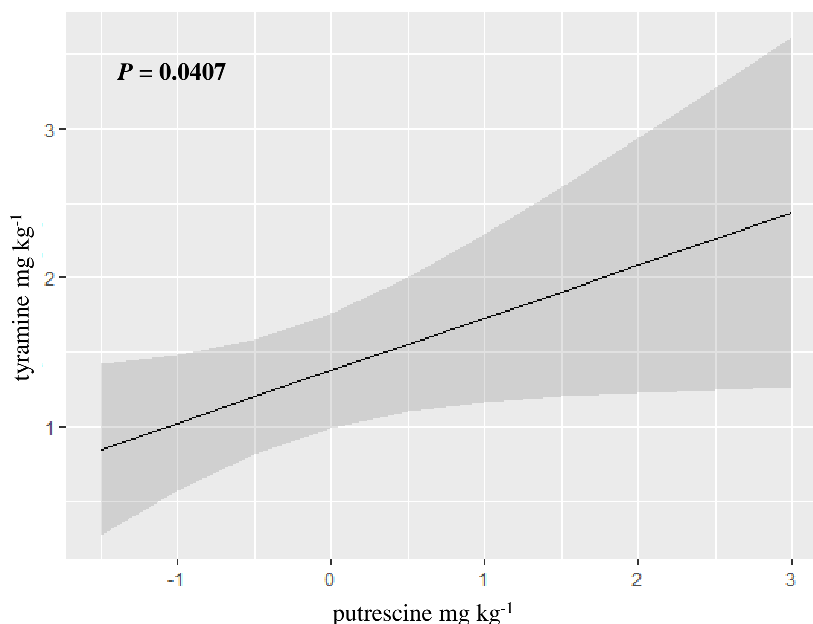

Figure 2.3: Linear regression of the correlation between putrescine and tyramine.

Discussion

Because of their methods of production, fermented sausages have an increased risk of achieving higher levels of BAs. The present study focused mainly on the concentration of tyramine and the direct or indirect influence on microorganisms, pH and other BAs in artisanally- and industrially-produced sausages on tyramine concentration to determine their potential health risk.

In this study, the levels of CNS observed were expected because CNS are often used as starter cultures (Martuscelli et al., 2000). Furthermore, the identified enterococci and CNS were identified as the main representatives of tyramine producers. It is in line with the findings that *S. xylosus* is the most frequently isolated *Staphylococcus* spp. from fermented sausages (Torriani et al., 1994) and that such *S. xylosus* isolates (Martuscelli et al., 2000) and similarly *S. carnosus* (Straub et al., 1995) were reported to produce BAs at a high frequency. Different studies reported that enterococci, particularly *E. faecalis*, are able to produce tyramine (Gardini et al., 2001; Ladero et al., 2012b; Liu et al., 2015). Former studies showed that the majority of enterococci isolated from food carry the *tdcA* gene to produce tyramine (Bover-Cid et al., 2001; Fernández et al., 2007a; Komprda et al., 2008; Ladero et al., 2012b). Furthermore, a higher concentration of tyramine-producing microorganisms was found in artisanally-produced sausages compared to industrially-produced ones. These results underscore the importance of controlling the microbial background of the meat before fermentation.

However, all six CPS-containing samples were above the recommended guidelines of the Swiss Meat Association (SFF), which specifies a level of 1000 cfu g^{-1} in salami-type fermented sausages. Although this may indicate a health risk for *in situ* enterotoxin production (Bellio et al., 2019; Guidi et al., 2018; Schweizer Fleisch Fachverband, 2017), evidence for a health risk from BA production was not found, considering the absence of the *tdcA* gene in all of these CPS isolates.

The finding of the high concentrations of lactococci/lactobacilli was also expected since these two bacterial groups often used as starter cultures (Latorre-Moratalla et al., 2012; Martuscelli et al., 2000; Suzzi and Gardini, 2003).

Tyramine was by far the most abundant BA detected in fermented sausages. There is currently insufficient information for tyramine to establish a no adverse health effect level (NOAEL) in humans. No adverse health effects have been observed in healthy individuals exposed to 600 mg tyramine per person per meal (EFSA, 2011). It is unlikely that this level would be exceeded, even by a combined intake of food sources containing tyramine during the same meal. However, for individuals taking monoamine oxidase inhibitor drugs, the NOAEL is set much lower and is between 6 and 50 mg of tyramine per person per meal (EFSA, 2011; Ladero et al., 2010a). Taking into account, the portion sizes according to the Swiss meat industry (Schweizerische Gesellschaft für Ernährung, 2018) and the observation that 10 of 36 artisanally- and 12 of 26 industrially-produced fermented salami-type sausages have a tyramine content over 200 mg kg^{-1}, consumption of the average amount of approximately 30 g of fermented sausages per meal in Switzerland is above the recommended value if 6 mg tyramine per meal for individuals taking classical monoamine oxidase inhibitors (EFSA, 2011). Therefore, as from a health point of view, only low levels or the absence of tyramine, as in group 4 and 5, would be acceptable. Nevertheless, it was shown that even samples with a tyramine concentration below the detection limit are possible (group 5).

The overall concentration of the four BAs ($P = 0.003$) and the measured pH after maturation in industrial sausages ($P = 0.035$) were significantly higher than in artisanal sausages although it is known, that LAB starters in industrial sausages are usually fast acidifiers to pH 4.8 at the beginning of the ripening process. Even though a low pH at the beginning of ripening time in industrial sausages induced by the starter cultures reduce the growth capacity of spoilage bacteria and possible BA-producers, a low pH might increase the production of BAs as a stress response to increase the pH to the optimum level. Turnover of the tyrosine-tyramine antiporter results in membrane potential of physiological polarity. The decarboxylation of tyrosine to tyramine consumes a proton, which results in an alkalised cytoplasm relative to the external medium. Therefore, the tyrosine decarboxylation pathway plays, because of the alkalinising effect of the decarboxylation reaction, a role in cytoplasmic pH homeostasis and resistance against acid stress (De Palencia et al., 2011; Wolken et al., 2006). Subsequently, pH could have an influence on the decarboxylation activity of the bacteria and consequently on the formation of BAs

(Fernández et al., 2007b, 2004; Moreno-Arribas, 2002) since, in *Enterococcus* and *Staphylococcus* spp., TdcA is most active at a pH near 5 as a consequence of stress (Fernández et al., 2004; Novella-Rodríguez et al., 2004; Ravyts et al., 2010).

In our study, a significant correlation between tyramine and putrescine was found. Other studies have confirmed that different bacteria can produce more than a single BA which can correlate with tyramine formation (Combarros-Fuertes et al., 2015; Gardini et al., 2006; José María Landete et al., 2007; Marcobal et al., 2012; Pugin et al., 2017). Furthermore, studies concerning the Spanish dry fermented sausages Sobrasada, Salsichon, Fuet and Chorizo report that tyramine was generally detected at higher concentrations (up to 600 mg kg^{-1}), while in some samples, putrescine was produced up to 450 mg kg^{-1}, which aligns with our findings in salami-type sausages (Bover-Cid et al., 2000; Hernández-Jover et al., 1997, 1996a, 1996b; Roig-Sagués et al., 1999). The concentration of histamine in our study was low in agreement with other studies in which no or low (below levels at which health issues arise) levels of histamine were detected (Paparella and Tofalo, 2019; Suzzi and Gardini, 2003). This may be attributed to unfavourable growth conditions for *Enterobacteriaceae*, regarded as major histamine producers in food (Halász et al., 1994; Roig-Sagués et al., 1999).

Fermented sausages provide a permissive environment for BA production and accumulation. However, differences have been observed in different sausages, which has been related to bacterial counts in the meat, duration of the ripening process and freshness of the meat source (Suzzi and Gardini, 2003). The use of starter cultures could also influence the production of BAs, either directly or indirectly, through interaction with the resident microbiota originating mainly from the meat and meat processing environment (Linares et al., 2012; Suzzi and Gardini, 2003). The presence and levels of tyramine-producing microorganisms report an essential condition for decarboxylation of tyrosine, but the accumulation of high levels of tyramine also relies on the coincidence of different factors during sausage manufacturing and storage (Joosten and Northolt, 1987; Ladero et al., 2008). In this study, no significant correlation between tyramine concentrations in the 62 fermented sausage samples and the level of a specific population of tyramine-producing microorganisms were found. This is in agreement with previous studies and may be due to two main causes: The use of non-specific methods for identifying

tyrosine-decarboxylating bacteria, and the fact that the capability to produce tyramine is mostly strain- and not species-specific (Linares et al., 2012; Novella-Rodríguez et al., 2010). It should also be taken into account that in this study, only the maturated product was examined, and therefore, it cannot be excluded, that microorganisms responsible for tyramine formation were not viable anymore. Nevertheless, it is essential to note that within the population of CNS, some strains have the potential to produce tyramine, but they are not the only responsible factor for high tyramine concentrations.

Conclusion

In conclusion, the findings of the present study indicate that BA concentrations in fermented sausages show variable levels of accumulation and tyramine being the major BA followed by putrescine. Based on the tyramine content, five groups of fermented sausages were identified. Low concentrations or even the absence of tyramine are regarded as a quality characteristic, whereas the occurrence of tyramine in a significant concentration is considered to be an adverse health effect and as an indicator of poor hygiene. Therefore, more than one-third of the analysed sausages are classified as unacceptable from a health and hygienic point of view (group 1, 2 and 3). Contrary to the assumptions, artisanal producers may not fulfil the requirements of food safety standards, artisanally-produced sausages showed high-quality products. Despite the higher level of tyramine-producing microorganisms in the artisanal sausages, industrially-produced ones have a higher concentration of all four measured BAs. This leads to the conclusion that not only the amount of the *tdcA*-positive resident microbiota of the meat influences tyramine concentration but also environmental as well as physicochemical factors. Since a higher pH was generally measured in industrially-produced sausages after maturation, a relationship between tyramine concentration and pH can be suspected with the explanation, that a fast decrease of the pH at the beginning of the fermentation leads to an activation of the tyramine production to increase the pH again. Therefore, the absence of *tdcA*-positive strains should be a criterion for selecting starter culture strains, along with the lack of ability to produce other BAs. Furthermore, high-quality raw materials and optimal technological conditions are critical factors to reduce tyramine production and accumulation in fermented sausages.

Acknowledgements

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Chapter 3

Determination of Biogenic Amines, Tyramine-Producing Bacteria and Factors influencing Tyramine Accumulation in Cheeses from Switzerland

Janine Anderegg, Dominik Hug, Céline Jenni, Alfonso Die, Christophe Lacroix and Leo Meile*

Laboratory of Food Biotechnology, Institute of Food, Nutrition and Health, ETH Zurich, Zurich, Switzerland

*Corresponding author

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Abstract

Cheese provides ideal conditions for the formation of biogenic amines (BA). Therefore, we investigated 274 cheese samples on decarboxylating bacterial strains and their content of the BAs cadaverine, histamine, putrescine and tyramine. Tyramine was by far the most abundant BA with concentrations up to 984.38 mg kg^{-1}, followed by cadaverine with a maximum tested concentration of 872.09 mg kg^{-1}. Histamine and Putrescine were found in fewer samples with a concentration of up to 859.85 mg kg^{-1} and 538.82 mg kg^{-1}, respectively. Moreover, the concentration of cadaverine in cheese was significantly correlated with the concentration of tyramine. Tyrosine decarboxylase-positive enterococci were with a prevalence of 78.04% the major tyramine producers in cheese and showed a significant correlation with tyramine concentration. Nevertheless, not only tyrosine decarboxylase-positive enterococci are responsible for a high tyramine concentration. It was shown that the factors of milk treatment, milk origin, and ripening time affecting BA biosynthesis and accumulation in cheese. A higher concentration of each of the four measured BAs was found in semi-hard and hard cheese made from raw milk. The obtained results open up the possibility to include this knowledge in cheese production to prevent BA accumulation.

Introduction

Cheesemaking is one of the major industries worldwide to enhance shelf life from milk (Kindstedt, 2018). Production methods of cheese are specified with varying degrees of details, e.g. the use of starter cultures, thermal milk treatment, ripening period, and the type of rennet. Primary or starter cultures are mainly lactic acid bacteria (LAB) which had been selected and added to the milk before cheese making and are responsible for the production of lactic acid from lactose during ripening. (Parente et al., 2017). Milk treatment processes influence the bacterial composition. Contrary to pasteurisation, thermisation at lower temperatures reduces only part of the microbiota but leaves the raw milk's properties unchanged (Panthi et al., 2017). Surviving microbes may metabolise during cheese ripening, e.g. in proteolysis as a fundamental process, which covers a series of biochemical events like the breakdown of casein to peptides and free amino acids (Ardö et al., 2017).

Biogenic amines (BA) are formed by microbial decarboxylation of free amino acids. BAs belong to a group of basic nitrogenous compounds, and according to their chemical structure, they can be classified as heterocyclic (histamine and tryptamine), aliphatic (cadaverine, putrescine, spermine and spermidine) or as aromatic (tyramine and phenylethylamine) (Linares et al., 2011). The most important BAs, because of their health effect and frequent occurrence in fermented foods, are cadaverine, histamine, putrescine and tyramine derived from decarboxylation of the free precursor amino acids lysine, histidine, ornithine and tyrosine, respectively (Fernández et al., 2007b). BAs can be found in fermented food products including fermented meat and fish (Moon et al., 2013; Suzzi and Gardini, 2003), dairy products (Linares et al., 2011), fermented vegetables (Lee et al., 2019) and fruits (Moreno-Arribas, 2002) and can thereby pose a risk of food intoxication (Ladero et al., 2012b). For example, histamine and tyramine can cause severe allergic reactions including an increase of cardiac output, respiration, high blood pressure, and blood glucose levels; and release of norepinephrine (Broadley, 2010; EFSA, 2011). Cadaverine and putrescine have a much lower toxicological impact compared to histamine and tyramine but can potentiate the toxic effect of other amines (Combarros-Fuertes et al., 2015).

Since milk is not a sterile product and the casein proteolysis in cheese ensures the availability of free substrate amino acids, BAs in cheese can reach a level up to 2000 mg kg^{-1} (Fernández et al., 2007a).

However, the BA content varies not only for different cheeses but also for the same variety due to differences in milk treatment and microbial milk quality regarding microbes (Novella-Rodriguez et al., 2006). The milk resident microbiota can influence the production of BAs directly or indirectly, either through interaction among each other or through the pH in the cheese. Moreover, different microorganisms are able to produce more than one BA. However, the accumulation of high levels of BAs depends not only on the presence of BA-producing microorganisms but also relies on a minimum of bacterial titres and the coincidence of different factors during cheese production and storage (Joosten and Northolt, 1987; Ladero et al., 2008).

Among fermented food products, tyramine appears with the highest frequency in cheese. Therefore, the term "cheese reaction" was coined to refer to the symptoms sometimes seen after cheese consumption (Fernández et al., 2006; Ladero et al., 2012b). Tyramine is produced by a variety of LAB including enterococci, carnobacteria, lactobacilli and lactococci and by some staphylococci. All of these bacteria may be present during the manufacture of cheese as either part of the starter cultures or may be introduced by contamination before, during or after processing (Linares et al., 2012; Torriani et al., 2016). The presence of tyrosine decarboxylase (TdcA, EC 4.1.1.25) and a transporter protein for tyrosine/tyramine interchange, is needed for tyramine production (Linares et al., 2011; Marcobal et al., 2012). The corresponding genes are clustered in an operon called TDC or TYR, on the chromosome or plasmids of tyramine producer strains (Fernández et al., 2004; Mohedano et al., 2015). One of the genes called *tdcA* or *tdc* encodes the tyrosine decarboxylase, while the gene *tyrP* encodes the tyrosine-tyramine antiporter. A gene responsible for the regulation of tyrosine decarboxylase is sometimes also part of the operon (Linares et al., 2011). This genetic setup and the fact that the production of tyramine is strain-specific and not species-specific supports the hypothesis of horizontal gene transfer (Bonnin-Jusserand et al., 2012; Linares et al., 2011; Marcobal et al., 2012). The tyrosine decarboxylase has been characterised extensively in a few LAB species, including *Enterococcus faecalis*, *E. faecium*, *Lactobacillus brevis* and *Lb. curvatus* strains (Linares et al., 2011).

The main source of decarboxylase-positive bacteria and therefore of BA accumulation in cheese is raw milk. Raw milk contains pyridoxal phosphate that can act as a co-factor for decarboxylase activity, and

it is inactivated by milk pasteurisation (Combarros-Fuertes et al., 2015). Pasteurisation is one of the main technological factors that can reduce the accumulation of tyramine or in general the accumulation of BAs, in cheese by heating up the milk to inhibit the growth of the bacteria (Ladero et al., 2010b). Another crucial factor for tyramine synthesis is the availability of the substrate tyrosine (Linares et al., 2011). While starter LAB contribute to the protein breakdown, the non-starter LAB are responsible for the peptinolysis and the release of free amino acids (Ardö et al., 2017). Furthermore, the microbiota of the milk must be carriers of the TDC operon to decarboxylate tyrosine to tyramine (Linares et al., 2011). A further factor involved in tyramine production is the pH of the media. Several studies reported, that an acidic environment favour tyramine formation and tyrosine decarboxylase enzymes have an optimum pH around 5 (Fernández et al., 2007b; Gardini et al., 2001; Moreno-Arribas, 2002).

In this study, bacterial counts of potential BA producers and concentrations of individual BAs in ripened cheese samples produced in Switzerland and available on the Swiss market were examined to determine the most relevant BAs in cheese products and to enhance the knowledge of tyramine formation and its factors influencing the accumulation in cheese. Special emphasis was given to tyramine and in particular, the correlation between tyramine concentration in different cheeses and the different factors that influence concentration. These factors include ripening time, milk treatment, milk origin, seasons, pH, the concentration of enterococci, lactobacilli, lactococci and staphylococci and the other BAs cadaverine, histamine and putrescine.

Material and Methods

Cheese samples

The experimental work contains the analysis of 274 Swiss cheese samples all produced in Switzerland and available in the Swiss market. The selection and number of cheese samples of the same type of production analysed are very heterogeneous as they represent the occurrence on the Swiss market and are either bought in a supermarket or obtained from different cantonal laboratories from Switzerland (Table 3.1).

Table 3.1: Number and distribution of the 274 cheese samples examined. 224 of 274 cheese samples were made from cow's milk, 30 from goat's milk and 20 from sheep's milk.

Ripening time	Total	Raw milk			Thermised milk			Pasteurised milk		
		Cow	Goat	Sheep	Cow	Goat	Sheep	Cow	Goat	Sheep
Cream cheese	3	0	0	0	0	1	0	1	1	0
Soft cheese	37	5	2	3	6	3	2	7	8	1
Semi-hard cheese	195	141	6	2	29	3	0	8	3	3
Hard Cheese	39	25	1	4	0	0	2	2	2	3

Enumeration and isolation of dominant microorganisms

To prepare the samples for the enumeration and isolation of dominant microorganisms, the cheese rind was removed, and 10 g of each sample was homogenised with 90 mL peptone solution (9% NaCl, 1% peptone from casein, pH 7) using a Stomacher Bag Mixer (BagMixer 400 P; Interscience, Saint Nom, France). Dilutions were prepared, and aliquots of 0.1 mL were spread in duplicate on different semi-selective agar media. Semi-selective media were used to enumerate specific bacteria, including Baird-Parker agar (BP; Biolife, Milano, Italy) for *Staphylococcus* spp., KF *Streptococcus* agar (KFS, Becton Dickinson, Allschwil, Switzerland) for *Enterococcus* spp., and De Man, Rogosa and Sharpe agar (MRS, Biolife, Milano, Italy) for *Lactobacillus* and *Lactococcus* spp. BP and KFS agar were incubated aerobically for 48 h at 37°C and 43°C, respectively whereas MRS agar was incubated anaerobically in 2.5 L culture jars with AnaeroGen AN 25 sachets (Oxoid, Basingstoke, UK) for 48 h at 37°C. The detection limit was 2 $\log_{10}$ cfu g^{-1} for BP and KFS and 4 $\log_{10}$ cfu g^{-1} for MRS colonies. Three randomly

selected single colonies of *Staphylococcus* spp., *Enterococcus* spp. and *Lactobacillus*/*Lactococcus* spp. were picked from different semi-selective agar media containing 10–300 cfu. Afterwards, isolates were streaked out for purification onto the corresponding agar media for following DNA isolation, genotypic identification and identification of the decarboxylating ability.

Identification of bacterial isolates and tyrosine-decarboxylating ability

Bacterial DNA isolation from single colonies was done using the Goldenberger B extraction method (Goldenberger et al., 1995). A multiplex-PCR for the identification of *Enterococcus* spp. was performed using their primers to generate amplicons of 215 bp for *Enterococcus faecium*, 295 bp for *Enterococcus durans*, 360 bp for *Enterococcus faecalis*, and 733 bp for the genus *Enterococcus* (Jackson et al., 2004). The expected size of the amplicons was visualised after electrophoresis in a 2% agarose gel.

All other isolates were identified by 16S rRNA gene sequencing. The target DNA was amplified using the primer pair bak4 (5'-AGGAGGTGATCCARCCGCA-3') and bak11w (5'-AGTTTGATCMTGGC TCAG-3'), with an expected amplicon size of approximately 1500 bp (Dasen et al., 1998). For amplification of the fragments, the PCR mixture (20 µL) was composed of 10 µL PCR Master Mix (2X) (Thermo Scientific, Reinach, Switzerland), 1 µM concentrations of each primer, 1 µL of extracted DNA and Milli-Q water. PCR analysis was conducted in a Biometra Professional Basic Gradient thermocycler (Biometra GmbH, Göttingen, Germany), under the following conditions: initial denaturation at 95°C for 5 min followed by 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 2 min; and a final extension at 72°C for 7 min. PCR amplicons were purified by the use of the Promega Wizard® SV Gel and PCR Clean-Up System (Promega, Fitchburg, USA) and DNA was analysed by Sanger sequencing at GATC (Konstanz, Germany).

The identification of the tyrosine decarboxylase capability in bacterial isolates was done pheno- and genotypically using the screening plate method (S. Bover-Cid & Holzapfel 1999) and by PCR. Strains were streaked on the pH-induced medium plates containing tyrosine and bromocresol purple as an indicator and were incubated for 2 days at 37°C under aerobic conditions for *Enterococcus* spp. and *Staphylococcus* spp., and under anaerobic conditions for *Lactobacillus*/*Lactococcus* spp.

The genotypic identification of the *tdcA* gene was done by PCR using the primer pair DEC3 (5'-CCGCCAGCAGAATATGGAAYRTANCCCAT-3') and DEC5 (5'-CGTTGTTGGTG TTGTTGGCACNACNGARGARG-3') developed by Torriani et al., (2016), which were tested for different presumable *Enterococcus* spp., *Lactobacillus* spp. and *Staphylococcus epidermidis* isolates. The expected size of about 350 bp of the amplicon was visualised after electrophoresis in a 2% agarose gel.

Determination of biogenic amines

For quantitative determination of BAs, ion exclusion chromatography with pulsed amperometric detection (IC-PAD) was used (Dionex, 2007) with modified changes in the eluent gradient (5–10 mM MSA from 0–6 min, 10–20 mM from 6–16 min, and 20–45 mM from 16–20 min). The IC-PAD apparatus used was Dionex™ ICS-5000+ Hybrid HPIC™ system and IonPac CG18, 2 x 50 mm were used as a precolumn and IonPac CS18, 2 x 250 mm as separation column, respectively (Thermo Fisher Scientific, Switzerland). To prepare the samples for determination of BAs, the cheese rind was removed, and 5 g of each sample was homogenised for 10 min with 22.5 mL 0.2 molar perchloric acid (Sigma-Aldrich, Buchs, Switzerland) and 22.5 mL acetonitrile (Sigma-Aldrich, Buchs, Switzerland) in a stomacher (BagMixer 400 P; Interscience, Saint Nom, France). After homogenisation, the samples were incubated for 30 min at room temperature and subsequently, 10 mL of the homogenate was transferred and centrifuged for 10 min at 6,000 x g at 4°C. A volume of 1.5 mL of the supernatant was transferred into a new tube and centrifuged again for 10 min at 10,000 x g at 4°C. The supernatant was stored at -20°C and used in a 1:50 dilution for the measurement of BAs.

Statistical analysis

All samples were prepared and analysed in duplicate. Statistical analysis with linear regression with interaction was performed using the statistic software R (R Core Team) using the lme4, sjplot, sjmisc and ggplot 2 packages and the statistical analysis of cell counts and concentrations of BAs were performed using the statistic software SigmaPlot 13 (Systat Software Inc., San Jose, California, USA).

A one-way unpaired Mann-Whitney Rank Sum Test ($P < 0.05$) and an all pairwise multiple comparison procedures (Dunn's Method) was used for calculations in SigmaPlot 13. All means of technical replications were first calculated prior to comparison. Samples with concentrations of BAs and cell counts below the detection limit were replaced with artificial values of 0.049 for the concentration of all four BAs and 99 for cell counts of *Staphylococcus* spp. and *Enterococcus* spp. and of 9999 for cell counts of *Lactococcus*/*Lactobacillus* spp. respectively, to allow statistical analysis (Garriga et al., 2002; Meier et al., 2018).

Results

Microbial counts of cheese analysis

The sources of relevant BA producers were expected to originate from dominant bacteria (starter cultures) in cheese samples and single non-dominant species regarded as contaminants often reaching remarkable titers. Therefore, 274 cheese samples were analysed by plating dilutions on semi-selective agar-media to determine the content of *Staphylococcus* spp., *Enterococcus* spp. and *Lactobacillus*/ *Lactococcus* spp. in considering the three different parameters milk treatment, milk origin, and ripening time, in cheese. Total counts of coagulase-negative *Staphylococcus* spp. (CNS) and coagulase-positive *Staphylococcus* spp. (CPS) were in the range of 2.00 to 6.91 $\log_{10}$ cfu g^{-1} and 2.00 to 6.15 $\log_{10}$ cfu g^{-1}, respectively (Table 3.2A) and each of them did not differ significantly ($P > 0.05$) in the three parameter groups of milk treatment, ripening time or milk origin (Table 3.3). In general, the factor “thermised milk” led to the highest titers of CPS with a mean value of 2.36 $\log_{10}$ cfu g^{-1} and was found in 13 of 46 thermised cheese samples (28.26%).

Table 3.2: Lowest and highest number, mean and median of (A) cell counts of CNS (BP agar), CPS (BP agar), enterococci (KFS agar) and lactobacilli/lactococci (MRS agar), and (B) of biogenic amine concentrations of the 274 analysed cheese samples.

A	lowest amount $\log_{10}$ cfu g^{-1}	highest amount $\log_{10}$ cfu g^{-1}	Mean $\log_{10}$ cfu g^{-1}	Median $\log_{10}$ cfu g^{-1}
CNS	2.00	6.91	3.44	3.39
CPS	2.00	6.15	2.21	2.00
enterococci	2.00	7.54	3.84	3.79
lactobacilli/lactococci	4.00	9.53	6.91	7.18
B	**lowest amount mg kg^{-1}**	**highest amount mg kg^{-1}**	**Mean mg kg^{-1}**	**Median mg kg^{-1}**
Cadaverine	0.05	872.09	42.09	5.30
Histamine	0.05	859.85	29.01	0.05
Putrescine	0.05	538.82	11.55	0.05
Tyramine	0.05	984.38	64.08	14.13

Colony counts of *Enterococcus* spp. were detected in 206 of 274 cheese samples (75.18%) in the range of 2.00 to 7.54 log_{10} cfu g^{-1} (Table 3.2A). In all of the three analysed parameter milk treatment, milk origin and ripening time, the difference of numbers of *Enterococcus* spp. were significant ($P < 0.05$) (Table 3.3). In the parameter of milk treatment, *Enterococcus* spp. showed a significantly higher ($P = 0.012$) number with a mean of 4.04 log_{10} cfu g^{-1} in thermised compared to pasteurised milk with a mean of 3.10 log cfu g^{-1}. The number of *Enterococcus* spp. were also significant between raw and pasteurised milk ($P = 0.004$) with a mean of 3.94 log_{10} cfu g^{-1} and 3.10 log_{10} cfu g^{-1}, respectively. Furthermore, there was a significantly higher ($P = 0.001$) number of enterococci in cow milk with a mean of 3.94 log_{10} cfu g^{-1} compared to sheep milk with a mean of 2.56 log_{10} cfu g^{-1} as well as in the number of enterococci between the factor goat and sheep milk ($P = 0.020$) with a mean of 3.93 log_{10} cfu g^{-1} for goat milk. Also, a significantly higher ($P = 0.001$) number of *Enterococcus* spp. was found between the group of semi-hard cheese with a mean of 4.08 log_{10} cfu g^{-1} and the group of hard cheese with a mean of 3.08 log_{10} cfu g^{-1}. In general, *Enterococcus* spp. were with approximately 80% much more prevalent in cheeses from raw and thermised milk compared to cheeses from pasteurised milk with a prevalence of 45%.

Lactococcus/*Lactobacillus* spp. were found in 226 of 274 cheese samples (82.48%) and were between 4.00 to 9.53 log_{10} cfu g^{-1} (Table 3.2B). In the parameter of milk treatment, *Lactococcus*/ *Lactobacillus* spp. showed a significantly higher ($P = 0.026$) number between thermised, with a mean of 7.41 log_{10} cfu g^{-1} compared to raw milk with a mean number of 6.81 log_{10} cfu g^{-1}. Furthermore, in the parameter of ripening time, a significantly higher ($P < 0.001$) number of *Lactococcus*/*Lactobacillus* spp. was found in soft cheese samples with a mean of 7.39 log_{10} cfu g^{-1}, than in hard cheese samples with a mean of 5.78 log_{10} cfu g^{-1}. Likewise, a significantly higher ($P < 0.001$) number was found in semi-hard cheese samples with a mean of 7.06 log_{10} cfu g^{-1} than in hard cheese samples with a mean value of 5.78 log_{10} cfu g^{-1}.

Table 3.3: Mean and significance of CNS, CPS, enterococci and lactobacilli/lactococci counts and the four BA concentrations of cadaverine, histamine, putrescine and tyramine in the three different factors milk treatment, milk origin and ripening time of the 274 analysed cheese samples.

	Milk treatment			Milk origin			Ripening time			
	Raw	Thermised	Pasteurised	Cow	Goat	Sheep	Cream Cheese	Soft Cheese	Semi-Hard Cheese	Hard Cheese
	Mean log_{10} cfu g^{-1} (t-test $P < 0.05$)			Mean log_{10} cfu g^{-1} (t-test $P < 0.05$)			Mean log_{10} cfu g^{-1} (t-test $P < 0.05$)			
CNS	3.43	3.54	3.41	3.50	3.27	3.09	2.00	3.37	3.56	3.03
CPS	2.20	2.36	2.07	2.22	2.23	2.00	2.00	2.11	2.26	2.06
enterococci	3.94 **	4.04 *	3.10 * **	3.94 ***	3.93 *	2.56 *** *	3.61	3.41	4.08 ***	3.08 ***
lactobacilli/ lactococci	6.81 **	7.41 **	6.78	6.93	7.08	6.38	5.57	7.39 ***	7.06 ***	5.78 *** ***

	Milk treatment			Milk origin			Ripening time			
	Raw	Thermised	Pasteurised	Cow	Goat	Sheep	Cream Cheese	Soft Cheese	Semi-Hard Cheese	Hard Cheese
	Mean mg kg^{-1} (t-test $P < 0.05$)			Mean mg kg^{-1} (t-test $P < 0.05$)			Mean mg kg^{-1} (t-test $P < 0.05$)			
Cadaverine	38.79	30.46	72.69	47.38 ***	26.90	5.70 ***	0.05	40.34	49.21	11.40
Histamine	38.26	13.13	2.02	33.67	2.79	16.16	0.05	0.05 *	38.48 *	11.37
Putrescine	13.97	4.05	8.51	10.54	6.91	29.74	0.05	2.01	13.12	13.62
Tyramine	74.47	43.27	37.35	56.63	106.44	84.03	3.22	44.51	69.36	60.97

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Identification of tyrosine-decarboxylating isolates

Since tyramine can cause severe health reactions and tyramine is a major BA in cheese, a total of 1608 bacterial isolates, segregated from the 274 cheese samples, were analysed geno- and phenotypically for the capacity to produce tyramine. Of these 1608 isolates, 465 were CNS, 9 CPS, 592 enterococci and 542 were lactobacilli/lactococci. Of these, 93 CNS isolates (20.0%), 5 CPS (55.6%), and 462 isolates (78.04%) of enterococci were PCR-positive for the 350-bp *tdcA* amplicon and their decarboxylating activity on the tyrosine-enriched agar medium. Of the 542 lactobacilli/lactococci isolates, only 12 (2.21%) showed a *tdcA*-specific amplification paired with a phenotypic decarboxylation activity. All of the six analysed bacterial isolates from the parameter cream cheese were *tdcA*- and phenotypically decarboxylase-positive. The four parameters raw milk, thermised milk, cow milk, and semi-hard cheese showed with 38.83% for raw milk, 35.37% for thermised milk, 40.68% for cow milk, and 39.56% for semi-hard cheese a high prevalence of *tdcA*-positive and phenotypically decarboxylating isolates. The parameter of pasteurised milk, goat milk, sheep milk, soft cheese, and hard cheese showed with 21.92% for pasteurised milk, 25.97% for goat milk, 14.71% for sheep's milk, 23.48% for soft cheese, and 26.37% for hard cheese a lower prevalence of *tdcA*-positive and phenotypically decarboxylating isolates.

The analysis of tyrosine decarboxylase-positive isolates performed by 16S rRNA gene sequencing showed that within enterococci, the most common *tdcA* gene-positive subspecies was *E. faecalis,* followed by *E. faecium* and rarely *E. durans*. A large variety of staphylococci containing the *tdcA* gene was found, mostly *S. saprophyticus*, *S. xylosus, S. simulans,* and *S. equorum*. Nevertheless, the *tdcA* gene could also be detected in *S. haemolyticus*, *S. vitulinus, S. warneri,* and S. *epidermidis*. Among the five analysed CPS, which were *tdcA* gene-positive, all belong to *S. aureus*. Eleven of twelve analysed lactobacilli/lactococci were *Lactococcus lactis* isolates, and one sample belonged to *Lactobacillus parabuchneri*.

Content and distribution of biogenic amines in different cheeses

All 274 cheese samples were analysed for BA content. All four analysed BAs cadaverine, histamine, putrescine and tyramine were detected in 27 of 274 (9.85%) while 64 of 274 (23.26%) cheese samples did not contain all of these BAs.

With a concentration up to 984.38 mg kg^{-1}, tyramine was by far the most abundant BA and was found in 165 (60.22%), followed by cadaverine, with a concentration up to 872.09 mg kg^{-1} in 160 (58.39%) cheese samples. Histamine was detected only in 63 (22.99%) and putrescine in 78 (28.47%) cheese samples and were therefore considered as minor BAs. Concentrations of histamine and putrescine ranged between 0.05 and 859.85 mg kg-1 for histamine and between 0.05 and 538.82 mg kg^{-1} for cadaverine, respectively (Table 3.2B).

Differences between the four BAs were observed depending on the type of cheese, milk treatment or milk origin. In the parameter of milk treatment, there was no significant correlation ($P > 0.05$) between BAs and the different treatment factors. Nevertheless, the factor raw milk was involved with the highest concentration of histamine, putrescine and tyramine with a mean value of 38.26 mg kg^{-1}, 13.97 mg kg^{-1}, and 74.47 mg kg^{-1}, respectively. The highest concentration of cadaverine was linked to the factor of pasteurised milk with a mean of 72.69 mg kg^{-1} (Table 3.3). In the parameter of milk origin, the factor cow milk showed the highest concentration of cadaverine and histamine with a mean value of 47.38 mg kg^{-1} and 33.67 mg kg^{-1}, respectively, whereas tyramine has the highest concentration in goat milk with a mean of 106.44 mg kg^{-1} (Table 3.3). In the parameter of ripening time, the highest concentration of cadaverine with a mean value of 49.21 mg kg^{-1} was detected in the factor of semi-hard cheese. Also in the factor of semi-hard cheese, a significantly higher ($P = 0.039$) concentration of histamine with a mean of 38.48 mg kg^{-1}, compared to soft cheese with a mean value of 0.05 mg kg^{-1} was found. Furthermore, the factor semi-hard cheese represented the highest concentration of tyramine with a mean of 69.36 mg kg^{-1} whereas cream cheese contains only tyramine in a small concentration with a mean of 3.22 mg kg^{-1} (Table 3.3).

Factors influencing tyramine accumulation in cheese

The production of tyramine can be influenced either directly or indirectly through interaction with the resident microbiota or the pH in the cheese. Likewise, particular microorganisms might be able to produce more than one BA; thus, different BAs occur together with tyramine. Table 3.4 and the supplementary Figure S 3.1–Figure S 3.25, summarises the *P*-value of the correlation of pH; the four bacterial groups (CNS, CPS; enterococci and lactobacilli/lactococci), and the three BAs cadaverine histamine and putrescine on tyramine. Furthermore, Table 3.4 presents the common interaction of enterococci and CNS and its influence on tyramine and the *P*-value of the correlation of tyramine and the interaction of pH and the four bacterial groups, the common interaction of enterococci and CNS, and finally, the three monitored BAs.

Table 3.4: Significant correlations (*P*-values) of interaction parameters with tyramine in the three different factors milk treatment, milk origin and ripening time.

	Milk treatment			Milk origin			Ripening time			
	Raw	Thermised	Pasteurised	Cow	Goat	Sheep	Cream Cheese	Soft Cheese	Semi-Hard Cheese	Hard Cheese
	P-Value			*P*-Value			*P*-Value			
pH	0.2871	0.4460	0.2840	0.2782	0.0375 *	0.8320	NA	0.0129 *	0.1774	0.1295
CNS	0.1964	0.8853	0.1002	0.6773	0.9306	0.8390	NA	0.1463	0.9078	0.2272
CPS	0.9726	0.6469	0.2883	0.7924	0.0451 *	0.8290	NA	0.1307	0.8576	0.0763
enterococci	1.11E-06 ***	0.0019 **	0.2628	1.55E-06 ***	0.2805	0.8270	NA	0.0466 *	8.61E-05 ***	0.0097 **
lactobacilli/ lactococci	0.4264	0.2348	0.2867	0.6347	0.2555	0.4840	NA	0.0307 *	0.4942	0.9578
enterococci vs. CNS	0.7113	0.7579	0.0880	0.6253	0.0528	0.8380	NA	0.0110 *	0.1964	0.2891
Cadaverine	0.0438 *	0.0400 *	0.5477	0.0014 **	0.5437	0.8320	NA	0.0476 *	0.0122 *	0.2870
Histamine	7.08E-04 ***	0.0501	0.9716	0.0091 **	0.6144	0.8360	NA	NA	0.0241 *	0.7255
Putrescine	0.0817	0.4056	0.2720	0.1919	0.5778	0.8560	NA	0.1305	0.0248 *	0.6822
pH vs. CNS	0.1127	0.8793	0.2582	0.2707	0.7184	0.8130	NA	0.2244	0.0023 **	0.4833
pH vs. CPS	0.4667	0.2484	0.2876	0.8091	0.0425 *	0.8300	NA	0.2104	0.8964	0.1013
pH vs. enterococci	0.0590	0.3209	0.2771	0.2451	0.8948	0.9320	NA	0.4711	0.4813	0.3304

	Milk treatment			Milk origin			Ripening time			
	Raw	Thermised	Pasteurised	Cow	Goat	Sheep	Cream Cheese	Soft Cheese	Semi-Hard Cheese	Hard Cheese
	P-Value			*P*-Value			*P*-Value			
pH vs. lactobacilli / lactococci	0.5347	0.4175	0.3167	0.7087	0.6089	0.7650	NA	0.3091	0.4427	0.4423
pH vs. enterococci & CNS	0.4177	0.4730	0.1458	0.8092	0.0306 *	0.8220	NA	0.0333 *	0.0350 *	0.3850
pH vs Cadaverine	0.2544	0.1449	0.2565	0.5209	0.0926	0.8460	NA	0.8156	0.5938	0.2881
pH vs Histamine	0.7856	0.0816	0.9698	0.8829	0.9500	0.8380	NA	NA	0.7033	0.1130
pH vs Putrescine	0.2247	0.6905	0.0981	0.8918	0.4436	0.8490	NA	0.1012	0.2642	0.8268

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

NA: not analysed

In the parameter of milk treatment enterococci counts ($P < 0.001$), the concentration of cadaverine ($P = 0.0438$) and histamine ($P < 0.001$) in raw milk cheese samples were significantly correlated with tyramine concentration, and a similar observation was found in cheese samples from thermised milk, but without a significant correlation with histamine concentration. In the group of cheese samples from pasteurised milk, no significant correlation ($P > 0.05$) between the different factors and tyramine was detected (Table 3.4).

In the parameter of milk origin, as in raw milk, tyramine concentrations of cow milk were significantly correlated with enterococci counts ($P < 0.001$), cadaverine ($P = 0.0014$) and histamine ($P = 0.0091$) concentrations.

Furthermore, in the parameter of goat milk, the number of CPS ($P = 0.0451$), the interaction of pH and CPS ($P = 0.0425$), as well as the common interaction of pH with enterococci and CNS ($P = 0.0306$) were significantly correlated to tyramine concentration. On the contrary, in the group of sheep milk, there was no significant correlation ($P > 0.05$) between the different analysed factors and tyramine concentration (Table 3.4).

In the parameter of milk origin as well as in the parameter of ripening time, significant correlations of $P = 0.0375$ and $P = 0.0129$ were found between pH and tyramine concentration in goat milk and soft cheese, respectively. Furthermore, in the parameter of ripening time, significant correlations of $P = 0.0466$ for soft cheese, $P < 0.001$ for semi-hard cheese and $P = 0.0097$ for hard cheese, between enterococci and tyramine concentration were found. Moreover, the factor soft cheese showed also a significant correlation ($P = 0.0307$) between *Lactobacillus/Lactococcus* spp. and between the common interaction of enterococci and CNS ($P = 0.0110$) on tyramine.

Significant correlations between tyramine and the three BAs cadaverine, histamine and putrescine were found for cadaverine in the factors of soft cheese ($P = 0.0476$) and semi-hard cheese ($P = 0.0122$) wherein semi-hard cheeses also concentrations of histamine ($P = 0.0241$) and putrescine ($P = 0.0248$) correlated with tyramine. The common interaction of pH and CNS in correlation to tyramine was significant ($P = 0.0023$) in the factor of semi-hard cheese as well as the pH versus the two bacteria enterococci and CNS in correlation on tyramine in soft cheese ($P = 0.0333$) and semi-hard cheese ($P = 0.0350$). The factor cream cheese showed no significant correlation on tyramine (Table 3.4).

Influence of seasons on biogenic amines and bacteria

To examine the influence of the two season's spring and autumn on numbers of bacteria and BA concentration, 62 of the 274 cheese samples, which belong all to a distinct semi-hard Swiss cheese variety, were analysed (Figure 3.1). Of these samples, 31 were produced in spring and 31 in autumn. One of each cheese sample was made in spring and one in autumn in the same dairy. Counts of CNS were slightly higher ($P > 0.05$) in cheese samples from autumn with a mean value of 4.45 $\log_{10}$ cfu g^{-1} compared to spring samples with a mean value of 3.97 $\log_{10}$ cfu g^{-1}. Counts of CPS were significantly higher ($P = 0.004$) in cheese samples from autumn with a mean of 2.57 $\log_{10}$ cfu g^{-1} compare to CPS in spring samples with a mean of 2.11 $\log_{10}$ cfu g^{-1}. In contrast, counts of enterococci were significantly higher ($P = 0.025$) in samples from spring with a mean value of 4.62 $\log_{10}$ cfu g^{-1} compared to the counts of enterococci from autumn samples with a mean of 3.56 $\log_{10}$ cfu g^{-1} whereas *Lactobacillus*/ *Lactococcus* spp. titers were comparable in both kinds of samples.

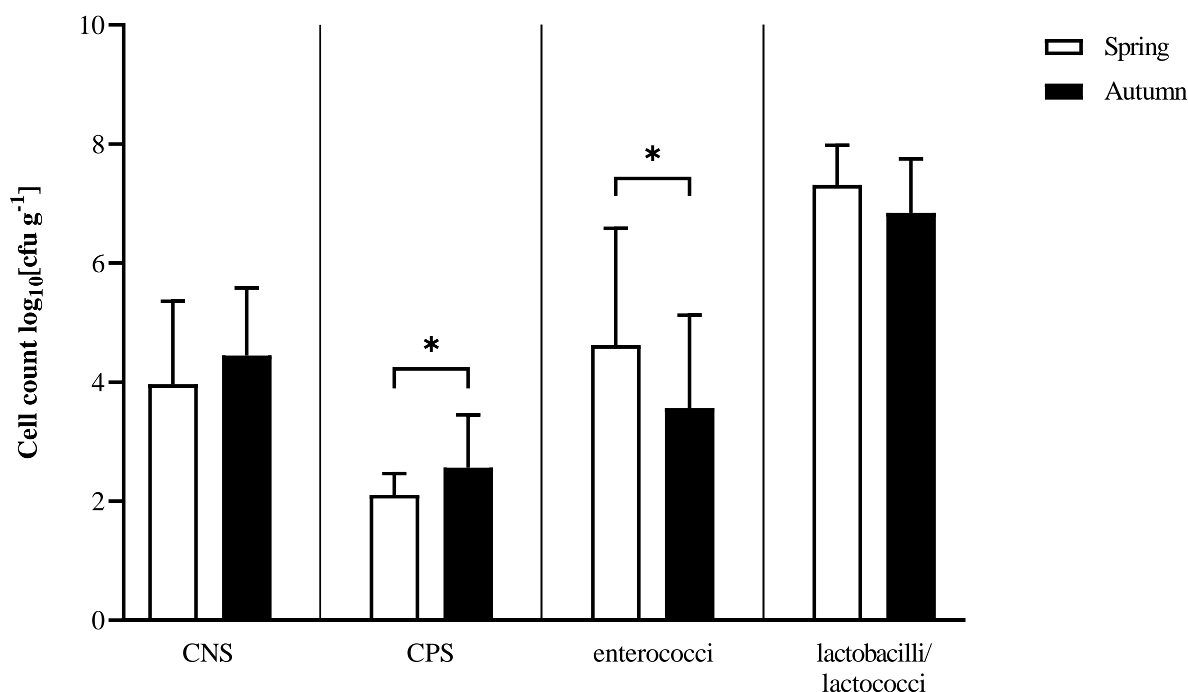

Figure 3.1: Cell count [$\log_{10}$ cfu g^{-1}] of CNS, CPS, enterococci and lactobacilli/lactococci in 62 analysed cheese samples from a distinct semi-hard Swiss cheese in spring (n = 31) and autumn (n = 31) cheese samples.

In the investigated BAs, histamine was significantly higher ($P = 0.029$) in autumn samples with a mean value of 35.57 mg kg^{-1} than in spring samples with a mean value of 32.85 mg kg^{-1}. Putrescine and tyramine showed a higher concentration in spring samples with a mean of 26.44 mg kg^{-1} and 112.09 mg kg^{-1}, respectively compared to autumn samples with corresponding values of 23.10 mg kg^{-1} and 70.79 mg kg^{-1}. Cadaverine, on the other hand, was present in higher concentrations with a mean of 99.69 mg kg^{-1} in autumn samples compared to spring samples with a mean of 56.56 mg kg^{-1} (Figure 3.2).

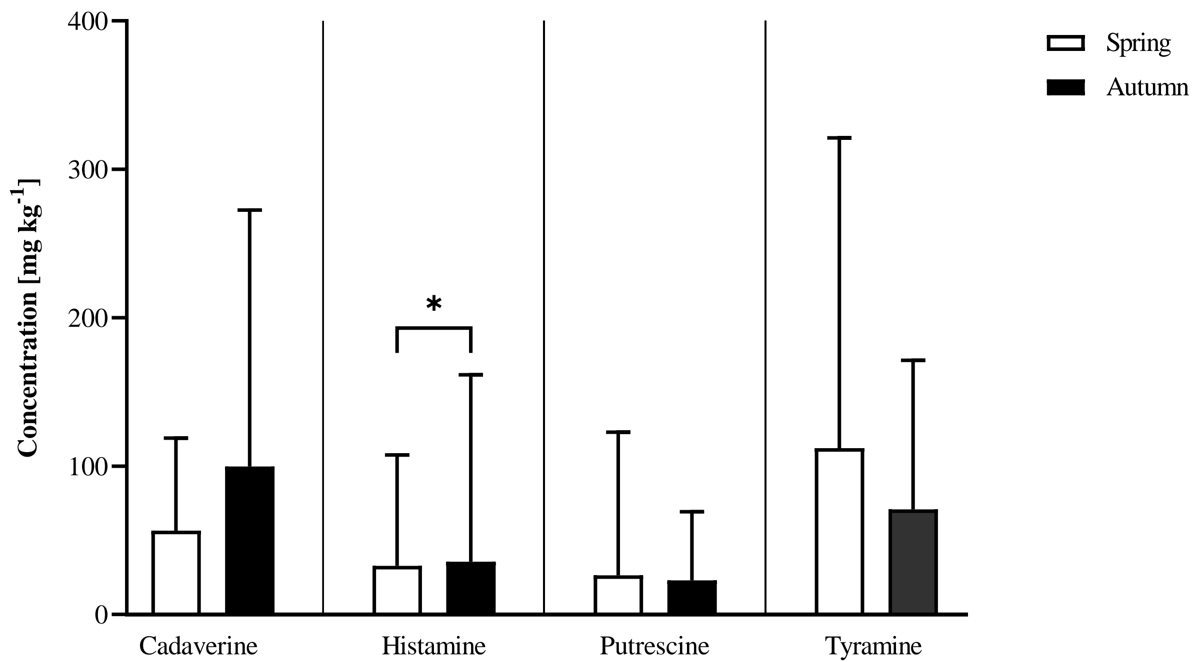

Figure 3.2: Concentration [mg kg^{-1}] of cadaverine, histamine, putrescine and tyramine in 62 analysed cheese samples from a distinct semi-hard Swiss cheese in spring (n = 31) and autumn (n = 31) cheese samples.

Discussion

Cheese manufacturing provides ideal conditions for the formation of BAs because milk is not necessarily deficient in microbes and the casein proteolysis ensures the availability of free amino acids. The present study focused mainly on the concentration of tyramine and the direct or indirect influence of microorganisms, pH and other BAs in the three different parameters milk treatment, milk origin, and ripening time in cheese.

In this study, levels of CNS showed only slight differences in the three different parameters milk treatment, milk origin, and ripening time. We were able to confirm that the levels of CNS do not differ from prior studies even though in our study different types of cheese and much more cheese samples were analysed (E. Coton et al., 2010; Irlinger, 2008; Soares et al., 2011). Among the microorganisms contaminating cheese, the coagulase-positive *Staphylococcus aureus* species is the most common one and is responsible for several health problems due to the production of endotoxins (Bellio et al., 2019; Guidi et al., 2018). Levels of CPS were found in 43 of 274 analysed cheese samples and nine of them (two raw, six thermised and one pasteurised milk) were above the Swiss legal limit of 10^5 cfu g^{-1} for raw milk cheese, and 10^4 cfu g^{-1} for the other two types of cheese, respectively (Eidgenössische Departement des Innern (EDI), 2013). The high numbers of lactobacilli/lactococci were expected and are associated with starter cultures in cheese (Flasarová et al., 2016; Parente et al., 2017). *Enterococcus* spp. were found in 75% of all analysed cheese samples. It could be confirmed that even in a large number of different cheese samples, which were analysed, enterococci can occur in a concentration of up to 10^7 cfu g^{-1} (Linares et al., 2011; Samelis et al., 2009). The dominant presence of *Enterococcus* spp. in thermised milk can be explained by the fact that thermisation reduces harmful bacteria but also have adverse effects on milk considering cheesemaking processes. According to suggestions of Samelis et al., (2009), the overall biodiversity of resident raw milk bacteria might be reduced, and potential protective LAB strains are inactivated, which allows the outgrowth of, e.g. enterococci. The lower number of enterococci in cheese samples from pasteurised milk compared to those from raw and thermised milk can be explained by the fact that pasteurisation results in a drastic reduction of enterococci and in general of the microbiota resident in raw milk (Fernández et al., 2007a; Ladero et al., 2010b; Novella-Rodríguez et al., 2004). A significant difference in enterococci between the factors semi-

hard and hard cheese samples was observed, which is due to two main causes. On the one hand, 92.82% of the semi-hard cheese samples were made with raw milk compared to the hard cheese samples in which only 82.05% were made with raw milk. On the other hand, there is the possibility that microorganisms are not viable anymore after the more extended ripening period for hard cheese samples compared to semi-hard ones. The significant difference in enterococci between cow and sheep's milk can be explained similarly. Compared with cow's milk, where 92% of the cheese samples were produced from raw milk, and 12.05% were hard cheese samples, sheep's milk cheese samples were only 65% made from raw milk and 45% corresponded to hard cheeses. These two reasons also explain the significant difference between goats and sheep's cheese. Only 10% of the goat cheese samples were hard cheese samples, compared to 45% for sheep cheese ones.

It is known that LAB are able to produce high concentrations of BAs in fermented food products and that a high concentration of tyramine may be present in cheese. Previous studies suggested that enterococci might be the main producers of tyramine (Bargossi et al., 2015b; Broadley, 2010; Combarros-Fuertes et al., 2015; Ladero et al., 2012b). Our study has confirmed that enterococci, especially *E. faecalis*, *E. faecium* and *E. durans*, are the main producers of tyramine in all cheese samples variety independent from milk treatment on the type of cheese, milk origin or the way of ripening time. CNS, as part of dominant starters in meat fermentation, were shown to be capable of tyramine production (Alfaia et al., 2018; Landeta et al., 2013a). In cheese, however, CNS are less dominant; nevertheless, previous studies reported frequently the occurrence of coagulase-negative tyramine-producing staphylococci, which is consistent with our study (Schirone et al., 2013; Torriani et al., 2016). Moreover, former studies reported the ability of *S. aureus* to produce histamine, and the ability of some CNS to produce tyramine, cadaverine or putrescine, but the ability of *S. aureus* to produce tyramine was not reported yet (Espinosa-Pesqueira et al., 2018). However, our data showed that coagulase-positive *S. aureus* strains could produce tyramine. In our study, all of the five *tdcA* gene-positive *S. aureus* strains were able to produce tyramine. The part of tyramine-producing isolates from MRS-media identified as *Lactococcus lactis* (n = 11) are representing only a minority of starters in our analysed cheese samples and confirm the observations by other studies in different cheese varieties (Buáková et al., 2011; Flasarová et al., 2016). It suggests that selected *Lactococcus* spp. applied as

starters are negative concerning tyramine production or that lactococci resident in milk, whenever outgrowing, do not play a significant role in tyramine production. One tyramine-producing MRS-isolate was identified as a *Lactobacillus parabuchneri* strain. Recent studies have shown the ability of *Lb. parabuchneri* to produce histamine, but no documentation for the capacity of tyramine production are available to the date (Diaz et al., 2016). The fact that single *Lb. parabuchneri* strains are able to produce tyramine supports the hypothesis of horizontal *tdcA* gene transfer (Linares et al., 2011).

Of the four analysed BAs, tyramine was by far the most abundant BA in the 274 analysed cheese samples. Although it is known that tyramine can be a health risk when occurring in high concentrations in cheese, there is no legal regulation limiting the amount of tyramine in foods. At present, however, there is also insufficient information available to determine a value for the no adverse health effect level (NOAEL) in humans. However, it is known that no adverse health effect has been observed in healthy individuals exposed to a tyramine concentration of 600 mg per meal (EFSA, 2011). The probability that the value of 600 mg will be exceeded, even in a combined intake of tyramine-containing foods during the same meal, is unlikely. However, for individuals taking monoamine oxidase inhibitor drugs or consuming alcohol, the NOAEL is much lower between 6 and 50 mg of tyramine per person per meal (EFSA, 2011; Galgano et al., 2001; Ladero et al., 2010a; Novella-Rodríguez et al., 2004). In consideration of the average amount of approximately 40 g of hard and semi-hard cheese and 50 g of soft cheese eaten by a person per meal in Switzerland according to the Swiss Nutrition Bulletin (Federal Food Safety and Veterinary Office (FSVO), 2019), 18 of 274 analysed cheese samples have a tyramine content over 200 mg kg^{-1}. Therefore, these cheese samples are above the recommended value of 6 mg tyramine per meal for individuals taking classical monoamine oxidase inhibitors or are sensitive against tyramine (EFSA, 2011). Our study confirmed previous studies that tyramine and cadaverine are the major BAs in cheese even for a large heterogeneous cheese group as this study has shown, followed by the minor amines histamine and putrescine (Linares et al., 2013; Schirone et al., 2013). The higher concentration of histamine, putrescine and tyramine in cheese made from raw milk compared to cheese made from thermised and pasteurised milk is due to the fact that raw milk has increased microbial counts, which can produce BAs (Novella-Rodríguez et al., 2004). Moreover, the presence of pyridoxal-phosphate, which acts as a co-factor for the decarboxylase activity is advantageous for the enhanced

production of tyramine (Combarros-Fuertes et al., 2015). Another factor that leads to a higher BA concentration is the ripening time, and it is well known that BA concentrations are increasing during cheese ripening(Linares et al., 2012). These findings can also be confirmed in our study, in which we found a higher concentration of all four measured BAs in semi-hard and hard cheese compared to cream and soft cheese with shorter ripening times. Furthermore, with the higher concentration of tyramine and putrescine in goat's and sheep's milk we could support former suggestions that the storage of milk under refrigerating can lead to an increase in BA concentrations since goat's and sheep's milk is often stored until a sufficient quantity is available to produce cheese (Novella-Rodríguez et al., 2004).

In this study, significant positive correlations between enterococci and tyramine concentration, in raw and thermised milk, cow milk, as well as in soft, semi-hard and hard cheeses, were found. These results confirmed prior studies based on less extended setups that enterococci are the main tyramine producers in cheese, regardless of the production method of the cheese or the milk used and their treatment (Bargossi et al., 2015b; Combarros-Fuertes et al., 2015; Ladero et al., 2012b). Interestingly, in goat cheese, a significant correlation between CPS and tyramine was found. This significance confirms our finding of the tyramine-producing CPS because all of the *tdcA* gene-positive CPS were isolated from goat cheese. Furthermore, in soft cheese samples, a significant correlation between tyramine and the amount of lactobacilli/lactococci as well as the amount of the interaction between the two bacterial species enterococci and CNS, respectively, was observed. The facts, that *Lactococcus* spp. were used as starter cultures and can produce tyramine (Buáková et al., 2011) and that microorganisms in soft cheese are still viable after the short ripening time can explain the significant correlation between tyramine and lactobacilli/lactococci numbers. Therefore, in our study, it could be shown that in cheese with a short maturation time such as soft cheese, the interaction between the two tyramine-producing bacterial groups CNS and enterococci have an enhancing effect on tyramine concentration. Furthermore, our data give evidence that the pH, which can influence the behaviour of bacteria as a stress factor (Fernández et al., 2007b; Gardini et al., 2001; Rousk et al., 2010), has an indirect, but significant influence on tyramine concentration in cheese in interaction with CPS or with enterococci and CNS. This significance was demonstrated in the factors of milk origin and maturation time. Besides, in the parameter of milk origin and cheese texture, a significant direct influence of the pH on tyramine could be demonstrated. This

confirms prior studies in cheese under experimental conditions, which demonstrated the highest activity of tyramine production at a pH around 5 (Fernández et al., 2007b; Gardini et al., 2001).

Previous studies have shown that tyramine and cadaverine often occur together (Fiechter et al., 2013; Standarová et al., 2010). In our study, we were able to prove that there is a significant correlation between the BAs cadaverine and tyramine in the factors raw and thermised milk, cow's milk as well as in soft and semi-hard cheese. In addition, we were able to prove that there is a significant correlation between the concentration of tyramine and histamine in the factors raw milk and cow's milk and that even the two BAs tyramine and putrescine can correlate significantly with each other in the factor of semi-hard cheese. Former studies have reported the occurrence of these amines, but no significant correlation has been found to date (Linares et al., 2012; Schirone et al., 2013). Since strains of *E. faecalis* and *E. faecium* are able to produce on species-level trait putrescine and histamine in addition to tyramine, the significant occurrence of tyramine and histamine and tyramine and putrescine support the hypothesis that the ability to produce BAs was probably acquired via horizontal gene transfer (Komprda et al., 2010; Ladero et al., 2012b).

Mean counts of enterococci were significantly higher in samples produced in spring compared to samples produced in autumn. The significant higher amount on enterococci in cheese samples produced in spring correlates with a higher level of tyramine, which confirms the influence of enterococci on tyramine formation (Combarros-Fuertes et al., 2015). Furthermore, a significant effect of the season on histamine could be shown. Cheese samples produced in autumn had a significantly higher histamine concentration than cheeses produced in spring. Moreover, mean counts of CPS were significantly higher and mean counts of CNS were slightly higher in cheese samples, which were produced in autumn than in samples, which were produced in spring. Former studies reported the ability of CNS and CPS to produce histamine (Espinosa-Pesqueira et al., 2018) and in our study, these two concentrations correlate positively in cheese samples produced in autumn. Therefore, it seems that seasonal effects affecting the milk quality in the number of staphylococci and histamine level. Unfortunately, in general, seasonal fluctuations are not easy to assess because of the high variability between different cheese samples from different dairies. It may have masked the seasonal effect of this type of cheese (Gaya et al., 2005).

Conclusion

Tyramine is by far the most abundant BA in cheese followed by cadaverine, histamine and putrescine. Enterococci, which was the main responsible bacterial group of tyramine producers in cheese, showed the highest decarboxylase activity, and there are numbers significantly correlated with tyramine concentration. Nevertheless, tyramine concentration depends not only on the amount of the *tdcA*-positive resident microbiota such as enterococci. In particular, tyramine content varied in different cheeses and even within the same type of cheese. The three parameters of milk treatment, milk origin and ripening time have a high effect on tyramine formation and in general on the formation of BAs. In particular, cheese made from raw milk has an increased tendency to produce high concentrations of BAs as more *tdcA*-positive enterococci are present, and the pyridoxal phosphate, which acts as a cofactor, is still present. Another essential role in BA production is the ripening time since proteolysis release during ripening time the free amino acids. Because cadaverine, histamine and putrescine can be significantly correlated with tyramine concentrations, it will be crucial that there is a low concentration of *tdcA*-positive enterococci available in milk, above all, because enterococci neither have a Generally Regarded as Safe (GRAS) nor a Qualified Presumption of Safety (QPS) status.

Acknowledgements

We are grateful to the cantonal laboratories of Thurgau, St. Gallen and Urkantone providing the cheese samples.

This project was funded by the Swiss Federal Food Safety and Veterinary Office (FSVO).

Supplementary information

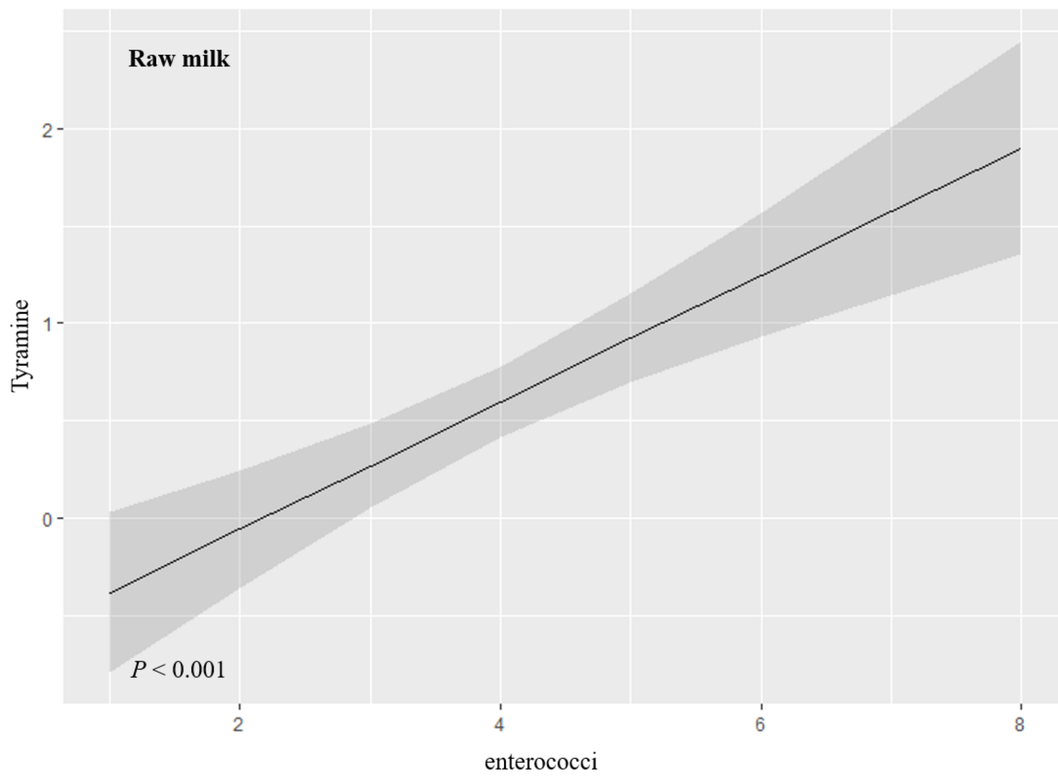

Figure S 3.1: Significant regression of correlation between enterococci and tyramine in raw milk.

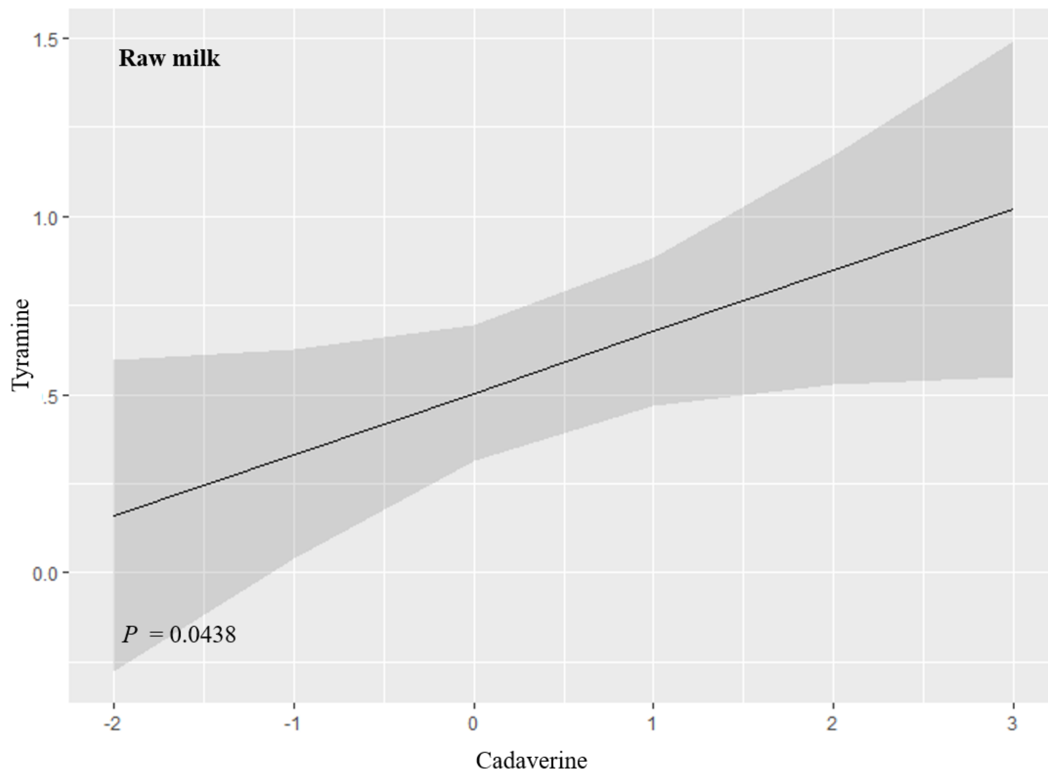

Figure S 3.2: Significant regression of correlation between cadaverine and tyramine in raw milk.

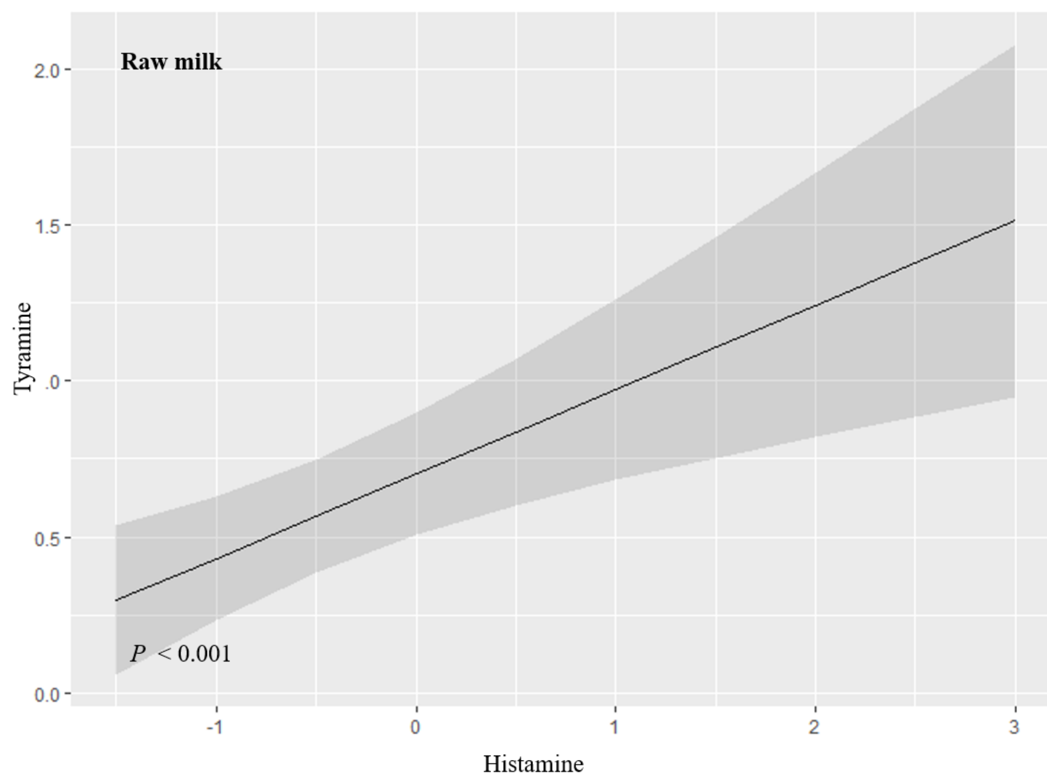

Figure S 3.3: Significant regression of correlation between histamine and tyramine in raw milk.

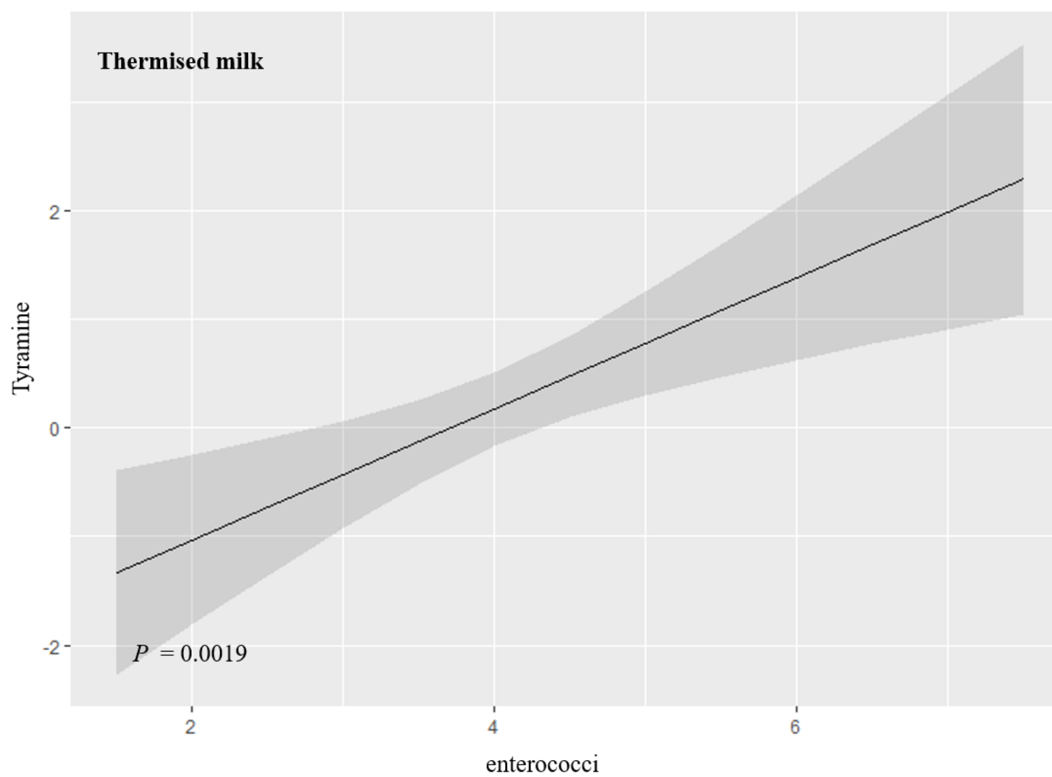

Figure S 3.4: Significant regression of correlation between enterococci and tyramine in thermised milk.

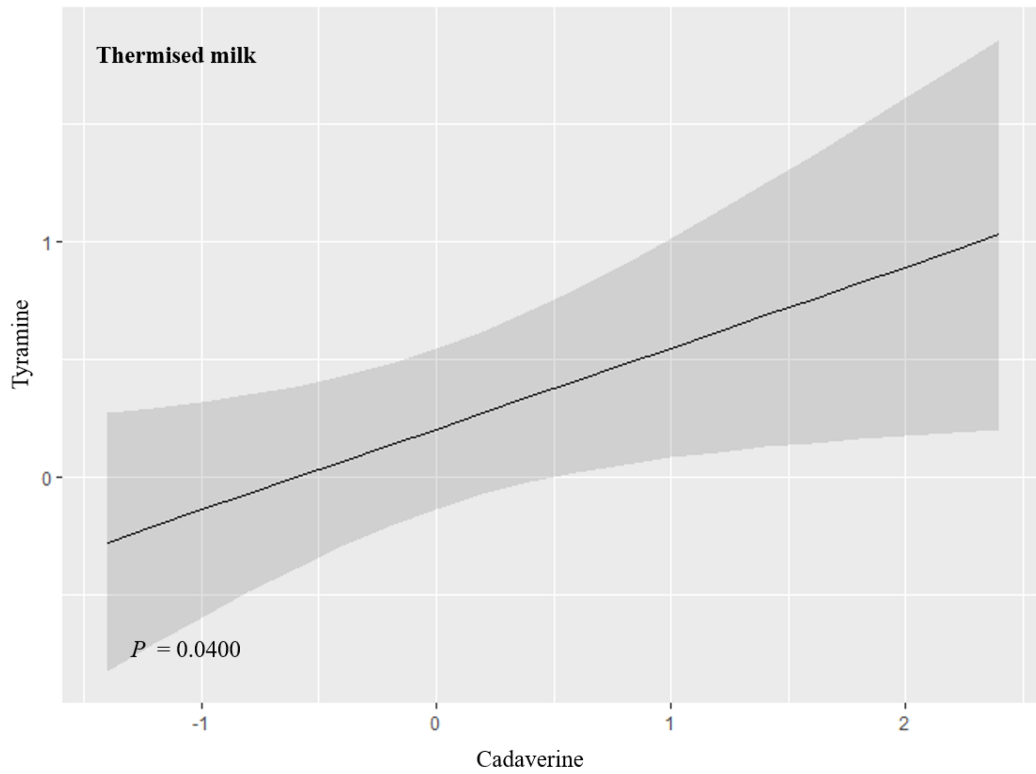

Figure S 3.5: Significant regression of correlation between cadaverine and tyramine in thermised milk.

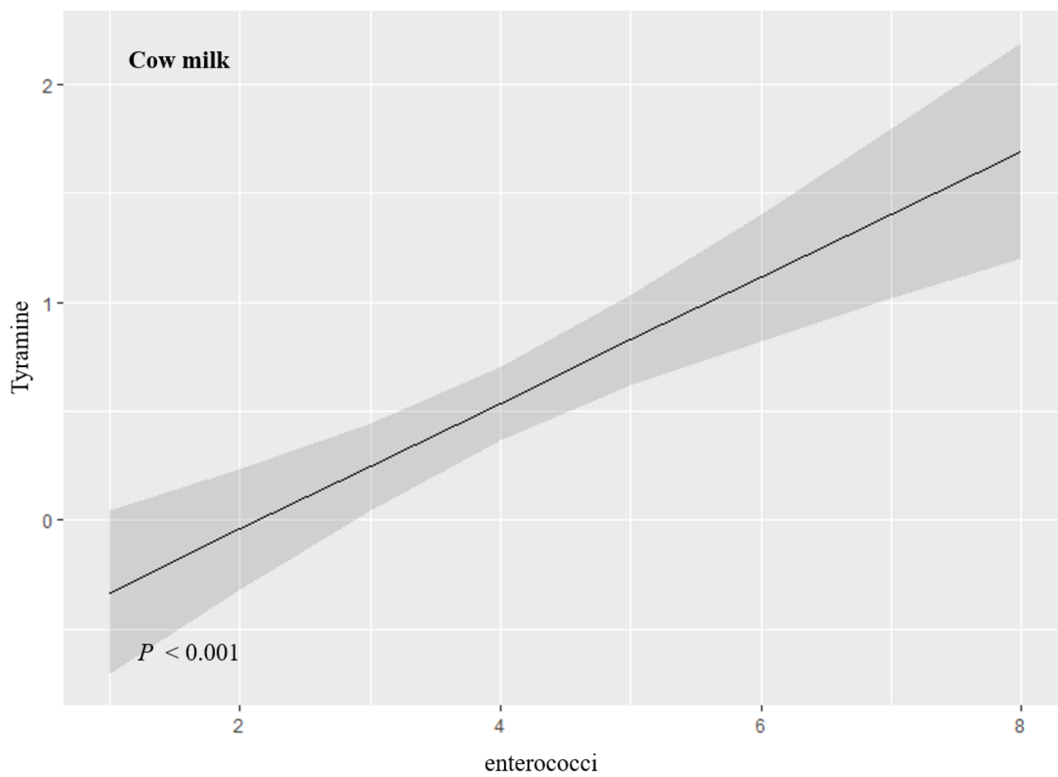

Figure S 3.6: Significant regression of correlation between enterococci and tyramine in cow's milk.

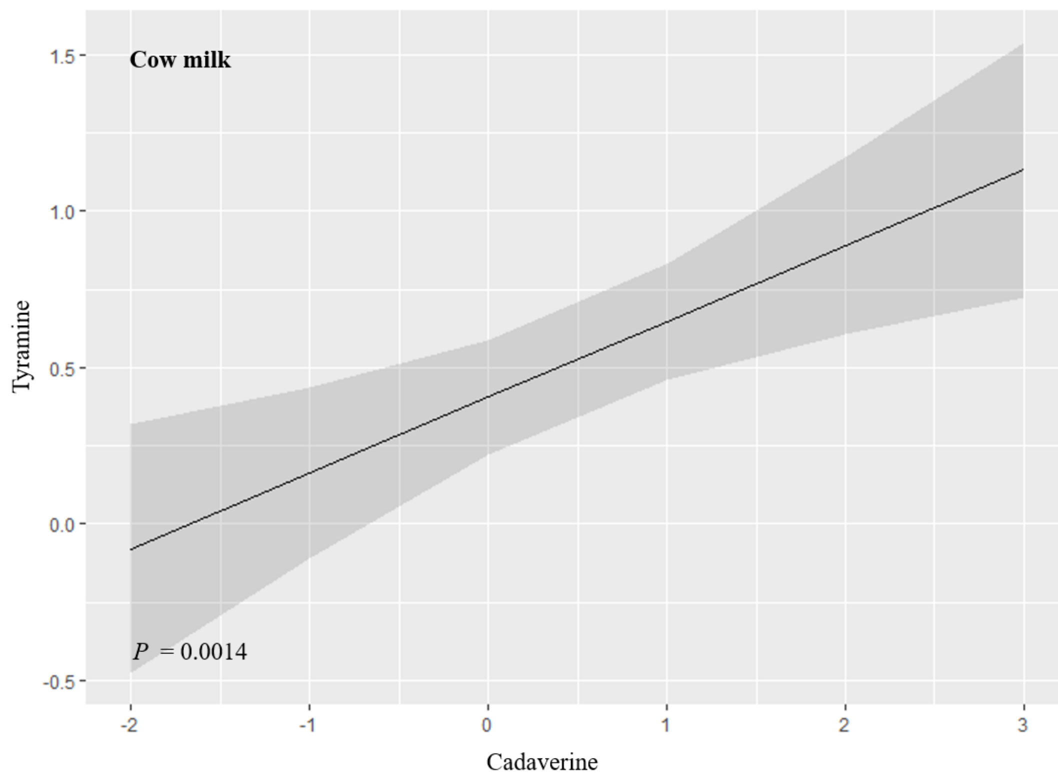

Figure S 3.7: Significant regression of correlation between cadaverine and tyramine in cow's milk.

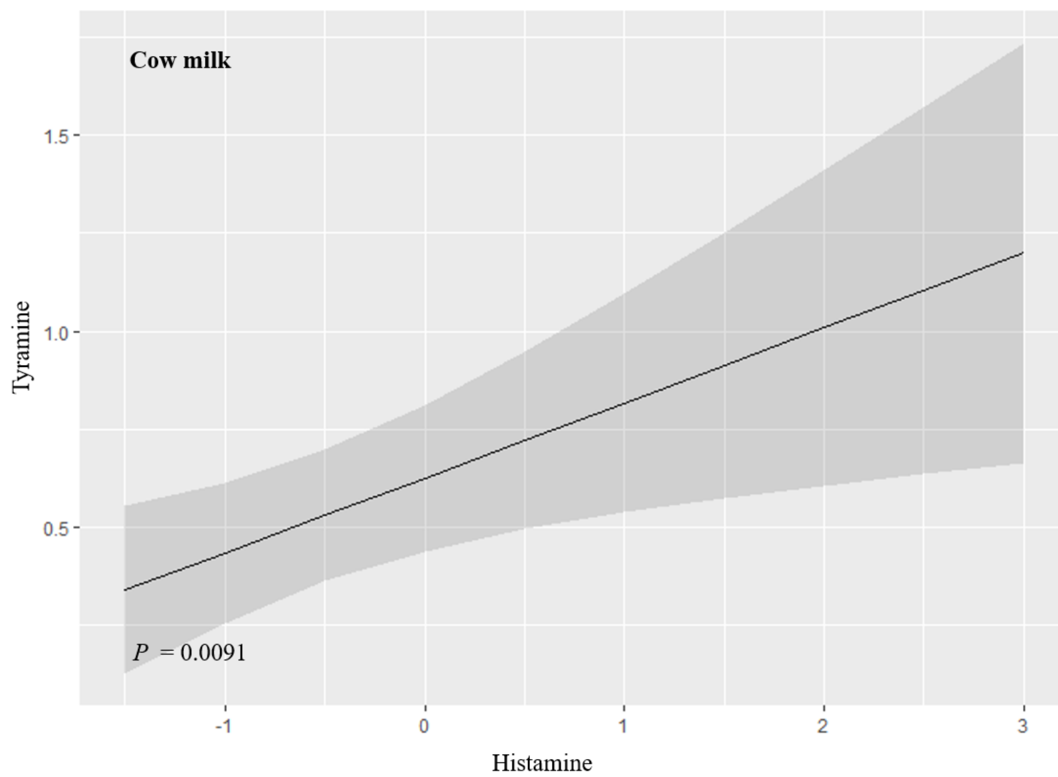

Figure S 3.8: Significant regression of correlation between histamine and tyramine in cow's milk.

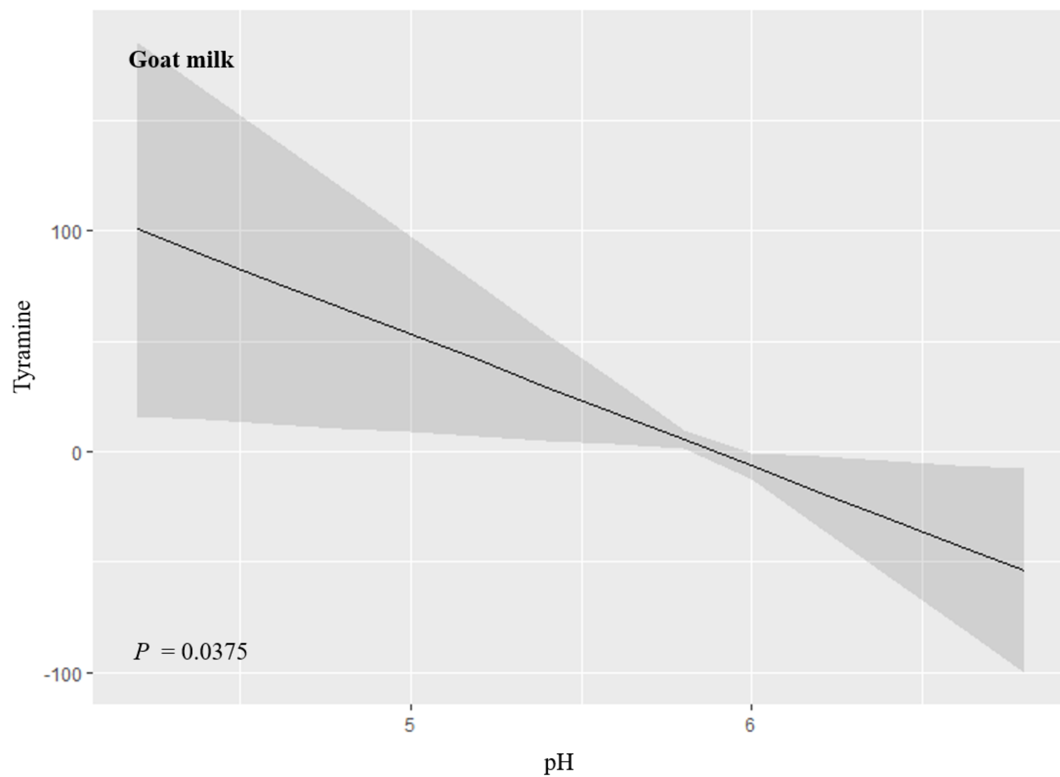

Figure S 3.9: Significant regression of correlation between pH and tyramine in goat's milk.

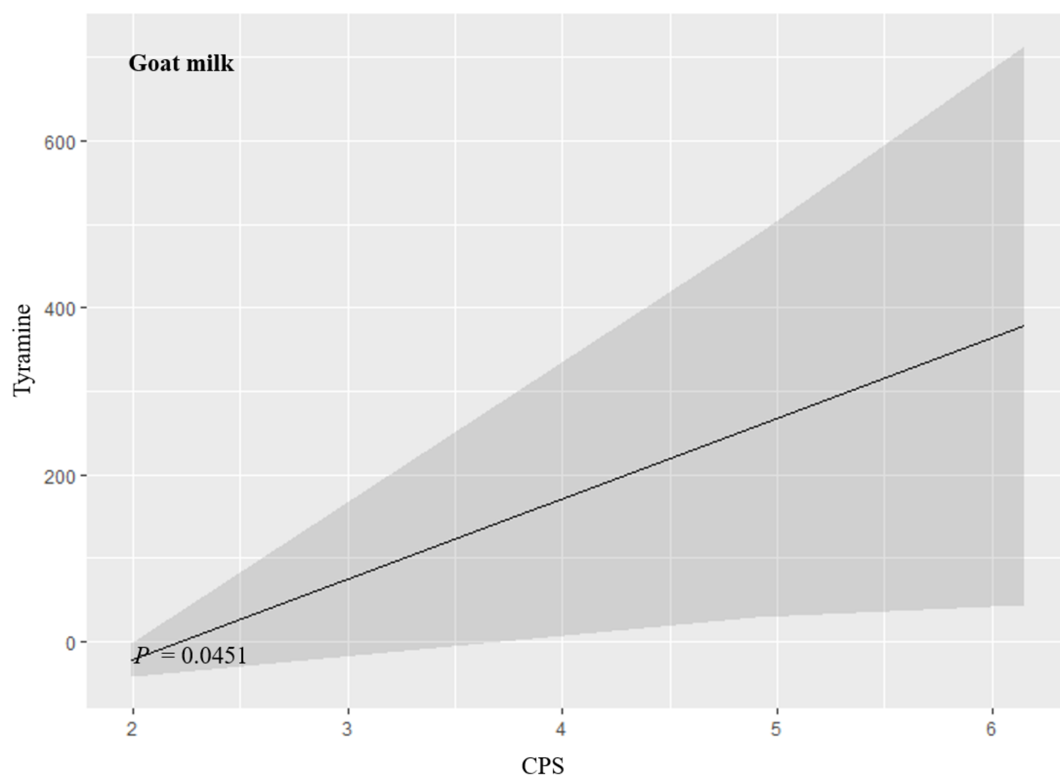

Figure S 3.10: Significant regression of correlation between CPS and tyramine in goat's milk.

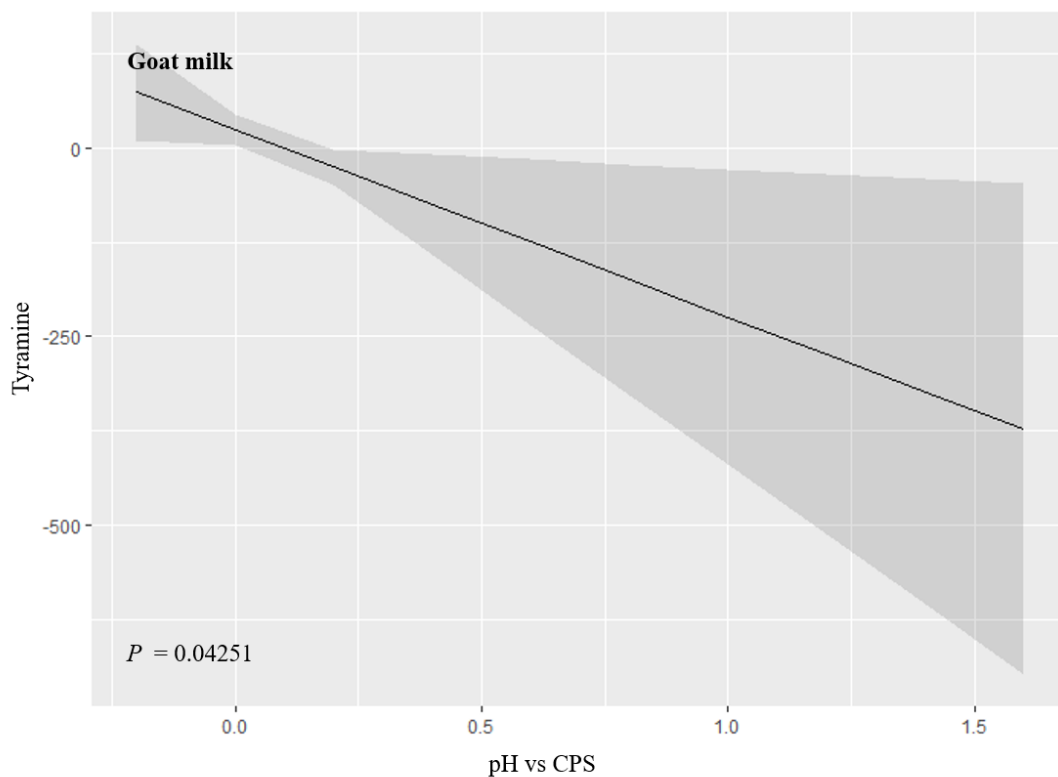

Figure S 3.11: Significant regression of correlation between pH vs CPS and tyramine in goat's milk.

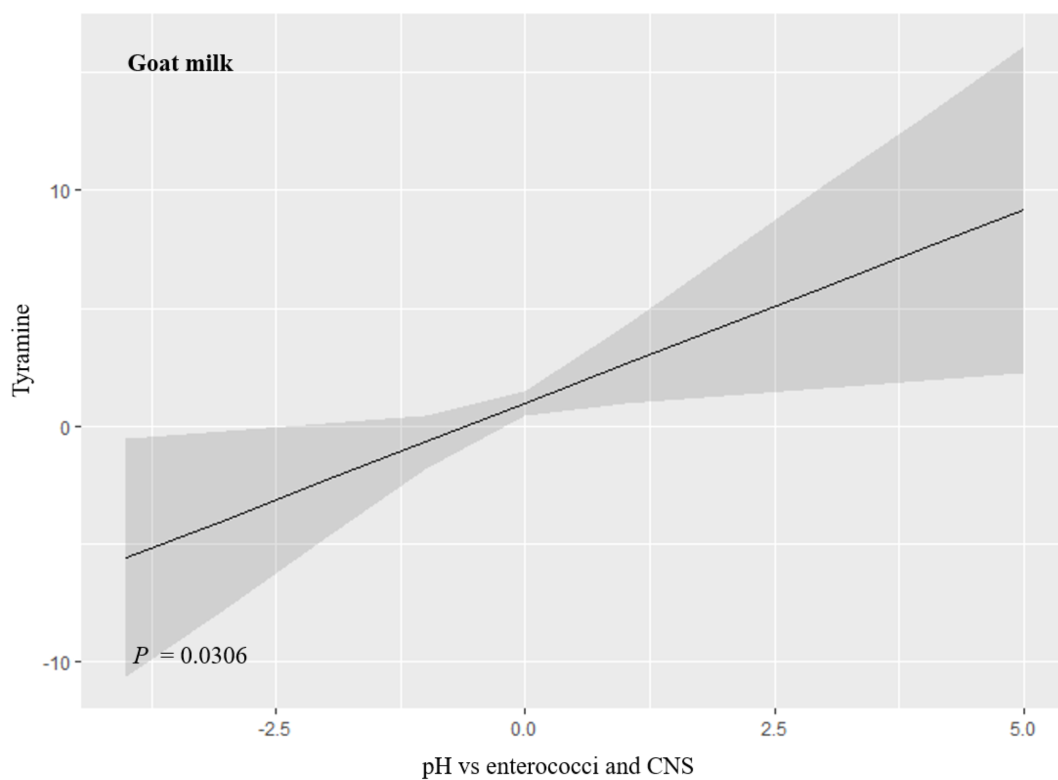

Figure S 3.12: Significant regression of correlation between pH vs enterococci/CNS and tyramine in goat's milk.

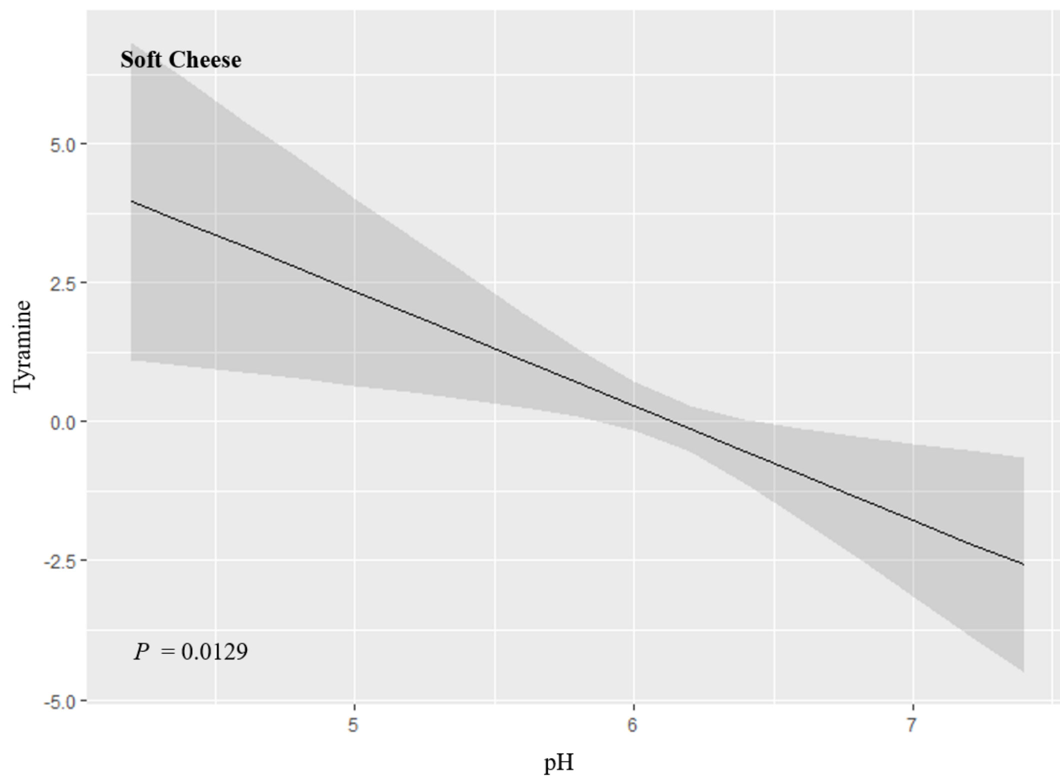

Figure S 3.13: Significant regression of correlation between pH and tyramine in soft cheese.

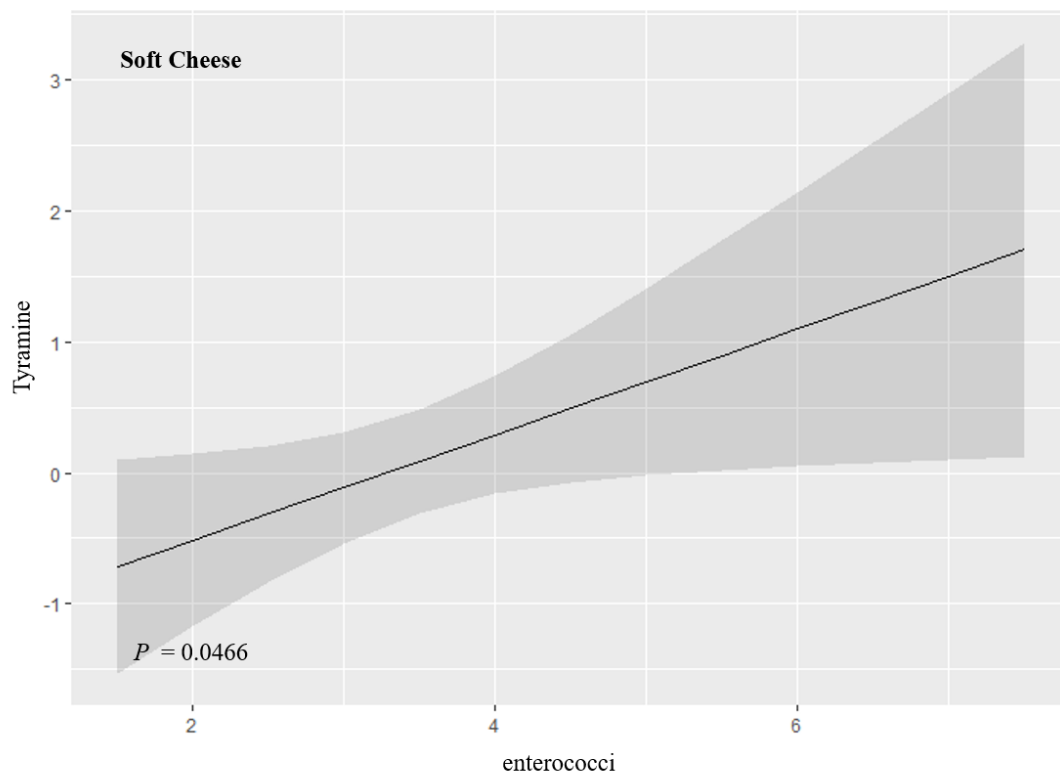

Figure S 3.14: Significant regression of correlation between enterococci and tyramine in soft cheese.

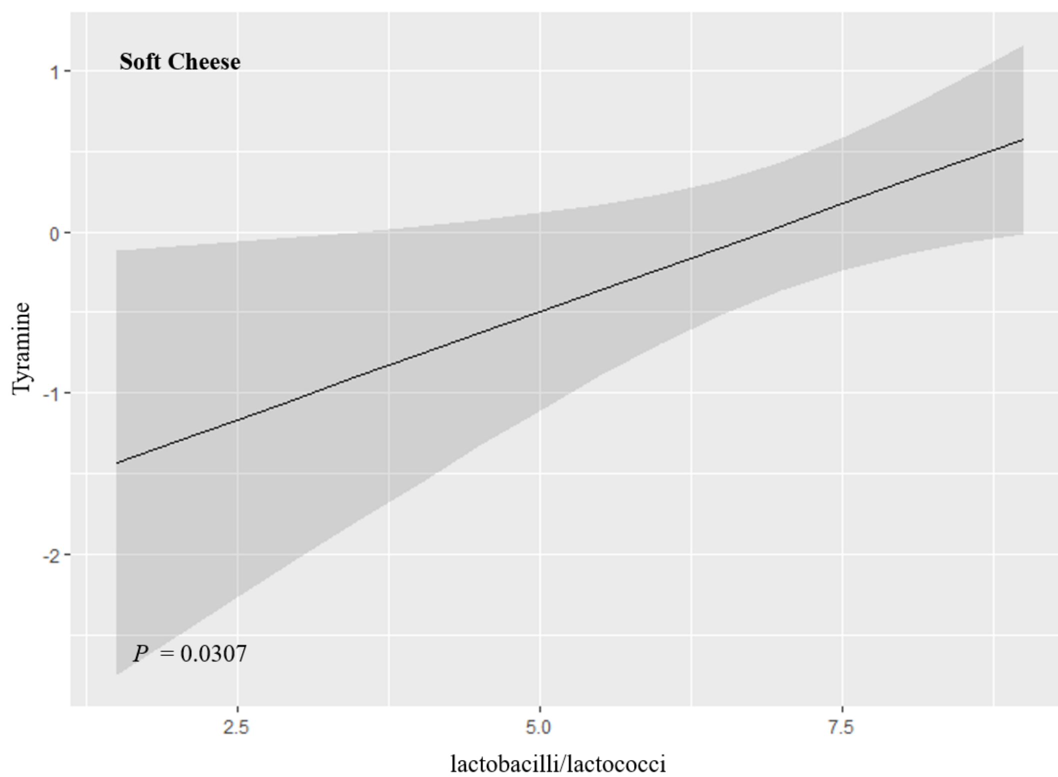

Figure S 3.15: Significant regression of correlation between lactobacilli/lactococci and tyramine in soft cheese.

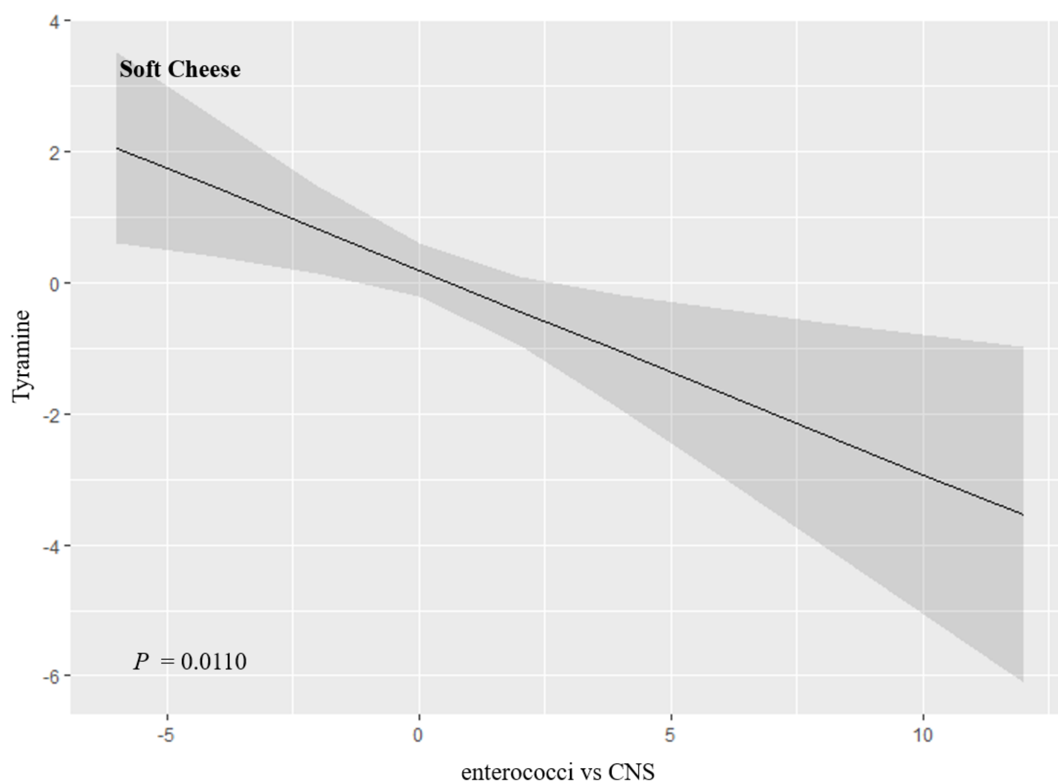

Figure S 3.16: Significant regression of correlation between enterococci vs CNS and tyramine in soft cheese.

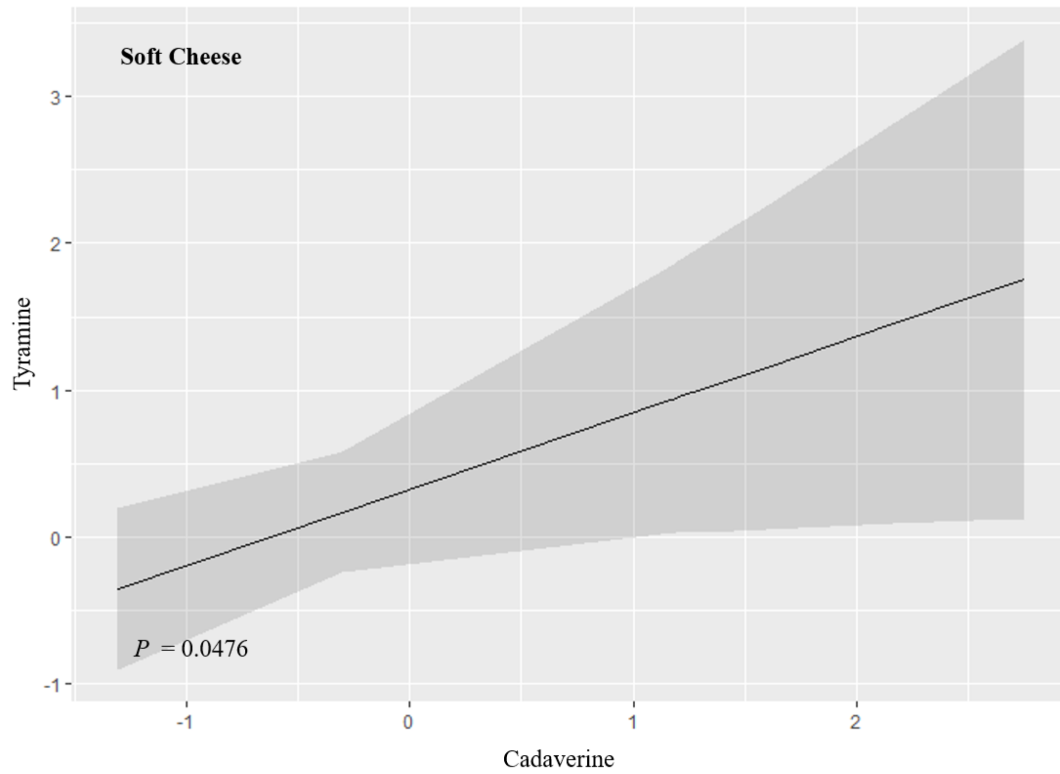

Figure S 3.17: Significant regression of correlation between cadaverine and tyramine in soft cheese.

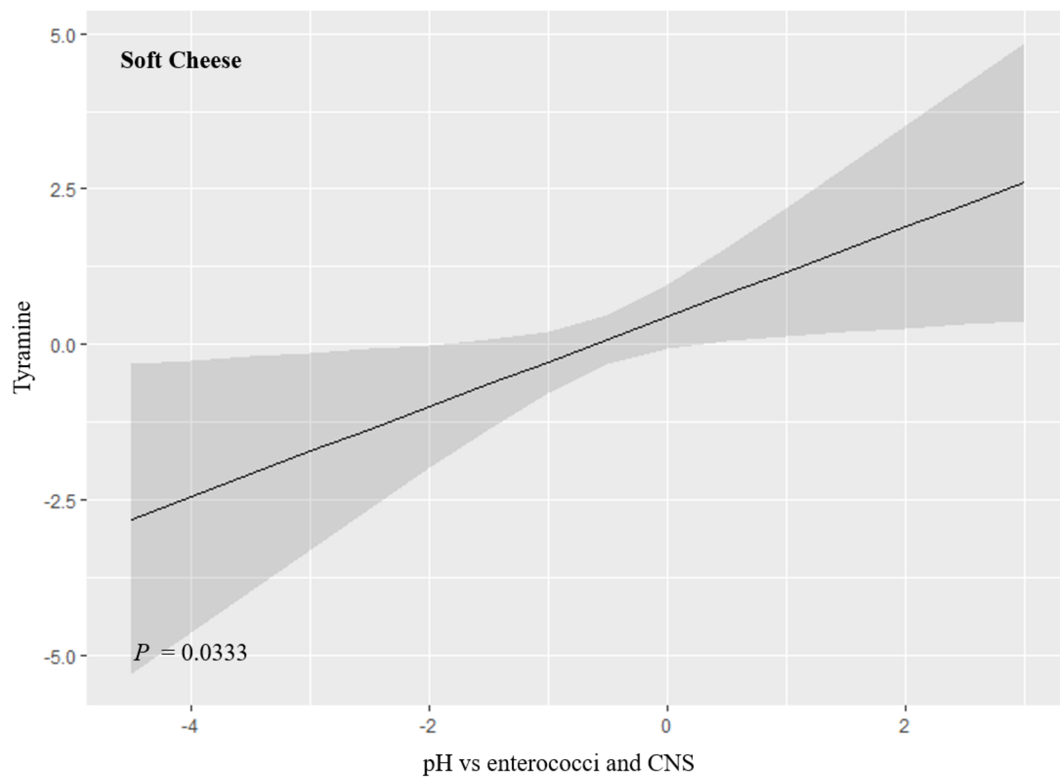

Figure S 3.18: Significant regression of correlation between pH vs enterococci/CNS and tyramine in soft cheese.

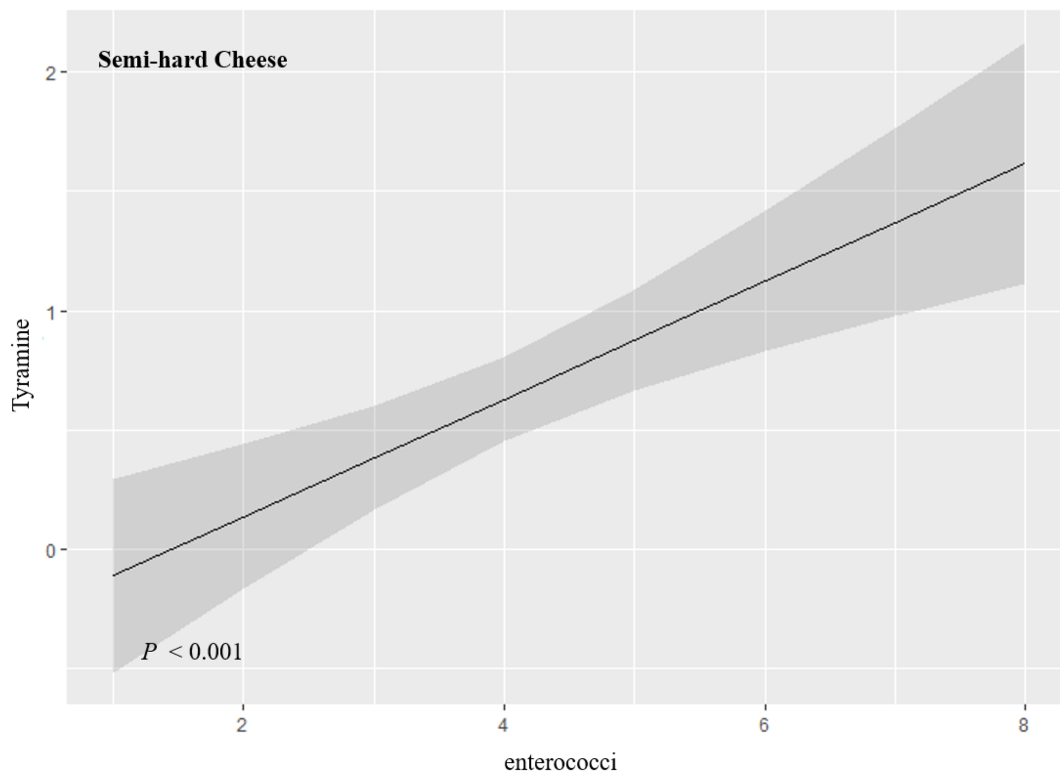

Figure S 3.19: Significant regression of correlation between enterococci and tyramine in semi-hard cheese.

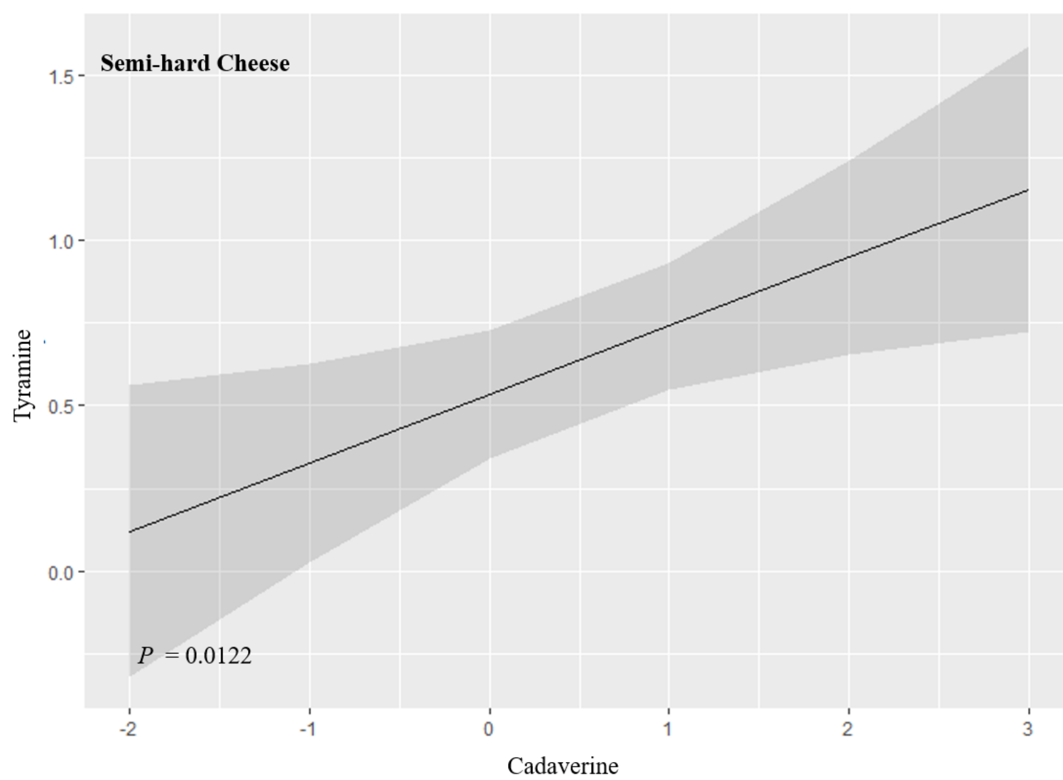

Figure S 3.20: Significant regression of correlation between cadaverine and tyramine in semi-hard cheese.

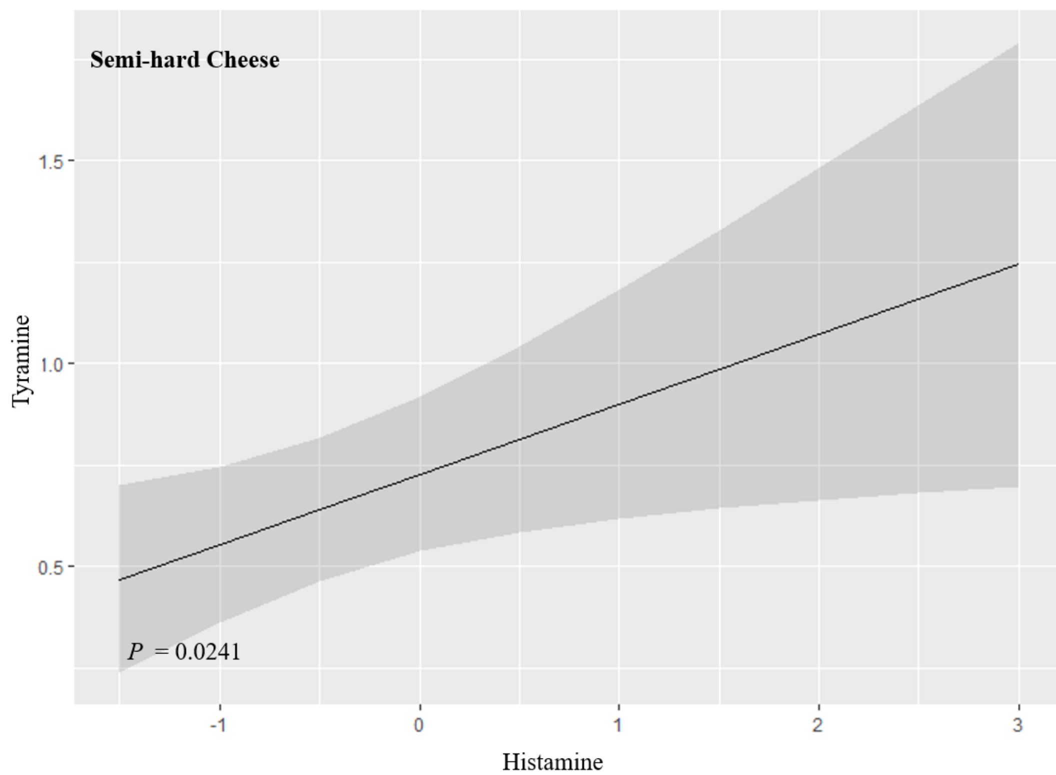

Figure S 3.21: Significant regression of correlation between histamine and tyramine in semi-hard cheese.

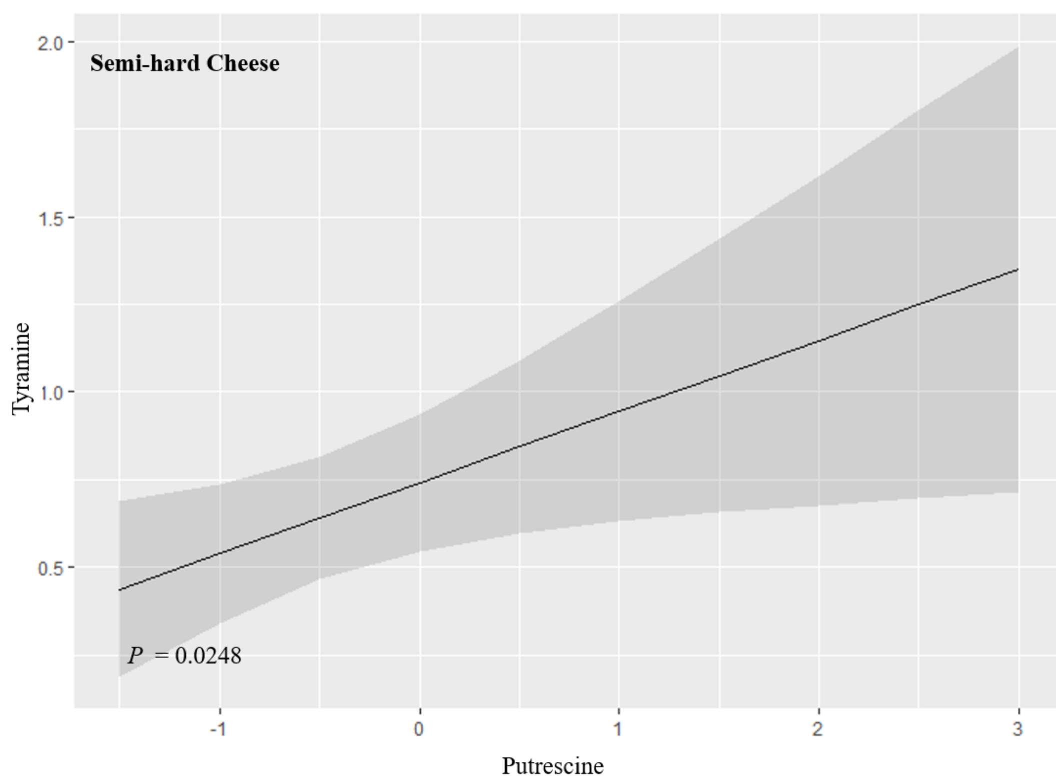

Figure S 3.22: Significant regression of correlation between putrescine and tyramine in semi-hard cheese.

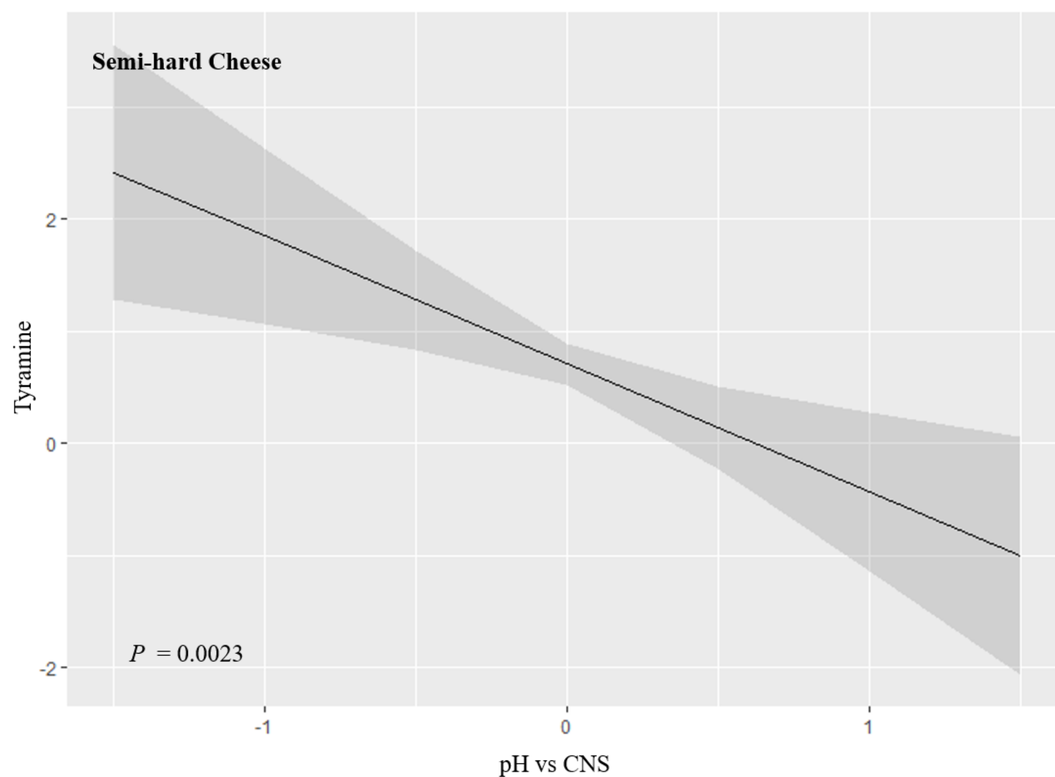

Figure S 3.23: Significant regression of correlation between pH vs CNS and tyramine in semi-hard cheese.

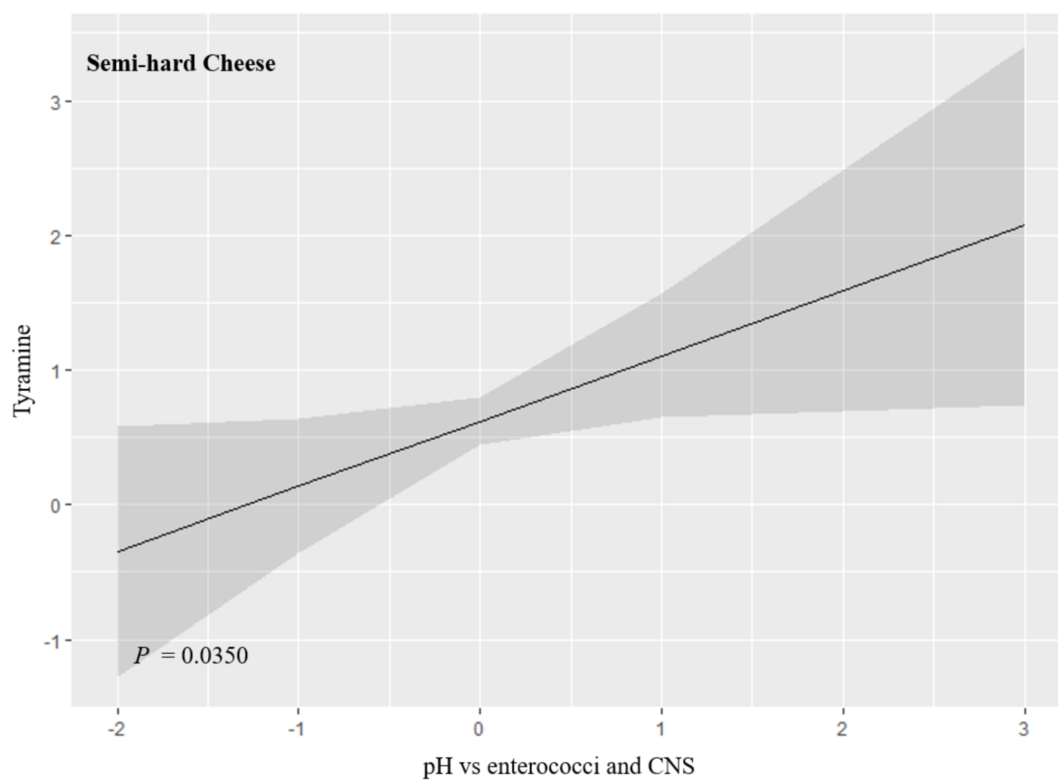

Figure S 3.24: Significant regression of correlation between pH vs enterococci/CNS and tyramine in semi-hard cheese.

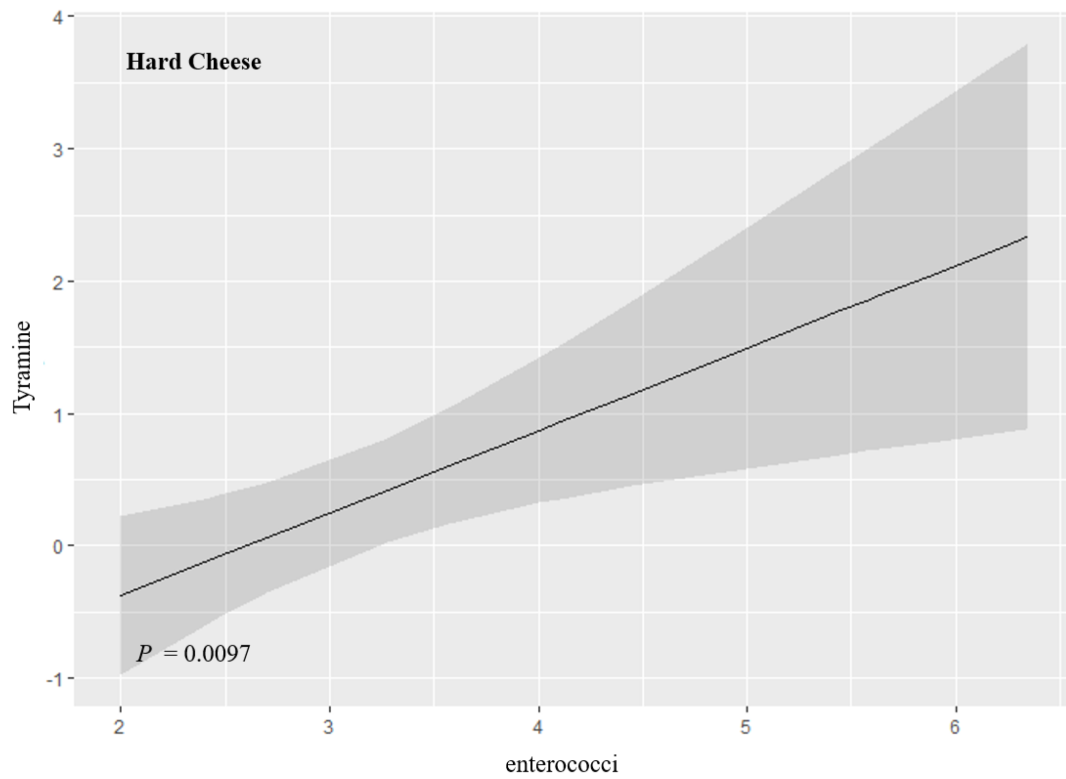

Figure S 3.25: Significant regression of correlation between enterococci and tyramine in hard cheese.

Chapter 4

Effects of Sodium Chloride on Tyramine Production in a Fermented Food Model and its Inhibition by Tyrosine-Degrading *Lactobacillus plantarum* JA-1199

Janine Anderegg, Florentin Constancias, Leo Meile*

Laboratory of Food Biotechnology, Institute of Food, Nutrition and Health, ETH Zurich, Zurich, Switzerland

*Corresponding author

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Abstract

Tyramine is a health adverse biogenic amine, which can accumulate in fermented foods like cheese by decarboxylation of the free amino acid tyrosine by either starter cultures or resident microbes such as lactic acid bacteria including *Enterococcus* spp., respectively. Our study aimed to show the effect of sodium chloride concentrations on tyramine production as well as to characterise bacterial strains as anti-tyramine biocontrol agents in a 2 mL micro-cheese fermentation model. The effect of sodium chloride on tyramine production was assayed with tyramine-producing strains from eight different species or subspecies. Generally, an increase in sodium chloride concentration enhanced tyramine production, e.g. from 0% to 1.5% of sodium chloride resulted in an increase of tyramine of 870% with a *Staphylococcus xylosus* strain. In the biocontrol screening among lactic acid bacteria, a *Lactobacillus plantarum* JA-1199 strain was screened that could consume in successful competition with other resident bacteria tyrosine in the micro-cheese model as a source of energy gain. Thereby tyramine accumulation was reduced between 4% up to 99%. The results of this study disclose a feasible strategy for decreasing tyramine concentration and increasing the safety level of fermented food.

Introduction

Biogenic amines (BA) are low-molecular weight organic bases formed by decarboxylation of free amino acids. According to their chemical structure, they can be classified as aliphatic (cadaverine, putrescine, spermine and spermidine), as aromatic (tyramine and phenylethylamine) or as heterocyclic (histamine and tryptamine) (Linares et al., 2011). The most well known BAs are cadaverine, histamine, putrescine and tyramine derived from decarboxylation of the free precursor amino acids lysine, histidine, ornithine and tyrosine, respectively (Fernández et al., 2007b; Herrero-Fresno et al., 2012). BAs have been reported in a variety of foods, including dairy products (Linares et al., 2011), fermented vegetables (Lee et al., 2015) and fruits (Moreno-Arribas, 2002) and also in fermented meat and fish (Moon et al., 2013; Suzzi and Gardini, 2003). Although BAs can have important physiological functions in plants, animals and humans (Boonstra et al., 2015; Borges et al., 2019; Dismukes and Rake, 1973; Kalač, 2014), the consumption of food with high concentrations of BAs can thereby pose a risk of food intoxication (Ladero et al., 2012b). For example, histamine and tyramine can cause severe allergic reactions as nausea and headaches; increase respiration, cardiac output, blood glucose level and high blood pressure; and release norepinephrine (Broadley, 2010; De Mey et al., 2014; EFSA, 2011). Compared to histamine and tyramine, cadaverine and putrescine have a much lower toxicological impact, but they are able to increase the toxic effect of other amines (Combarros-Fuertes et al., 2015). Even though BAs can induce severe health reactions, only histamine in fishery products covers specific legislation for BAs (EFSA, 2005).

Tyramine derives through decarboxylation of the free amino acid tyrosine and is one of the most abundant, frequent BA in food, especially in cheese and fermented sausages (Fernández et al., 2006; Latorre-Moratalla et al., 2012; Linares et al., 2011). There is currently insufficient information for tyramine to establish a no adverse health effect level (NOAEL) in humans. No adverse health effects have been observed in healthy individuals exposed to 600 mg tyramine per person per meal (EFSA, 2011). However, for individuals consuming alcohol or taking monoamine oxidase inhibitor drugs, the NOAEL is set much lower between 6 and 50 mg of tyramine per person per meal (EFSA, 2011; Galgano et al., 2001; Ladero et al., 2012b; Novella-Rodríguez et al., 2004). Tyramine is produced by a variety of

lactic acid bacteria (LAB) including carnobacteria, enterococci, lactobacilli and lactococci and by some staphylococci (Linares et al., 2012; Suzzi and Gardini, 2003; Torriani et al., 2016). The key enzyme, a tyrosine decarboxylase (EC 4.1.1.25), together with a transporter protein for tyrosine/tyramine interchange, are involved in tyramine enrichment (Coton et al., 2011; Linares et al., 2011; Marcobal et al., 2012). All corresponding genes are clustered in an operon called TDC or TYR, on the plasmids or chromosomes of tyramine producer strains (Fernández et al., 2004; Mohedano et al., 2015). The gene called *tdcA* or *tdc* encodes the tyrosine decarboxylase, while the gene called *tyrP* encodes the tyrosine-tyramine antiporter influx/efflux system. Tyrosine decarboxylase has been characterised extensively in a few LAB species, including *Enterococcus faecalis*, *Enterococcus faecium*, *Lactobacillus brevis* and *Lactobacillus curvatus* (Linares et al., 2011; Suzzi and Gardini, 2003). The production of tyramine is possible only when at least three external factors converge. At first, the substrate amino acid tyrosine must be available; secondly, the presence of microorganisms with the appropriate catabolic pathway activated and thirdly, the environmental conditions must be favourable to the decarboxylation activity (Linares et al., 2012). The external factors can be influenced by pH and temperature of the environment, sodium chloride concentration in the media and availability of carbon sources. For example, a key factor for the production of tyramine is the tyrosine decarboxylase enzyme, which has an optimum around pH 5 and sodium chloride stress could upregulate the expression of the tyrosine decarboxylase and the tyrosine-tyramine antiporter genes (Bover-Cid et al., 2001; Emborg and Dalgaard, 2008a, 2008b; Fernández et al., 2007b; Gardini et al., 2001; Liu et al., 2015).

Traditional methods to prevent tyramine formation are primarily by limiting microbial growth through chilling and freezing. Further approaches to limit microbial growth and therefore, tyramine concentration include thermisation or pasteurisation, hydrostatic pressures, irradiation, controlled atmosphere packaging, or the use of food additives (Ardö et al., 2017; Fernández et al., 2007a; Naila et al., 2010; Novella-Rodríguez et al., 2004). Another technological measurement to control tyramine during fermentation is the use of selected tyrosine decarboxylase negative starter cultures (Latorre-Moratalla et al., 2012; Novella-Rodríguez et al., 2010).

A further approach, however rarely applied, for the reduction of tyramine is the use of selected bacterial

strains provided with a specific amino oxidase, reducing *in situ* tyramine during food production (Guarcello et al., 2016). In the presence of oxygen, for example, such amino oxidases are able to deaminate BAs by the production of ammonium (NH_3), hydrogen peroxide (H_2O_2) and aldehydes (Jalkanen and Salmi, 2001). Enzymes, providing this oxidase activity belong to the multicopper oxidase family (EC 1.10.3), a family of enzymes comprising oxidases such as ascorbate oxidase (EC 1.10.3.3), ferroxidases (EC 1.16.3.1), laccases (EC 1.10.3) as well as tyrosinase (EC 1.14.18.1) (Hoegger et al., 2006; Zaidi et al., 2014). Multicopper oxidases are found in different bacterial species, and previous studies reported the application of *Lactobacillus* spp. containing a multicopper oxidase to decompose BAs (Callejón et al., 2014; Li et al., 2018). These aldehydes are further reduced to the corresponding acids, which can be transferred to the central metabolism of the cells (Cooper, 1997). Another approach reducing tyramine in fermented foods is the use of selected bacterial strains, which can use tyrosine as an energy source and thus acting in competition with tyramine-producing strains for the substrate tyrosine. Such enzymes are called aminotransferases, and they can catalyse the reversible pyridoxal phosphate-dependent transfer of amino groups from amino acids to oxo acids (Hasegawa et al., 2019).

The accumulation of tyramine and other BAs in fermented food products is not only a consequence of bacterial contaminants harbouring decarboxylase activity but also an uncontrolled metabolic activity of natural starter cultures, e.g. in traditionally-produced cheese (Latorre-Moratalla et al., 2012; Novella-Rodríguez et al., 2010). The addition of a non-tyramine-producing but tyramine- or tyrosine-oxidising lactic acid bacterium as part of the starter culture would be an interesting strategy to reduce the accumulation of tyramine in traditionally-produced foods while preserving the sensory properties (Callejón et al., 2014; Herrero-Fresno et al., 2012).

In this context, starting from many lactic acid bacteria previously isolated from food and screened for amino acid oxidising ability, this study aimed to explore whether a selected non-tyramine-producing lactic acid bacterium strain, is able to (1) use tyrosine to deplete this substrate for potential tyramine production or (2) to oxidase tyramine. Furthermore, this study aimed to identify the responsible enzyme *in silico* and to apply the selected strain in a micro-cheese model containing tyrosine and different sodium chloride concentration in combination with different tyramine-producing microorganism.

Material and Methods

Strains

Lactobacillus plantarum JA-1199, previously isolated from fermented sausages, was used in this study as a tyrosine degrader and *Lb. plantarum* WCFS1 as a non-degrader, was used as a negative control (Kleerebezem et al., 2003). Both of these *Lb. plantarum* strains were routinely propagated anaerobically at 37°C for 24 h in De Man-Rogosa-Sharpe (MRS) broth. In this study, strains of *Enterococcus faecalis* 201701784.1.K, *Enterococcus faecium* VF21.2-1122.3.K, *Enterococcus durans* 201702044.1.K, *Staphylococcus equorum* 201701784.3.B, *Staphylococcus simulans* 201702044.2.B, *Staphylococcus xylosus* 201702052.2.B, *Lactococcus lactis* P.6.2.M and *Lactobacillus parabuchneri* P.12.3.M, respectively, were used as tyramine producers. These strains were previously isolated from cheese or fermented sausages by our research group and tested geno- and phenotypic for the presence of the *tdcA* gene and tyrosine decarboxylase activity, respectively. *Enterococcus* spp. and *Staphylococcus* spp. were incubated aerobically at 37°C in brain-heart infusion (BHI) broth while *Lactobacillus* and *Lactococcus* spp. were incubated anaerobically at 37°C in MRS broth.

In silico screening of *Lb. plantarum* JA-1199

Tyrosine decarboxylase enzyme EC 4.1.1.25

The absence of the tyrosine decarboxylase gene (*tdcA*) in *Lb. plantarum* JA-1199 was investigated using an *in silico* screening approach. The genome of *Lb. plantarum* JA-1199 was sequenced with Illumina MiSeq (pairwise reads of 150 bp) with coverage of 30-fold. Genes were then identified using prokka v.1.12 (http://www.vicbioinformatics.com/software.prokka.shtml), and the absence of tyrosine decarboxylase was screened by aligning amino acids sequences of *Lb. plantarum* JA-1199 against tyrosine decarboxylase enzyme EC 4.1.1.25 sequence in the GeneBank Genome Net (https://www.genome.jp/), UniProtKB (https://www.uniprot.org/help/uniprotkb), and Brenda enzymes (https://www.brenda-enzymes.info/index.php) databases with the BLAST program included CLC Genomics workbench (version 10.0.1) program.

Identification of tyrosine-degrading enzymes of *Lb. plantarum* JA-1199

Genes were also annotated using prokka v.1.12, and pathway analysis were conducted using the KEGG pathway web services (https://www.genome.jp/kegg/kaas/), the Laccase Engineering Database (LccED https://lcced.biocatnet.de/) and the NCBI gene bank (https://www.ncbi.nlm.nih.gov/).

Phenotypic identification of tyrosine degradation

Analysis of metabolites by Biolog

Carbon and nitrogen metabolism of selected strains were analysed by the Biolog microarray systems PM3B (Nitrogen Sources) and PM1 (Carbon Source), respectively (Biolog, 21124 Cabot Blvd., Hayward, CA 94545, USA). The 96-well plates were inoculated with 0.1 mL of the calculated concentration (turbidity of 75%) of the overnight bacterial suspension of *Lb. plantarum* JA-1199 and *Lb. plantarum* WCFS1, respectively, with an IF-0a GN/GP base inoculating fluid and a tetrazolium redox dye mix according to the manual. Plates were analysed at 30°C for 3 days every 15 min, and data were analysed using the program "DuctApe" (https://combogenomics.github.io/DuctApe/index.html).

Milk and minimal chemically defined medium (mCDM)

Lb. plantarum JA-1199 was phenotypically assayed for his capacity to degrade tyrosine upon cultivation in pasteurised skim milk and in minimal chemically defined medium (mCDM) (Guarcello et al., 2016). Slight modifications by adding sterile-filtered copper (II) chloride to a final concentration of 1 mM solution after sterilisation of mCDM and by changing the glucose concentration to 5 g L^{-1} were done. The overnight pre-cultures of *Lb. plantarum* JA-1199 and *Lb. plantarum* WCFS1 were centrifuged at 7'000 x g for 10 min, washed with peptone solution (9% NaCl, 1% peptone from casein, pH 7) and inoculated at a calculated $OD600_{nm}$ value of 0.1 in milk or mCDM broth each containing 4 mM tyrosine (Sigma Aldrich, Buchs, Switzerland) as a substrate for potential tyrosine-degrading bacteria. Afterwards, samples were incubated at 37°C for 96 h, and tyrosine and tyramine were measured at the time points of 0, 4, 6, 24, 48, 72, and 96 h. Bacterial growth was monitored by plating serial dilutions on the corresponding semi-selective MRS agar-medium incubated anaerobically for 48 h at 37°C.

Experimental micro-cheese model manufacture

A micro-cheese model, containing 2 mL pasteurised skimmed milk, 1 mM $CaCl_2$ and 0.02% Fromase 220 TL rennet (Winkler AG, Konolfingen, Switzerland) was prepared. All tyramine producer strains were tested in these micro-cheese models in triplicate under four different sodium chloride concentrations (0%, 1.5%, 3% and 4.5%) each with and without adding 2.5 mM tyrosine, either the strains alone or combined with *Lb. plantarum* JA-1199 to test its capacity to reduce tyramine accumulation in a cheese-like environment. Overnight cultures of the used strains were centrifuged at 7’000 x g for 10 min and resuspended at a calculated OD_{600} of 0.2 in pasteurised skimmed milk. The casein degradation step was started through the added 1 mM calcium chloride and 0.075% rennet, followed by incubation at 30°C for 1 h. The curd was cut by using a sterile inoculation loop and incubated for 15 min at 37°C, after which cutting was repeated followed by incubation at 37°C for 40 min again. To simulate the pressing process of the cheese, micro-cheese samples were centrifuged at 6'000 x g for 10 min. The supernatant was used for tyrosine, tyramine and pH measurement, and the micro-cheese pellet was incubated at 30°C for 7 days to simulate the ripening time. After the maturation step, the pellet was resuspended in 1.8 mL Milli-Q water, and 0.1 mL were used for colony counting. Afterwards, the resuspension was centrifuged at 10'000 x g for 5 min, and the supernatant was used for tyrosine, tyramine and pH measurement. Colony counting was performed by plating serial dilutions of the 0.1 mL portion on the corresponding semi-selective agar-media Baird-Parker agar (BP; Biolife, Milano, Italy) for *Staphylococcus* spp., KF *Streptococcus* agar (KFS, Becton Dickinson, Allschwil, Switzerland) for *Enterococcus* spp. and De Man, Rogosa and Sharpe agar (MRS, Biolife, Milano, Italy) for *Lactobacillus* and *Lactococcus* spp. BP and KFS agar were incubated aerobically for 48 h at 37°C and 43°C, respectively whereas MRS agar was incubated anaerobically for 48 h at 37°C in 2.5 L culture jars with AnaeroGen AN 25 sachets (Oxoid, Basingstoke, UK).

Determination of tyrosine and tyramine

For the quantitative determination of tyrosine and tyramine, ion exclusion chromatography with pulsed amperometric detection (IC-PAD) was used (Dionex, 2007) with using IC-PAD apparatus Dionex™ ICS-5000+ Hybrid HPIC™ system and IonPac CG18, 2 x 50 mm as a precolumn and IonPac CS18, 2 x 250 mm as separation column, respectively (Thermo Fisher Scientific, Switzerland). The Dionex method was modified by changing the eluent gradient: 5-10 mM methanesulfonic acid (MSA) from 0–6 min, 10–20 mM from 6–16 min, and 20–45 mM from 16–20 min. The samples were used in a 1:10 dilution for determination of tyrosine and tyramine concentrations with a detection limit of 0.13 mg L^{-1} and 0.05 mg L^{-1}, respectively.

Statistical analysis

All samples were measured in triplicate, and all means of replications were first calculated prior to comparison. Samples with concentrations of tyramine or tyrosine below the detection limit were replaced with artificial values of 0.049 for the concentration of tyramine and 0.129 for the concentration of tyrosine, respectively, to allow statistical analysis (Garriga et al., 2002). Statistical analysis was performed by using a t-test including Shapiro-Wilk normality and Brown-Forsythe equal variance test ($P < 0.05$) in the statistic software SigmaPlot 13 (Systat Software Inc., San Jose, California, USA).

Results

In silico screening of *Lb. plantarum* JA-1199

In a preliminary test, different *Lb. plantarum* strains, previously isolated from cheese and sausage samples, were tested on the ability to degrade tyrosine. Because of *Lb. plantarum* JA-1199 showed the highest capacity to degrade tyrosine and were under phenotypic assay conditions unable to produce tyramine; this strain was selected for further experiments. Indeed, its genome analysis revealed a genome-size of 3.33 Mb, a G+C content of 44.3% and showed a high level of similarity to *Lb. plantarum* WFCS1, which is one of the best characterised *Lb. plantarum* strains, and both strains, either at the nucleotide or at the protein level, lack a tyrosine decarboxylase gene (*tdcA*) or tyrosine decarboxylase enzyme (EC 4.1.1.25), respectively.

In the *in silico* screening of *Lb. plantarum* JA-1199 for a tyrosine-degrading enzyme, a multicopper oxidase family protein of 501 as well as a histidinol-phosphate transaminase (EC 2.6.1.9) of 365 amino acid residues were found. The detected enzymes showed on the protein blast level a 100% identity and an E-value of 0.0 to *Lactobacillus* multicopper oxidase domain-containing proteins (WP_003641930) and to the histidinol-phosphate transaminase protein (WP_046947669.1), which confirmed, that the compared amino acid sequences have the same residues at the same positions in an alignment.

Phenotypic identification of tyrosine degradation

For a phenotypic confirmation of the *in silico* screening, an analysis of metabolites by a Biolog microarray assay was done. This assay seemed to have the capacity to test the *Lb. plantarum* JA-1199 strain as a tyrosine degrader. The strain was able to use tyrosine as a nitrogen source, whereas the negative control strain *Lb. plantarum* WCFS1 showed no activity (Figure 4.1). Furthermore, the ability of the strains to use tyramine as a carbon source was negative for both strains (Figure 4.2).

In a further experiment and for a second phenotypic confirmation of the capacity of *Lb. plantarum* JA-1199 to degrade tyrosine, a phenotypic assay in pasteurised skimmed milk and upon the cultivation in mCDM, both containing tyrosine was done. In addition, IC-PAD mediated measurement of tyrosine and

tyramine concentrations after growth in both mCDM and milk confirmed the ability of *Lb. plantarum* JA-1199 to degrade tyrosine up to 54.68% and 40.72%, respectively, whereas the tyramine concentration was below the detection limit or in a minimum concentration of 6.05 mg L^{-1}, respectively (Figure S 4.1).

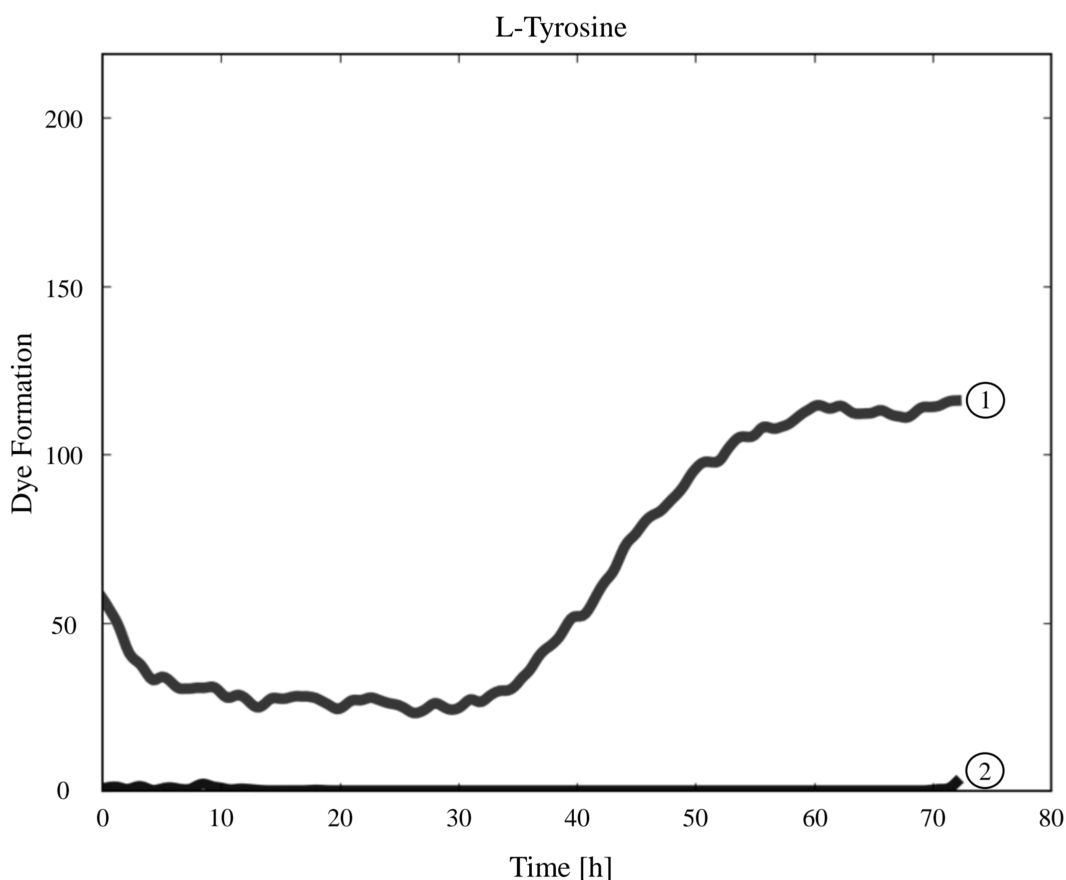

Figure 4.1: Biolog activity of *Lb. plantarum* JA-1199 (1) as a tyrosine utilizer and *Lb. plantarum* WCFS1 (2) as a negative control on tyrosine medium. The activity was quantified by colour intensity. The more NADPH was reduced, the used dye formation increase its intensity.

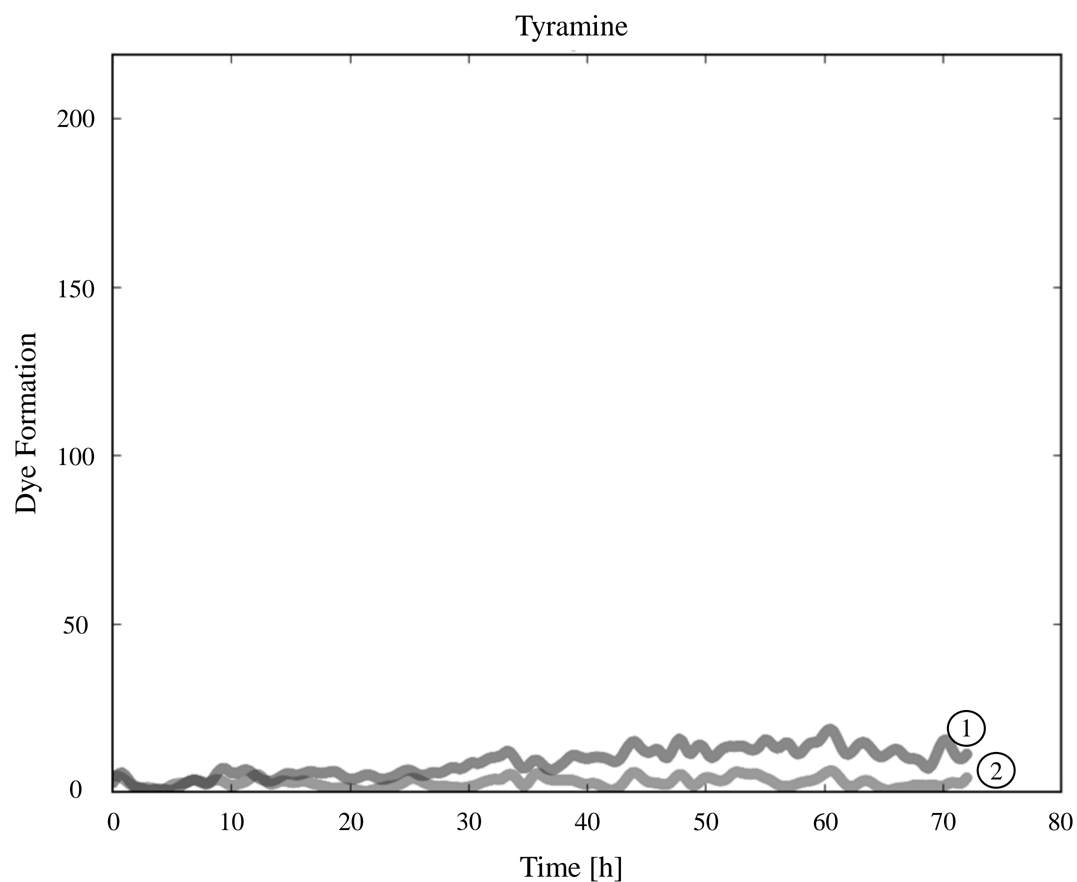

Figure 4.2: Biolog activity of *Lb. plantarum* JA-1199 (1) and *Lb. plantarum* WCFS1 (2) on tyramine medium. The activity was quantified by colour intensity. The more NADPH was reduced, the used dye formation increase its intensity.

Tyramine formation and tyrosine degradation in a micro-cheese model with different sodium chloride concentrations

To investigate tyrosine degradation and tyramine formation, in an environment similar to cheese with different sodium chloride concentration, a micro-cheese model was performed. Therefore, sodium chloride concentration in a micro-cheese fabrication process at an initially fixed concentration of tyrosine (2.5 mM) and variable sodium chloride concentrations were tested. Indeed sodium chloride markedly influenced tyramine formation of all eight bacterial strains, all selected before as tyramine producers, belonging to the species *E. faecalis*, *E. faecium*, *E. durans*, *S. equorum*, *S. simulans*, *S. xylosus*, *L. lactis*, and *L. parabuchneri*, respectively (Table 4.1).

Table 4.1: Production of tyramine in mg L^{-1} by eight different tyramine-producing bacterial strains in micro-cheese models containing 2.5 mM tyrosine and four different sodium chloride concentrations.

		Sodium chloride concentration			
		0%	**1.5%**	**3%**	**4.5%**
Species		**Tyramine production [mg L^{-1}]**			
Enterococcus faecalis	201701784.1.K	716.10	635.90	598.52	701.08
Enterococcus faecium	VF21.2-1122.3.K	195.22	325.75	566.20	653.26
Enterococcus durans	201702044.1.K	301.15	248.16	342.42	685.32
Staphylococcus equorum	201701784.3.B	107.92	575.93	613.11	600.66
Staphylococcus simulans	201702044.2.B	72.12	349.82	143.73	337.65
Staphylococcus xylosus	201702052.2.B	67.91	659.17	322.92	573.75
Lactococcus lactis	P.6.2.M	264.96	349.32	417.40	517.15
Lactobacillus parabuchneri	P.12.3.M	262.71	369.25	429.85	475.86

Importantly, all of the eight strains were able to grow in a micro-cheese model, containing up 4.5% of sodium chloride and initial tyrosine concentration at 2.5 mM, and form concentrations of tyramine up to 701.08 mg L^{-1} in the cheese-model containing *E. faecalis* 201701784.1.K. In addition, this strain formed a relatively stable and high tyramine concentration in the micro-cheese models at all four sodium chloride concentrations (0%, 1.5%, 3% and 4.5%) (Figure S 4.2). The highest increase of tyramine concentration was 870.72% and was produced by *S. xylosus* 201702052.2.B in the two micro-cheese models containing 0% and 1.5% sodium chloride and tyramine concentrations of 67.91 mg L^{-1} and

659.17 mg L^{-1}, respectively (Table 4.1). All tyramine-producing bacteria, except *E. faecalis* 201701784.1.K, *S. simulans* 201702044.2.B, and *S. xylosus* 201702052.2.B accumulated a higher concentration of tyramine in micro-cheese models at sodium chloride concentrations of 3 and 4.5% than at lower sodium chloride concentrations of 0 and 1.5%, respectively. Furthermore, the three tyramine-producing bacteria *L. lactis* P.6.2.M, *Lb. parabuchneri* P.12.3.M, as well as *E. faecium* VF21.2-1122.3.K, showed with an increase of sodium chloride a continuous increase of tyramine concentration (Table 4.1 and Figure S 4.2–Figure S 4.9).

In micro-cheese models started without the addition of tyrosine, tyramine concentrations were negligible during production and were between "below detection limit" (*S. equorum* 201701784.3.B at 0% sodium chloride and *S. simulans* 201702044.2.B at 0 and 3% sodium chloride) and 17.55 mg L^{-1} (*E. faecalis* 201701784.1.K at 0% sodium chloride) (Table S 4.1).

Interestingly, tyramine accumulation could be reduced in all setups containing one of the eight tyramine producer strains by the addition of *Lb. plantarum* JA-1199 except in the setup containing *L. lactis* P.6.2.M at 4.5% sodium chloride. However, there were significant differences between different tyramine-producing strains as well as between the different sodium chloride concentrations within a tyramine producer itself (Table 4.2, Figure S 4.10–Figure S 4.17).

Table 4.2: Reduction ability of tyramine accumulation in percentage by *Lb. plantarum* JA-1199 in micro-cheese models containing 2.5 mM tyrosine and four different sodium chloride concentrations inoculated with eight different tyramine-producing strains.

		Sodium chloride concentration			
		0%	**1.5%**	**3%**	**4.5%**
Species		**Reduction [%]**			
Enterococcus faecalis	201701784.1.K	27.65*	18.90*	31.30*	4.80
Enterococcus faecium	VF21.2-1122.3.K	33.44*	32.79*	41.00*	5.51
Enterococcus durans	201702044.1.K	31.96*	15.04	12.76*	6.57
Staphylococcus equorum	201701784.3.B	72.80*	49.16*	9.39	4.17
Staphylococcus simulans	201702044.2.B	99.93*	82.56*	75.75*	4.94
Staphylococcus xylosus	201702052.2.B	99.93*	53.73*	40.85*	9.23
Lactococcus lactis	P.6.2.M	50.26*	69.12*	6.25	-1.37
Lactobacillus parabuchneri	P.12.3.M	41.44*	37.65*	7.57	4.45

* $P < 0.05$

At a sodium chloride concentration of 4.5%, the ability of *Lb. plantarum* JA-1199 to reduce tyramine accumulation significantly decreased in all eight micro-cheese model setups and showed a maximum of 9.23% of reduction at *S. xylosus* 201702052.2.B with tyramine concentrations of 520.77 mg L^{-1} in combination with *Lb. plantarum* JA-1199 and 573.75 mg L^{-1} without, respectively. Moreover, at 4.5% sodium chloride, *L. lactis* P.6.2.M was even able to produce in a minimal concentration more tyramine in combination with *Lb. plantarum* JA-1199 compare than without adding *Lb. plantarum* JA-1199. In combination with *S. simulans* 201702044.2.B or *S. xylosus* 201702052.B, *Lb. plantarum* JA-1199 showed the highest reduction efficiency of tyramine accumulation of 99.93% at 0% of sodium chloride. In combination with *S. simulans* 201702044.2.B, tyramine concentrations of "below detection limit", 61.00 mg L^{-1} and 34.86 mg L^{-1} in 0%, 1.5% and 3% sodium chloride were detected and without *Lb. plantarum* JA-1199 concentrations of 72.12 mg L^{-1}, 349.82 mg L^{-1} and 143.73 mg L^{-1}, respectively. In combination with *S. xylosus* 201702052.2.B, tyramine concentrations of "below detection limit", 305.01 mg L^{-1}, and 191.01 mg L^{-1} in 0%, 1.5% and 3% sodium chloride could be detected and without *Lb. plantarum* JA-1199 concentrations of 67.91 mg L^{-1}, 659.17 mg L^{-1} and 322.92 mg L^{-1}, respectively, which is a reduction of tyramine accumulation of 99.93%, 82.56% and 75.75% for *S. simulans*

201702044.2.B and 99.93%, 53.73%, and 40.85% for *S. xylosus* 201702052.2.B, respectively. As without *Lb. plantarum* JA-1199, *E. faecalis* 201701784.1.K also produced a relatively stable and high tyramine concentration in combination with *Lb. plantarum* JA-1199 in all four tested sodium chloride concentrations. Moreover, *Lb. plantarum* JA-1199 showed a lower activity, with a maximum reduction of tyramine accumulation of 31.30% in a micro-cheese with 3% sodium chloride, compared to the reduction in combination with other tested tyramine producers (Table 4.2).

In comparison, tyramine concentrations in micro-cheese models in combination with *Lb. plantarum* JA-1199 without initial addition of 2.5 mM tyrosine could be neglected since concentration reached only values between "below the detection limit" (*S. equorum* 201701784.3.B, and *S. xylosus* 201702052.2.B at 0% sodium chloride and *S. simulans* 201702044.2.B at 0%, 1.5% and 3% sodium chloride) and 18.89 mg L^{-1} (*E. faecalis* 201701784.1.K at 0% sodium chloride) (Table 4.3).

Table 4.3: Production of tyramine in mg L^{-1} by eight different tyramine-producing bacterial strains in combination with *Lb. plantarum* JA1199 in micro-cheese models without tyrosine at four different sodium chloride concentrations.

		Sodium chloride concentration			
		0%	**1.5%**	**3%**	**4.5%**
Species		**Tyramine production [mg L^{-1}]**			
Enterococcus faecalis	201701784.1.K	18.98	9.14	11.60	9.81
Enterococcus faecium	VF21.2-1122.3.K	5.24	9.80	12.50	13.76
Enterococcus durans	201702044.1.K	6.02	7.51	11.71	9.81
Staphylococcus equorum	201701784.3.B	b.d.	2.09	6.35	4.25
Staphylococcus simulans	201702044.2.B	b.d.	b.d.	b.d.	3.60
Staphylococcus xylosus	201702052.2.B	b.d.	2.32	2.56	2.79
Lactococcus lactis	P.6.2.M	6.15	4.21	11.98	10.90
Lactobacillus parabuchneri	P.12.3.M	7.08	8.87	12.26	10.94

b.d.: below detection limit

In order to confirm the previous data of tyrosine degradation of *Lb. plantarum* JA-1199 and to verify its ability to degrade tyrosine in a micro-cheese model, *Lb. plantarum* JA-1199 was incubated with and without 2.5 mM tyrosine at different sodium chloride concentrations (0%, 1.5%, 3% and 4.5%). It could

be shown that *Lb. plantarum* JA-1199 has the ability to reduce tyramine concentration up to 98.66%, independent of sodium chloride concentration (Figure S 4.18). However, after incubation, a small concentration of tyramine was found. Tyramine concentration increased with sodium chloride concentration and had a maximum at 4.5% sodium chloride with a value of 57.08 mg L^{-1} (Table S 4.2A). The incubation of *Lb. plantarum* JA-1199 without tyrosine at different sodium chloride concentrations showed negligibly small values with a maximum of 14.46 mg L^{-1} (4.5% sodium chloride after 7 days of ripening time) for tyrosine and 8.66 mg L^{-1} (3% sodium chloride after 7 days of ripening time) for tyramine, respectively (Table S 4.2B). All tyramine concentrations prior to incubation were below the detection limit of 0.05 mg L^{-1}. Furthermore, it was verified that *Lb. plantarum* JA-1199 does not inhibit *per se* the growth of tyramine-producing bacteria, and therefore less tyramine would be produced. Tyramine-producing bacteria were thus plated without and in combination with *Lb. plantarum* JA-1199 on the corresponding semi-selective media-agar and incubated. It could be confirmed that the growth of the tyramine-producing strain in combination with *Lb. plantarum* JA-1199 was in the same log count as in growth without, respectively (Figure S 4.19).

To confirm, tyrosine concentrations in the micro-cheese model remain stable during incubation without bacterial strains, micro-cheese models were incubated with initial 2.5mM tyrosine at four different sodium chloride concentrations (0%, 1.5%, 3% and 4.5%). It was shown that the tyrosine concentration remained stable with a marginal variation of 3.95% at 0%, 1.19% at 1.5% and 1.26% at 3% sodium chloride, respectively. At a sodium chloride concentration of 4.5%, a slight increase of tyrosine of 12.93% from 448.61 mg L^{-1} up to 506.62 mg L^{-1} was observed. Importantly, all tyrosine and tyramine concentrations before and all tyrosine concentrations after ripening without tyrosine addition at different sodium chloride concentrations were below the detection limit of 0.13 mg L^{-1} and 0.05 mg L^{-1}, respectively. After 7 days of ripening, only very low tyramine concentrations of 7.33 mg L^{-1} at 0% sodium chloride, 5.84 mg L^{-1} at 1.5% sodium chloride, 7.42 mg L^{-1} at 3% sodium chloride and 2.34 mg L^{-1} at 4.5% sodium chloride were detected.

Discussion

The accumulation of BAs in fermented foods is a matter of public health concern, and it is important to know, which factors affecting the production of BAs and how to prevent or reduce the accumulation in fermented foods (Özogul and Özogul, 2020).

It is known that mono- and diaminooxidase catalyse the detoxifying oxidation of BAs in the gastrointestinal tract of higher organisms (Youdim et al., 2006). Furthermore, amino oxidases were identified in *Arthrobacter kristallopoes*, *Bacillus amyloliquefaciens*, *Candida boidinii, Klebsiella aerogenes*, *Lactobacillus* and *Pediococcus* spp., *Micrococcus rubens*, *Rhodococcus erythropolis*, *Sarcina lutea* and *Staphylococcus carnosus*. Although the exact mechanism of action is still unclear, amine-degrading bacteria are used to reduce BAs in food (Capozzi et al., 2012; Cooper, 1997; García-Ruiz et al., 2011; Ota et al., 2008; Van Hellemond et al., 2008; Yagodina et al., 2002; Zaman et al., 2011). Within lactic acid bacteria *Lb. plantarum* J16 and its amine-degrading activity were attributed to a protein annotated as laccase (EC 1.10.3.2) (Callejón et al., 2016). This enzyme belongs to the multicopper oxidase superfamily, and their catalytic centres contain four reactive copper atoms, giving them a characteristic blue colour (Reiss et al., 2013). Furthermore, it is known that aminotransferases catalyse the transfer of an amino group from the amino acid, acting as a substrate to an acceptor oxo acid (Mehta et al., 1989). Therefore, aminotransferases play an essential role in the metabolism of amino acids as the resulting product 4-hydroxy-phenylpyruvate can be further metabolised to succinate and finally used as an energy source in the citrate cycle as an energy gain (Kyoto Encyclopedia of Genes and Genomes, 2019). In this study, the *in silico* screening of *Lb. plantarum* JA-1199 identified a multicopper oxidase as well as a histidinol-phosphate transaminase (EC 2.6.1.9). The biological roles of multicopper oxidases in bacteria are poorly elucidated, but it is known that laccases oxidise phenols by a radical-generating reaction mechanism (Claus, 2003; Sharma et al., 2007). Furthermore, in this study, the use of tyrosine by *Lb. plantarum* JA-1199 was phenotypically demonstrated using a Biolog microarray assay and through incubation in mCDM and milk both containing tyrosine. Tyrosine metabolism contains various enzymatic pathways via tyrosinases and laccases to L-dopa or via aminotransferases to 4-hydroxy-phenylpyruvate. It may be hypothesised that the capacity of

Lb. plantarum JA-1199 and its histidinol-phosphate transaminase (EC 2.6.1.9), which shows homology to the aspartate aminotransferase and tyrosine aminotransferase, namely aspartate aminotransferase (EC 2.6.1.1) and tyrosine aminotransferase (EC 2.6.1.5) (Mehta et al., 1989), to use tyrosine are based on this metabolism.

Previous studies reported that sodium chloride has an influence on decarboxylase activity and thus on BA accumulation in fermented foods (Buáková et al., 2011). A higher sodium chloride concentration increases the concentration of BAs produced by increased enzyme activity and the expression of genes involved in BA production (Buáková et al., 2011; Buňková et al., 2012; Ladero et al., 2016b). In this study, the influence of sodium chloride on tyramine production could be confirmed. However, a high variability on tyramine concentration of different bacteria and their response to different sodium chloride concentrations was observed. While *E. faecalis* 201701784.1.K due to its osmophilia showed a relatively stable and high tyramine concentration for all four sodium chloride concentrations (Foulquié Moreno et al., 2006), the other seven tyramine-producing strains showed an elevated tyramine concentration at an increased sodium chloride concentration. However, due to the sensitivity to sodium chloride, *S. simulans* 201702044.2.B and *S. xylosus* 201702052.2.B showed at a sodium chloride concentration of 3% and 4.5% a smaller concentration of tyramine and a lower growth rate compares to 1.5% sodium chloride. Thus, it could be demonstrated that representatives of different tyramine-producing bacterial species react differently to an increased sodium chloride concentration.

Because of his use of the substrate tyrosine as a nitrogen source, in this study, *Lb. plantarum* JA-1199 was successfully used to reduce tyramine accumulation in different environments. Compared to the results without *Lb. plantarum* JA-1199, the micro-cheese models containing this strain showed significantly lower concentrations of tyramine with a reduction of up to 99% in co-incubation with *S. simulans* 201702044.2.B and *S. xylosus* 201702052.2.B. However, this study also showed a drastic decrease in the ability of *Lb. plantarum* JA-1199 to reduce tyramine accumulation as a response to an increase in sodium chloride concentration up to 4.5%. This effect can be confirmed by the potential role of osmoprotection of decarboxylase gene expression as a part of complex metabolic responses in the presence of stress conditions. The TDC cluster in tyramine-producing bacteria contains a Na^+/H^+

transporter encoding gene and sodium ions (Na^+) are known to be involved in the regulation of intracellular pH and are important in sodium/proton antiporter systems by exchange with H^+ ions that are removed from the cell. Therefore, sodium ions play an essential role in the tyrosine decarboxylation pathway and may explain the higher tyramine production at a higher sodium chloride concentration as proposed by other authors (Buáková et al., 2011; Pereira et al., 2009; Tabanelli et al., 2012). However, since sodium chloride is used in fermented food products to prevent spoilage and food poisoning (Linares et al., 2012), reducing sodium chloride concentration in cheese is not an option to reduce the tyramine content in cheese. The minimal concentration of tyramine found in the micro-cheese model incubated only with *Lb. plantarum* JA-1199, as well as in the control samples, can be explained by the fact that milk may already contain tyramine at low dosages (EFSA, 2011; Novella-Rodríguez et al., 2004).

Lb. plantarum is a common member of the non-starter lactic acid bacteria in cheeses and given the concerns over the presence of tyramine concentrations in different cheeses, this study showed a feasible strategy for decreasing tyramine accumulation and therefore increasing the safety level of fermented foods by application of a food-grade biocontrol strain.

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We are grateful to the Swiss Federal Food Safety and Veterinary Office (FSVO) for funding this study. The authors also extended their acknowledgements to Dr. Ueli von Ah, and the whole Agroscope team, for their support. We would also like to thank Iris Häsuermann, Serafina Plüss and Shera Ly for their work during their Bachelor thesis and Simon Gürber for his work during his Master thesis.

Supplementary information

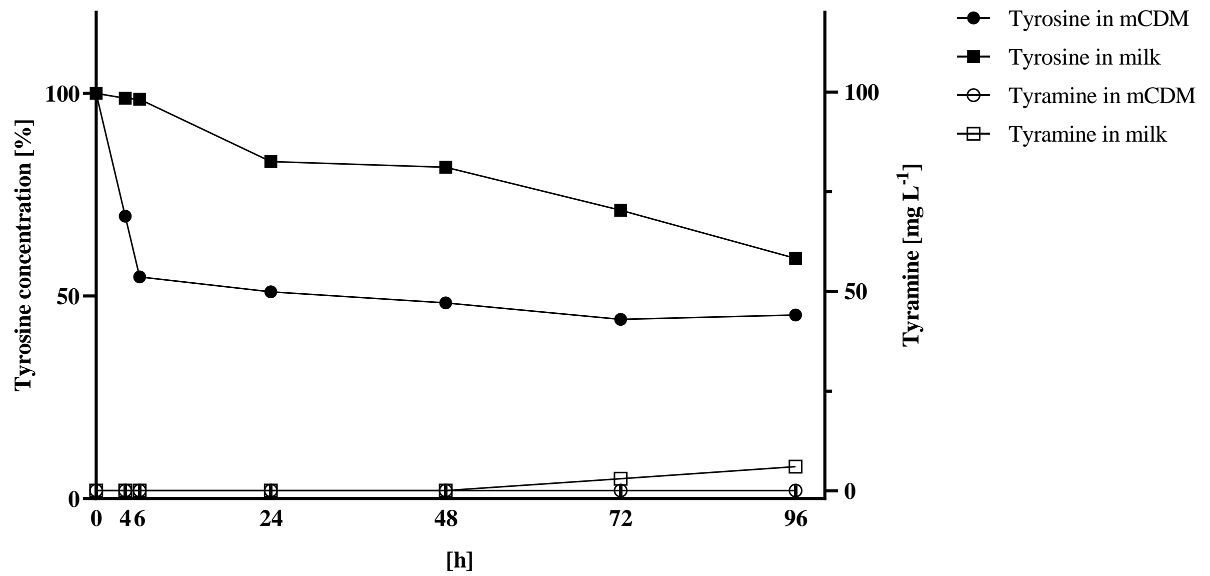

Figure S 4.1: The ability of *Lb. plantarum* JA-1199 in the reduction of tyrosine in [%] and tyramine production in [mg L^{-1}] in mCDM and milk containing 4 mM tyrosine.

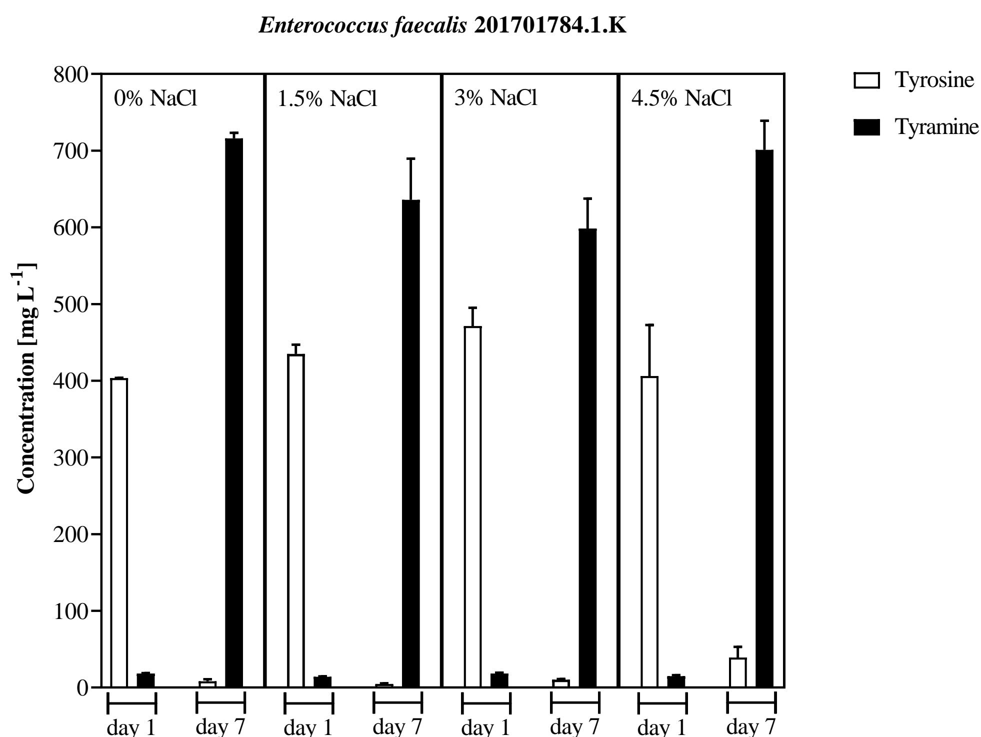

Figure S 4.2: Mean values of tyrosine (white) and tyramine (black) concentrations [mg L^{-1}] produced by *E. faecalis* 201701784.1.K at 0%, 1.5%, 3% and 4% sodium chloride incubated with 2.5 mM tyrosine.

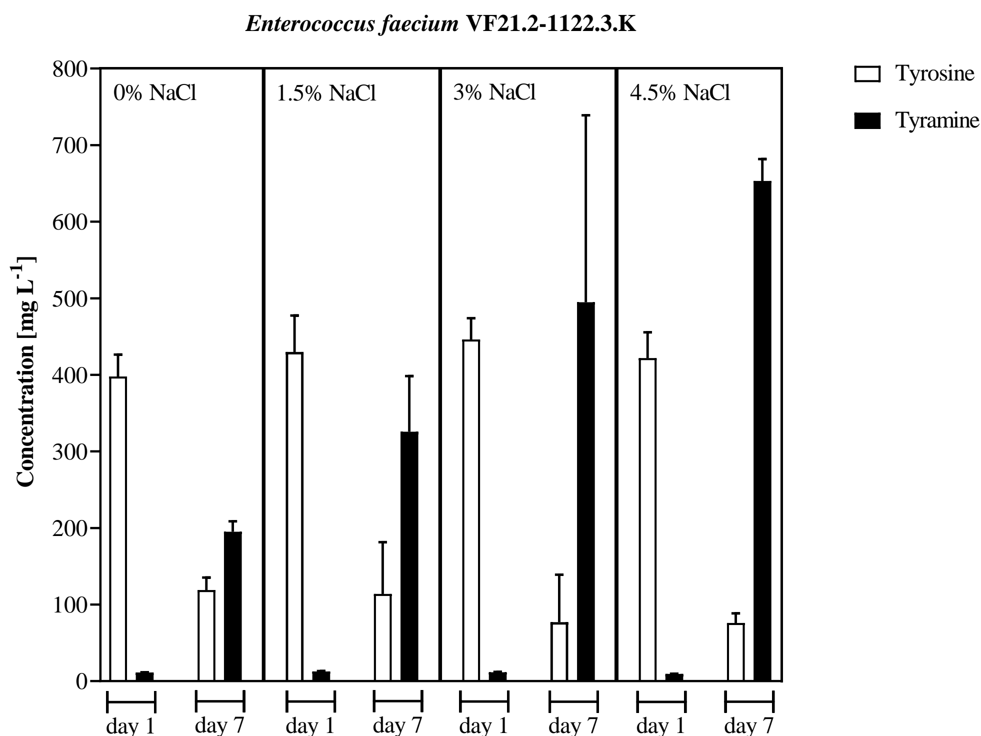

Figure S 4.3: Mean values of tyrosine (white) and tyramine (black) concentrations [mg L^{-1}] produced by *E. faecium* VF21.2-1122.3.K at 0%, 1.5%, 3% and 4% sodium chloride incubated with 2.5 mM tyrosine.

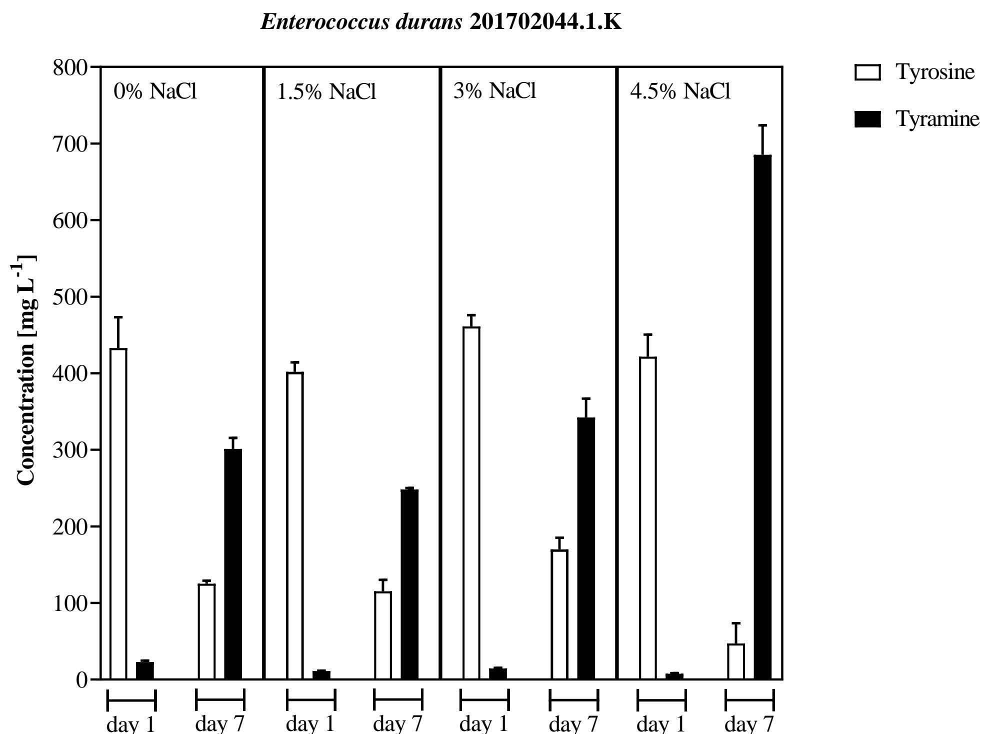

Figure S 4.4: Mean values of tyrosine (white) and tyramine (black) concentrations [mg L^{-1}] produced by *E. durans* 201702044.1.K at 0%, 1.5%, 3% and 4% sodium chloride incubated with 2.5 mM tyrosine.

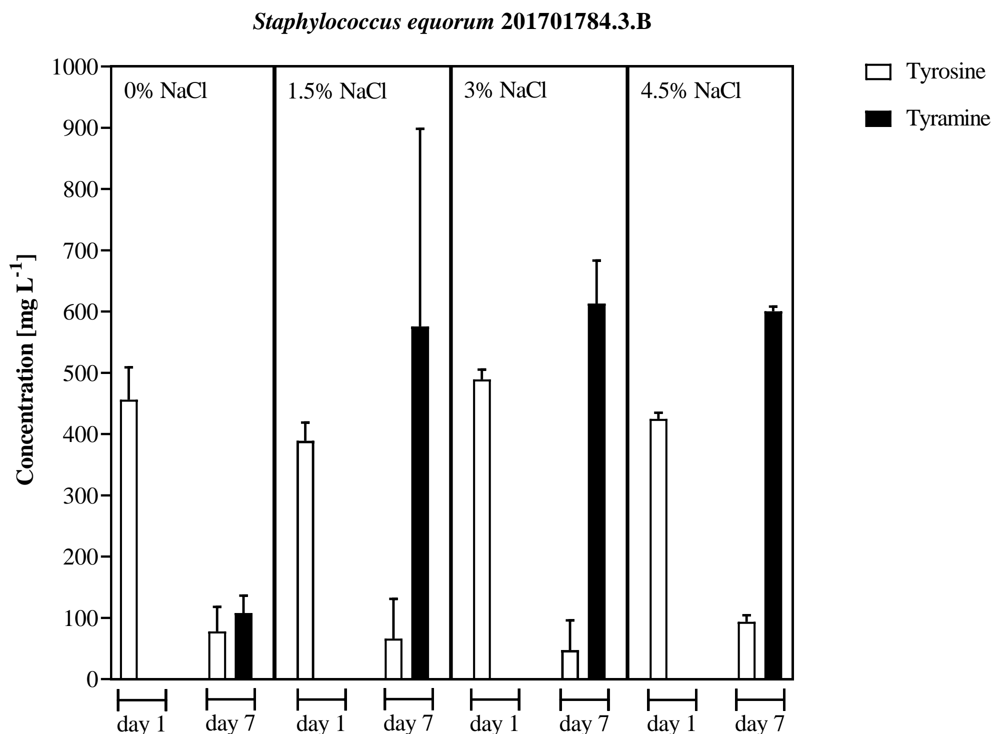

Figure S 4.5: Mean values of tyrosine (white) and tyramine (black) concentrations [mg L^{-1}] produced by *S. equorum* 201701784.3.B at 0%, 1.5%, 3% and 4% sodium chloride incubated with 2.5 mM tyrosine.

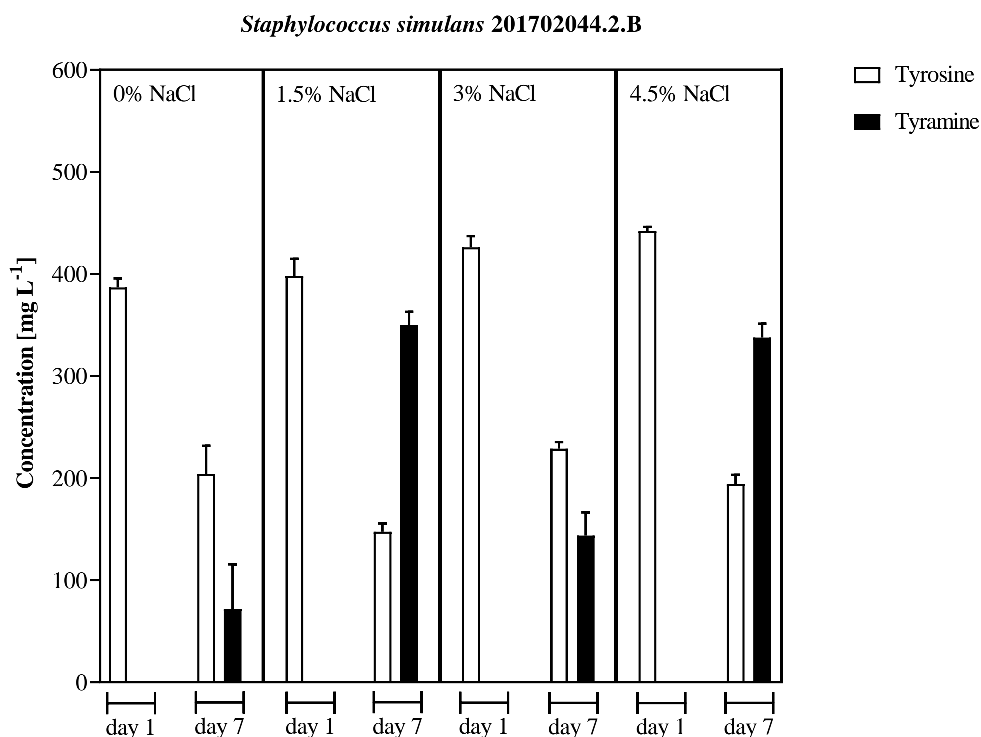

Figure S 4.6: Mean values of tyrosine (white) and tyramine (black) concentrations [mg L^{-1}] produced by *S. simulans* 201702044.2.B at 0%, 1.5%, 3% and 4% sodium chloride incubated with 2.5 mM tyrosine.

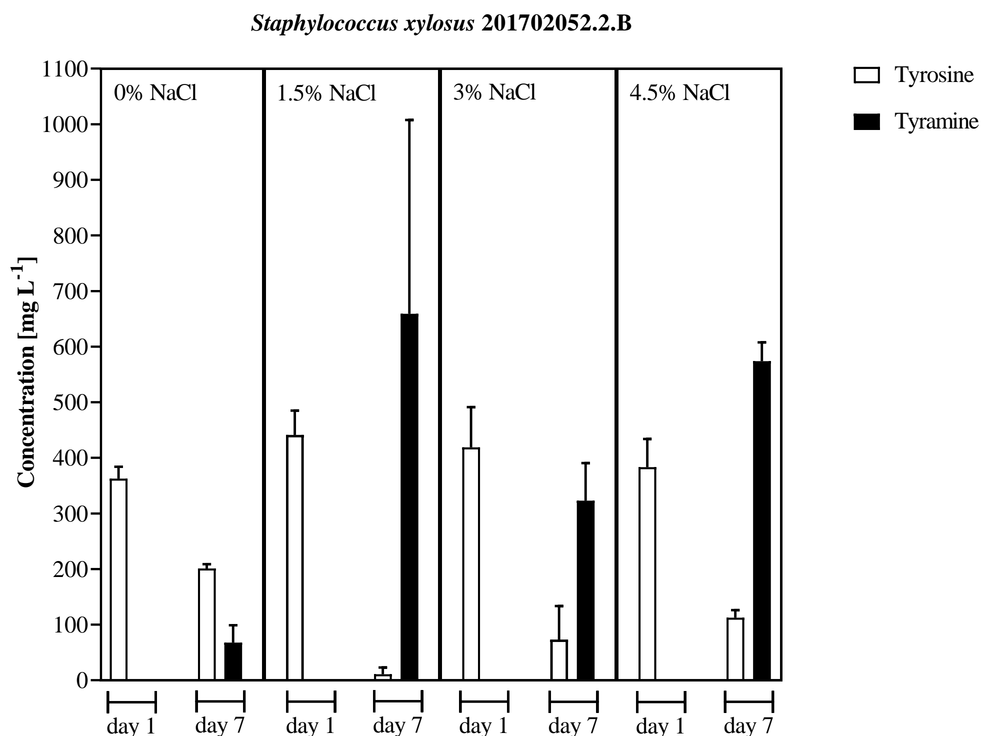

Figure S 4.7: Mean values of tyrosine (white) and tyramine (black) concentrations [mg L^{-1}] produced by *S. xylosus* 201702052.2.B at 0%, 1.5%, 3% and 4% sodium chloride incubated with 2.5 mM tyrosine.

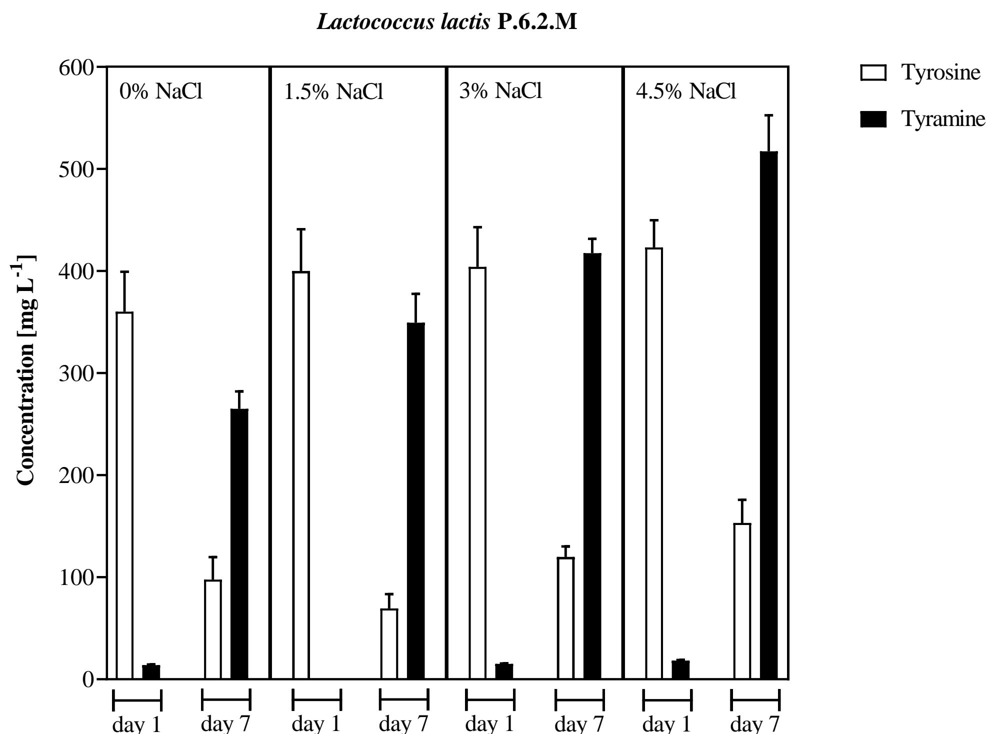

Figure S 4.8: Mean values of tyrosine (white) and tyramine (black) concentrations [mg L^{-1}] produced by *L. lactis* P.6.2.M at 0%, 1.5%, 3% and 4% sodium chloride incubated with 2.5 mM tyrosine.

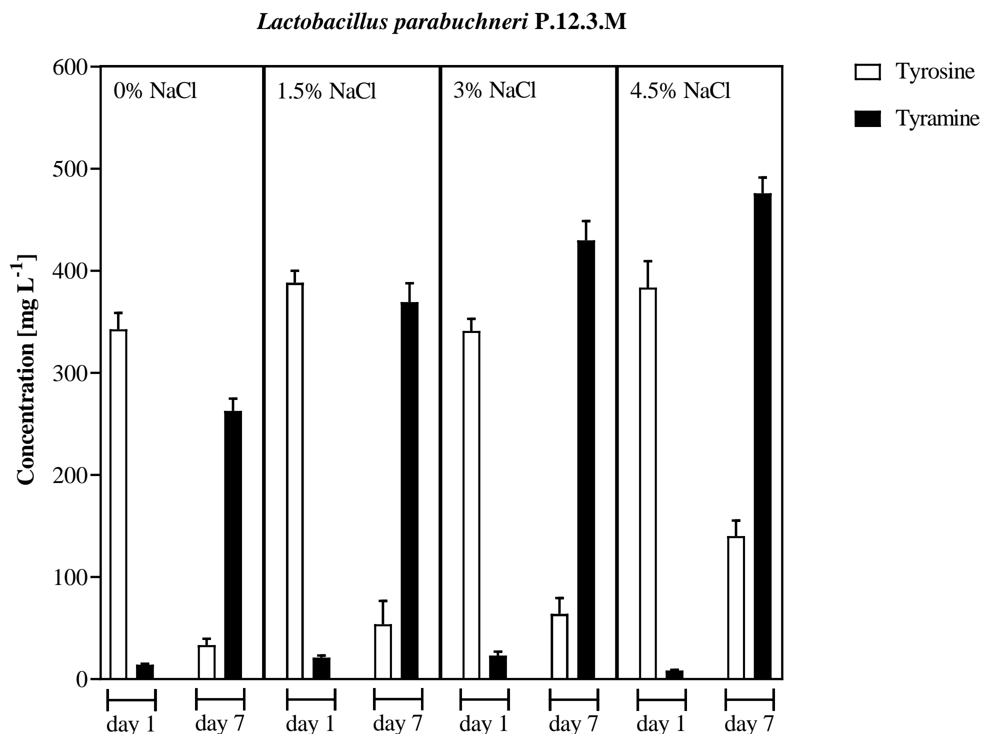

Figure S 4.9: Mean values of tyrosine (white) and tyramine (black) concentrations [mg L^{-1}] produced by *Lb. parabuchneri* P.12.3.M at 0%, 1.5%, 3% and 4% sodium chloride incubated with 2.5 mM tyrosine.

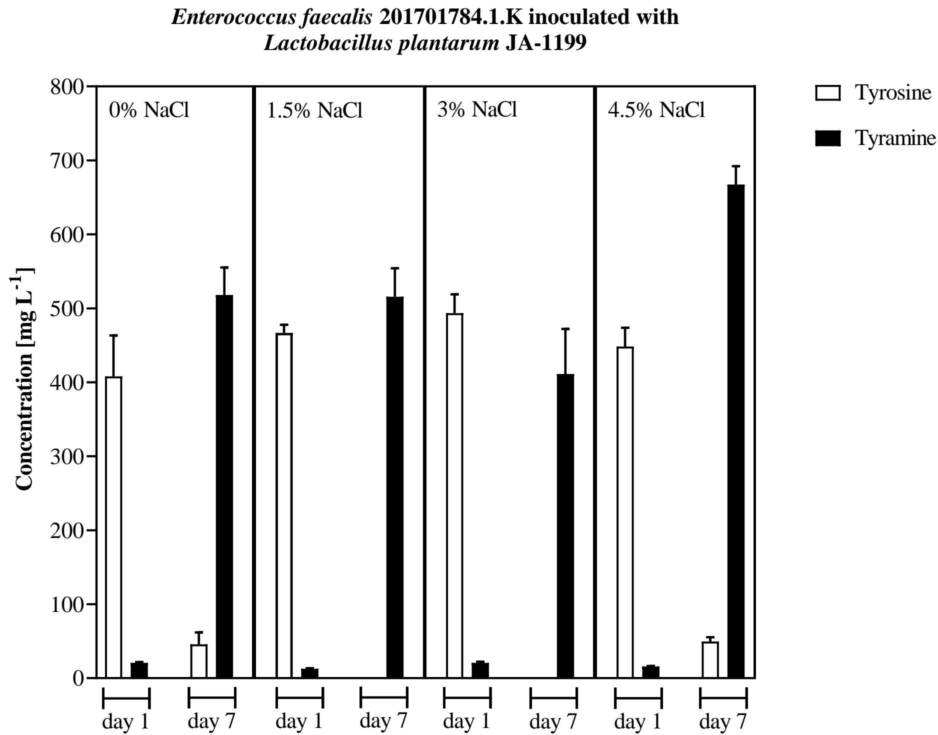

Figure S 4.10: Mean values of tyrosine (white) and tyramine (black) concentrations [mg L^{-1}] produced by *E. faecalis* 201701784.1.K at 0%, 1.5%, 3% and 4% sodium chloride containing 2.5 mM tyrosine inoculated with *Lb. plantarum* JA-1199.

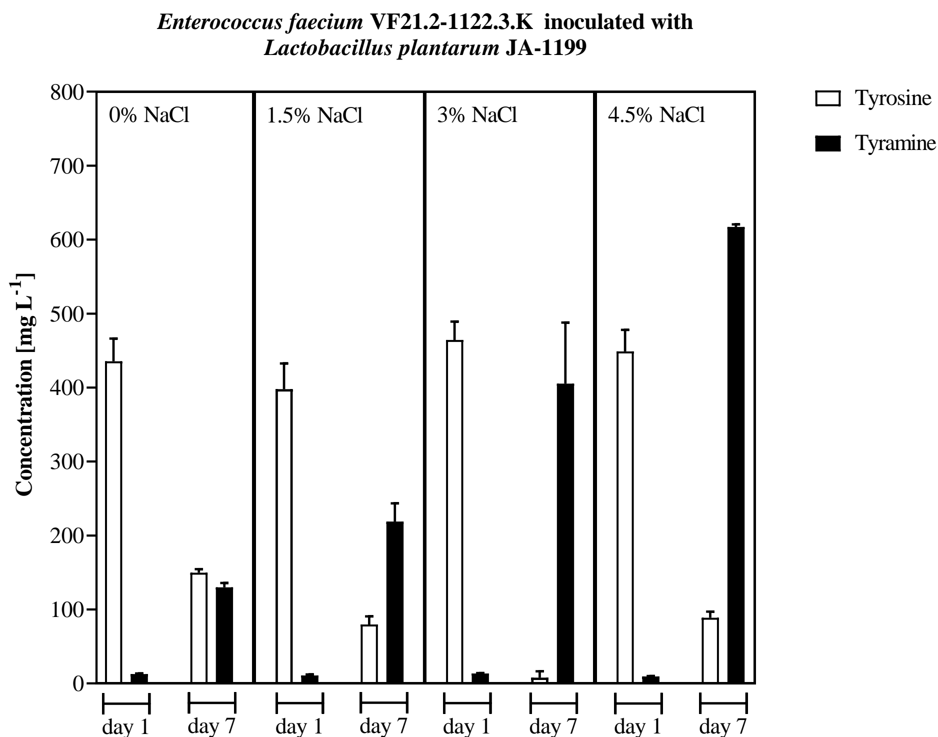

Figure S 4.11: Mean values of tyrosine (white) and tyramine (black) concentrations produced by *E. faecium* VF21.2-1122.3.K at 0%, 1.5%, 3% and 4% sodium chloride containing 2.5 mM tyrosine inoculated with *Lb. plantarum* JA-1199.

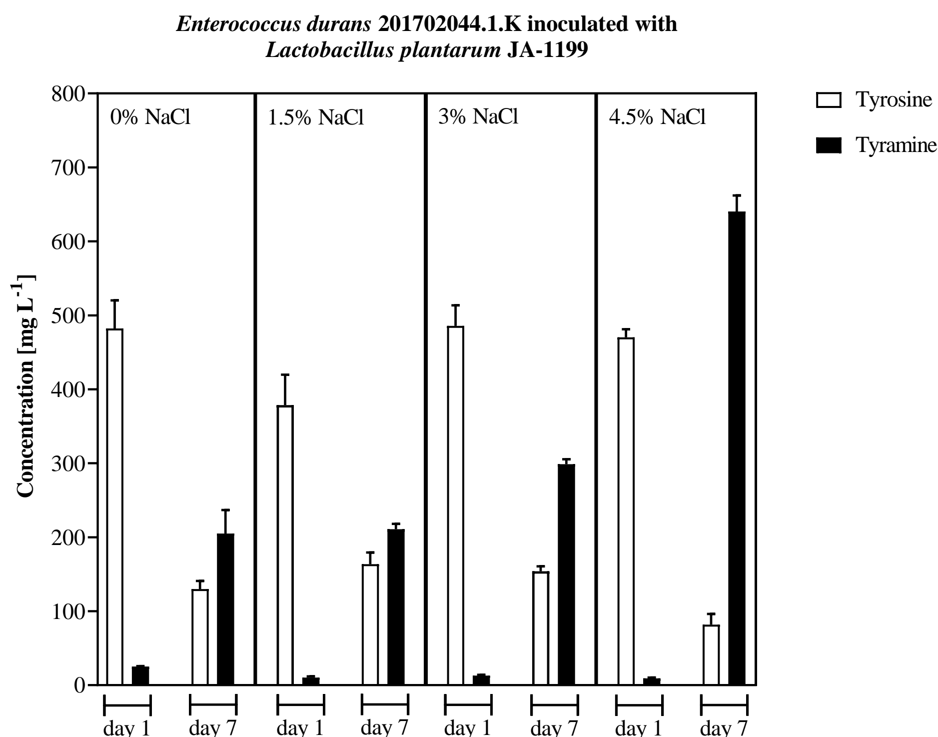

Figure S 4.12: Mean values of tyrosine (white) and tyramine (black) concentrations [mg L^{-1}] produced by *E. durans* 201702044.1.K at 0%, 1.5%, 3% and 4% sodium chloride containing 2.5 mM tyrosine inoculated with *Lb. plantarum* JA-1199.

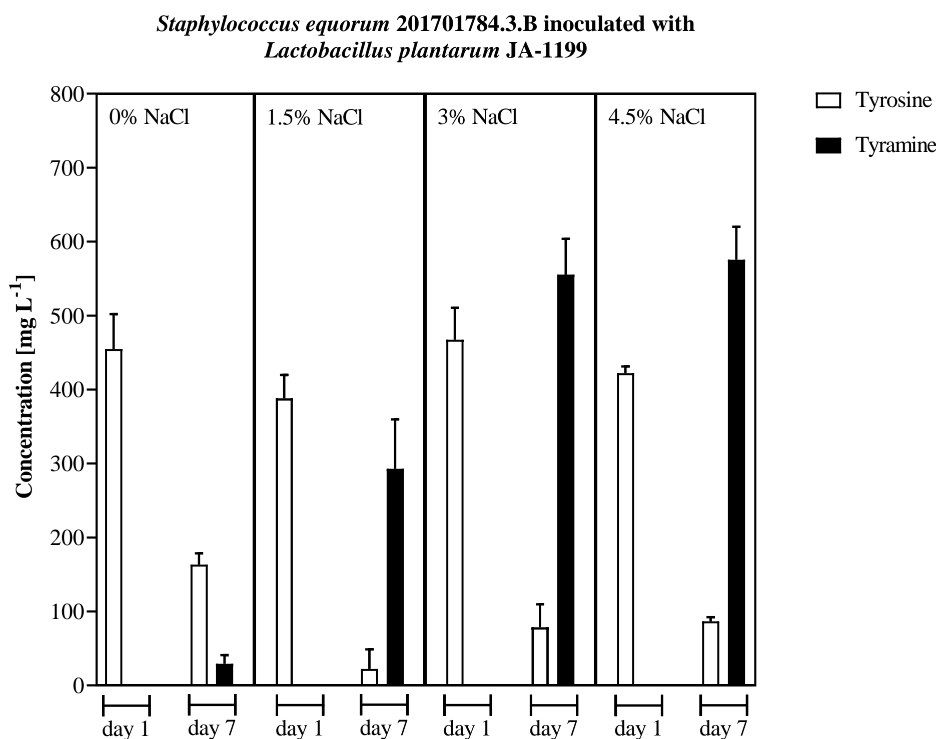

Figure S 4.13: Mean values of tyrosine (white) and tyramine (black) concentrations [mg L^{-1}] produced by *S. equorum* 201701784.3.B at 0%, 1.5%, 3% and 4% sodium chloride containing 2.5 mM tyrosine inoculated with *Lb. plantarum* JA-1199.

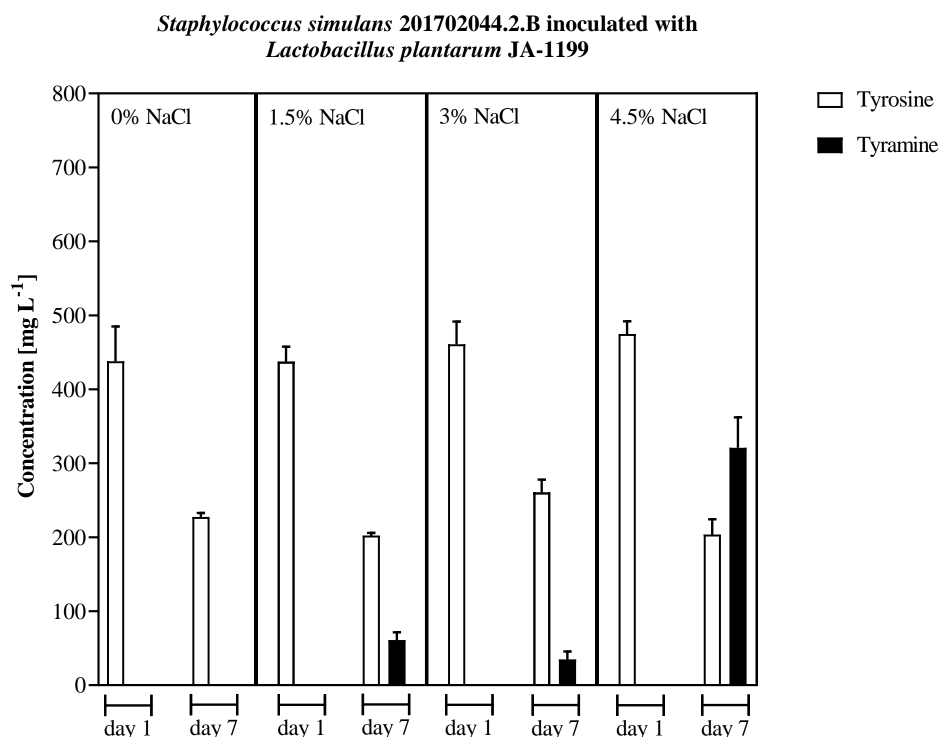

Figure S 4.14: Mean values of tyrosine (white) and tyramine (black) concentrations [mg L^{-1}] produced by *S. simulans* 201702044.2.B at 0%, 1.5%, 3% and 4% sodium chloride containing 2.5 mM tyrosine inoculated with *Lb. plantarum* JA-1199.

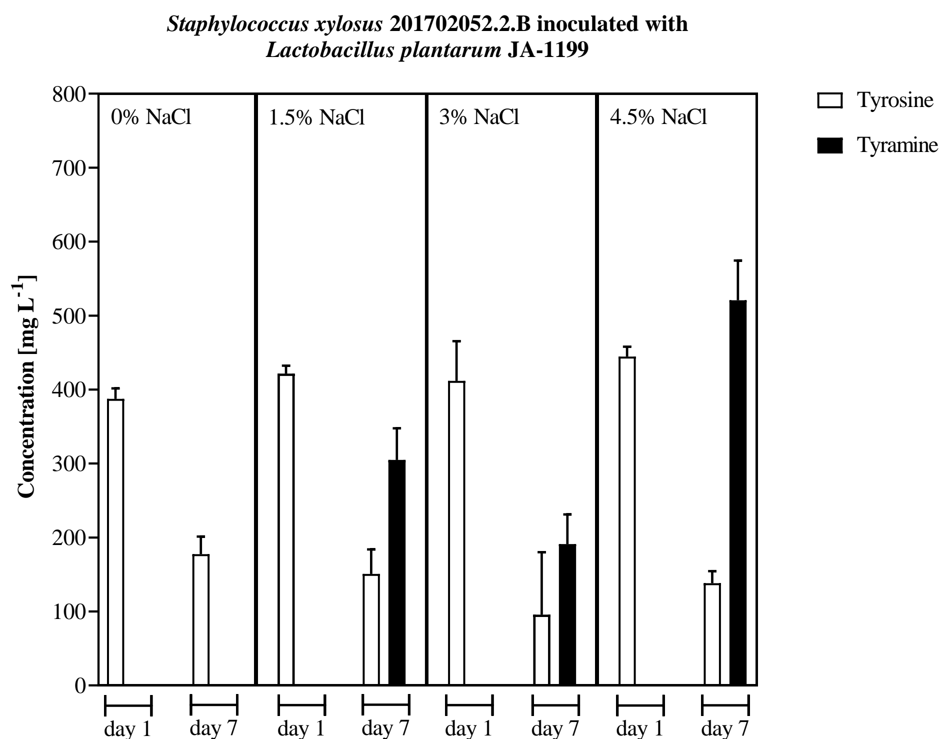

Figure S 4.15: Mean values of tyrosine (white) and tyramine (black) concentrations [mg L^{-1}] produced by *S. xylosus* 201702052.2.B at 0%, 1.5%, 3% and 4% sodium chloride containing 2.5 mM tyrosine inoculated with *Lb. plantarum* JA-1199.

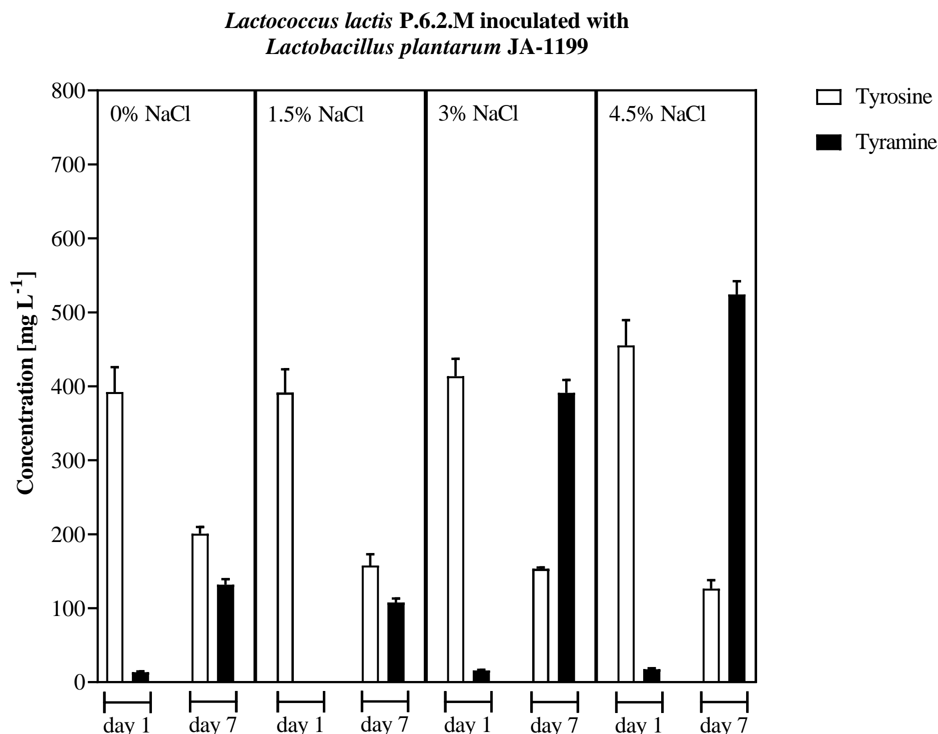

Figure S 4.16: Mean values of tyrosine (white) and tyramine (black) concentrations [mg L^{-1}] produced by *L. lactis* P.6.2.M at 0%, 1.5%, 3% and 4% sodium chloride containing 2.5 mM tyrosine inoculated with *Lb. plantarum* JA-1199.

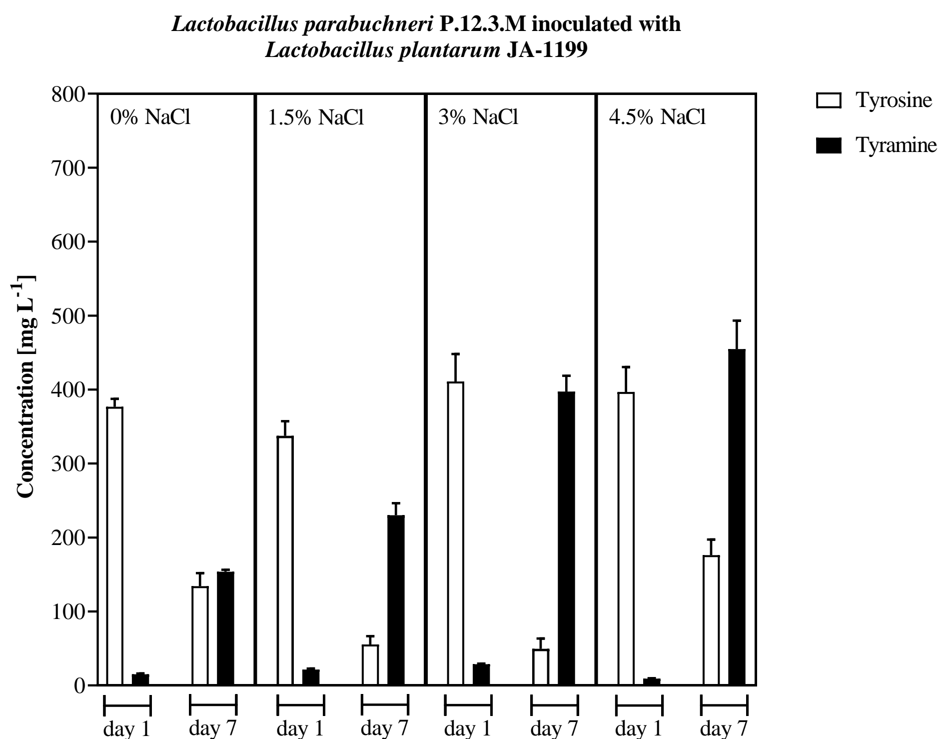

Figure S 4.17: Mean values of tyrosine (white) and tyramine (black) concentrations [mg L^{-1}] produced by *Lb. parabuchneri* P.12.3.M at 0%, 1.5%, 3% and 4% sodium chloride containing 2.5 mM tyrosine inoculated with *Lb. plantarum* JA-1199.

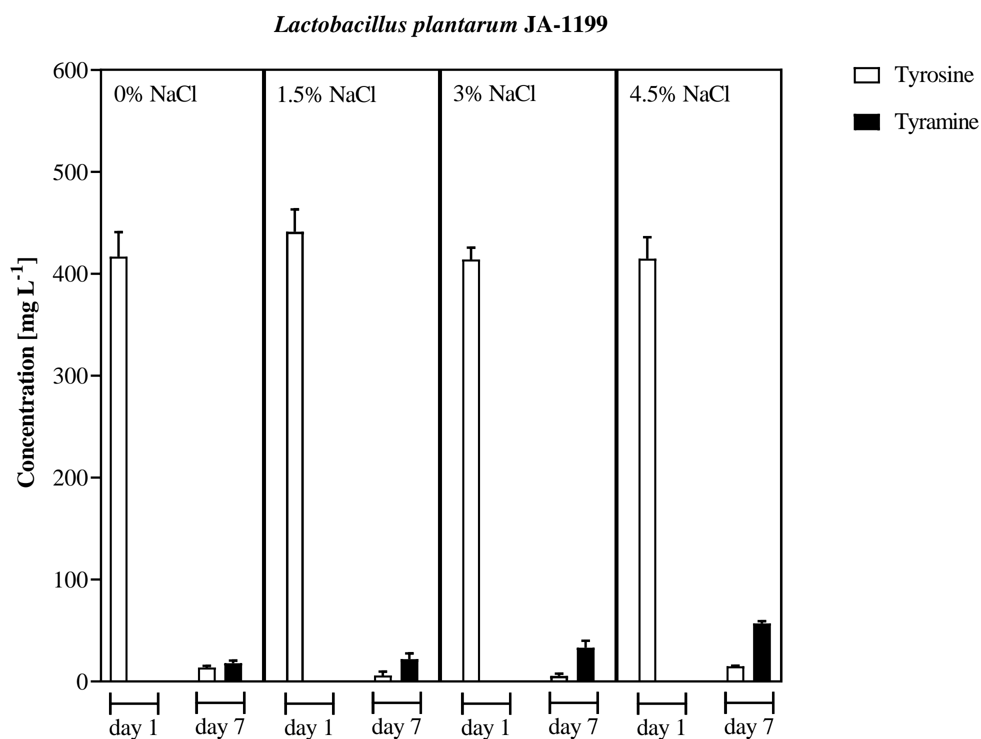

Figure S 4.18: Mean values of tyrosine (white) and tyramine (black) concentrations [mg L^{-1}] produced by *Lb. plantarum* JA-1199 at 0%, 1.5%, 3% and 4% sodium chloride incubated with 2.5 mM tyrosine.

Table S 4.1: Production of tyramine in mg L^{-1} by eight different tyramine-producing bacterial strains in micro-cheese models without tyrosine at four different sodium chloride concentrations.

		Sodium chloride concentration			
		0%	**1.5%**	**3%**	**4.5%**
Species		**Tyramine production [mg L^{-1}]**			
Enterococcus faecalis	201701784.1.K	17.35	17.55	10.44	7.27
Enterococcus faecium	VF21.2-1122.3.K	9.26	7.39	10.38	8.79
Enterococcus durans	201702044.1.K	10.22	8.88	11.79	7.32
Staphylococcus equorum	201701784.3.B	b.d.	3.13	5.31	3.37
Staphylococcus simulans	201702044.2.B	b.d.	2.80	b.d.	3.38
Staphylococcus xylosus	201702052.2.B	7.98	4.88	3.78	3.64
Lactococcus lactis	P.6.2.M	9.94	6.51	10.93	8.62
Lactobacillus parabuchneri	P.12.3.M	10.10	7.95	11.06	7.90

b.d., below detection limit

Table S 4.2: Production of tyramine in mg L^{-1} by *Lb. plantarum* JA-1199 in micro-cheese models containing 2.5 mM tyrosine (A) or without tyrosine (B) at four different sodium chloride concentrations.

A		**Sodium chloride concentration**			
		0%	**1.5%**	**3%**	**4.5%**
Species		**Tyramine production [mg L^{-1}]**			
Lactobacillus plantarum	JA-1199	17.94	21.84	33.06	57.08

B		**Sodium chloride concentration**			
		0%	**1.5%**	**3%**	**4.5%**
Species		**Tyramine production [mg L^{-1}]**			
Lactobacillus plantarum	JA-1199	2.89	5.48	8.66	3.70

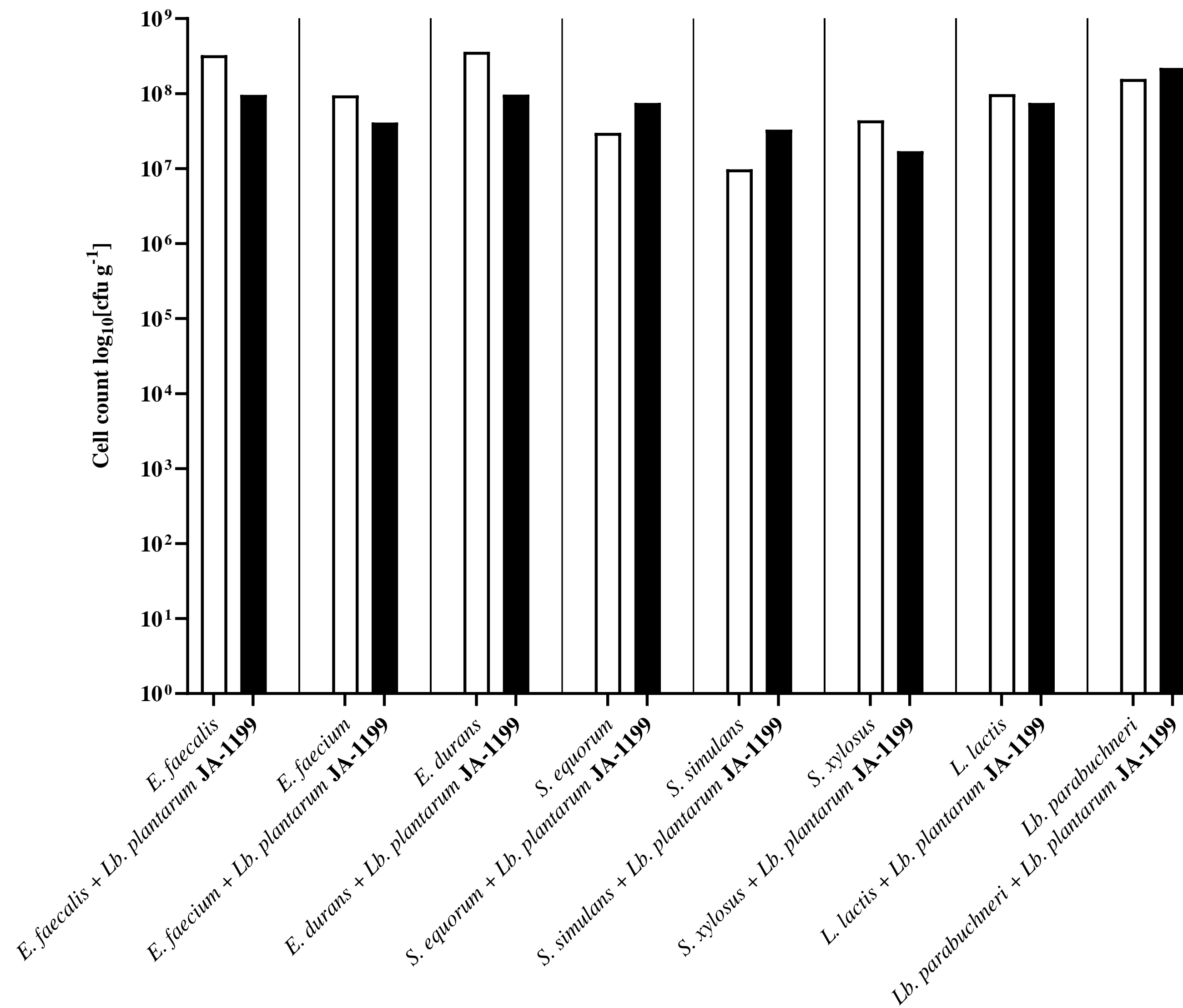

Figure S 4.19: Growth ability of tyramine-producing strains without (white) or in combination with *Lb. plantarum* JA-1199 (black).

Chapter 5

General conclusion and perspectives

General conclusion

Biogenic amines (BA) in fermented food products pose serious risks for human health, and fermented food products contain favourable conditions for BA accumulation. They are formed by microbial decarboxylation of free amino acids, and the responsible microorganisms are even part of the starter culture or may be introduced by contamination during food processing and storage. Tyramine is one of the main BAs found in fermented foods, and a higher intake can trigger a range of toxic effects such as cardiac failure, intracranial haemorrhage, hypertensive crises, pounding heart and palpitations, pulmonary oedema and even death. Although despite the frequent occurrence in high concentrations and the range of toxic effects, there is no legal regulation of tyramine concentrations in food. An explanation might be the lack of knowledge about the conditions of tyramine production as well as about the main responsible tyramine producers in different fermented food products. In those circumstances, it is important to fill these gaps in knowledge in order to develop strategies to reduce tyramine in fermented foods.

Therefore, **chapter 2** aimed to investigate tyrosine-decarboxylating bacterial strains and the content of different BAs and their influence on tyramine accumulation in salami-type fermented sausages to determine their potential health risk. Results of this chapter showed, that more than one-third of the analysed sausages are classified as unacceptable from a health and hygienic point of view. Contrary to the assumptions, artisanal producers may not fulfil the requirements of food safety standards, artisanally-produced sausages showed high-quality products. Moreover, despite the higher level of tyramine-producing microorganisms, namely enterococci and coagulase-negative staphylococci, in the artisanally-produced sausages, industrially-produced ones contain a higher concentration of all four measured BAs. This leads to the conclusion that tyramine concentrations did not only depend on the amount of tyramine-producing resident microorganisms but also on the chosen starter-culture strains in combination with pH. Although starter cultures decrease the pH to inhibit the growth of pathogenic bacterial strains and therefore increase the food safety level, a low pH induce stress conditions to several bacteria and might increase the production of BAs via the induction of gene clusters for BA synthesis as a mean of defence to improve the chances of survival in acidic habitats by restoring the pH to a less

acidic level. Therefore, the absence of tyramine-producing strains should be a criterion for selecting starter culture strains, along with the lack of ability to produce other BAs. Furthermore, high-quality raw materials and optimal technological conditions are critical factors to reduce tyramine production and accumulation in fermented sausages.

In order to expand our knowledge of tyramine production in fermented food products, in **chapter 3,** tyrosine-decarboxylating bacterial strains, the content of different BAs and factors affecting tyramine accumulation in cheese were investigated. We have demonstrated, that tyramine is by far the most abundant BA and enterococci significantly represent the main responsible bacterial group of tyramine producers in cheese. Furthermore, a variety of factors influencing the tyramine concentration could be determined. It could be shown that milk treatment, milk origin and ripening time have a significant impact on tyramine concentration and that other BAs potentially correlate positively to tyramine and therefore, factors, which increase tyramine concentrations, increase other BA concentrations.

In **chapter 4**, we demonstrated the influence of sodium chloride on tyramine production. It could be shown that an increase in sodium chloride concentration leads to an increase in tyramine concentrations in a micro-cheese model. This model revealed differences in the extent of tyramine production, and this suggests that our strains representing different species have a different counteracting activity of defence mechanisms.

To maintain low levels of BAs in fermented foods, it is important to reduce the initial microbial load and growth of decarboxylase-positive bacteria. An effective way therefor is to decrease production, transport, and storage temperature as well as maintain a neutral pH. Unfortunately, in fermented food products, the range of temperature and pH that can be modulated is extremely limited and cannot be applied. Therefore, we presented (also in **chapter 4**) the application of the *Lactobacillus plantarum* JA-1199 strain that decreased tyramine accumulation in a cheese-like model. Our data disclose a feasible strategy to decrease tyramine accumulation and increasing the safety level of fermented foods.

Overall, the results obtained during this doctoral thesis increased the knowledge of tyramine-producing microorganisms and their factors that influence tyramine concentrations. Our findings suggest that the absence of *tdcA*-positive strains should be a criterion for selecting starter culture strains. Furthermore,

high quality of raw materials and optimal technological and hygienic conditions are prerequisites for the production. The results on the reduction of tyramine highlight the potential of *Lb. plantarum* JA-1199 in applications as an additional starter culture to reduce tyramine in fermented food products and thereby increase the safety level. The application of *Lb. plantarum* JA-1199 is cheap, natural and sustainable, and our approach should be further investigated.

Perspectives

In this doctoral thesis, salami-type fermented sausages and cheeses were analysed to investigate tyramine-producing microorganisms and their influencing factors. To increase the knowledge on tyramine production and further BAs, other fermented food samples should be analysed, which might confirm or neglect our findings in cheese where the high prevalence of enterococci correlates with tyramine contents. Furthermore, different physicochemical factors such as temperature, pH, sugar concentrations, as wells as other factors so far unknown, influencing tyramine concentration should be investigated to increase the knowledge of factors affecting tyramine production and therefore optimise the production method of different fermented food products. These further investigations lead to the facts, which enable to create legal regulations in order to determine a maximum concentration of tyramine in fermented foods.

The selected *Lb. plantarum* JA-1199 strain was tested in a micro-cheese model. For the application as an additional starter culture, *in vivo* studies are needed to demonstrate the efficacy of *Lb. plantarum* JA-1199 in conditions of different fermented food products without negatively affecting their properties.

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