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Characterization of tyrosine decarboxylase (tyrDC) activity in genus *Enterococcus*

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Chapter 1

Biogenic amines

1.1. Biogenic amines

Biogenic amines are organic bases with low molecular weight that can be easily found in a wide variety of raw or processed food, and, in particular, in fermented foods.

Low concentrations of these compounds are normally present in human organisms because they play important physiological functions. Norepinephrine, serotonin and dopamine are important monoamines that are neurotransmitter for human organisms. In fact they are involved in the regulation of the sympathetic nervous system and in the coordination of bodily movement. Another example is represented by epinephrine that is a stress hormone and a neurotransmitter.

These compounds are totally different from those produced from the decarboxylation of free amino acids by microbial enzymes for this reason it must be clearly distinguish.

Biogenic amines with microbial origin, in fact are considered anti-nutritional factors because they are implicated in different food poisoning episodes and it is reported that they can interfere with some pharmacological reactions (Önal, 2007).

In Figure 1.1 are reported the different biogenic amines that can be found in foods and their precursor amino acids.

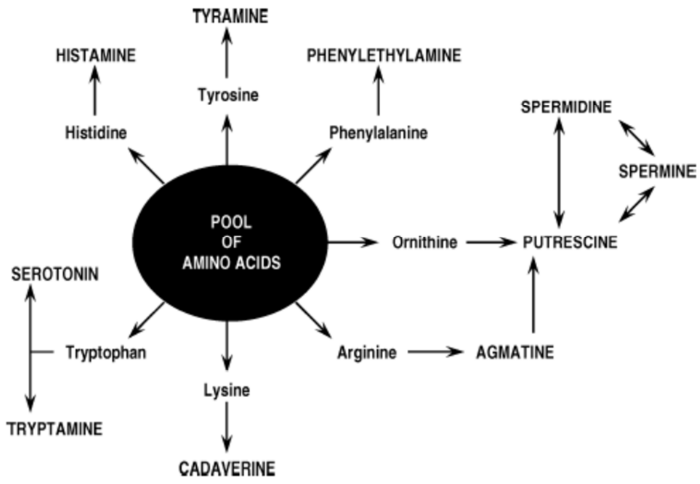

Figure 1.1: Precursor amino acids of biogenic amines (Ancìn-Azplicueta *et al*., 2008)

The decarboxylation process causes the substitution of one, two or three hydrogens of ammonia with alkyl or aryl groups (Shalaby, 1996). Three different chemical structures characterize these compounds as shown in Figure 1.2 (Silla-Santos, 1996):

- Aliphatic structure → putrescine, cadaverine, spermine and spermidine;
- Aromatic structure → tyramine and 2-phenylathylamine;
- Heterocyclic structure → histamine and tryptamine.

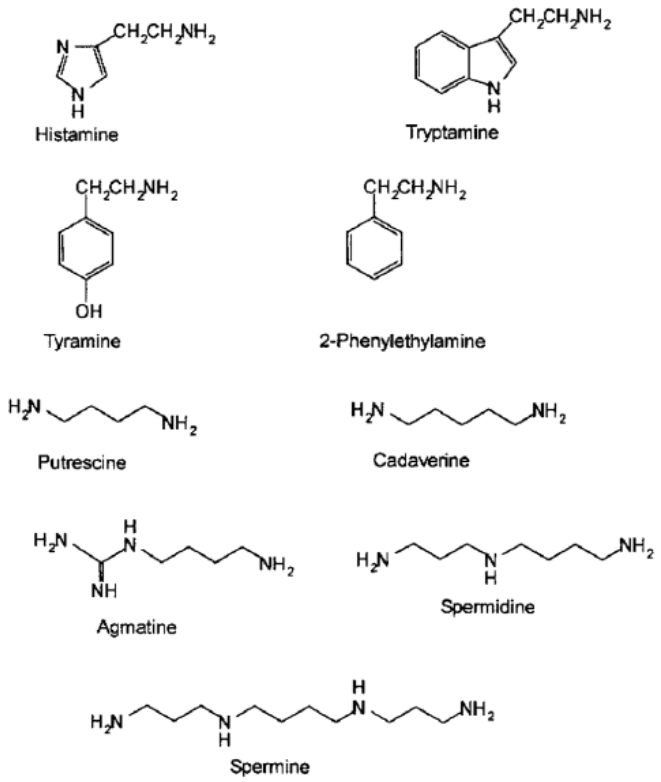

Figure 1.2: Chemical structures of different biogenic amines (Önal, 2007)

Based on the number of amine groups these compounds can be classified into monoamines (tyramine and 2-phenylethylamine), diamines (cadaverine and putrescine) and polyamine (spermine and spermidine) (Spano *et al*., 2010).

Fresh foods can present low concentrations of different biogenic amines, but the presence of dangerous amounts of these compounds can be associated with a massive growth of microorganisms able to decarboxylate the amino acids (>7 log CFU/ml). For this reason, some researchers proposed the use of biogenic amine content as a quality index for fresh and processed foods (Al Bulushi *et al*., 2009; Özogul and Özogul, 2006; Baixas-Nogueras *et al*., 2005; Ruiz-Capillas and Jiménez-Colmenero, 2004; Karmas, 1981). Nevertheless, fermented foods are the product of a massive growth of microorganisms which can often cause a biogenic amine accumulation (Linares *et al*., 2012b, Rabie *et al.*, 2011; Ancín-Azpilicueta *et al*., 2008; Suzzi and Gardini, 2003), especially during the ripening period (when the selected starter cultures can be replaced by wild strains) or when natural (spontaneous) fermentations occur.

Biogenic amines can be produced both by Gram-positive and Gram-negative bacteria. In particular, Gram-negative spoilage microorganisms belonging to the Genus *Enterobacteria* and *Pseudomonas*, are known as the main producers of histamine, cadaverine and putrescine (Lorenzo *et al*., 2010; de las Rivas *et al*., 2007; Pircher *et al*., 2007; Ben-Gigirey *et al*., 2000).

Regarding Gram-positive bacteria, the attention has been mainly focused on lactic acid bacteria (LAB), which are commonly present in the ripening microflora of several fermented foods. These microorganisms can produce different biogenic amines, but, in particular, they are the most efficient producers of tyramine and 2-phenylethylamine (Ladero *et al*., 2012; Bunková *et al*., 2011; Kuley and Özogul, 2011; Buňková *et al*., 2009; Pircher *et al*., 2007; Suzzi and Gardini, 2003; Arena and Manca de Nadra, 2001; Pereira *et al.*, 2001).

The most important biogenic amines in foods, in relation to their amounts and their toxicological effects, are histamine, tyramine, 2-phenylethylamine, tryptamine, putrescine and cadaverine (Wunderlichová *et al*., 2014; Marcobal *et al*., 2012; Landete *et al*., 2008; Tanaka *et al*., 2008; Silla-Santos, 1996). In addition, other polyamines (spermine and spermidine) can be produced with pathways more complex, which starts from putrescine (Kalač and Krausová, 2005; Bardócz, 1995b).

1.2. Biogenic amines production

Biogenic amines are the products of the decarboxylation of free amino acids carried out by specific microbial enzymes of the family of the decarboxylase. Chemically the decarboxylation involves the α-carboxylic group of the amino acid that is removed from the structure to obtain the amine as reported in Figure 1.3.

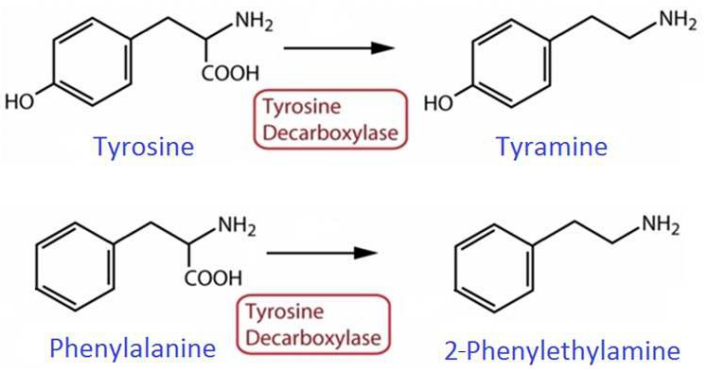

Figure 1.3: Tyrosine and phenylalanine decarboxylation

The capacity to decarboxylate amino acids is generally recognized as strain dependent (Bover-Cid and Holzapfel, 1999), but in some cases it is a characteristic of the entire species, as shown for *Enterococcus faecalis* and it is extremely widespread in *E. faecium and E. durans* species (Ladero *et al*., 2012).

Microbial decarboxylases can be divided in two groups:

- Pyridoxal-phosphate dependent and
- Pyruvoyl dependent.

The first group, which includes almost all the decarboxylase enzymes, require the pyridoxal-5-phospate as cofactor, in fact this compound reacts with the amino group of the amino acid to obtain a Schiff base that is the final substrate of the decarboxylation with the production of the biogenic amine, CO_2 and H_2O (Guirard and Snel, 1987).

The enzymes of the second group, that are represent by the histidine decarboxylases, utilize a covalently bound pyruvoyl moiety as prosthetic group (Landete *et al*., 2008; Recsei and Snell, 1984).

Generally microbial decarboxylase enzymes are pyridoxal-phosphate dependent, so the presence of this cofactor is necessary for the production of biogenic amines.

In addition to the enzyme there are some other proteins that play an important role in this pathway, the first one is the membrane antiport transporter that absorbs the amino acids and excretes the biogenic amine that is toxic for the microbial cell. This transport protein is located in the cellular membrane and for its correct activity the membrane must be in a perfect status because a delocalization of the transporter alter the entire process. The second membrane protein is a Na^+/H^+ pump that regulate the balance of H^+ between the cytoplasm and the external environment.

The process is schematically reported in Figure 1.4.

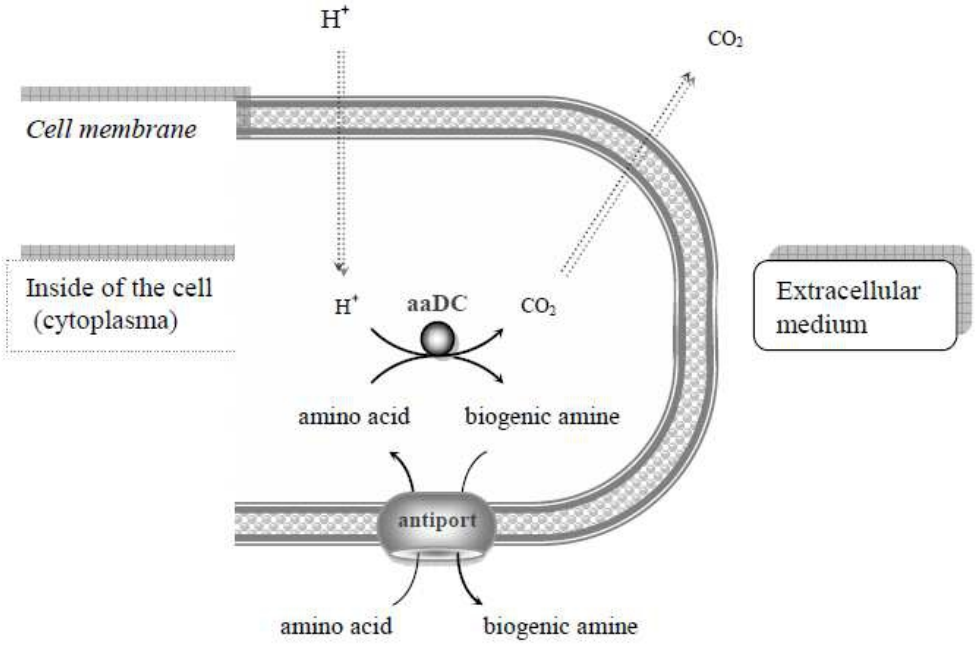

Figure 1.4: Bacterial biosynthesis pathways of biogenic amines (EFSA, 2011)

1.3. Physiological role of the production of biogenic amines for microbial cells

Microbial cells can be characterized by the presence of two different amino acid decarboxylase enzymes with two different physiological roles: constitutive and induced. The first role that these enzymes can play is strictly related with the cells growth, in fact some decarboxylase enzymes are

constitutive and they are part of some biosynthetic pathways as reported by Kamio *et al*. (1986) and Kamio and Nakamura (1987). These types of enzymes represent the minority, in fact most of them are inducible. These last are the most common in microbial cells, they are synthetized in response to different environmental factors, as biodegradative mechanisms.

The decarboxylation represent a defence mechanism used by bacteria to withstand acidic environments as reported by Lee *et al*. (2007) and Rhee *et al*. (2002). In fact, the consumption of protons and the excretion of neutral biogenic amines and CO_2 through a specific electrogenic amino acid/amine antiport, restore the internal pH and produce energy as ATP by the generation of proton motive force as reported by Marcobal *et al*. (2012) for tyramine production in Figure 1.5.

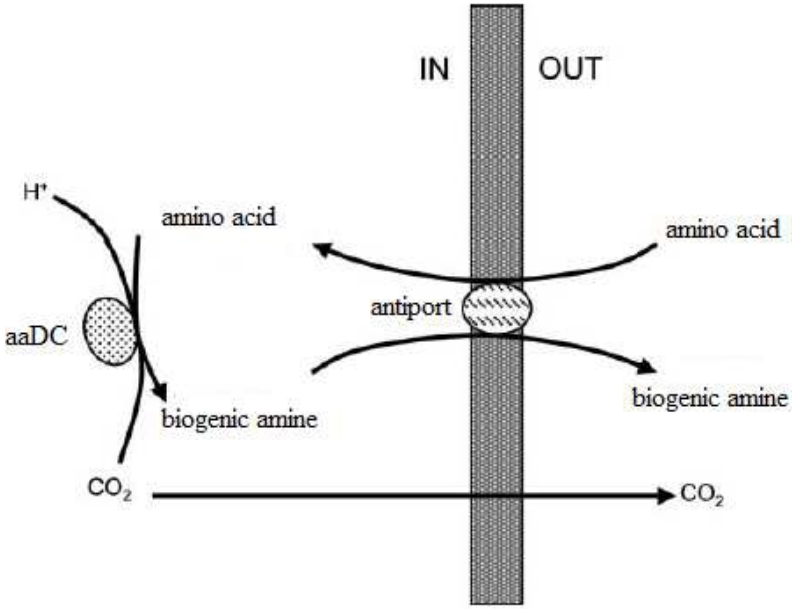

Figure 1.5: scheme of the decarboxylation process (adapted from Marcobal *et al*., 2012)

For example, de Palencia *et al*. (2011) have reported that the production of tyramine contribute to the survival of *Enterococcus durans* in human colon, so this metabolism characterize the pathogenesis of some bacteria. This mechanism can be also apply to the other biogenic amines and other bacteria, in particular the ones characterized by low yields of ATP caused by a lacking respiratory chain (Vido *et al*., 2004).

Tkachenko *et al*. (2001) reported that some microorganisms, like *Escherichia coli* use biogenic amines as an enhancer of the expression of *oxyR*, the gene that contrast oxidative stress. Another hypothesis regards the role of the putrescine as a protector of DNA from the damage caused by oxygen species.

It is also know that some biogenic amines play an important role in living cells as fundamental components for the stability of cellular membranes and regulators of the synthesis of nucleic acids and proteins, therefore these compounds are precursor of hormones (Silla-Santos, 1996).

Generally, microorganisms that can produce biogenic amines could overcome or reduce the effects of the stress generated from different environmental factors such as NaCl concentration, oxygen, temperature and pH.

1.4. Factors influencing the decarboxylation process

There are different factors that influencing the production of biogenic amines, some of these are linked to the specific characteristics of the substrate and others depend on the environmental and process production conditions. The different factors can modify directly the production of biogenic amines or indirectly by the influence on the microbial growth.

1.4.1. Substrate characteristics

The production of biogenic amines is strictly related to the concentration and the availability of free amino acids in the substrates, therefore the proteolysis is a crucial factor in fermented foods because it is directly related to the availability of free amino acids. High proteolysis, induces an increase of biogenic amines formation in foods (Komprda *et al*., 2008a,b; Fernàndez *et al*., 2007a,b; Innocente and D'Agostin, 2002; Leuschner *et al*., 1998), for this reason fermented foods are more subjected to high accumulation of biogenic amines as shown by many authors for different type of fermented foods (de las Rivas *et al*., 2008; Gardini *et al*., 2008; Suzzi and Gardini, 2003; Ansorena *et al*., 2002; Halàsz *et al*., 1994).

The presence of free amino acids is fundamental for the production of biogenic amines, but it is also necessary the presence of pyridoxal-5-phospate that is the cofactor of decarboxylase enzymes.

1.4.2. Environmental and process production characteristics

The main environmental factors affecting microbial activities in foods are temperature, a_w and pH. These factors can influence the formation of biogenic amines in two different ways: in first instance they are responsible for the overall metabolism of the cells and in addition, the activity of decarboxylase enzyme depends on the same parameters.

Temperature

The activity of the enzyme involved in biogenic amines formation is related to the temperature. In fact, the production of biogenic amines increase with temperature within specific values that characterize the enzyme. Temperatures close to the optimum growth value promote the proliferation and the metabolism of the cells, thus favouring the production of biogenic amines that is often related to the number of cells that are present in the system. This is the reason for which low

temperatures, that slow down or inhibit microbial growth, cause a reduction of the activity of decarboxylase enzymes.

Studies carried out by Gardini *et al*., 2001 with a strain of *Enterococcus faecalis* in a model system demonstrated that an increase of temperature from 16 to 44°C promote the growth of the strain and enhances the kinetic and the final amount of tyramine produced. Marcobal *et al*., 2006b, using an experimental design characterized by several factors, have shown that the redox condition play a crucial role in the definition of the optimum temperature of tyramine production of *Enterococcus faecium* and *Lactobacillus brevis*. They reported that aerobic condition requires higher temperature (32°C) that the anaerobic one, where the maximum tyramine concentrations were reached at 22.0-24.5°C. By contrast, Zhang and Ni (2014) have reported that the tyrosine decarboxylase of *Lb. brevis* had an optimum of temperature of activity temperature at 50°C, but it is inactivated at higher temperatures.

Other studies have been conducted on the histidine decarboxylase activity by Tabanelli *et al*., 2012. In these studies it is showed that a cell free extract of a strain of *Streptococcus thermophilus* had its maximum decarboxylase activity at 50°C and rapidly decreased at higher temperatures. The same study was conducted using living cells and the results showed a different kinetic in histamine production, in fact the most rapidly histamine accumulation was found at 40°C. It was also reported that the histamine production was limited or negligible at 25 and 20°C.

Few information about the production of others biogenic amines in relation to the temperature can be found in literature. Generally, it can be assumed that the production of biogenic amines increase with the temperature (Wunderlichova *et al*., 2014). Psychrotrophic pseudomonadas represent an exception to this general rule, in fact they can produce biogenic amines at low temperature (Paulsen and Bauer, 1997).

All these examples highlight that the time and the temperature that characterize the storage period of some foods represent a key factors for the control of biogenic amines accumulation. In fact, abuse temperatures can cause an increase of the concentrations of tyramine, putrescine and cadaverine as shown by some authors (Ferreira and Pinho, 2006; Suzzi and Gardini, 2003; Bover-Cid *et al*., 2001b). This implies that the control of the cold chain plays an important role to avoid the accumulation these molecules, in particular in not fermented foods, such as fishery products (Knope *et al*., 2014).

Regarding fermented foods, the temperature of fermentation and ripening is established to allow the microbiological activity of the desired microflora by the protocols for the production of the different fermented foods. For example, the temperature applied during the first three days of fermentation of dry sausages determined the concentrations and the balance of the different biogenic amines (in

particular, tyramine, 2-phenylethylamine, cadaverine and putrescine) also during all the ripening period (one month) as reported by Gardini *et al*. (2008) and Bover-Cid *et al*. (2009).

The application of thermal treatments (when possible) to raw material such as the milk before fermentation (pasteurization) can contribute to the elimination of the wild decarboxylating microflora. For this reason, usually cheeses from pasteurized milk are characterized by lower biogenic amines content (Marino *et al*., 2008; Novella-Rodriguez *et al*., 2004; Novella-Rodriguez *et al*., 2003; Schneller *et al*. 1997). However, Ladero *et al*. (2011) and Tabanelli *et al*. (2012) have found some strains of *L. curvatus* and *S. thermophilus* that maintain a residual activity after thermal treatment over 70°C.

The effects of temperature on biogenic amines production is the result of the influence on the enzyme activity and on the balance of the growth of the different strains and species that compose the specific microflora.

pH

The pH level is one of the main factors that influence microorganism growth and their enzymatic activity (Silla-Santos, 1996). Since the decarboxylation is a mechanism that the cells activate to counteract acidic stress, it is clear that there is an important relationships between pH and biogenic amine accumulation. As for the temperature, also the effect of pH is different if the focus is directed towards the activity of the pure enzyme or to the activity of the living cells. In any case, it has been extensively demonstrated that the transcription of genes of many decarboxylase clusters are induced by low pH and improves the fitness of cells subjected to acidic stress (Perez *et al*., 2015; Romano *et al*., 2014; Marcobal *et al*., 2012; Romano *et al*., 2012; Pessione *et al*., 2009).

For example, histidine decarboxylase of *S. thermophilus* has its optimum value of pH at 4.5, measured in cell free extract, while histamine accumulation by viable cell cultures was very low at the same pH, due to the negative effect of acidity on the overall metabolism of the strain (Tabanelli *et al*., 2012).

The effect of this factor on the production of biogenic amines is twofold because, first it influence the growth of microorganisms and then the activity of the enzyme, so the balance of these regulation results in the production of biogenic amines.

a_w and NaCl concentration

Generally, the presence of NaCl causes the reduction of the activity of decarboxylase enzyme, for example the rate of biogenic amines production of some strains of *Lactobacillus* is reduced when sodium chloride concentration in the medium increase from 0% to 6%. This effect can be explained

by the reduction of cell yields and the alteration of the stability of cellular membrane where the specific amino acids transport proteins are located.

In particular, Gram negative bacteria are more inhibited by increasing salt concentrations than Gram positive microflora. For example, Gardini *et al*., 2001 demonstrated that the ability to accumulate tyramine and 2-phenylethylamine of a strain of *E. faecalis* was inversely related to NaCl concentration, in a range comprised between 2 and 6%. It is also demonstrated that in fermented sausages inoculated with the same tyraminogenic *E. faecalis* strain, increasing amounts of salt reduced the concentration of tyramine, 2-phenylethylamine, but also limited cadaverine and putrescine production by enterobacteria (Bover-Cid *et al*., 2009; Gardini *et al*., 2008).

Recent studies demonstrate that the production of histamine by the histidine decarboxylase of living cells of a strain of *S. thermophilus* can be significantly reduced with NaCl concentrations of 2.5%, but the activity of the enzyme in cell free extract was not influenced up to NaCl concentration of 5% and the activity was reduced at NaCl concentration of 20-30% (Tabanelli *et al*., 2012). Another study reported that the presence of NaCl cause an upregulation of the histidine decarboxylase gene in the same strain grown on skim milk, suggesting a potential role of this enzyme in osmoprotection mechanisms (Rossi *et al*., 2011) and confirming that the activation of decarboxylase system is a part of a complex metabolic responses in presence of different stress conditions (Pessione *et al*., 2009).

Kimura *et al*. (2001) reported that a halophilic strain of *Tetragenococcus muriaticus* isolated from fish sauce produced histamine during the late exponential phase and reached the maximum production at 5-7% of NaCl and was able to maintain an histidine decarboxylase activity also in presence of 20% of salt. In literature is reported that the histamine decarboxylase activity of two strains of *Photobacterium phosphoreum* decreased rapidly with the increase of salt from 2-5% to 10%, but *Raoultella planticola*, *Photobacterium damselae* and *Morganella morganii* are more resistant (Kanki *et al*., 2007).

Regarding putrescine and cadaverine it is reported that they were produced with more efficiency by *Serratia marcescens* in the presence of 1-3% NaCl (3-5 in the yield factor was applied) (Buňka *et al*., 2015). In other words, stressed cells seem to activate the decarboxylating pathways in the framework of a more complex response system. This make the potential of biogenic amines production by each single cell more efficient.

In fermented sausages biogenic amines are accumulate during ripening. However, the rate of accumulation decreases with the decrease of water activity due to the water losses. Products packaged under modified atmosphere, in which the weight losses were inhibited, continued to accumulate biogenic amines when the water activity at the moment of the packaging was high (0.92 and more) (González-Tenorio *et al*., 2013; Tabanelli *et al*., 2013). The Greek cheese Feta,

characterized by a high salt content, with a ripening carried out in brine and with a low pH, was characterized by a noteworthy amine concentration (about 200 mg/kg of tyramine, 90 mg/kg of histamine and 200 mg/kg of putrescine) (Valsamaki *et al*., 2000).

It can be assumed that the effects of sodium chloride on the inhibition and stimulation biogenic amines production is strain specific (Hernandez-Herrero *et al*., 1999; Rodriguez-Jerez *et al*., 1994; Taylor and Speckard, 1984; Taylor and Woychik, 1982).

Carbon sources

The concentration of carbon sources, such as glucose, in the food matrix plays a fundamental role in the regulation of the production of biogenic amines. Biogenic amines are produced when microbial cells require energy to survive in harsh environment characterized by low concentration of energetic substances, so the presence of carbon sources can minimize the production of amines.

Oxygen

The effect of the oxygen on the production of biogenic amines is controversial and less studied respect the other factors. The availability of oxygen has a significant effect on the biogenic amines production, it can reduce the quantity of some amines and increase the concentrations of others (Halàsz *et al*., 1994). Generally, the oxygen availability has little influence on the production of tyramine, putrescine and 2-phenylethylamine while it influences the quantity of cadaverine and histamine.

Additives

The addition of additives during the production of fermented foods can significantly reduce the final amount of biogenic amines. Bover-Cid *et al*. (2001c) reported that an addition of sodium sulphite can inhibit cadaverine accumulation but not the tyramine ones. Cantoni *et al*. (1994) highlighted that the addition of sodium nitrite can reduce the production of cadaverine and putrescine in sausages, but it cause an increase of histamine concentration.

1.5. Biogenic amines toxicology

Biogenic amines are normally synthesize by human body because they are hormone and neurotransmitter, but high consumption of these compounds with foods can cause health problems. The appearance and the severity of toxicological effects (flushing, headaches, nausea, cardiac palpitation and increase and decrease blood pressure) of all the biogenic amines depends not only on the intake with foods, but also on the consumption of monoamine oxidase inhibiting (MAOI) drugs, alcohol and other food amines. In fact, the concomitant intake of diamines (putrescine and

cadaverine) and polyamines (spermine and spermidine) favour the intestinal absorption ad decrease the catabolism of the other amines (Bardócz, 1995a,b).

Despite these knowledge it is very difficult to determinate the exact toxicity threshold of every biogenic amine for human because the toxic dose is variable in relation to the personal sensibility to these molecules. In fact, the efficiency of human detoxification mechanisms depends on each individual, as reported by Halasz *et al*. (1994). Nout (1994) reported that the maximum concentration of histamine and tyramine in foods must be in the range of 50-100 mg/kg and 100-800 mg/kg respectively, in fact over 1080 mg/kg of tyramine becomes toxic.

1.5.1. Detoxification mechanisms

Normally, low daily intake of biogenic amines do not represent a problem for human health because in human liver and gut there are specific detoxification system that metabolise these molecules to physiologically less active degradation products as shown in Figure 1.6.

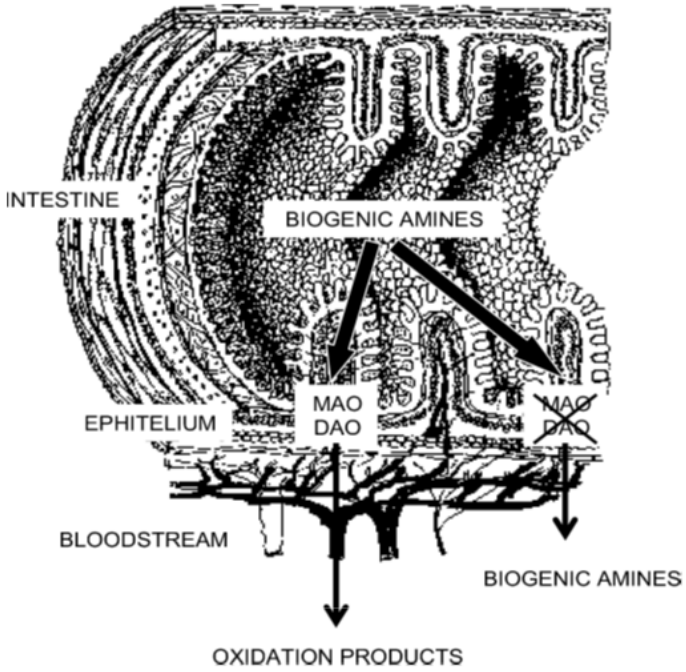

Figure 1.6: Gut MAO and DAO systems (Ancín-Azpilicueta *et al*., 2008)

These systems include enzymes named monoamineoxidase (MAO) and diamineoxidase (DAO).

Unfortunately, these systems can metabolise only reduced concentrations of biogenic amines, and high consumptions can cause their saturation. Another characteristic of the activity of MAO and DAO consist in the reduction of effectiveness caused by genetic predisposition, gastrointestinal disease or consumption of medicines and alcohol. For these reasons, the sensibility to biogenic amines can be really different between subjects.

All these molecules generally, and tyramine and 2-phenylethylamine in particular, are considered anti-nutritional compounds that can be a serious problem for sensitive individuals.

1.5.2. Toxic effects and dose-response relationships of biogenic amines

The most dangerous biogenic amines for human health are histamine and tyramine. Histamine causes a symptomatology known as “fish (scombroid) poisoning” (because it is often associated with the consumption of contaminated fish products) (Hungerford, 2010; Lehane and Olley, 2000), while tyramine is the responsible of the syndrome known as “cheese reaction” (Marcobal *et al*., 2012; McCabe-Sellers *et al*., 2006; Shalaby 1996).

1.5.2.1. Histamine

Histamine is the most implicated in outbreaks of food poisoning, in fact the symptoms cause by this amine are recognized as “fish poisoning”. The intolerance caused by histamine appears when DAO system is saturated or less active due to the consumption of specific drugs, genetic predisposition or gastrointestinal diseases. In these cases, also the ingestion of small amounts can cause an allergic reaction (Maintz and Novak, 2007). First symptoms can appear after few minutes or some hours and can last for few hours. In particular, histamine cause headache, nasal secretion, tachycardia, hypotension, edema, flushing and asthma (Maintz and Novak, 2007; Jarisch, 2004). As reported by EFSA (2011), a consumption of 25-50 mg of histamine with solid foods or non-alcoholic drinks by healthy persons doesn’t produce any effects, but an intake ranging between 75 and 300 mg could provoke headache and flushing as shown in Table 1.1.

Administration	Histamine *amount ingested	Symptoms	Number of subjects showing symptoms / total number of subjects	Reference
Solid foods				
tuna	25 mg	no symptoms	0/8 healthy volunteers	Motil and Scrimshaw, 1979
herring paste	45 mg	no effect level	0/8 healthy volunteers	Van Gelderen et al., 1992
tuna	50 mg	no symptoms	0/8 healthy volunteers	Motil and Scrimshaw, 1979
herring paste	90 mg	warm face, flushing, headache	2/8 healthy volunteers	Van Gelderen et al., 1992
tuna	100 mg	mild headache, flushing	1/8 healthy volunteers	Motil and Scrimshaw, 1979
tuna	150 mg	mild headache, flushing	2/8 healthy volunteers	Motil and Scrimshaw, 1979
tuna	180 mg	mild to severe headache, flush	4/8 healthy volunteers	Motil and Scrimshaw, 1979
mackerel	300 mg	headache, flushing, oral tingling (no significant effects)	n.i.[a] /7 healthy volunteers	Clifford et al., 1989
Non-alcoholic drinks				
apple juice	25 mg	no statistically significant effects	n.i.[a] /25 healthy volunteers and 2 migraine patients	Lüthy and Schlatter, 1983
grapefruit juice	25 mg	no significant effect	0/4 healthy volunteers	Motil and Scrimshaw, 1979
grapefruit juice	50 mg	no significant effect	0/4 healthy volunteers	Motil and Scrimshaw, 1979
peppermint tea	75 mg	diarrhoea, headache, sneezing, flatulence	5/10 healthy females	Wöhrl et al., 2004
grapefruit juice	100 mg	mild headache, flushing	2/4 healthy volunteers	Motil and Scrimshaw, 1979
grapefruit juice	150 mg	mild headache, flushing	2/4 healthy volunteers	Motil and Scrimshaw, 1979
grapefruit juice	180 mg	severe headache, flushing	1/4 healthy volunteers	Motil and Scrimshaw, 1979
Alcoholic drinks				
wine	0.12 - 4.2 mg	no statistically significant effects	n.i.[a] /20 healthy volunteers	Lüthy and Schlatter, 1983
wine	100 mg	no effects	0/2 healthy volunteers	Lüthy and Schlatter, 1983
sparkling wine	4 mg	dizziness, headache, nausea, itching	12/40 patients with histamine intolerance	Menne et al., 2001
Digestive histamine challenge				
Instillation into the duodenum	120 mg	No symptoms	0/8 healthy volunteers	Kanny et al., 1993
Instillation into the duodenum	120 mg	urticaria, headache, accelerated heart rate, drop in blood pressure, nausea, diarrhoea	26/32 patients with chronic urticaria	Kanny et al., 1993; 1996

[a] n.i. = not indicated

Table 1.1: dose-response relationship of histamine (EFSA, 2011)

1.5.2.2. Tyramine, 2-phenylethylamine and tryptamine

Generally, high consumption of tyramine, 2-phenylethylamine and tryptamine cause hypertension, headache, perspiration, vomiting and pupil dilatation. Also in this case the consumption of MAOI drugs can interact with detoxification system and increase the sensibility of some person. These symptoms appear between 30 minutes and few hours after the consumption and disappear completely after 24 hours. In particular, tyramine and 2-phenylethylamine have been recognized as agent of dietary-induced migraines and initiators of hypertensive crisis in some patients.

Few information about the dose-response for these biogenic amines can be found in literature so, actually, no dose-response curve has been estimated. Some study suggest that a consumption from 600 mg up to 2000 mg of tyramine in a meal is necessary to cause minimal blood pressure increase.

1.5.2.3. Cadaverine, putrescine and polyamines

Cadaverine, putrescine and the polyamines seem not be responsible of direct poisoning, but they can react with nitrite to form carcinogenic nitrosamines as reported from many author (Eerola *et al*., 1997; Hernandez-Jover *et al*., 1997). In particular, cadaverine and putrescine seems to have less potent pharmacological activity, in fact no data about the dose-response for humane are now available. Even if these amines do not show a directly toxicity they are recognized as enhancer of the effect of other biogenic amines (Hui and Taylor, 1985; Chu and Bjeldanes, 1981).

1.5.3. Legislation

Nowadays there is not a specific legislation about the content of biogenic amines in foods in Europe. Only for histamine exist a dose governed by law, but this limit regard specific fishery products. The European Commission Regulation (EC) n. 2073/2005 reports the safety criteria for histamine in fresh fish and fermented fish products of some specific species of the families *Scombridae, Clupeidae, Engraulidae, Coryphenidae, Pomatomidae* and *Scombreresosidae*. Sampling plan for fresh fish placed on market comprising nine units of which two can contain histamine between 100 and 200 mg/kg and no one over this value. For fermented fish products the sampling plan is composed by nine units of which two can present a content of histamine between 200 and 400 mg/kg, and no one over 400 mg/kg.
No limit has been established for other biogenic amines, only some information about the dose-response can be found in literature as reported previously.

1.6. Biogenic amines producing microorganisms

Different group of microorganisms are characterized by the ability to produce biogenic amines, for example the *Enterobacteria* are known as producer of putrescine and cadaverine, while enterococci are the major producer of tyramine. Normally, this characteristic is strain-specific, but in the case of the specie *Enterococcus* seems to be a characteristic of the specie.

1.6.1. Tyramine and 2-phenylethylamine

Tyramine producing bacteria are usually Gram positive microorganisms isolated from cheese and fermented sausages. The main genera that produce relevant quantity of tyramine in these foods are: *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Lactococcus* and *Carnobacterium* as reported by Fernández *et al*. (2007a,b), Fernández *et al*. (2004), Bover-Cid *et al*. (2001b), Bover-Cid *et al*. (2000) and Masson *et al*. (1999).

An important characteristic of the enzyme that decarboxylate tyramine is the ability of this enzyme to decarboxylate also phenylalanine to obtain 2-phenylethylamine as reported by Marcobal *et al*., 2012.

1.6.2. Histamine

Histamine producing bacteria have been found in Gram positive and Gram negative groups, but is the Gram negative group the most important for food industries. Some of the major producer of histamine in foods are *Photobacterium phosphoreum* and *psychrotolerans*, *Klebsiella pneumonia*, *Morganella morganii* and *psychrotolerans* that can be easily isolated from spoilage fish (Dalgaard *et al*., 2008; Özogul and Özogul, 2006; Emborg *et al*., 2006; Kanki *et al*., 2004).

1.6.3. Putrescine and cadaverine

Putrescine and cadaverine producing bacteria have been found especially in Gram negative spoilage bacteria such as *Enterobacteriaceae* (in particular *Citrobacter*, *Klebsiella*, *Escherichia*, *Proteus*, *Salmonella* and *Shigella*), *Pseudomonadaceae* and *Shewanellaceae* as reported by Lopez-Caballero *et al*. (2001). Not only spoilage microorganisms are responsible of the production of these biogenic amines, in fact in fermented food also some genera of lactic acid bacteria, such as *Lactobacillus* and *Staphylococcus*, are recognized as relevant producer of putrescine and cadaverine (Beneduce *et al*., 2010; Coton *et al*., 2010; Arena *et al*., 2001).

1.7. Biogenic amines in foods

Biogenic amines can be found in a wide range of foods, in fact all the foods that contain proteins and/or free amino acids are good substrates for the growth of microorganisms that can decarboxylate these substrates. Normally, biogenic amines are produced by the spoilage microflora, because starter cultures should be selected so they do not produce these compounds during the fermentation. In any case the production of biogenic amines require also the presence of favourable conditions for the microorganisms growth and for the activity of decarboxylase enzymes (ten Brink *et al*., 1990; Stratton *et al*. 1991).

The concentrations of the different biogenic amines and the total amount depends on different factors, first the specific composition of the food, second the characteristics of the microbial flora and finally the storage conditions. Some data about the concentration of different biogenic amines in foods are shown in Table 1.2 (EFSA, 2011).

			Biogenic amines									
			Histamine		Tyramine		Putrescine		Cadaverine		Sum of BAs	
Food class	Sub-category	n	Mean (mg/kg)	P95 (mg/kg)	Mean (mg/kg)	P95 (mg/kg)	Mean (mg/kg)	P95 (mg/kg)	Mean (mg/kg)	P95 (mg/kg)	Mean (mg/kg)	P95 (mg/kg)
Alcoholic beverages	Beer	188	1.4	4.8	6.1	24.7	3.3 - 3.5	8.3	1.3 - 1.5	5.3	12.1 – 12.4	36.7
	Fortified and liqueur wines	28	1.1	2.8	6	21.3	1.4	3.6	0.1	0.3	8.6	26.4
	Wine, white, sparkling	45	1	5.2	4.9	26.4	5.2	15	<0.1	0.2	11.1	46.1
Condiment	Fish sauce	71	198 - 199	597	105 - 107	421	98.1 - 99	167	180 - 182	502	582 - 588	1500
	Other savoury sauces	27	0.5 - 10.1	<13.3	1.5 - 10	18.6	6 - 13.6	24.2	3 - 12.7	<17	11 - 47	24.2 - 56
Fish and fish products	Fermented Fish products	68	7.7 - 11.4	31.5	45.5 - 47	136	12.2 - 15	75.1	14.4 - 17	34.5	79.8 - 91	552 - 572
Meat products	Fermented sausages	369	23.2 - 23	149	136	397	84.2 - 84	334	37.4 - 38	154	281 - 283	889
	Other ripened meat products	92	6 - 6.2	35	44 - 44.2	149	32.8	136	17.2 - 17	84.1	100 - 101	342
	Other meat products	75	3.9 - 4.4	4.8	16.1 - 16	67	17.4 - 17	123	6.7 - 6.8	25	44 - 45	151
Dairy products	Cheese	2136	20.6 - 61	127	59 - 98	420	25.4 - 65	143	72.2 - 109	472	177 - 334	1050
	Fresh cheese	98	3.2 - 38	20 - 50	12.8 - 48	89	5.5 - 41	4 - 50	10.7 - 45	33.8 - 50	32.1 - 172	323 - 464
	Hard cheese	1062	25 - 65	136	67.1 - 103	475	26.6 - 65	132	47.8 - 83	235	167 - 318	940 - 1030
	Washed rind cheese	676	8.5 - 54	46 - 50	31.6 - 76	240	32.3 - 72	182	147 - 186	989	220 - 388	1420 - 1516
	Blue cheese	296	21.3 - 63	149	63.2 - 10	453	20.9 - 62	149	83.1 - 12	519	188 - 351	1100 - 1184
	Acid curd cheese	4	51.3 - 55	102	335	480	449	648	628	980	1460	2140
	Yoghurt	7	0.5	1	1.9	5.2	0.7	1.1	3.2	10.3	6.3	12
	Other dairy products	4	0.3	0.6	0.3	0.4	0.7	0.9	1.9	3	3.1	4.8
Vegetables and vegetable products	Fermented vegetables	9	39.4 - 42	92	45 - 47.4	91	264	549	26 - 35.4	94	375 - 390	747
	Other vegetables	14	5.4	75.7	1.8	25.4	37.2	310	17	85	61.4	422

The statistics are presented using a bounded approach for the handling of non-detected/non-quantified data (therefore they are displayed as ranges). The upper bound of the range estimates the non-detected/non-quantified values using the reported limit of detection (LOD) or limit of quantification (LOQ) respectively. The lower bound of the range instead assumes the non-detected/non-quantified values as zero. When the lower bound and the upper bound of the range are coincident, only one number is presented. When the lower bound is zero, the range is represented by the upper bound prefixed by '<'. The table contains the number of samples (n), the mean and the 95-percentile (P95).

Table 1.2: Sum of biogenic amine in different food sample (EFSA, 2011).

1.7.1. Non-fermented foods

Non-fermented foods are normally characterize by the presence of low concentration of biogenic amines of endogenous origin, but sometimes is possible to find high concentrations of these compound caused by undesired microbial activity. For this reason, in these products, they can be used as an indicator of food spoilage.

Fish

In particular Scombroid fish has been associated with incidents of histamine intoxication, commonly named "scombroid fish poisoning". The production of this biogenic amine in marine fish containing high concentration of endogenous histidine has been attributed to microbial action rather than to endogenous histidine decarboxylase activity (Halàz *et al*., 1994).

Different biogenic amines can be found in mackerel, herring, tuna and sardines such as histamine, putrescine, cadaverine, tyramine, spermine and spermidine as reported by different authors (Lebiedzinska *et al*., 1991; Middlebrooks *et al*., 1988; Ramesh and Venugopalan, 1986).

These biogenic amines are produced in particular during fish spoilage and usually the concentrations of histamine, putrescine and cadaverine increase, while the contents of polyamines decrease (ten Brink *et al*., 1990).

Fruit and vegetables

Juices and nectar produced from a wide range of fruit (oranges, raspberries, lemons, grapefruit, mandarins, strawberries, currants and grapes) can contain different biogenic amines (Maxa and Brandes, 1993). Halàz *et al*. (1994) have reported high concentration of biogenic amines in orange

juice (noradrenaline, tryptamine), tomato (tyramine, tryptamine, histamine), banana (tyramine, noradrenaline, tryptamine, serotonin), plum (tyramine, noradrenaline) and spinach leaves (histamine).

Meat

Normally, fresh meat correctly stored contain only low level of spermine, spermidine and putrescine. An improper storage can promote the growth of an environmental contaminant microflora that can produce high concentration of cadaverine and histamine. In particular, fresh minced meat is a problematic matrix because the increase of the surface expose to the deterioration factors can cause an increase of the concentration of the different biogenic amines. For these reasons the biogenic amines content can be used as freshness quality index for unprocessed meat (Vinci and Antonelli, 2002)

Milk

In general, the concentration of biogenic amines in milk of different species are very low and normally regards only polyammines.

1.7.2. Fermented foods

Fermented foods are characterized by the presence, at the end of the fermentation, of different biogenic amines. The presence of these compounds is caused by the decarboxylation of free amino acids that are produced from the proteolysis that characterize these foods. In fact during the preparation of these products a lot of different microorganisms, not only the starter culture added during manufacture can be present in the raw materials and some of them can produce biogenic amines during the fermentation. These are the reasons that explain the concentration of putrescine, cadaverine, histamine and tyramine that can be found especially in sausages and cheese.

Cheese and dairy products

Cheese is one of the fermented foods most involved in biogenic amine poisoning, in fact the syndrome caused by the consumption of high concentration of tyramine is named “cheese reaction”. In literature are presents many studies that characterize dairy products regarding the amount of biogenic amines, and all of these are agree that in these foods all the biogenic amines known can be present. In particular, histamine and tyramine can be present in different amount in relation to the type of cheese and to the quality of raw materials and ripening conditions.

The key role of the quality of the milk is demonstrated from the study of Lau *et al*., 1991, where they showed that cheese obtained from raw milk contain higher concentrations of biogenic amine than the one obtained from pasteurized milk.

Vegetables

Generally, in fermented vegetables very low levels of biogenic amines can be detected. Fermented vegetables obtained by high quality raw materials are not characterized by relevant concentration of biogenic amine, but a contamination or a thermal abuse can cause a relevant increase of the concentration of tyramine.

Fermented meat products

Fermented meat products are the mainly food products studied for their content in biogenic amines. These products are characterized by an important proteolysis process that produce high quantity of free amino acids that are available for the decarboxylation, in fact fresh meat (utilized to produce for example dry fermented sausages) can contain only minimal concentrations of spermidine and spermine, sometimes also putrescine (Hernandez-Jover *et al*., 1997). These amines are produced by contaminant microorganisms, so they are an index of the freshness of the meat.

The type and the quantity of the biogenic amines present in fermented sausages are characterized by a great variability even if the microbiological profile are similar between the products, as reported by Gardini and Suzzi (2003). This variability is the result of the complex interaction of different factors that influence the fermentation process.

Many authors (Komprda *et al*., 2001; Eerola *et al*., 1998; Maijala *et al*., 1995) confirmed the key role of the quality of raw materials and chemical-physical factors (pH, a_w, NaCl concentration, temperature, etc.) to obtain products with low concentrations of biogenic amines.

Tyramine and putrescine are the principal amines that can be found at the end of the ripening of fermented dry sausages due to the activity of lactic acid bacteria, but sometimes can be found also cadaverine and histamine, generally deriving from low quality raw materials or environmental contamination. In fact, during the producing process raw material can be contaminated by environmental microorganisms and some of them can decarboxylate amino acids causing an increasing amount of some amines.

To obtain fermented products with low concentration of these compounds it is necessary to control the quality of raw materials, to choose starter cultures that do not produce biogenic amines and to select the best conditions in terms of chemical-physical factors that characterize these products.

Fermented fish products

Fermented fish products are characterize by a great variability in the content of biogenic amines as demonstrated from the study of Yankah *et al*. (1993) on Ghananian fish and Ayensa, (1993) on anchovies. In this study only few sample present detectable quantity of histamine, in fact the decarboxylation of histidine is very variable and dependent on time, temperature and on the specific characteristics of the microflora present.

Wine and beer

In this type of product relevant quantities of agmantine, ethanolamine, cadaverine, histamine, tyramine and putrescine can be found as products of microorganisms that operate the alcoholic fermentation. Numerous authors including Bravo *et al*. (1983) and Dumont *et al*. (1992) have showed the presence of histamine and tyramine in red wines and beer.

As reported by EFSA (2011) it is possible to create two different ranks for these foods, one based on the mean content in tyramine and histamine (the most toxic amines) and one on the consumer exposure.

Based on the mean concentration foods can be ranked in following increasing order:

- histamine → fermented sausages (23.0 – 23.6 mg/kg), other fish and fish products (26.8 – 31.2 mg/kg), cheese (20.9-62 mg/kg), fermented vegetables (39.4 – 42.6 mg/kg), fish sauce (196-197 mg/kg) and dried anchovies (348 mg/kg);
- tyramine → fermented vegetables (45 – 47.4 mg/kg), fermented fish (47.2 – 47.9 mg/kg), cheese (68.5 – 104 mg/kg), fish sauce (105 – 107 mg/kg) and fermented sausages (136 mg/kg).

Based on the consumer exposure foods can be ranked in following increasing order:

- histamine → fermented vegetables, fish sauce (0.4 – 29.9 mg/day), cheese (13 – 32.1 mg/day), fermented sausages (6.4 – 37.1 mg/day) and other fish and fish products (8.8 – 41.4 mg/day).
- tyramine → preserved meat, fermented fish meat (2 – 94.4 mg/day), fermented sausages (17.2 – 99.3 mg/day), cheese (44 – 108 mg/day) and beer (18.5 – 124.6 mg/day).

Chapter 2

The genus *Enterococcus*

2.1. Characteristics of the genus *Enterococcus*

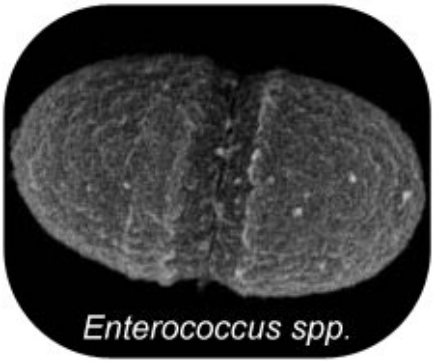

Figure 2.1: *Enterococcus* spp. (www.sourcemolecular.com)

Enterococci are ubiquitous microorganisms that have as predominant habitat the gastrointestinal tract of humans and animals. Generally, they are characterized by a high heat tolerance and a relevant survival under adverse environmental conditions. For these reasons, they can easily colonize a wide variety of habitats, including foods. Their origin, ubiquity and resistance justify the use of their microbial count as a quality index for fresh and processed foods. In fact, their presence is considered negative in fresh foods and fermented sausages in which they can promote the spoilage process and in which they are often considered indicators of faecal contamination. Nevertheless, they are fundamental in the production of some cheeses because they play an important role in the development of organoleptic characteristics during the ripening. For these reasons they are sometime utilized in the formulation of the starter cultures for cheese production (Giraffa, 2003). Others important characteristics of these microorganisms are their resistance towards several antibiotic (such as ampicillin, vancomycin, aminoglycosides and glycopeptides) and the ability to exchange genetic material that can enhance their survival. For example it has been demonstrated that the 25% of the DNA of *E. faecalis* is exogenously acquired (Polidori *et al*., 2011). It is also demonstrated that these transferred genes derive from different genus like *Streptococcus* and *Staphylococcus* (Gilmore *et al*., 2014).

In the last two decades *Enterococcus* species have acquired an important role in the clinical microbiology, in fact they are considered nosocomial pathogens and often they are involved in food-borne illnesses due to their virulence factors (Foulquié Moreno *et al*., 2006).

2.2. Taxonomy

The identification of the microorganisms belonging to the genus *Enterococcus* has always been problematic, because this genus is composed by a heterogeneous group of Gram-positive cocci that

are characterized by many proprieties typical of other genera, such as *Streptococcus* and *Lactococcus*.

During the last century the classification of the genus *Enterococcus* has been refined and in 1984, with the use of DNA hybridization and 16s rRNA sequencing, the most significant changes were made. Until some decades ago, the homo lactic coccal shaped cells of LAB were classified in the genus *Streptococcus*. The evidence of the heterogeneity of this genus (both physiological and genetic) determined its subdivision into in three genera: *Streptococcus*, *Lactococcus* and *Enterococcus* (Giraffa, 2002) as reported in Figure 2.2.

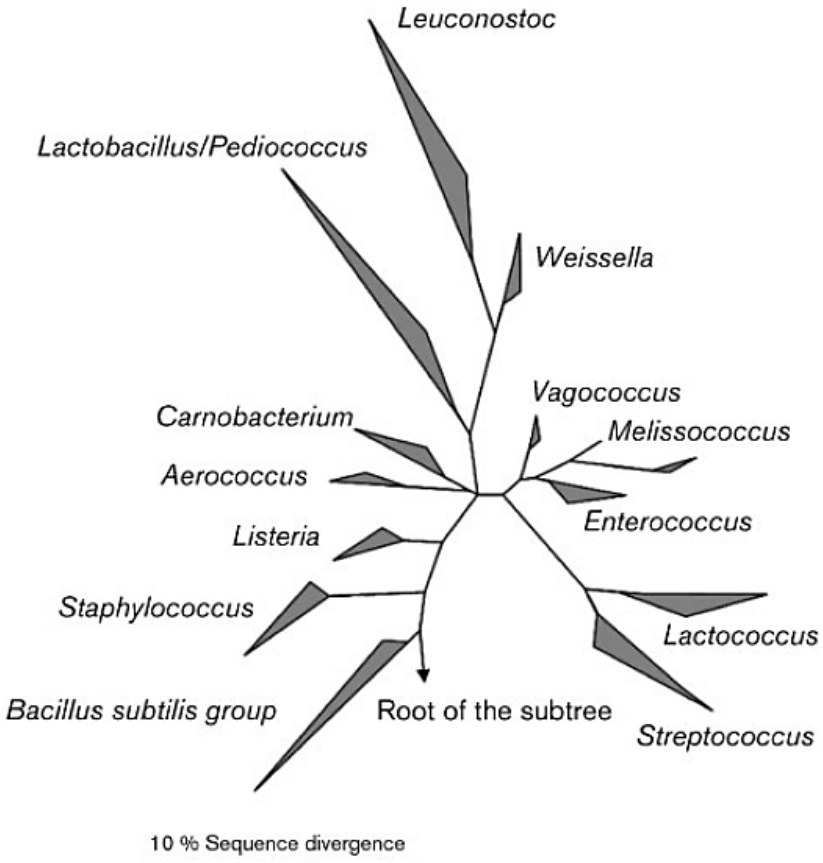

Figure 2.2:16s rRNA dendrogram (Fisher and Phillips, 2009)

Even if this classification is accepted, today still difficult to define the phylogenetic system for *Enterococcus*. Nowadays, the genera *Enterococcus* consists of 28 species: *E. faecalis*, *E. faecium*, *E. durans*, *E. mundtii*, *E. asini*, *E. avium*, *E. canis*, *E. casseliflavus*, *E. cecorum*, *E. columbae*, *E. dispar*, *E. flavescens*, *E. gallinarum*, *E. gilvus*, *E. haemoperoxidus*, *E. hirae*, *E. malodoratus*, *E. moraviensis*, *E. pallens*, *E. phoeniculicola*, *E. pseudoavium*, *E. raffinosus*, *E. ratti*, *E. saccharolyticus*, *E. saccharominimus*, *E. solitarius*, *E. sulfureus* and *E. villorum* (Foulquié Moreno *et al*., 2006).

2.3. General aspects

Enterococci are Gram positive microorganisms, non-spore forming, catalase-negative that can occur singly, in pairs or in short chains. They are facultative anaerobes, their metabolism is homo-fermentative and they produce only L-lactic acid. They can grow in a wide range of temperature from 10 to 45°C, but their optimum is around 35°C. Enterococci can survive to a heat treatment at 60°C for 30 minutes. Generally, their growth is scarcely influenced by osmotic pressure and pH, in fact they can grow in hypotonic, hypertonic, acidic and alkaline environments. As facultative anaerobes they can grow also under reduced or oxygenated conditions (Fisher and Phillips, 2009).
They can survive to a range of stress and hostile environments, characterize by extreme values of temperature (5-65°C), pH (4.5-10.0) and concentrations of NaCl (up to 6.5%) (Fisher and Phillips, 2009). Not all the strains are able to grow in all the reported conditions, there are some exception that include *E. dispar*, *E. sulfureus*, *E. malodoratus* and *E. moraviensis* which do not grow with a temperature of 45°C (Svec *et al*., 2001; Martìnez-Murcia and Collins, 1991; Collins *et al*., 1984), and *E. cecorum* and *E. columbae* that do not grow at 10°C, as reported by Devriese *et al*., 1993. Other exception regard the ability to grow in presence of 6.5% of NaCl, in fact *E. avium*, *E. saccharominimus*, *E. cecorum* and *E. columbae* do not grow in this condition (Vancanneyt *et al*., 2004; Devriese *et al*., 1993).
These general aspects are the reasons for which the species of this genus can be isolated in a large variety of habitats, such as fresh vegetables, processing foods, water, soil and so on.

2.4. Genome

In literature there are different studies about the genome of *Enterococcus*, but the majority regards the genome of *Enterococcus faecalis* V538, because it is a pathogenic antibiotic-resistant bacteria. These studies have highlighted that the main chromosome of *E. faecalis* V583 is composed by 3.218.031 bp and contains 3.182 open reading frames (ORFs). The genome of this microorganism is characterized by the presence of three plasmids of 66.320, 57.660 and 17.963 bp, respectively. Also it has been identified a “pathogenicity island”, that is composed by 150 kbp and contains the genes that help microbial cells in the colonization and infection processes.

2.5. Physiology and metabolism

Enterococci are bacteria characterized by a vast metabolic potential that promote their growth in a wide variety of environments characterized by different stress factors. This complex metabolism is under investigation from the last century and in the last few years with the tools of molecular

biology a lot of new information has been obtained. These researches have shown that many metabolic genes and pathways vary even within single species, for this reason the knowledge of the principal metabolic pathways that characterize the genus is fundamental.

2.5.1. Sugar metabolisms

The sugar metabolism is the most implied in the survival of enterococci in extremely different habitats. All the *Enterococcus* species are able to metabolize 13 sugars and it is demonstrated that at least two members can metabolize other 30 sugars. Studies about the *Enterococcus* metabolism have highlighted that these species can be able to utilize carbon sources that characterize the metabolism of different genus or species thanks to mobile elements. Anyway, they can easily metabolize carbohydrate monomers and polymers.
Enterococci can metabolize sugars by two different pathways:

- Embden-Meyerhof-Parnas
- Pentose phosphate.

An important characteristic of the specie *Enterococcus faecalis* is the ability to metabolize glucose also by Entner-Doudoroff pathway. This pathway normally is associated only to Gram negative microorganism, but *E. faecalis* represent an exception, in fact is the only Gram positive microorganism that can follow this way (Gilmore *et al*., 2014). Peykov *et al*. (2012) have demonstrated that the genes involved in this pathway can be used to identify *E. faecalis* strains by a novel method based on a PCR (polymerase chain reaction) with specific primers for these genes.
In addition to simple sugars, enterococci are able to metabolize some biological polymers resistant to human digestive system. One of these sugars is cellulose, it is demonstrated by different studies that *E. saccharolyticus*, *E. faecalis* and *E. gallinarum* can grow on substrate where the only sugar source is represented by the cellulose (Chassard *et al*., 2010; Adav *et al*., 2009; Wang *et al*., 2009). Other sugars that can be metabolized by enterococci are: raffinose (Zhang *et al*., 2011), maltose (Mokhtari *et al*., 2013) and trehalose (Andersson *et al*., 2001).

<u>Embden-Meyerhof-Parnas</u>

In general, lactic acid bacteria do not possess a respiratory system, so they produce energy through different metabolic pathway. Enterococci, in particular, obtain energy by an homo-lactic fermentation based on the Embden-Meyerhof-Parnas pathway (glycolysis). This fermentation produce 2 moles of ATP for every mole of glucose used and lactic acid as shown in Figure 2.3.

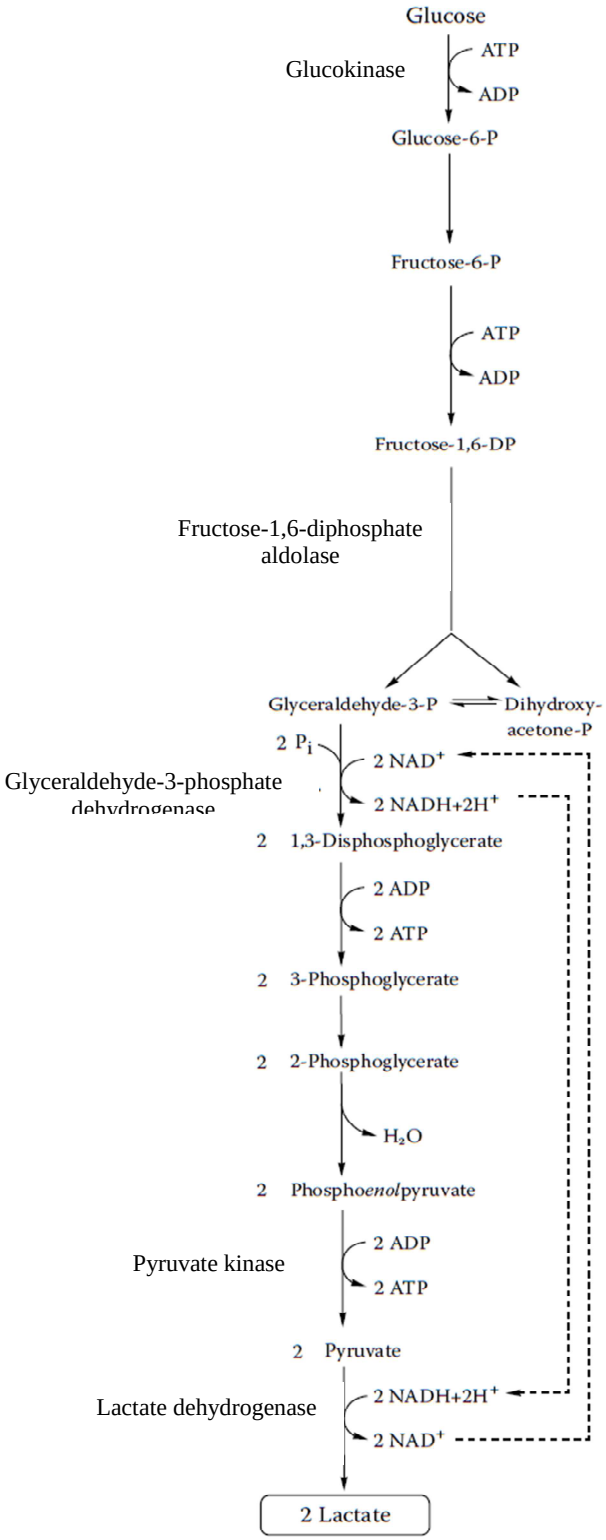

Figure 2.3: Glycolysis and homo lactic fermentation pathways (Von Wright and Axelsson, 2012)

The glycolysis pathway is composed by ten reactions that can be divided in two phases:

1. From glucose to glyceraldehyde-3-phosphate;
2. From glyceraldehyde-3-phosphate to pyruvate.

The first reaction consist in the phosphorylation of the glucose to glucose-6-phophate by the action of the enzyme glucokinase. This reaction requires ATP for the phosphorylation of the substrate that is necessary to maintain it inside the cells. The second reaction is catalysed by glucose-6-phosphate isomerase that made the isomerization to fructose-6-phosphate, in this way the carbon in position 3 is activated for the action of the phosphofructokinase (PFK). The third reaction represent the regulation step of the entire metabolic pathway (Figure 2.4), in fact the PFK is highly regulated. The enzyme is activated when the cells need energy (high concentration pf AMP) or in presence of fructose-2,6- diphosphate (that is the product of the reaction) and is inhibited when the concentration of ATP is high, as reported in Figure 2.5.

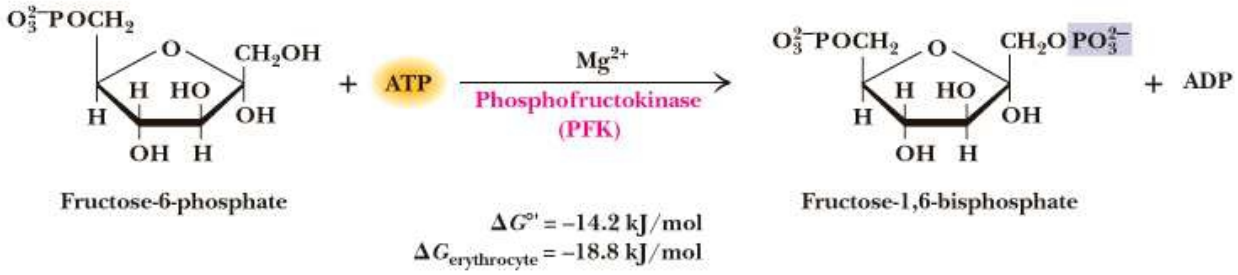

Figure 2.5: PFK reaction (Garrett and Grisham, 2012)

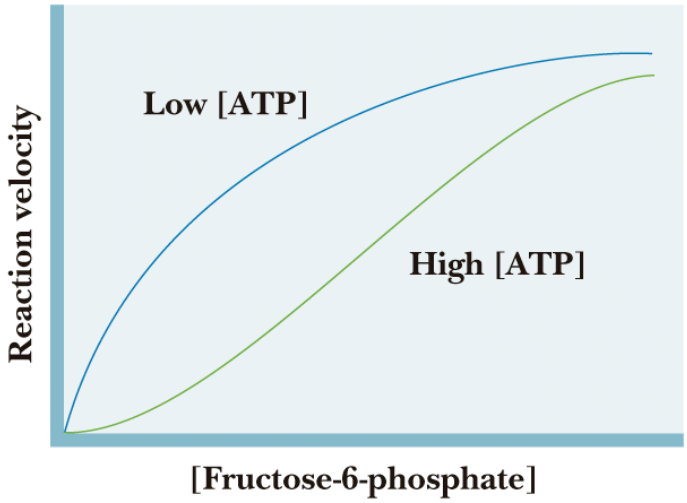

Figure 2.4: Regulation of PFK by ATP and AMP (Garrett and Grisham, 2012)

Moreover, PFK is allosterically inhibited by citrate. The next reaction is catalysed by fructose-1,6-diphosphate aldolase, that "cut" the molecule of fructose-1,6-diphosphate and produce

dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. The dihydroxyacetone phosphate obtained must be converted to glyceraldehyde-3-phospate to be metabolized by the triosephosphate isomerase.

The second part of glycolysis is the energetic one, in fact it is characterized by the production of two phosphorylated compounds with high energy, from which are obtained four ATP.

This phase starts with the oxidation of the glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate through the action of glyceraldehyde-3-phosphate dehydrogenase. This is the first molecule with high content of energy that is used to synthetize ATP by a process of substrate phosphorylation made by the enzyme phosphoglycerate kinase, as shown in Figure2.5.

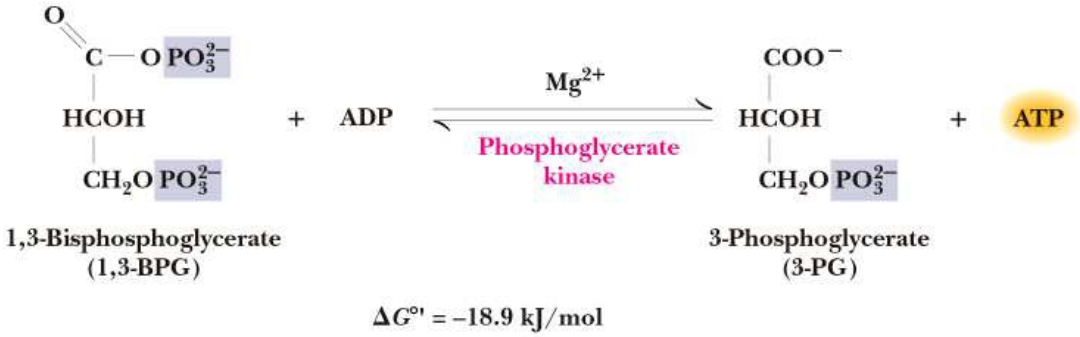

Figure 2.5: Phosphoglycerate kinase reaction (Garrett and Grisham, 2012)

Then the phosphoric group of 3-phosphoglycerate is replaced on the C-2 atom to obtain 2-phosphoglycerate, that is the substrate for the following reaction that produces phosphoenolpyruvate (PEP) through the action of enolase. Finally, PEP is converted in pyruvate by pyruvate kinase, with the production of ATP, as shown in Figure 2.6.

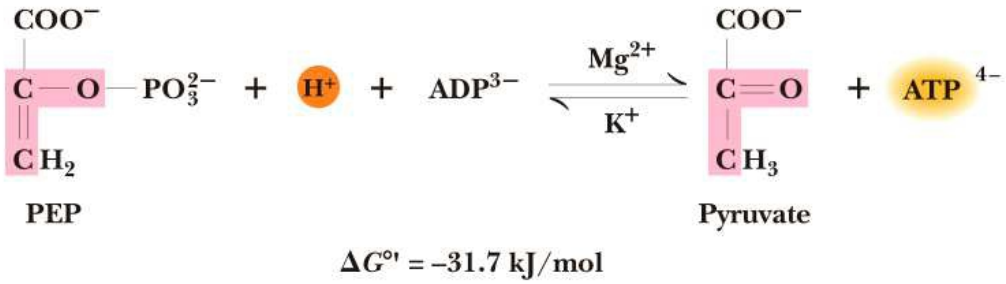

Figure 2.6: Pyruvate kinase reaction (Garrett and Grisham, 2012)

In the last reaction of the homo-fermentative pathway the pyruvate obtained is reduced to lactate by the enzyme lactate dehydrogenase. This enzyme can catalyse the conversion of pyruvate to lactate, but also the conversion of lactate to pyruvate, for this reason it exhibits a feedback inhibition when lactate is present in high concentration in the medium. The inhibition result in a reduction of the

conversion of pyruvate and of the regeneration of NAD^+. In this case the pyruvate is metabolized by other pathways which will be shown later (Von Wright and Axelsson, 2012). Another important characteristic of the lactate dehydrogenase is its enantiospecificity, in fact it can produce D-lactate or L-lactate in relation to its specificity (Kim and Whitesides, 1988).

Other hexose, for example fructose, galactose and mannose, are metabolized by this pathway after an isomerization and a phosphorylation. In particular, galactose can follow two different pathways depending on the form in which it enters in the cells, as reported in Figure 2.7. Galactose, in fact, can be phosphorylated by a phosphoenolpyruvate-dependent phosphotransferase system (PEP:PTS) and absorbed, or can be imported as free galactose by a specific membrane permease.

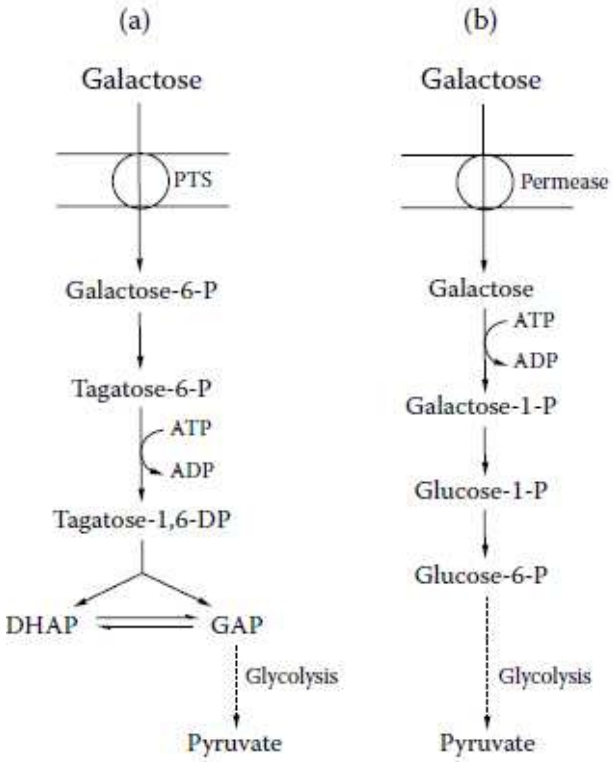

Figure 2.7: Galactose metabolisms a) tagatose-6-phosphate pathway; b) Leloir pathway (Von Wright and Axelsson, 2012)

Pentose phosphate

The pentose phosphate is an alternative pathway of glucose. This is not an energetic metabolism, but it is a way to obtain sugars with five atoms of carbon that are precursors of important molecules, such as deoxyribose and nicotinamide adenine dinucleotide phosphate. They are fundamental for the synthesis of nucleic acids and for the process of reduction of some biosynthetic pathways, respectively.

This metabolism produce also some compounds, such as fructose-6-phosphate and glyceraldehyde-3-phosphate.

2.5.2. Pyruvate metabolism

The pyruvate obtained by these and other metabolisms are not converted totally in lactate, but it can be utilized to obtain different compounds important for microbial cells as shown in Figure 2.8.

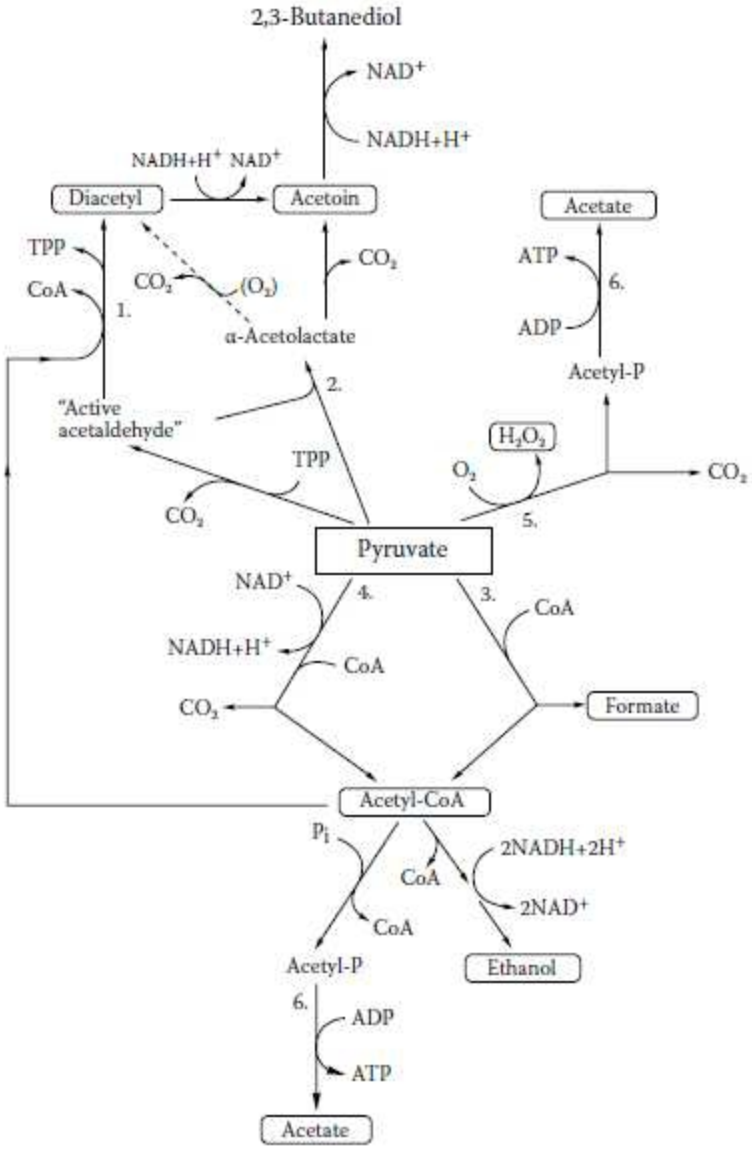

Figure 2.8: Alternative metabolisms of pyruvate (Von Wright and Axelsson, 2012)

These alternative metabolisms do not produce high quantity of energy for cells, but they are important when the activity of lactate dehydrogenase is suppressed and the growth of cells are slow. In fact, these pyruvate metabolism are activated in particular when the microbial cells are in nutritional stress (Von Wright and Axelsson, 2012).

Four are the alternative pathways of pyruvate:

1. Diacetyl/acetoin pathway;
2. Pyruvate-formate lyase system;
3. Pyruvate dehydrogenase pathway;
4. Pyruvate flavodoxin/ferredoxin oxidoredeuctase;
5. Pyruvate oxidase pathway.

Diacetyl/acetoin pathway

This is one of the most important pathway for the construction of the aromatic profile of cheeses and fermented sausages. This metabolism is activated only if there is an excess of pyruvate (respect the amount needed to produce NAD^+ from the homo-fermentative pathway), that is normally obtained from other pathway, for example the breakdown of citrate (Von Wright and Axelsson, 2012). The pyruvate can follow two different route to form diacetyl, but the most common is the one that involves the α-acetolactate, in fact this compound is converted spontaneously to diacetyle without any enzymatic reaction (Von Wright and Axelsson, 2012).

Pyruvate-formate lyase system

Another use of the pyruvate is the formate lyase system. This pathway is activated by enterococci in anaerobic condition and when the substrate is limited (Von Wright and Axelsson, 2012). In this metabolism pyruvate is used to obtain acetyl-CoA and formate by the enzyme pyruvate-formate lyase (named also as formate acetyltransferase) (Kandler, 1983; Thomas *et al*., 1979). These compounds are then used to obtain different end products (lactate, acetate, formate and ethanol) and, in particular, acetyl-CoA is used as an electron acceptor in the production of ethanol and as a substrate for the production of ATP in the reaction that has as end product acetate (Von Wright and Axelsson, 2012).
An important characteristic of the pyruvate-formate lyase is its high sensibility to the oxygen, in fact it cause an alteration of the bound between the sub-units of the enzyme and completely inactivate the protein (Gilmore *et al*., 2014).

Pyruvate flavodoxin/ferredoxin oxidoredeuctase

This is a specific pathway that characterize the specie *E. faecalis* (Yamazaki *et al*., 1976) and produce acetyl-CoA in reduced condition by the action of the enzyme flavodoxin/ferredoxin oxidoreductase. The products are the same of the pyruvate-formate lyase system, but this enzyme is not sensitive to catabolite repression.

Pyruvate dehydrogenase pathway

The dehydrogenation of pyruvate represent another metabolic pathway that lead to the production of acetyl-CoA that is utilized in the lipid biosynthesis (Von Wright and Axelsson, 2012). This pathway is composed by different oxidative reactions, so it require oxygen to be active.

Pyruvate oxidase pathway

This pathway take place only in presence of oxygen because the main reaction is an oxidation of the pyruvate by the enzyme pyruvate oxydase, to obtain acetate. Sedewitz *et al*., 1984 have reported that this metabolism can lead to the aerobic formation of acetic acid.

2.5.3. Glycerol metabolism

Another important metabolism that characterize this genus is the glycerol catabolism from which microbial cells can obtain fundamental molecules for the synthesis of lipids and lipoteichoic acids (Coyette and Hancock, 2002). In particular, Bizzini *et al*., 2009 reported that the metabolism of the specie *E. faecalis* is very different from the one that characterize the genus, in fact the members of this specie can metabolize glycerol under aerobic conditions, but also in anaerobic conditions. The two different pathways are reported in Figure 2.9.

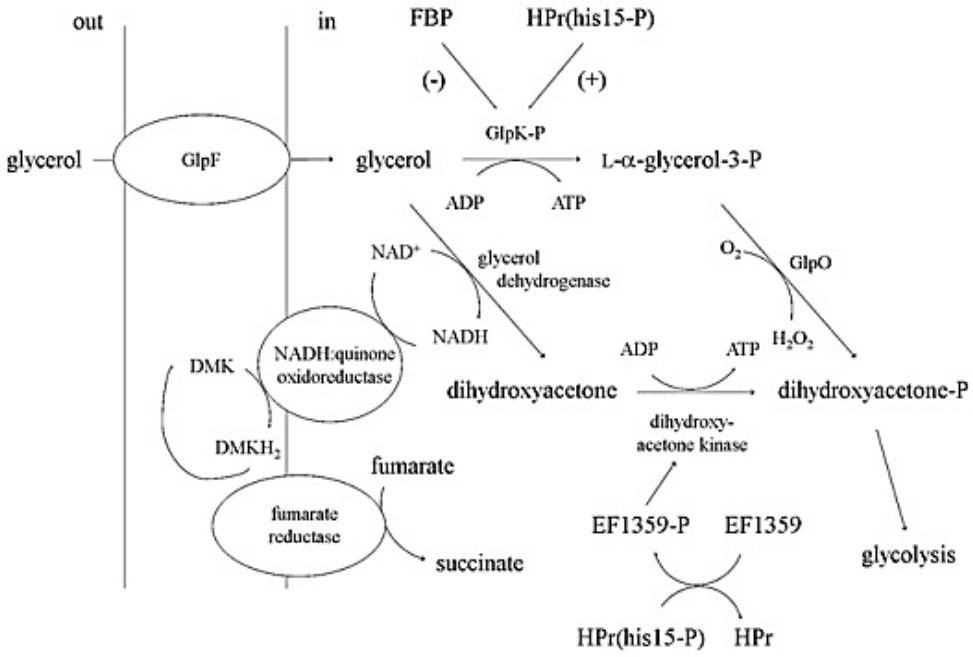

Figure 2.9: Different pathways of glycerol metabolism in *Enterococcus faecalis* (Gilmore *et al*., 2014)

The first pathway involves two enzymes that phosphorylate and oxidate the glycerol to obtain a product that can be used in the glycolysis. In particular, the glycerol absorbed by the specific

membrane protein (GlpF) from substrate is firstly phosphorylated to glycerol-3-phosphate by glycerol kinase (Glpk) and then it is oxidized to dihydroxyacetone-phosphate by the action of the enzyme glycerol-3-oxidase (Glpo). These two enzymes, along with the specific carrier, are encoded in a single operon that is highly conserved among enterococci, without any differences between species.

The second pathway starts with the oxidation of glycerol by glycerol dehydrogenase (GldA) to obtain dihydroxyacetone that is subsequently phosphorylated to dihydroxyacetone phosphate by the enzyme dihydroxyacetone kinase (Dhak). As for the enzymes of the first pathway, also in this case the enzymes are encoded in a single operon for *E. faecalis*. The same operon can be found in the genome of *E. faecium*, but it lacks of the enzyme GldA and this justifies the inability of the species to grow and metabolize glycerol in anaerobic conditions.

2.5.4. Citrate metabolism

Citrate is an important organic tribasic acid that can be found in several raw materials and is often used as an additive (in form of salt of sodium or potassium) in some food production. The metabolism of this compound has a principal role in the construction of the characteristic flavour of different fermented food, in particular cheeses. For this reason the microorganisms that can metabolize citrate are particularly important in this production. Unfortunately, not all the lactic acid bacteria are able to metabolize citrate (Kennes *et al*., 1991), in fact this ability is linked to the presence of a specific gene encoding for the transporter citrate permease, that control the uptake of citrate from the medium, as shown by Bandell *et al*. (1998). This gene is not normally present in the genome of lactic acid bacteria but it is acquired by an endogenous plasmid (Bandell *et al*., 1998). In literature there are some studies that demonstrated that enterococci isolated from different dairy products are able to metabolize citrate with an high efficiency (Vaningelgem *et al*., 2006; Sarantinopoulos *et al*., 2001b).

In Figure 2.10 is reported the citrate pathway, in particular the first step of this pathway require the transport of the citrate from the substrate, then it must be cleavage to acetate and oxaloacetate by the action of the enzyme citrate lyase, as shown by Hugenholz, (1993). The oxaloacetate obtained is then decarboxylated to pyruvate and CO_2. The pyruvate that is produced by this pathway is then utilized as substrate for different reaction that produce important end products, such as diacetyle, acetaldehyde, acetoin, acetate, formate, lactate, ethanol, α-acetolactate and 2,3-butanediol, that characterize the organoleptic profile of some foods (Vaningelgem *et al*., 2006). Moreover, the carbon dioxide produced with the decarboxylation of oxaloacetate is important in the definition of the structure of dairy products.

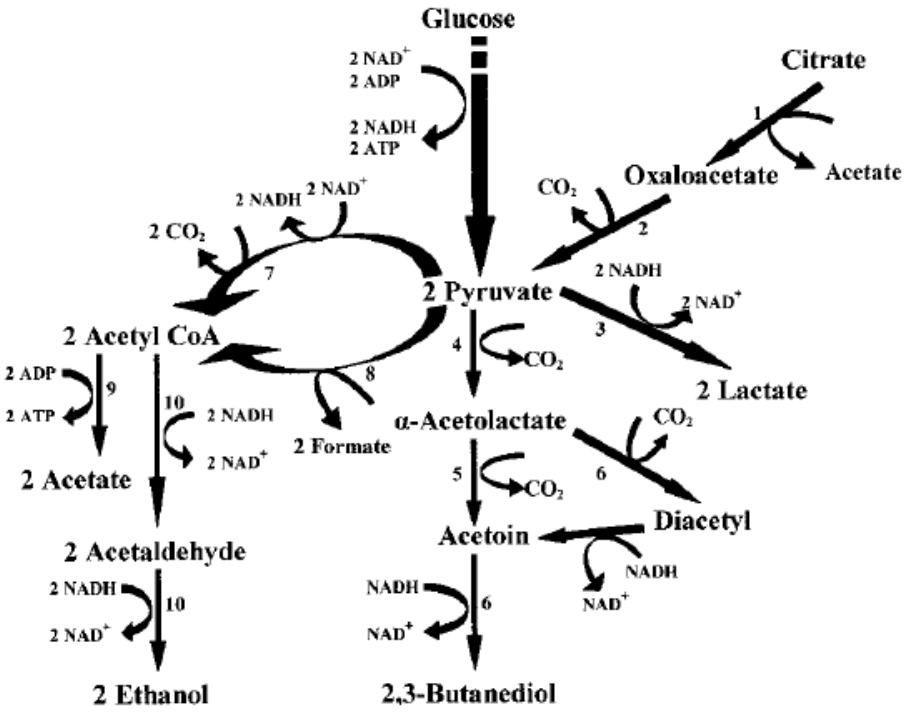

Figure 2.10: Citrate metabolism (Sarantinopoulos *et al*., 2001b)

Recently, the interest of some researchers is focalized on citrate metabolism of *Enterococccus* spp. because the molecules that are produced by this metabolism can be used as a quality index for fermented foods (Rea and Cogan, 2003a,b; Sarantinopoulos *et al*., 2001a,b) and represent a criteria in the selection of *Enterococcus* strains that can be used in the formulation of starter cultures for dairy and meat industry.

2.5.5. Respiration

Some enterococcal species are able to produce energy through a partial respiration metabolism. This pathway is not completely characterize for all the species, but some information can be found about *E. faecalis*. Given the inability of LAB (and enterococci among them) to synthetize heme, the enterococcal electron transport chain can work only when cells has aa exogenous supply of this important cofactor needed for cythocrome activity (Pritchard and Wimpenny, 1978; Ritchey and Seeley, 1974). In *E. faecalis* the respiration chain involves: demethylmenaquinone (the electron carrier), cytochrome bd (the protein which oxidizes the quinone and generate the protons that release outside the cell provide the proton-motive force), fumarate reductase (the enzyme that catalyzes the reduction of fumarate) and F0F1-ATP synthase (primary ion pump that control the

electrochemical gradient of protons). In Figure 2.11 is reported the respiration pathway in *E. faecalis*.

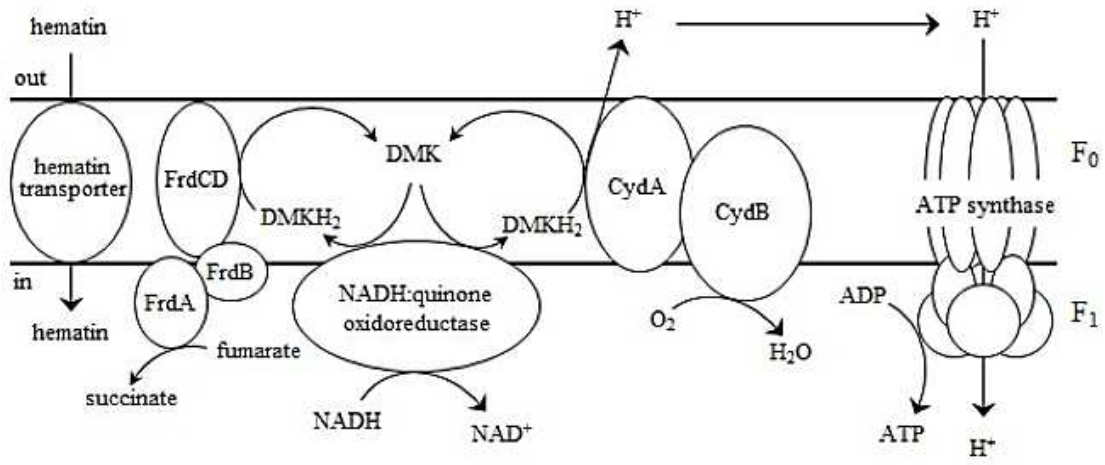

Figure 2.11: Respiration pathway of *E. faecalis* (Gilmore *et al*., 2014)

2.5.6. Redox metabolism

Another important metabolism for enterococci is the redox one. In fact, they are recognized as potent producers and scavengers of reactive oxygen species. These compounds are dangerous for the cell viability because they can react with the cell membrane and irreversibly damage it. There are different systems that provide the inactivation of these compounds and they are represent by: peroxidases, oxidases, peroxiredoxins, alkyl hydroperoxidases and glutathione.
The activity of these systems justify the resistance to oxidative stress that characterize enterococci, this is the reason for which they can survive inside phagocytic cells (Gentry Weeks *et al*., 1999).

2.5.7. Deiminase catabolism

Arginine and agmatine are alternative energy sources for few species of enterococci, in particular *E. faecalis*. Obviously this metabolism produce less energy respect to the glycolysis, in fact one mole of substrate produces one mole of ATP. This pathway starts with the deiminase of argine and agmatine to obtain, respectively, citrulline and carbamoylputrescine. These reactions are catalysed respectively by the enzymes arginine and agmatine deiminase. Then two different reactions take place, one is the phosphorolysis of the citrulline carried out by the ornithine carbamoyltransferase to form carbamoyl phosphate and ornithine and the other regards the carbamoylputrescine that reacts with putrescine carbamoyltransferase to obtain putrescine and carbamoyl phosphate. The carbamoyl phosphate is characterized by a high-energy phosphate bond that can be used to produce ATP. Figure 2.12 shows the deiminase pathway.

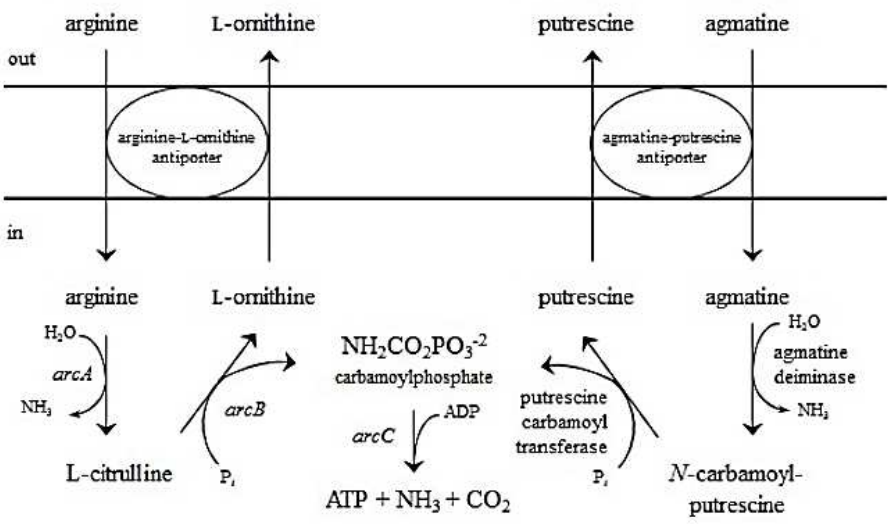

Figure 2.12: Deiminase pathway (Gilmore *et al*., 2014)

2.5.8. Ion metabolism

Enterococci are able to survive in harsh environments characterized by extreme pH and high salt concentration. This ability implies that microbial cells must have an efficient system of transport that maintain a constant ion concentration, essential for the homeostasis of the cells.

The survival of cells in environments characterize by high salt concentration require some system that can regulate the intracellular concentration of cations and anions. It is demonstrated that *E. hirae* has two systems that act to control the Na^+ concentrations. These systems are represented by a Na^+/H^+ antiporter and a vacuolar-type ATPase. It is worthy to note that not all the cations must be strictly bound to the concentration between the inside and the outside of the cells, for example, potassium is a cation fundamental for cells and it must be present in high concentration inside the cells. This ion is necessary to neutralize intracellular anions, to activate some enzymes and to regulate the cytosolic pH. It is obvious that high intracellular proton concentrations is a problem when low concentration of potassium is available in the cells. To avoid this problem the cells are provided with specific active transporter for this ion, as reported for *E. hirae* by Kawano *et al*., 2001.

Another important ion for microbial cells is copper. It is a cofactor of redox-active metabolic enzymes because of the redox activity between its two oxidation states (Cu^+ and Cu^{2+}). The high reactivity that characterize this ion can cause important problem to microbial cells, in particular the ones of enterococci. To reduce the toxicity of the copper the cells have specific proteins, named copper chaperones and copper-ATPase, that regulate its uptake and export as reported in Figure 2.13.

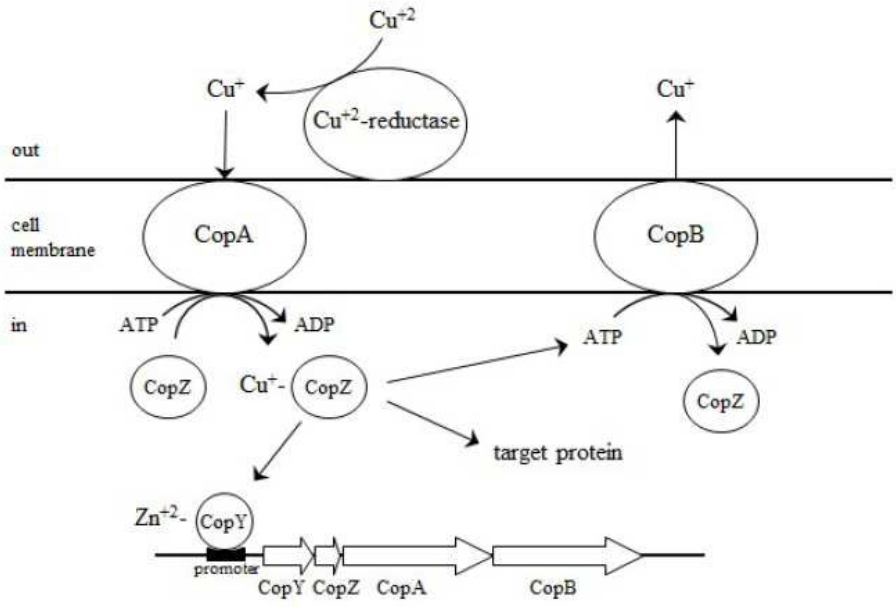

Figure 2.13: Copper metabolism (Gilmore *et al*., 2014)

Iron is a fundamental ion for microbial cells, it can be considered an essential nutrient (Lisiecki and Mikucki, 2006). Due to the physiological pH the concentration of this ion in cells is usually low, for this reason enterococci are able to synthesize and secret specific binding compounds named siderophores. These molecules bind iron and then the cells with specific membrane receptors absorb it and utilize the iron bind (Lisiecki *et al*., 2000).

Another important ion for microbial cells is the manganese. It is a cofactor of numerous enzymes and a regulator of some metabolic pathways related to signal transduction and oxidative stress (Jakubovics and Jenkinson, 2001).

2.6. Stress response general aspects

Enterococci are recognized as members of the intestinal microbiota and for this reason they are considered faecal contaminants. To survive in the intestine these microorganisms must be resistant to a wide range of stress conditions and stress factors, such as change of pH, high salt concentrations, presence of bile salt. In particular, their resistance to high temperature, low and high pH and high NaCl concentration are important characteristics that should be taken into consideration for industrial production of fermented foods such as sausage and cheese. Giard *et al*. (2003) and Bøhle *et al*. (2010) have reported that the synthesis of over 200 polypeptides is enhanced when the microorganisms grow in stress conditions. These stress proteins represent the tool that enterococci use to survive in inhospitable habitats. Two of the principal stress proteins correspond to GroEL and DnaK chaperones and are named Gsp 66 and Gsp 67 respectively (Rince

et al., 2001). Some other stress proteins are found from the analysis on *E. faecalis* such as Gsp 65, that is homologous to bacterial hydroperoxide reductases, and Gls 24 that is related to the virulence.

2.7. Functional properties of enterococci

Enterococci are characterized by many positive functional properties, that can play an important role in some food productions.

2.7.1. Production of bacteriocins

The bacteriocins are small cationic peptides with important antimicrobial activity that are synthesised in the ribosomes. These compounds are characterized by different mode and target of activity, moreover they have different molecular mass, chemical structure, thermostability, pH of activity and genetic determinants (Riley and Wertz, 2002; Cleveland *et al*., 2001; McAuliffe *et al*., 2001; Ennahar *et al*., 2000).

Bacteriocins are classified into three classes characterized by different structural, molecular and physicochemical properties (Nes *et al*., 1996; Klaenhammer, 1993):

- Class I: bacteriocins named lantibiotics, that are post-translationally formed;
- Class II: bacteriocins not post-translationally modified divided into three subclasses:
    - Class IIa or pediocin–like bacteriocins;
    - Class IIb bacteriocins require two polypeptide chains to be functional;
    - Class IIc contain the molecules that can be part of other groups.
- Class III: hydrophilic and heat-labile peptides.

Enterococci are able to produce enterocins (Fontana *et al.*, 2015; Beshkova and Frengova, 2012), that are bacteriocins that belong to class I, class IIa, class IIc and class III.

In literature there are many studies about the activity of enterocins towards *Listeria* spp.. For example Garcia *et al*. (2004) studied the inhibition of *Listeria monocytogenes* by enterocin EJ97, that is produced by *E. faecalis* EJ97, against these microorganisms.

Today many different bacteriocins produced by enterococci are known as shown in Table 2.1.

Enterocin	Actual enterocin	Producer strain	Reference
Enterocin 4	Enterocin AS-48	*E. faecalis* INIA 4	Joosten et al., 1996
Enterococcin EFS2		*E. faecalis* EFS2	Maisnier-Patin et al., 1996
Bacteriocin 21		*E. faecalis* OG1X	Tomita et al., 1997
Enterocin 7C5		*E. faecium* 7C5	Folli et al., 2003
Enterocin 900	Enterocin A	*E. faecium* BFE 900	Franz et al., 1999b
Enterocin 1146		*E. faecium* DPC1146	O'Keeffe et al., 1999
Enterocin EFM01		*E. faecium* EFM01	Ennahar and Deschamps, 2000
Enterocin P21		*E. faecium* P21	Herranz et al., 2001
Enterocin 81		*E. faecium* WHE 81	Ennahar et al., 2001
Enterocin N15		*E. faecium* N15	Losteinkit et al., 2001
Enterocin BC25		*E. faecium* BC25	Morovský et al., 2001
Enterocin CCM 4231		*E. faecium* CCM 4231	Foulquié Moreno et al., 2003a
Enterocin 900	Enterocin B	*E. faecium* BFE 900	Franz et al., 1999b
Enterocin P21		*E. faecium* P21	Herranz et al., 2001
Enterocin 81		*E. faecium* WHE 81	Ennahar et al., 2001
Enterocin RZS C13		*E. faecium* RZS C13	Foulquié Moreno et al., 2003a
Enterocin RZS C5		*E. faecium* RZS C5	Foulquié Moreno et al., 2003a
Enterocin I	Enterocin L50	*E. faecium* 6T1a	Floriano et al., 1998
Enterocin B2		*E. faecium* B2	Moreno et al., 2002
Enterocin AA13	Enterocin P	*E. faecium* AA13	Herranz et al., 1999
Enterocin G16		*E. faecium* G16	Herranz et al., 1999
Enterocin B1		*E. faecium* B1	Moreno et al., 2002
Enterocin B2		*E. faecium* B2	Moreno et al., 2002
Enterocin 1071A	Enterocin 1071A	*E. faecalis* FAIR-E 309	Franz et al., 2002
Enterocin 1071B	Enterocin 1071B	*E. faecalis* FAIR-E 309	Franz et al., 2002
Enterocin B2	Enterocin 1071	*E. faecium* B2	Moreno et al., 2002

Table 2.1: Enterocins present in literature (Foulquié Moreno *et al*., 2006).

Only few of these molecules have been purified to homogeneity, in fact the majority still not be purified, for example some enterocins produce by: *E. faecalis* E-1 (Bottone *et al*., 1971), E23 (Nakagawa, 1979), K 4 (Kühnen *et al*., 1985), DS16 (Ike *et al*., 1990), 226 NWC (Villani *et al*., 1993) and *E. faecium* E1 (Krämer and Brandis, 1975), S-34 (Nakagawa and Matsuo, 1981), 3 (Krämer *et al*., 1983), 25 (Reichelt *et al*., 1984), 100 (Kato *et al*., 1993), NA01 (Olasupo *et al*., 1994), 7C5 (Torri Tarelli *et al*., 1994), L1 (Lyon *et al*., 1995).

The target of these compounds is the cytoplasmic membrane In fact they create pores that alter its functionality in terms of transmembrane potential and pH gradient. Moreover these pores cause the loss of important intracellular molecules (Cleveland *et al*., 2001).

2.7.2. Proteolysis

The studies about the proteolytic activity of enterococci are scarce, in fact the majority of the researches concerns other genus of lactic acid bacteria such as *Lactococcus* and *Lactobacillus*.

The proteolysis is an important microbial metabolic process because it produces peptides of different molecular weight, that contribute to the organoleptic characteristics of some foods, in particular the fermented ones. These peptides can give to products good flavour, but also undesirable bitter taste, for this reason is necessary to control the proteolysis through the selection of the correct microorganisms.

The first study about the proteolysis of enterococci was performed by Somkuti and Babel (1969), they analyse the activity of an extracellular proteinase of a strain of *E. faecalis* var. *liquefaciens*.

This research revealed that this proteinase can degrade with high activity the casein, but it is less active on α-lactalbumin and β-lactoglobulin. Another study on *E. durans* protease highlight its activity only on casein and β-lactoglobulin (Wallace and Harmon, 1970).
Carrasco de Mendoza *et al.*, (1989) from the characterization of 61 strains of *Enterococcus* report that proteolytic activity is strain and time dependent. Moreover, Wessels *et al.*, (1990) found that some strains of *E. faecium*, *E. faecalis* and *E. durans* are characterized by a relevant proteolytic activity at psychrotrophic temperatures.
In a recent study performed by Sarantinopoulos *et al*., (2001a) is reported that *E. faecalis* strains, in general, are more active respect strains of *E. faecium* and *E. durans*.

2.7.3. Lipolysis

Lipolysis, such as proteolysis, is a fundamental process for the construction of the typical sensory profile of some fermented foods. Lipolysis contribute to the formation of organoleptic profile by the production of short chain fatty acids (that are precursors of sapid compounds such as methyl ketones and lactones), the oxidation of fatty acids (that produce strongly flavoured compounds) and the solubilisation of aromatic compounds that are derived from other metabolisms.
Also in this case the information that can be found in literature are few, probably because lactic acid bacteria are known as weakly lipolytic. The first study on the lipolysis of enterococci, that was known as Streptococci yet, was performed by Lund (1965), he found some esterases in the cell free extracts of *E. faecalis*, *E. faecium* and *E. durans* and, in particular, the one identified from *E. faecalis* exhibited higher activity respect the other. Another study reported that the lipolityc activity of enterococci (also in this case these strains was known as streptococci and only after new genetic study they are recognized as enterococci) is higher respect the activity of streptococci (Dovat *et al*., 1970). Carrasco de Mendoza *et al*., (1992) reported that the lipolytic activity of enterococci is strain dependent, in fact the majority of enterococci strains are not lipolytic, but some strains of *E. faecalis* shown important lipolytic activities. Sarantinopoulos *et al*., (2001a) confirmed the data of the previous studies, they reported that in their trials *E. faecalis* strains were the most lipolytic and esterolytic, followed by *E. faecium* and *E. durans*.

2.7.4. Probiotics

Probiotics are living microorganisms that improve the health of the host, if they are ingested in adequate amounts, through the modification of the intestinal microbiota. The beneficial effects that these microorganisms can produce on the host are different, including: improvement of the immune response, modification and improvement of the composition of the colonic microbiota, reduction of

the enzyme implied in cancer initiation, treatment of diarrhea caused by travel and antibiotics, vaccine adjuvant effects and prevention of ulcers caused by *Helicobacter pylori*.
To define a microorganism as probiotic some specific characteristics are required:

- Ability to adhere to the intestinal epithelium;
- Prevent or reduce the adherence of pathogens;
- Persistence and multiplication;
- Production of important microbial compounds, such as acids, hydrogen peroxide and bacteriocins;
- Be safe for human organisms (Salminen *et al*., 1996).

The best known probiotics belong to the genus *Bifidobacterium* and *Lactobacillus*, but there are some other genus of lactic acid bacteria that are characterized by typical probiotic features. Among them there is the genus *Enterococcus* and, in particular the species *E. faecium* and *E. faecalis*.
The most studied strain is *E. faecium* SF 68, that is actually produced in Switzerland. It is reported that this strain can prevent antibiotic associated diarrhea (Wunderlich *et al*., 1989) and treat the children's diarrhea (Bellomo *et al*., 1980). Other strains that are considered as probiotics are *E. faecium* CRL 183 (Rossi *et al*., 1999) and *E. faecium* PR88 (Allen *et al*., 1996).
The use of enterococci as probiotics is still a controversial topic of discussion in microbiology research. The major problem is related to the increasing association of enterococci with some diseases and their multiple antibiotic resistance.

2.8. Safety status of enterococci

Enterococci are known as common nosocomial pathogens, in fact they are associated to numerous cases of endocarditis, bacteremia and infections. One of the most worrisome characteristics of these microorganisms is their wide antibiotic resistance (Rossi *et al*., 2014; Klein, 2003; Giraffa, 2002), in particular respect to vancomycin. There are also some other virulence factors that characterize the genus (Hollenbeck and Rice, 2012; Foulquié Moreno *et al*., 2006), for example the important ability, that characterize some of the strains of the genus *Enterococcus*, consist in the possibility to produce biogenic amines, such as tyramine and 2-phenylethylamine.

2.8.1. Antibiotic resistance

The antibiotic resistance that characterize enterococci can be intrinsic or acquired by plasmids (Klare *et al*., 2001; Clewell, 1990).

Enterococcal antibiotic resistance is not important only for the clinical arena, but also for the food industry (Verraes *et al*., 2013; Nawaz *et al*., 2011; Andersson and Hughes, 2010). This microorganisms are resistant to different antibiotics, such as: vancomycin, isoxazolylpenicillins, cephalosporins, monobactams, aminoglycosides, lincosamides, polymyxins, ampicillin, tetracyclines, macrolides, chloramphenicol, quinolones and streptogramins (Vignaroli *et al*., 2011; Foulquié Moreno *et al*., 2006, Cocconcelli *et al*., 2003). In particular, the strains resistant to vancomycin are known as VRE (vancomycin resistant *Enterococcus*), and their number is increased in the last years. This resistance is related to the presence in the genome of some microorganisms of specific genes, in fact there are six different gene clusters that induce this resistance: *van*A, *van*B, *van*C, *van*D, *van*E, *van*F and *van*G. Of the different VRE phenotypes known, the *van*A and *van*B are the most important from a clinical point of view and they are the phenotypes most frequently observed in *E. faecium* and *E. faecalis*.

2.8.2. Biogenic amines production

Enterococci are known as the most efficient tyramine producers among lactic acid bacteria (Ladero *et al*., 2012; Capozzi *et al*., 2011; Kuley and Özogul, 2011; Özogul and Özogul, 2007; Suzzi and Gardini, 2003). This metabolism is particularly important foe these microorganisms and justify their high survival rate in harsh environments, in fact the decarboxylation is a secondary metabolic pathways to obtain energy for cells, moreover it is a way to contrast the acidic stress, as reported by Molenaar *et al*. (1993) and Fernández and Zúñiga (2006).

Usually this enzyme and the related metabolism can be considered a strain characteristic, but the species *E. faecalis* represent an exception because all the strains that belong to the species are characterized by the presence of this metabolism, also many strains belongings to *E. faecium* and *E. durans* species possess this ability (Ladero *et al*., 2012).

E. faecalis is the first species for which the tyrDC locus was studied and described, in fact in literature can be found different researches, for example the one about the genome of *E. facealis* JH2-2 (Connil *et al*., 2002), *E. faecium* RM58 (Marcobal *et al*., 2006a) and *E. durans* IPLA 655 (Ladero et al., 2013). Marcobal *et al*. (2012) have reported that the gene that encode for the enzyme tyrosine decarboxylase (tyrDC) is part of an operon in which four open reading frames (ORFs) can coexist, as reported in Figure 2.7.

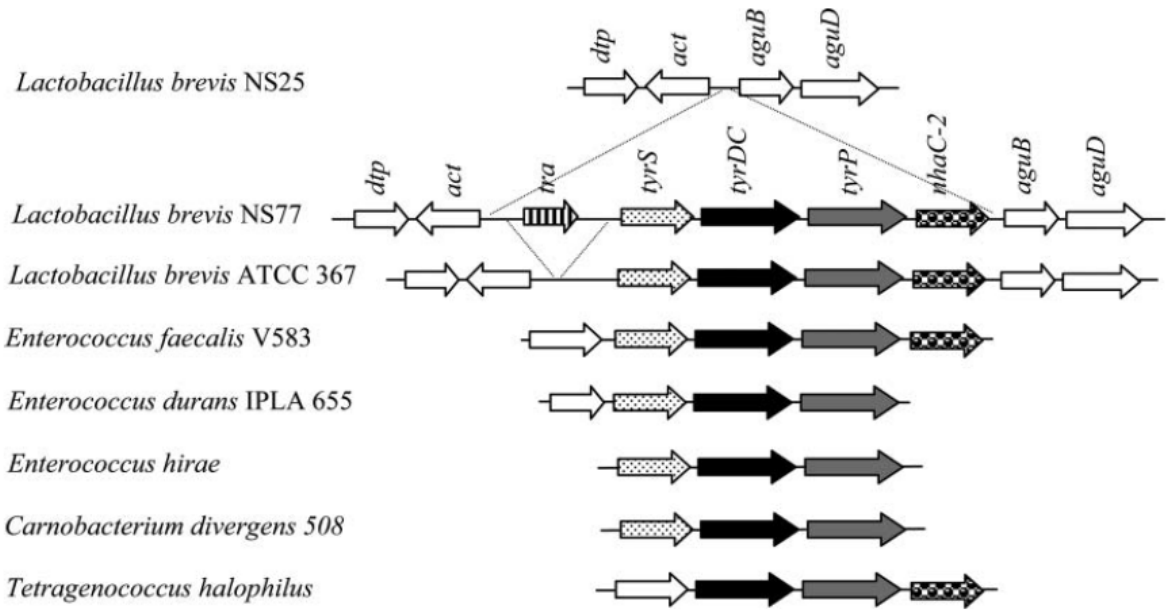

Figure 2.7: Genetic organization of genome region that contain the operon encoding for the proteins involved in the decarboxylating metabolism of different species (Marcobal *et al*., 2012).

The tdc operon (cluster) of *E. faecalis* is formed by:

- *tyrS* → gene encoding for tyrosyl tRNA synthetase;
- *tyrDC* → gene encoding for the enzyme tyrosine decarboxylase;
- *tyrP* → gene encoding for the specific tyrosine/tyramine antiporter;
- *nhaC-2* → gene encoding for a Na^+/H^+ antiporter.

The role of these proteins are different but their complex lead to the formation of biogenic amines. In particular, the role of the protein tyrosyl tRNA synthetase is not totally clear but is known that it is involved in a mechanism of activation of tyrosine by the formation of a bound between the enzyme and the intermediate tyrosyl-adenylate (Marcobal *et al*., 2012). TyrP is the specific transporter that catalyse the exchange between tyrosine and its decarboxylation product tyramine. This protein is fundamental for the energization of the cell membrane, in fact the tyrosine/tyramine exchange is electrogenic and the energy produced is represent by a proton motive force. The activation of the proton motive force is also related to the activity of the nhaC-2 pump that regulate the exchange of Na^+ and H^+ (Marcobal *et al*., 2012).

Connil *et al*. (2002) and Marcobal *et al*. (2012) have reported that the first three genes in some species are co-transcribed. It is important to highlight that the transcriptional organization can be different between the strains, for example in some cases *tyrS* is transcribed independently.

Tyrosine decarboxylase is an enzyme located in the cellular membrane characterized by large hydrophobic regions and a wide range of physico-chemical conditions in which can work efficiently, also outside the cells. This ability is already reported by different study regarding species *Lactobacillus brevis* (Moreno-Arribas and Lonvaud-Funel, 2001), *E. faecalis* and *E. faecium* (Liu *et al*., 2014a).

An important characteristic of tyrosine decarboxylase is its ability to decarboxylate also phenylalanine to obtain 2-phenylathylamine. Obviously the affinity of the enzyme with phenylalanine is lower than tyrosine, this results in a lower efficiency of the decarboxylation.

2.8.3. Virulence factors

This factors can be explained as molecules or cell products that can enhance the ability to create a damage to an organism (Kayaoglu and Orstavik, 2004). Enterococci are able to produce some substances that can act in this way, such as: gelatinase, aggregation substances and extracellular surface proteins. These virulence factors are easily transfer from a microorganism to another by gene transfer mechanisms as reported by Rossi *et al*. (2014). These mechanisms can be called "horizontal gene transfer" (HGT) and it includes all the non-genealogical transmission of genetic materials between organisms, as reported by Golden and Woese, (2007).

There are three different mechanism of HGT:

- conjugation;
- transduction and
- transformation (Rossi *et al*., 2014).

The first mechanism requires the direct contact between the two bacterial cells, different studies have reported that this is the principal way of mobilization of the genes involved in antibiotic resistance and toxin production (Gazzola *et al*., 2012; Van der Auwera *et al*., 2007; Cocconcelli *et al*., 2003).

The transduction require the activity of specific bacteriophages that transfer the DNA of a microorganism to another as a carrier and a receiver microorganism that can acquire the phage. Brabban *et al*. (2005) have reported that the transmission by phage is restricted because the microorganisms that can receive this genic information are few.

The last mechanism is a transmission that are carried out by virus-like gene transfer agents, that are small bacteriophage with a genome composed by their DNA and a part of the DNA of another microorganism. Also in this case it represent a minor way to transmit genetic information in a food system.

Cytolytic activity

The studies performed by Granato and Jackson (1969 and 1971a,b) highlighted the ability of some strains of *E. faecalis* to produce a particular type of molecules with hemolytic effect, named cytolysins. Cytolysins are classified as bacteriocins, of the group of the lantibiotics, that are characterized by important bactericidal effect, but they are also able to cause the lysis of eukaryotic cells (Fisher and Phillips, 2009; Foulquié Moreno *et al*., 2006; Cox *et al.,* 2005).
The production of cytolysins require the presence in the genome of six genes (Foulquié Moreno *et al*., 2006; Saris *et al.,* 1996; Gilmore *et al*., 1994) and only the strains that are able to express and secrete the subunits that are encoded in these genes can produce these molecules. Unfortunately the cytolysin genes are located on highly transmissible plasmids, for this reason this production is a virulence factor related in particular to the specie *E. faecalis*.

Gelatinase

Gelatinase are extracellular metallo-endopeptidases that cause the hydrolysis of collagen, gelatin, haemoglobin and other important peptides. They are considered a virulence factor, in particular in nosocomial condition, in fact it is demonstrated that gelatinase contributes to the pathogenesis of enterococcal endocarditis (Thurlow *et al*., 2010; De Fátima *et al*., 2006). Eaton and Gasson (2001) and Franz *et al.* (2001) found some strains of *E. faecalis* isolated from food positive to the production of gelatinase, but they also found that the presence of the gene is not necessarily related to the production of the enzyme. In fact there are some phenotypes that are negative to the production even if they have the gene.

Aggregation substance

Enterococcus faecalis strains are characterized by the presence of pheromone-inducible surface proteins that promote the aggregation during bacterial conjugation, as reported by Clewell (1993). These proteins can contribute to the pathogenesis of enterococcal infection, for example trough the increasing of the hydrophobicity of cells and the enhancement of the activity of the cytolysins (Süβmuth et al., 2000).

Extracellular surface protein

These proteins play a relevant role in enterococcal infection, in fact they act on the adhesion and the evasion of the immune response of the host. Eaton and Gasson (2001 and 2002) and Franz *et al*. (2001) reported an high incidence of this protein in strains of *E. faecalis* and *E. faecium* isolated from food.

2.9. Enterococci in foods

The genus *Enterococcus* is the most controversial group of lactic acid bacteria, in fact they are ubiquitous microorganisms that have as predominant habitat the gastrointestinal tract of humans and animals, in particular the species *E. faecalis* and *E. faecium*. For this reason is common to find these microorganisms in products of animal origins, such as meat and milk, and in products such as sausages and cheeses. It is worthy to note, as demonstrated by Mundt (1986), that the presence of these microorganisms is not always related to a direct faecal contamination, in fact enterococci can be easily isolated from soil, water, plants, vegetables and insects.

Despite this research, usually the presence of enterococci in raw matrices is considered a faecal contamination and often is related to their hygienic condition, but in European Union no legal limits are reported for this microorganisms. The reason for which do not exist these limits is probably related to the important role that they play in the definition of the typical organoleptic profile (aroma and texture) of cheeses and sausages. In fact, enterococci are important during the ripening processes due to their typical proteolysis, lipolysis and citrate breakdown. These positive characteristics is the reason for which in recent years the use of enterococci in the formulation of some starter cultures is rising.

In addition, some strains are able to produce bacteriocins (enterocines) that can contrast the growth of some undesirable microorganisms (in particular *Listeria* spp., *Clostridium* spp., *Escherichia coli* and *Vibrio colerae*) and some other can be used as probiotics.

In Table 2.2 are reported some of the studies about the application of enterocins producing strains in cheeses and meat products.

Strain	Enterocin produced	Application	Reference
E. faecalis B114	Enterocin not known	Camembert cheese	Sulzer and Busse, 1991
E. faecium 7C5	Enterocin 7C5	Taleggio cheese	Giraffa et al., 1995b
E. faecalis INIA 4	Enterocin 4	Manchego cheese	Joosten et al., 1995
E. faecalis INIA 4	Enterocin 4	Hispano cheese	Garde et al., 1997
E. faecalis INIA 4	Enterocin 4	Manchego cheese	Núñez et al., 1997
E. faecalis INIA 4	Enterocin 4	Hispano cheese	Oumer et al., 2001
E. faecium CCM 4231	Enterocin CCM 4231	Saint-Paulin cheese	Lauková et al., 2001
E. faecium CCM 4231	Enterocin CCM 4231	Spanish-style dry fermented sausages	Callewaert et al., 2000
E. faecium RZS C13	Enterocin RZS C13	Spanish-style dry fermented sausages	Callewaert et al., 2000
E. faecium CTC492	Enterocins A and B	Dry fermented sausages	Aymerich et al., 2000b
E. faecium CTC492	Enterocins A and B	Cooked pork	Aymerich et al., 2002
E. faecalis TAB 28	Enterocin AS-48	Raw milk cheese	Rodríguez et al., 2001
E. faecium RZS C5	Enterocin RZS C5	Cheddar cheese production	Foulquié Moreno et al., 2003b
E. faecium DPC 1146	Enterocin DPC 1146	Cheddar cheese production	Foulquié Moreno et al., 2003b
E. faecium FAIR-E 198	Enterocins A and/or P	Feta cheese	Sarantinopoulos et al., 2002b
E. casseliflavus IM 416K1	Enterocin 416K1	Italian sausage (Cacciatore)	Sabia et al., 2003

Table 2.2: Application of enterocin producing strains (Foulquié Moreno *et al*., 2006)

Obviously their adaptability to a lot of different stress conditions (high treatment temperature, high salt concentration and acid pH) with the antibiotic resistance which characterize them represent for some products an important problem.

2.9.1. Enterococci in dairy products

The presence of enterococci is usually related to traditional European cheeses, in particular the ones produced in Mediterranean countries (Italy, Spain, Greece and Portugal).

Some studies about the characterization of the microflora of these cheeses report concentrations of enterococci between 10^4 and 10^6 CFU/g in curds and concentrations between 10^5 and 10^7 CFU/g in ripened cheeses (Razavi *et al*., 2007; Giraffa, 2003; Franz *et al*., 2003; Manolopoulou *et al*., 2003; Giraffa, 2002; Sarantinopoulos *et al*., 2001a; Xanthopoulos *et al*., 2000; Suzzi *et al*., 2000), with a prevalence of the species *E. faecium* and *E. faecalis*. These values are different in relation, not only to the type of cheese considered, but also to the season of production, to the initial milk contamination and to the specific survival rate related to the productive process and to the ripening variables.

Researcher opinion about these value are in contrast, because some sustain that enterococci can cause only a deterioration of sensory characteristics of the product, but some other researchers have recognized to these microorganisms an important and fundamental role the definition of the organoleptic characteristics of the final.

In recent years, the important role of these microorganisms in the cheese ripening has led producers to include them in the formulation of the starter cultures. Some studies have reported that the use of *E. faecalis* and *E. durans* as starter cultures in dairy products can enhance the proteolysis and the lipolysis process leading to an overall improvement of the production of some important component such as: volatile free fatty acids (in particular short chain free fatty acids), acetoin and diacetyl (Franz *et al*., 2003; Sarantinopoulos *et al*., 2001a,b).

The use of enterococci in the production of cheese can be further favoured by recent studies concerning the production in situ of enterocins. It is known that enterocins are effective on *Listeria* spp. and *Clostridium* spp. in vitro or in pilot-scale conditions, but no information about their activity in large industrial scale conditions are now available.

2.9.2. Enterococci in meat products

Enterococci can be isolated from many different habitats and they are often contaminant in food of animal origin because of their association with gastrointestinal tract. Stiles *et al*., (1978) reported that the predominant species that can be isolated in pork and beef cuts are *E. faecalis* and *E.*

faecium. Also in the research of Franz *et al*., (2003 and 2011) is recognized *E. faecalis* as the predominant specie isolated in beef, poultry and pig carcasses.

Their tolerance to high concentrations of NaCl and their ability to grow over a wide range of temperature and pH, are the reasons of their competitiveness, especially when the environmental conditions become harsher (Gardini *et al*., 2001). In fact, these microorganisms can survive to the fermentation process and can be isolated also in fermented meat products, such as sausages (Franz *et al*., 2003; Hugas *et al*., 2003; Giraffa, 2002). For example Hugas *et al*., (2003) analysed 31 types of Spanish naturally fermented sausages and they found concentrations of enterococci between 1.30 and 4.48 log CFU/g. Also Ferreira *et al*. (2006 and 2007) studied the presence of enterococci in fermented sausages of north Portugal and found concentrations ranging between 10^4 and 10^8 CFU/g. These microorganisms can be also isolated in thermal processed meat, in fact the heat treatments do not inactivate enterococci and confer to them a selective advantage in the colonization of the products. This is the reason for which *E. faecalis* and *E. faecium* are frequently related to the spoilage of cured meat products (Magnus *et al*., 1986; Magnus *et al*., 1988).

The role of enterococci in meat products are important because they contribute to the development of the aroma and the structure of fermented meat products (Latorre-Moratalla *et al*., 2011; Giraffa, 2002). Moreover they are able to produce enterocins, that can be used to prevent the growth of pathogens, such as *Listeria monocytogenes*, during the fermentation process or in the final products, especially in sliced vacuum packed cooked meat (Hugas *et al*., 2003). For these reasons the use of selected enterococcal strains in the formulation of starter cultures is rising in the last few years.

On the other hand, several studies reported that enterococci are characterized by different virulence factors important for consumers health (Cariolato *et al*., 2008; Valenzuela *et al*., 2008; Mannu *et al*., 2003; Semedo *et al*., 2003a,b; Eaton and Gasson, 2001; Franz *et al*., 2001).

Another important characteristic of these microorganisms id the ability to produce high concentrations of biogenic amine, in particular in fermented meat products (Tabanelli *et al*., 2015; Foulquié Moreno *et al*., 2006; Suzzi and Gardini, 2003; Joosten and Northolt, 1989).

2.9.3. Enterococci in vegetable products

Enterococci are normally present in a large variety of products that includes vegetables, olives and plant material (Ben Omar *et al*., 2004; Giraffa, 2002; Franz *et al*., 1999). Some studies report that during the Spanish-style green olive fermentation is possible to isolate different species of *Enterococcus*, in particular the predominantly are *E. faecium* and *E. faecalis*. Randazzo *et al*., (2004) studied the microflora of naturally fermented green olives from different areas of Sicily. The

phenotypic characterization and the analysis of 16S rDNA of the isolates revealed the presence of *E. faecium*, *E. hirae* and *E. casseliflavus*.

In literature the information about the presence of enterococci in vegetable products is scarce also regarding minimally processed foods and ready-to-eat products.

Chapter 3

Objectives

Even if the presence of biogenic amines in foods (and the risks associated with them) is known since a long period (Gale, 1946), systematic studies regarding their presence have been carried out only in relatively recent times. The reviews of Shalaby (1996) and Silla-Santos (1996) had the merit to collect the fragmented information about this issue and were the starting point for a drastic multiplication of scientific publications regarding the presence of biogenic amine in food products and the elucidation of the metabolic and genetic drivers of their production by microorganisms.

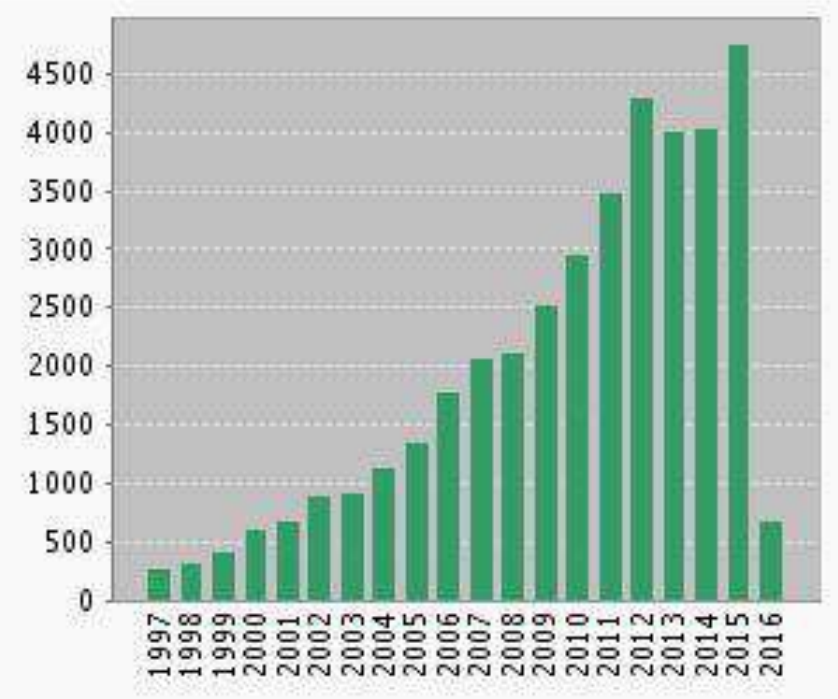

Figure 3.1: Number of citations in Web Of Science responding to the key words "biogenic", "amine" and "food" (date of acquisition 26/02/2016).

A strong impulse to this growth was given also by the publication of some papers, which proposed analytical and microbiological methods accepted by the scientific community to standardize the results of the researches allowing the possibility to compare the data obtained by food technologists and microbiologists (Bover-Cid and Holzapfel, 1999; Moret and Conte, 1996; Eerola *et al.*, 1993; Maijala, 1993).

On the other hand, the interest about the presence of these molecules increased not only to assure a simple and generic “quality” of food, but also in relation with the numeric increase of the more susceptible consumers to biogenic amines (*i.e*. more exposed to the adverse effects). This category includes elder and younger people, immunocompromised patients and consumers using specific drugs, which may potentiate the negative effects of some biogenic amines. The consequence of this amplification of the possible target of the adverse effect of biogenic amines was well highlighted by a recent scientific opinion of EFSA addressed mainly to fermented foods (EFSA, 2011).

In the last decades, the presence of biogenic amines has been screened in several foods. In particular, several recent papers have been addressed to review these aspects in foods like cheese (Buňkova *et al*., 2010; Komprda *et al*., 2008a,b; Marino *et al*., 2008; Innocente and D'Agostin, 2002), sausages (Tabanelli *et al*., 2013; Gonzáles-Tenorio *et al*., 2013; de las Rivas *et al*., 2008; Gardini *et al*., 2008; Pircher *et al*., 2007; Suzzi and Gardini, 2003); wine (Beneduce *et al*., 2010; Ancín-Azpilicueta *et al*., 2008; Ferreira and Pinho, 2006), beer (Kalac and Krízek, 2003; Anli *et al*., 2006; Loret *et al*., 2005) and fish products. Similarly, genetic and metabolic aspects has been reviewed for histamine (Rossi *et al*., 2011; Landete *et al*., 2008), tyramine and 2-phenylethylamine (Zhang and Ni, 2014; Marcobal *et al*., 2012; Torriani *et al*., 2008; Connil *et al*., 2002), cadaverine and putrescine (Pircher *et al*., 2007; de las Rivas *et al*., 2006).

In the fermented foods, the production of biogenic amines is often related to the activity of the microbiota responsible for secondary fermentation or better for the transformation characterizing the ripening and maturation processes. This microbiota is in many cases completely different from those responsible for the fermentation steps, which occur at the beginning of the production.

Within this microbiota, enterococci have often a crucial role, due to their ubiquity, their ability to survive and multiply in harsh environments and their capacity to produce biogenic amines. In particular, enterococci are known to be the major producer of tyramine and 2-phenylethylamine, together with other LAB species. The Laboratory in which I have carried out my PhD thesis has a fifteen year experience in this field. The main topics in this field interested the concentration in fermented foods of these substances (Tabanelli *et al*., 2013; Bover-Cid *et al*., 2009; Gardini *et al*., 2008), the study of the factors influencing their accumulation (Tabanelli *et al*., 2012; Gardini *et al*., 2001) as well as the development of genetic tools for improving the detection and the study of amino positive strains (La Gioia *et al*., 2011; Rossi *et al*., 2011; Torriani *et al*., 2008).

My PhD thesis is the prosecution of some of these studies and, in particular, of the researches concerning the tyraminogenic properties of enterococci. Previous works interested the relationships between the activity of enterococci and tyramine in food systems (Bover-Cid *et al*., 2009), the studies of the possible influence of some technological factors on tyrosine decarboxylation activity in an *E. faecalis* strain (Gardini *et al*., 2008) and the set up of a genetic probe able to recognize the presence of the *tyrDC* gene also in enterococci (La Gioia *et al*., 2011; Torriani *et al*., 2008).

Starting from these acquisitions, the activity of this PhD thesis was addressed to have a deeper insight on the genetic and metabolic characteristic of enterococci in relation to their ability to produce biogenic amines and, in particular, tyramine. Strains belonging to the species *E. faecalis* and *E. faecium* with different tyraminogenic potential were compared. The kinetics of tyramine (and 2-phenylethylamine) accumulation were studied in nutritionally rich as well as poor substrates.

In addition, the tyraminogenic activity was analysed in cells suspended in buffered systems at different pH, temperature and NaCl concentrations and the results were compared with the result of the activity of the pure enzyme, in order to better understand the relationships between decarboxylating activity and the integrity and viability of microbial cells. From a genetic point of view, the tyrosine decarboxylase cluster were sequenced in order to study their similarity and evidence the possible genetic reasons for the differences observed. As a further investigation, also the transcription of the tyrosine decarboxylase gene was quantified following the growth on a specific medium containing defined amounts of tyrosine and phenylalanine.

Another field of study regarded the characterization of the tyramine production activity of strains belonging to the species *E. mundtii*. For the first time, the tyrosine decarboxylase cluster of this species was sequenced and revealed differences with the same cluster in *E. faecalis* and *E. faecium*.

Finally, a part of this work was addressed to exploit the possibility to use bioprotective cultures (*Lactococcus lactis* producing bacteriocins) against biogenic amine producing LAB strains.

Chapter 4

Results

4.1. Tyrosine decarboxylase activity of enterococci grown in media with different nutritional potential: tyramine and 2-phenylethylamine accumulation and *tyrDC* gene expression.

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Running title: Accumulation of BAs by enterococcal strains in cultural media

ABSTRACT

The ability to accumulate tyramine and 2-phenylethylamine by four strains of *Enterococcus faecalis* and *Enterococcus faecium* was evaluated in two cultural media added or not with tyrosine. Enterococci differed in rate and level of biogenic amines accumulation in relation to substrate availability and strain. *E. faecalis* EF37 and *E. faecium* FC12 produced tyramine in high amount early and 2-phenylethylamine when tyrosine was depleted. The behavior of *E. faecium* FC12 and *E. faecalis* ATCC 29212 differed as they accumulated gradually tyramine during 72 h incubation and ATCC 29212 produced 2-phenylethylamine in both media without tyrosine added. The *tyrDC* gene expression was high during the exponential phase in rich medium for all the strains and subsequently decreased except for *E. faecium* FC12. Even if tyrDC presence is common among enterococci, this study underlines the extremely variable decarboxylating potential of strains belonging to the same species, suggesting strain-dependent implications in food safety.

Key words: Enterococci, Tyramine, 2-phenylethylamine, *tyrDC* gene expression, intraspecific variability

4.1.1. Introduction

Tyramine is a biogenic amine (BA) deriving from the microbial decarboxylation of tyrosine. It can have severe acute effects if ingested in excessive amounts with food, causing an hypertensive syndrome known as “cheese reaction”, which consists in peripheral vasoconstriction, increased cardiac output, increased respiration, elevated blood glucose and release of norepinephrine (Marcobal *et al.*, 2012; McCabe-Sellers *et al.*, 2006).

Lactic acid bacteria (LAB) are among the most efficient producers of tyrosine decarboxylase (tyrDC), the enzyme responsible for tyramine formation. In LAB, BA formation provides metabolic energy and/or acid resistance (Molenaar *et al.*, 1993; Fernández and Zúñiga, 2006). The presence of this enzyme is widespread among all LAB species (Marcobal *et al*., 2012). However, LAB belonging to the genus *Enterococcus* are recognized as the most efficient tyramine producers (Suzzi and Gardini, 2003; Ladero *et al*., 2012; Kuley and Özogul, 2011; Özogul and Özogul, 2007).

Tyramine production is considered a species characteristic of *Enterococcus faecalis* and also many strains of *Enterococcus faecium* possess this ability (Ladero *et al*., 2012). The *E. faecalis* tyrDC region was the first tyrosine decarboxylase locus described in prokaryotes (Connil *et al.*, 2002). In *E. faecalis*, upstream the *tyrDC* gene, an ORF can be found (*tyrS*), responsible for a tyrosyl tRNA synthetase involved in an ATP-dependent activation of tyrosine by forming an enzyme-bound tyrosyl-adenylate intermediate (Marcobal *et al.,* 2012). This tyrS could act as a sensor of the intracellular tyrosine pool to regulate tyrosine decarboxylation (Linares *et al*., 2012). The ORF (*tyrP*) located downstream of *tyrDC* encodes a tyrosine-tyramine antiporter. The three genes are co-transcribed in some strain (Connil *et al*., 2002¸ Marcobal *et al*., 2012). In addition, in enterococci downstream of *tyrP* an ORF was found related to a gene encoding for an Na^+/H^+ antiporter (*nhaC-2*) (Marcobal *et al.* 2012; Pessione *et al*. 2009). It has been demonstrated that the tyrDC of many tyraminogenic LAB, and especially enterococci, can decarboxylate, although with a lower efficiency, phenylalanine producing 2-phenylethylamine, a BA with characteristics very similar to tyramine (Marcobal *et al.*, 2006a).

Enterococci occur in many different habitats and, due to their association with the gastrointestinal tract, they are often contaminant in food of animal origin (Franz *et al.*, 2003; Franz *et al.*, 2011). When present in the raw material, enterococci can survive to the fermentation process and can be found in fermented foods such as sausages and cheeses in which they can have a relevant role during ripening (Franz *et al*., 2011; Giraffa, 2003). Due to their salt and pH tolerance, as well as their ability to grow over a wide temperature range, these LAB are particularly competitive especially when the environmental conditions become harsher, and can be a relevant component of the ripening microbiota of fermented foods. Their beneficial technological properties and their

positive impact on ripening and aroma formation in fermented sausages, cheeses and olives are reported by several authors. In addition, some strains showed probiotic features, while many enterococci produce bacteriocins able to limit the growth of pathogenic and spoilage microorganisms (Fisher and Phillips, 2009; Franz *et al.*, 2011).

On the other hand, enterococci are among the most common nosocomial pathogens and they can be responsible for endocarditis, bacteremia, as well as urinary tract, central nervous system, intra-abdominal and pelvic infections. In addition, enterococci are also known for their multiple antibiotic resistance (including vancomycin), which is in some case carried on mobile genetic elements transferable to other microorganisms (Klein, 2003). Moreover, several enterococci virulence factors have been described, such as cytolysins, aggregation substances and gelatinase extracellular surface proteins (Foulquié Moreno *et al.*, 2006). Finally, the presence of excessive content of tyramine in cheese and fermented meat is often attributed to these microorganisms (Foulquié Moreno *et al*., 2006; Joosten and Northolt, 1989; Suzzi and Gardini, 2003; Komprda *et al.*, 2008b).

The aim of this research was to study the tyramine and 2-phenylethylamine accumulation by four tyraminogenic strains of *Enterococcus*, two belonging to the species *E. faecalis* (EF37 and ATCC 29212) and two to the species *E. faecium* (FC12 and FC643). The ability to accumulate BAs was tested in a rich cultural medium, which does not limit enterococcal growth, and in a poor medium enhancing BA production (Bover-Cid and Holzapfel, 1999). Both media were tested with or without the addition of the precursor (tyrosine). In addition, the *tyrDC* gene expression of the four enterococci was analyzed by reverse transcription-quantitative real time PCR (RT-qPCR) during growth in rich medium in presence or not of the precursor.

4.1.2. Materials and methods

4.1.2.1. Enterococcal strains and growth conditions

The strains *E. faecalis* EF37 and ATCC 29212, *E. faecium* FC12 and FC643 were stored in 20% (w/v) glycerol at -80°C and pre-cultivated for 24 h at 37°C in BHI Broth (Oxoid, Basingstoke, UK) added with 800 mg/l of tyrosine (Sigma-Aldrich, Gallarate, Italy).

After 24 h of pre-cultivation, the microorganisms were inoculated, at a concentration of approximately 6.5 log CFU/ml, in BHI Broth and in Bover-Cid and Holzapfel broth, a medium proposed to highlight the biogenic amine formation (BAM) (Bover-Cid and Holzapfel, 1999), added or not with 800 mg/l of tyrosine and incubated at 37°C for 96 h. At defined times (1, 2, 3, 4, 5, 6, 7, 8, 24, 48, 72 and 96 h), the changes of optical density at 600 nm (OD_{600}) were monitored.

The modification of pH was determined by a pHmeter Basic 20 (Crison Instruments, Barcelona, Spain).

The maximum cell concentration reached in stationary phase was determined after 24 h of incubation by plate counting enterococci onto BHI agar. In addition, 2 ml aliquots of each culture was centrifuged at 3000 rpm for 10 min and the obtained cell pellets were frozen at -80°C.

4.1.2.2. Growth parameters

The evaluation of enterococcal growth in the different media was performed by measuring the OD_{600} with a UV-VIS spectrophotometer (UV-1204, Shimadzu Corporation, Kyoto, Japan) with plastic cuvettes (1.5 ml). The OD_{600} data were fitted with the Gompertz equation as modified by Zwietering, Jongenburger, Rombouts and van't Riet (1990).

$$y = k + Ae^{-e^{\left[\left(\frac{\mu_{\max} e}{A}\right)(\lambda - t)+1\right]}}$$

where y is the OD_{600} at time t, A represent the maximum OD_{600} value reached, μ_{max} is the maximum OD_{600} increase rate in exponential phase and λ is the lag time.

4.1.2.3. Biogenic amine determination

The BA were determined after 4, 8, 24, 48, 72 and 96 h of incubation. The cultures were centrifuged at 10000 rpm for 10 min at 10°C, and the supernatants were used for biogenic amines (BAs) determination by HPLC after derivatization with dansyl-chloride (Sigma-Aldrich, Gallarate, Italy) according to Martuscelli, Crudele, Gardini and Suzzi (2000). The BA content was analyzed using a PU-2089 Intelligent HPLC quaternary pump, Intelligent UV–VIS multiwavelength detector UV 2070 Plus (Jasco Corporation, Tokio, Japan) and a manual Rheodyne injector equipped with a 20 µl loop (Rheodyne, Rohnert Park, CA). The quantification was performed according to Tabanelli, Torriani, Rossi, Rizzotti, and Gardini (2012) and the amount tyramine and 2-phenylethylamine were expressed as mg/ml by reference to a calibration curve obtained with standard solutions.

4.1.2.4. Nucleic acid extraction from enterococcal cultures

Total DNA was isolated from cell pellets by using the Wizard Genomic DNA purification system (Promega Corporation, Madison, WI), following the manufacturer's instructions.

For total RNA extraction, cells were washed twice with 500 µl of sterile diethyl pyrocarbonate (DEPC)-treated water and shaken three times at the maximum speed for 30 s at 10-s intervals with

500 µl of LETS (200 mM LiCl, 20 mM EDTA, 20 mM Tris, 0,4% SDS, 0,1% DEPC), 500 mg of 450 µm-diameter glass beads (Sigma-Aldrich), 500 µl of phenol pH 4.7-chloroform-isoamyl alcohol (25:24:1 v/v; Sigma-Aldrich) in a cell disrupter (Mini-BeadBeater, BioSpec Products, Bartlesville, Okla). After centrifugation (4°C, 13000 rpm, 10 min), the supernatant was twice treated with 600 µl of chloroform-isoamyl alcohol (24:1 v/v; Sigma-Aldrich), added with 60 µl of 3 M sodium acetate, 1 ml of ice-cold absolute ethanol and left for 1 h at -80 °C. Total RNA was pelleted by centrifugation at 13000 rpm for 5 min at 4°C, washed with 200 µl of ethanol 70%, and dissolved in 30 µl of sterile water (RNAse- and DNAse-free).

DNA elimination was performed using 50 U of RNase-free DNase I recombinant (Roche Diagnostic, Germany) in 50 µl of DNAse reaction buffer (40 mM Tris-HCl, 10 mM NaCl, 6 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.9) for 30 min at 25°C. A PCR assay was carried out to check for any contaminating DNA, and, when necessary, the DNase treatment was repeated.

DNA and RNA integrity, concentration, and purity were checked by electrophoresis on a 1,5% (wt/vol) agarose gel and by measurement with the NanoDropTM Lite Spectrophotometer (Thermo Fisher Scientific Inc. MA, USA).

DNA and DNA-free RNA samples were stored at -20°C and -80°C, respectively, until use.

4.1.2.5. PCR amplification and expression of the *tyrDC* gene

A *tyrDC* fragment of about 336 bp was amplified using the primers DEC5 (5'-CGT TGT TGG TGT TGT TGG CAC NAC NGA RGA RG-3') and DEC3 (5'-CCG CCA GCA GAA TAT GGA AYR TAN CCC AT-3'), following the PCR conditions described previously (Torriani *et al*., 2008). PCR product was visualized on a 2% agarose gel.

Total cDNA was synthesized from 1 µg of RNA using the ImProm-IITM Reverse Transcriptase kit (Promega, USA), following the manufacturer's recommendations.

The expression level of the *tyrDC* gene was analyzed by a RT-qPCR assay with primers TYR3f (5'-CGT ACA CAT TCA GTT GCA TGG CAT-3') and TYR4r (5'-ATG TCC TAC TTC TTC TTC CAT TTG-3'); thermo cycler, reaction mixture, and amplification program were described in Torriani *et al*. (2008), as well as the procedure of the absolute quantification of the *tyrDC* copies number.

4.1.2.6. Statistical analysis

The growth model was fitted using the statistical package Statistica for Windows 6.1 (Statsoft Italia, Vigonza, Italy).

4.1.3. Results

4.1.3.1. Growth and pH modification in cultural media

The growth of four enterococcal strains, i.e. *E. faecalis* EF37 and ATCC 29212, and *E. faecium* FC12 and FC643, was monitored by measuring the OD_{600} increase in the absence or in the presence of tyrosine (800 mg/l) added in BHI and BAM media. The OD_{600} changes were modelled with the Gompertz equation (Zwietering *et al*., 1990) and the estimates of the parameters are reported in Table 1.

Strain	**Cultural medium**	**Gompertz equation parameters[a]**			**R^2**	**Residual mean square error (RMSE)**	**Maximum cell concentration (log CFU/ml)**
		A	**μ_{max}**	**λ**			
EF37	BHI + tyr[b]	0.947	0.767	2.532	0.977	0.042	9.40 (±0.13)
	BHI	1.029	0.601	2.030	0.954	0.068	9.42 (±0.13)
	BAM + tyr[c]	0.600	0.171	2.619	0.991	0.024	8.95 (±0.14)
	BAM	0.803	0.192	2.121	0.993	0.031	9.04 (±0.11)
ATCC 29212	BHI + tyr	0.899	0.494	2.399	0.989	0.013	9.30 (±0.15)
	BHI	1.014	0.632	2.109	0.990	0.069	9.48 (±0.19)
	BAM + tyr	0.544	0.191	3.455	0.989	0.049	8.52 (±0.15)
	BAM	0.788	0.252	2.544	0.992	0.043	8.80 (±0.14)
FC12	BHI + tyr	1.119	1.170	3.589	0.996	0.029	9.34 (±0.12)
	BHI	1.095	0.559	2.848	0.995	0.054	9.35 (±0.16)
	BAM + tyr	0.362	0.132	1.882	0.989	0.037	8.33 (±0.14)
	BAM	0.425	0.124	2.077	0.993	0.039	8.31 (±0.17)
FC643	BHI + tyr	1.114	0.566	2.876	0.983	0.047	9.38 (±0.09)
	BHI	1.191	0.584	2.158	0.989	0.036	9.52 (±0.13)
	BAM + tyr	0.739	0.177	1.635	0.992	0.028	8.46 (±0.20)
	BAM	0.807	0.187	1.445	0.993	0.028	8.72 (±0.11)

Table 1: Gompertz equation parameters for enterococcal growth measured as OD_{600}. R^2 and RMSE are given as diagnostics of the regression. The maximum cell concentrations (expressed as log CFU/ml) at the beginning of the stationary phase is also reported.

[a] *A*: maximum OD_{600} value reached; μ_{max}: maximum OD_{600} increase rate in exponential phase (OD_{600}/h); λ: lag phase duration (h); [b] BHI Broth plus 800 mg/l tyrosine; [c] Bover-Cid and Holzapfel medium (BAM) plus 800 mg/l tyrosine

When inoculated in BHI medium, all the strains reached the maximum value of OD_{600} after 8 h of incubation at 37°C, independently on the presence of tyrosine. Given the high initial inoculums (about 6 log CFU/ml), the lag phase (λ) was always very short and it was followed by a sharp increase of OD_{600}, whose maximum values (estimated by the *A* parameter of the equation) ranged between 0.9 and 1.2. In particular, the absence of tyrosine favoured the reaching of higher OD_{600} values for the two *E. faecalis* strains, and for *E. faecium* FC643. By contrast, no differences in the maximum OD_{600} were found for *E. faecium* FC12 in relation to the presence of tyrosine added.

Table 1 reports also the cell counts detected in the stationary phase (determined after 24 h incubation). In BHI no differences were detected in relation to the strain and to the addition of tyrosine. In fact, the counts revealed final cell concentrations comprised between 9.30 and 9.52 log CFU/ml.

All the strains, as expected, showed lower growth extent in BAM if compared with BHI. BAM is considered a poor medium and the energetic supply provided by aminoacid decarboxylation (and, consequently, by BA formation) became fundamental to support microbial growth. The more stringent conditions provided by this medium are reflected also in the lower OD_{600} reached in the stationary phase. Anyway, the OD_{600} was always higher in the BAM not supplemented with tyrosine (Table 1). Also cell counts in stationary phase confirmed this behaviour and were always higher in the medium without tyrosine, with the exception of the strain *E. faecium* FC643 in which no differences related to the presence of the aminoacid were found.

During incubation, the pH of the media was also monitored and the data are reported in Figure 1.

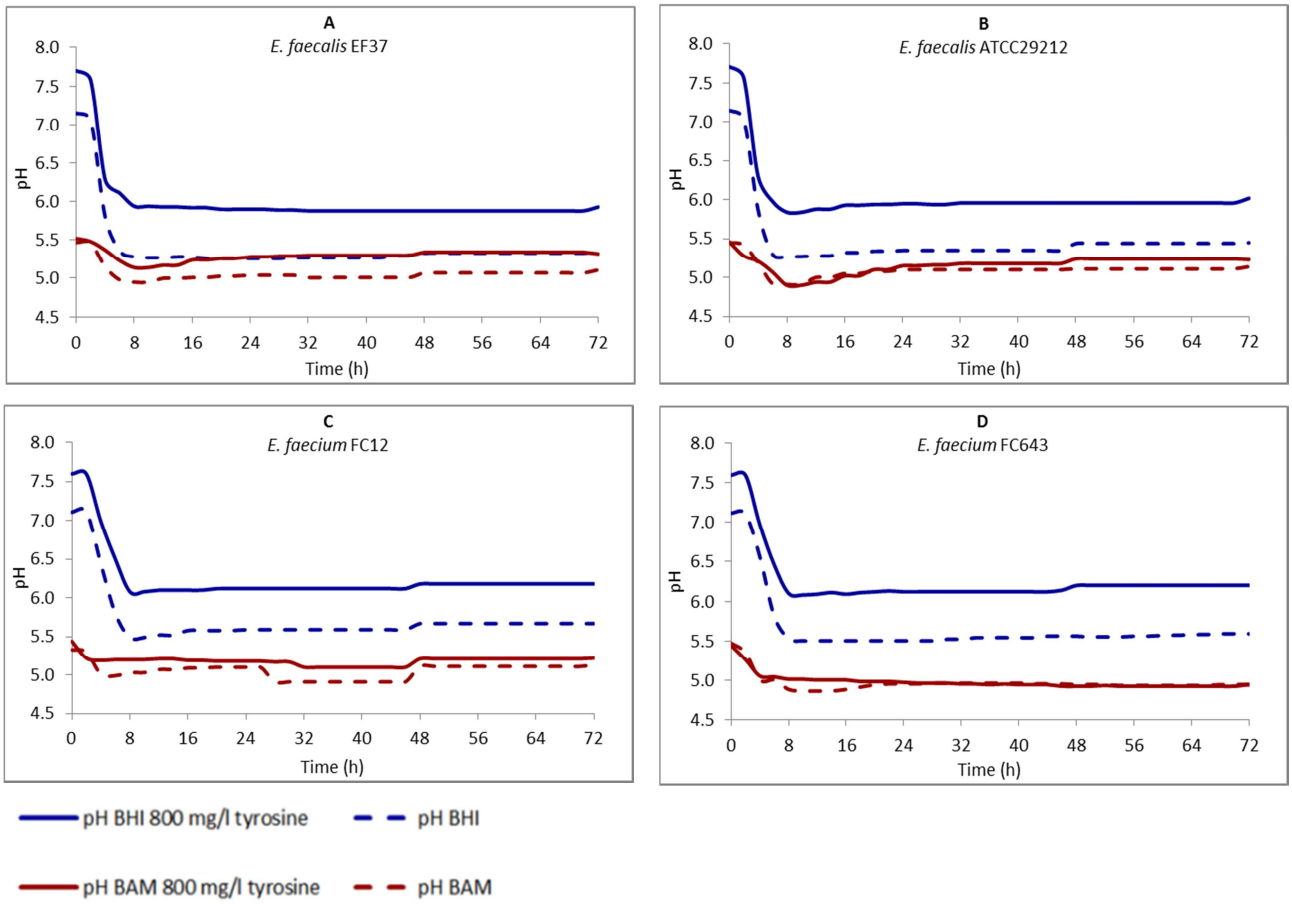

Figure 1: Modification of the pH in BHI and Bover-Cid medium (BAM) with or without tyrosine. (A) *E. faecalis* EF37, (B) *E. faecalis* ATCC 29212, (C) *E. faecium* FC12, (D) *E. faecium* FC643.

The results in BHI were specular to the growth curves for all the strains, and the pH decrease within the first 8 h was of about 1.5 units, and was quite constant during the remaining incubation period. In the samples added with tyrosine the pH value was higher of about 0.5 units, both at the beginning and at the end of incubation.

As far as BAM, the initial pH was 5.5 and only a slight decrease was observed in the first 8 h of incubation. This behavior can be attributed to the higher buffering potential of the medium. After the first 8 h, the pH showed a slight increase determined by the accumulation of BAs.

4.1.3.2. Biogenic amine production in the cultural media not added with tyrosine

Both the media used for the trials contained, in different amount, aminoacid sources (proteins and peptides) among which precursors for tyrDC were present, allowing a decarboxylase activity of the strains also in the absence of tyrosine added. The amounts of tyramine and 2-phenylethylamine produced during the growth in these media are reported in Figures 2 and 3.

Enterococcus faecalis EF37 was able to produce tyramine in both media. However, the final amount after 72 h did not exceed 70 mg/l in BHI and 90 mg/l in BAM (Figure 2A). These values

did not significantly change prolonging the incubation up to 96 h (data not shown). The maximum tyramine concentration was observed in the samples taken after 24 h of incubation. Also 2-phenylethylamine was produced under the same conditions and this BA was gradually accumulated reaching a concentration of about 270 mg/l in BHI and 130 mg/l in BAM after 72 h. *Enterococcus faecalis* ATCC 29212 showed an analogous decarboxylating activity even if lower tyramine amounts were produced in both media (Figure 2C).

A similar trend was observed for *E. faecium* FC12, though this strain accumulated in BHI higher concentrations of both the BAs while an opposite trend was observed for BAM (Figure 3A). Finally, *E. faecalis* FC643 showed the minor BA production in both the media and produced only traces of 2-phenylethylamine only in BHI (Figure 3C).

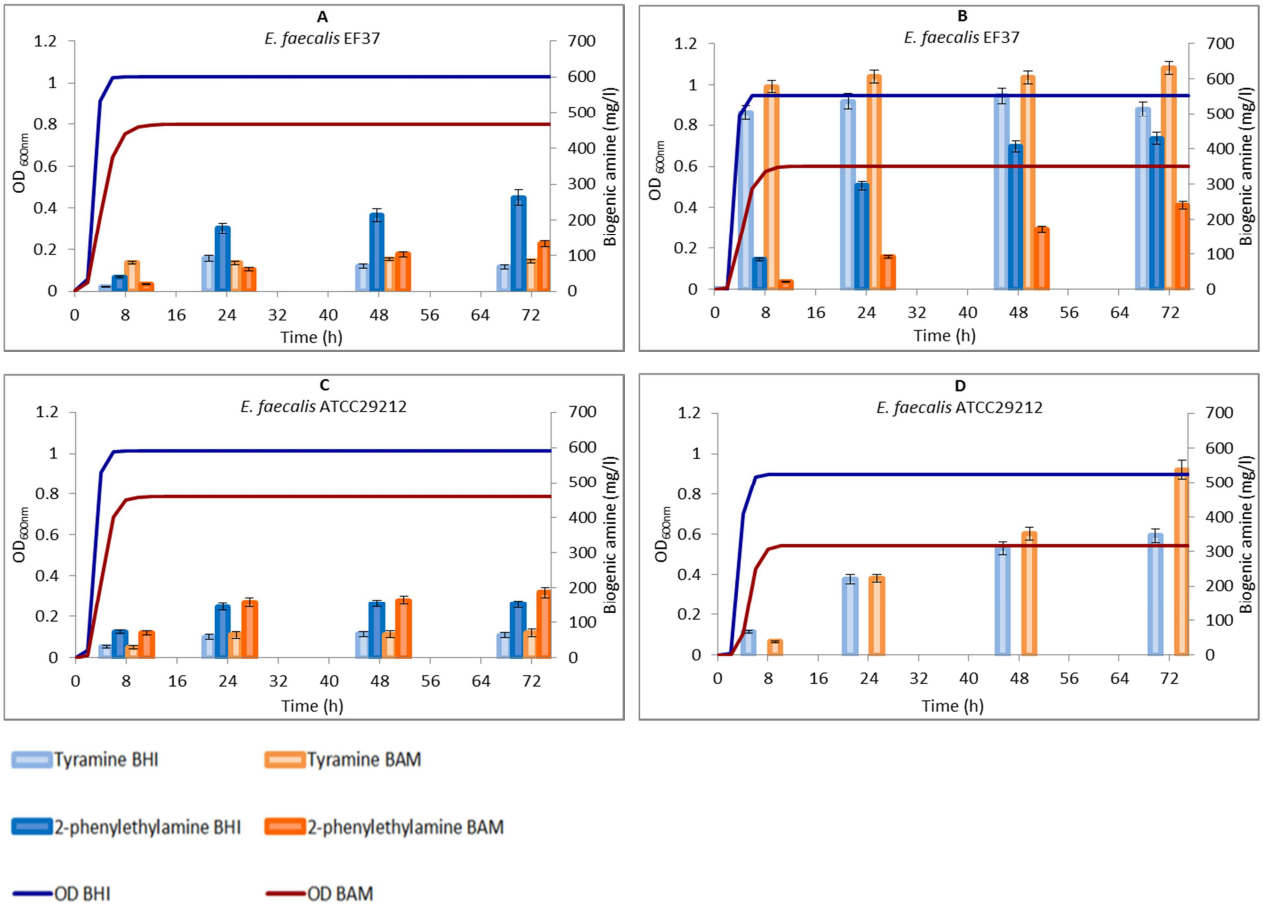

Figure 2: Amounts of tyramine and 2-phenylethylamine produced during the growth in BHI and Bover-Cid medium (BAM) without (A, C) and with (B, D) tyrosine addition. (A, B) *E. faecalis* EF37 (C, D) *E. faecalis* ATCC 29212.

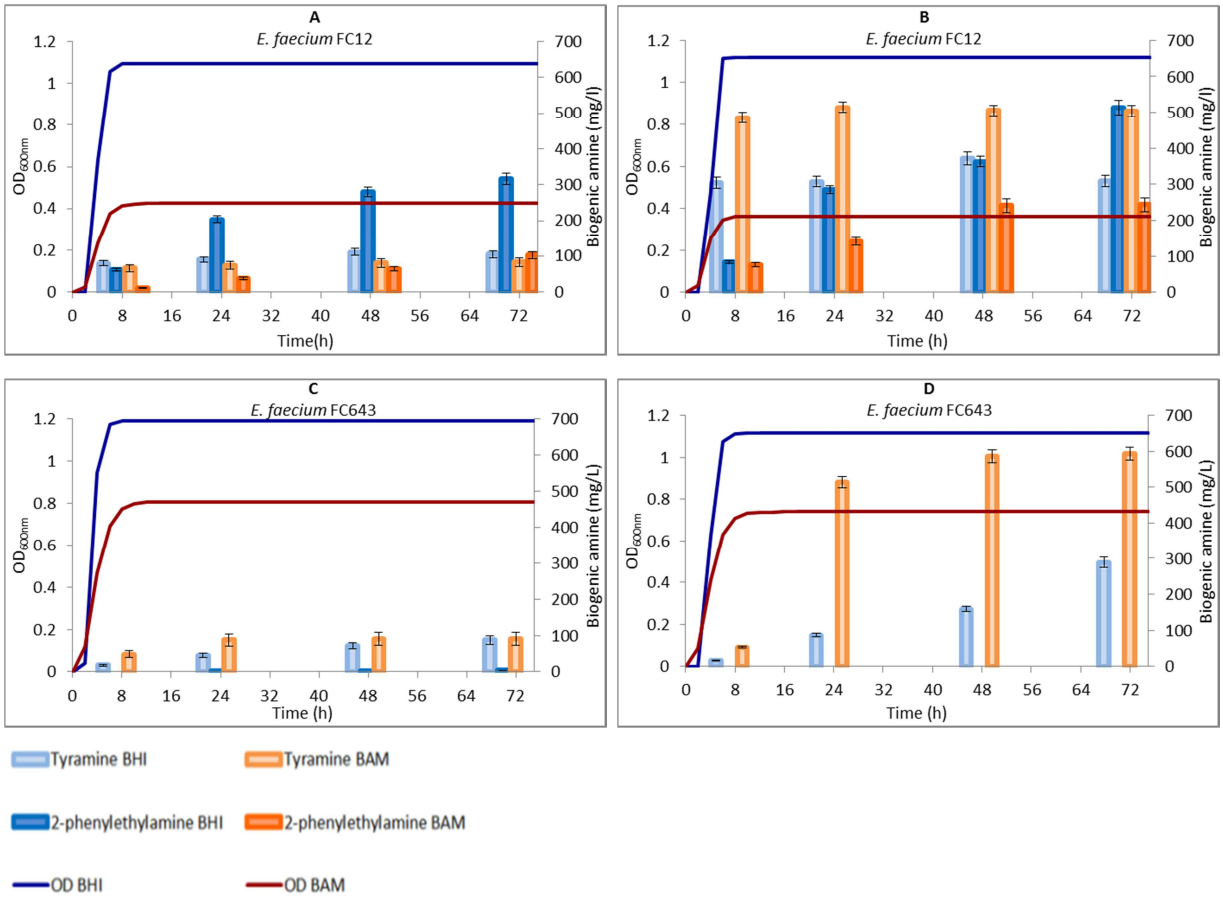

Figure 3: Amounts of tyramine and 2-phenylethylamine produced during the growth in BHI and Bover-Cid medium (BAM) without (A, C) and with (B, D) tyrosine addition. (A, B) *E. faecium* FC12 (C, D) *E. faecium* FC643.

4.1.3.3. Biogenic amine production in the cultural media added with tyrosine

Figures 2 and 3 report also the tyramine and 2-phenylethylamine accumulation when tyrosine (800 mg/l) was added to the two media. *Enterococcus faecalis* EF37 and *E. faecium* FC12 accumulated the maximum tyramine concentration within the first 8 h of incubation both in BHI and BAM and the reaching of the stationary phase did not further increase significantly these amounts (Figure 2B and 3B, respectively).

Enterococcus faecalis EF37 was the most efficient strain in the conversion of tyrosine to tyramine and the final concentration of this BA was about 515 mg/l in BHI and 620 mg/l in BAM. The behaviour of *E. faecium* FC12 was similar, but the final amounts of tyramine were lower than *E. faecalis* EF37, i.e. 505 and 360 mg/l of BA were produced in BAM and BHI, respectively. In addition, both the strains began to produce 2-phenylethylamine only after 8 h of incubation, i.e. when stationary phase and the maximum amount of tyramine have been reached. The 2-phenylethylamine accumulation increased during subsequent incubation and reached its maximum level at 72 h. About 513 and 428 mg/l of this amine were produced in BHI by *E. faecium* FC12 and

E. faecalis EF37, respectively, while lower concentrations were detected in BAM (about 230 mg/l for both the strains). In any case, these amounts were higher if compared with the 2-phenylethylamine produced in the same media in the absence of the tyrosine addition.

The presence of tyrosine determined a different behaviour in the sample inoculated with *E. faecalis* ATCC 29212. Indeed, the growth of this strain was characterized by a slowed rate of accumulation of tyramine. This BA was mainly produced when the cells reached the stationary phase and amounts of about 325 and 510 mg/l were detected only after 72 h of incubation in BHI and BAM, respectively. In addition, no 2-phenylethylamine was produced in both the conditions, even if this ability was displayed in the media without the addition of precursor (Figure 2D).

Enterococcus faecium FC643 showed a similar behaviour and reached in BAM a double concentration of tyramine with respect to BHI (592 mg/l *vs.* 288 mg/l). Also *E. faecium* FC643 did not accumulate 2-phenylethylamine when tyrosine was added to the media (Figure 3D).

4.1.3.4. Expression of the *tyrDC* gene in BHI added or not with tyrosine

All the enterococcal strains produced a 336-bp fragment characteristic of the *tyrDC* gene with the degenerate primers DEC5/DEC3 (data not shown), in accordance with their ability to accumulate tyramine. Therefore, the expression of the *tyrDC* gene could be evaluated by RT-qPCR during a period of 72 h. The expression of *tyrDC* gene has been evaluated during the enterococcal growth in BHI with or without tyrosine added.

As shown in Table 2, the *tyrDC* gene expression level differed considerably depending on the the strains and the growth phase. In general, the two strains with the most effective tyraminogenic activity showed since the beginning of incubation a level of transcript considerably higher than the other strains. It is important to note that the amount of *tyrDC* transcription is given as absolute value and the higher amount detected after 2 h did not correspond with the reaching of the maximum cell counts during incubation. Thus, the cell showed a considerably higher transcription activity during their early exponential phase.

Enterococcus faecalis EF37 displayed the highest level of *tyrDC* transcription [up to 5-6 log (copies/µg cDNA)] during the exponential phase of growth (2-5 h) in both media, according with its great ability to accumulate tyramine. After that, a significant decrease was found in BHI without tyrosine, while the *tyrDC* mRNA was rather high in BHI added with tyrosine, thus supporting the sequential 2-phenylethylamine production.

The *tyrDC* gene expression of *E. faecalis* ATCC 29212 was always lower (about two log units in the first 8 h) if compared with *E. faecalis* EF37. Its expression was higher in BHI without added tyrosine at the beginning of the growth and transcription decreased drastically after 8 h.

The strain *E. faecium* FC12, although exhibiting a phenotypic behavior analogous to *E. faecalis* EF37, showed a very different trend of the *tyrDC* gene expression. Indeed, the transcript level in both media was not related to the phase of growth, but it was rather constant during the entire incubation period. For this strain the high level of transcript after 24 h could determine the increase of 2-phenylethyamine in both the media.

Finally, *Enterococcus faecium* FC643 exhibited a *tyrDC* gene expression behavior similar to *E. faecalis* ATCC 29212. However, it was characterized by the lowest levels of *tyrDC* transcription after 4 h of incubation.

Time (h)	Log (copies/µg cDNA)							
	***E. faecalis* EF37**		***E. faecalis* ATCC 29212**		***E. faecium* FC12**		***E. faecium* FC643**	
	BHI	BHI + tyr	BHI	BHI + tyr	BHI	BHI + tyr	BHI	BHI + tyr
2	5.08 (±0.02)	4.79 (±0.06)	3.11 (±0.07)	2.60 (±0.08)	4.98 (±0.22)	3.91 (±0.18)	3.86 (±0.02)	2.79 (±0.02)
4	4.87 (±0.01)	6.11 (±0.02)	3.06 (±0.14)	3.25 (±0.02)	3.45 (±0.01)	3.49 (±0.07)	2.19 (±0.11)	2.65 (±0.01)
8	5.22 (±0.05)	5.03 (±0.05)	2.23 (±0.09)	2.07 (±0.09)	3.37 (±0.42)	2.98 (±0.19)	1.98 (±0.25)	0.98 (±0.34)
24	2.42 (±0.07)	4.15 (±0.05)	1.10 (±0.45)	1.61 (±0.17)	3.42 (±0.07)	4.61 (±0.24)	1.65 (±0.29)	0.84 (±0.06)
48	2.81 (±0.03)	3.38 (±0.03)	1.02 (±0.04)	1.83 (±0.01)	3.08 (±0.63)	4.37 (±0.02)	2.04 (±0.02)	1.24 (±0.02)
72	1.01 (±0.29)	4.10 (±0.12)	1.01 (±0.29)	1.61 (±0.02)	3.52 (±0.27)	3.47 (±0.68)	1.63 (±0.02)	1.11 (±0.20)

Table 2: *TyrDC* gene expression level for enterococcal strains grown in BHI Broth and BHI Broth added with 800 mg/l tyrosine during 72h.

4.1.4. Discussion

All the enterococcal strains used in these trials possessed an active tyrDC which determined tyramine accumulation (even if at different level) in all the conditions tested, independently on the addition of high concentration of free tyrosine. This fact clearly indicated the possibility of the enterococci to decarboxylate amino acids present in the proteic and peptidic ingredients used for media preparation.

Recently, Liu *et al*. (2014a) showed that the activity of purified recombinant tyrDCs of the strains *E. faecalis* R612Z1 and *E. faecium* R615Z1 was similar, and that the enzymes exhibited higher specificity for tyrosine than for phenylalanine. However, in our trials, different behaviours of

tyramine and 2-phenylethylamine accumulation were observed in relation to the strain. In particular, strains belonging to the same species (*E. faecalis* or *E. faecium*) were characterized by different responses indicating that, if the presence of tyrDC is common among enterococci, the decarboxylating potential can be extremely variable. The transcriptional analyses of the gene *tyrDC* confirmed these observations. In recent years, a number of studies have been conducted to evaluate the gene expression level of amino acid decarboxylases on the BAs accumulation in different food and model systems (Gardini *et al*., 2008; La Gioia *et al*., 2011; Rossi *et al*., 2011; Arena *et al*., 2011; Liu *et al*., 2014b). However, up to now no studies have compared the *tyrDC* transcript levels in enterococcal strains of the same species. In the present study a variability of the *tyrDC* gene expression in different strains of *E. faecalis* and *E. faecium* was evidenced for the first time. The two strains with the higher decarboxylating potential (*E. faecalis* EF 37 and *E. faecium* FC12) were characterized by a higher transcription of *tyrDC* after 2 h of incubation and the maintenance of a remarkably higher transcription level throughout all the incubation period considered here.

In contrast with Arena *et al*. (2011), who observed an increase of *tyrDC* transcription in the presence of tyrosine for *Lactobacillus brevis* IOEB 9809 in wine, in our conditions the presence of the precursor in high amounts (800 mg/l) did not enhance the transcript at the beginning of growth, but stimulated a higher transcription during the successive incubation.

Linares *et al*. (2009) evidenced that the maximization of the transcription of *tyrDC* and *tyrP* in *E. durans* was caused by a tyrosine concentration comprised between 2 mM and 5 mM. In this framework, the presence of tyramine added (800 mg/kg, i.e. 4.42 mM) should be sufficient to reach a high level of *tyrDC* transcript. Nevertheless, similar and often higher transcripts were obtained also in the medium without the addition of the precursor.

Enterococcus faecalis EF37 showed the higher tyramine production and *tyrDC* gene expression in the presence of tyrosine added to the media. In addition, independently on the media, *E. faecalis* EF37 and *E. faecium* FC12 produced also high amount of 2-phenylethylamine, which were significantly higher in the presence of tyrosine added. On the other hand, *E. faecalis* ATCC 29212 and *E. faecium* FC643 were not able to accumulate significantly 2-phenylethylamine when tyrosine was added; however, the same strains produced this BA in reduced amounts in the absence of tyrosine in BHI if compared with the other two strains and in the same magnitude in the poor medium (BAM). The absence of 2-phenylethylamine in BAM and BHI added with tyrosine and inoculated with *E. faecalis* ATCC 29212 and *E. faecium* FC643 reflected the lower efficiency of their tyrDC and could indicate that for these strains the increasing amount of tyramine can lower or inhibit further decarboxylase activities. Concerning transcriptional analysis, the maintenance of the

tyrDC gene expression in the stationary phase of growth could contribute to enhance 2-phenylethylamine biosynthesis in enterococcal strains when the preferred precursor was depleted.

However, in the case of the strains *E. faecalis* ATCC 29212 and *E. faecium* FC643, that accumulated gradually tyramine, the *tyrDC* gene expression was still present at the end of the incubation period, but 2-phenylethylamine was not produced because tyrosine was not entirely consumed after 72 h growth.

The early production of tyramine by *E. faecalis* EF37 and *E. faecium* FC12 confirms the results of Pessione *et al*. (2009), who demonstrated that tyrDC activity in *E. faecalis* DISAV1022 reached its maximum level during the exponential growth phase, suggesting that tyrosine decarboxylation was not simply a response to starvation or nutrient depletion typical of the stationary phase.

By contrast, the strains *E. faecalis* ATCC 29212 and *E. faecium* FC643 accumulate great part of tyramine after they reached the stationary phase, independently on the addition of precursor. However, these strains, although their transcript levels were much lower respect to *E. faecalis* EF37, showed a *tyrDC* transcription trend similar to *E. faecalis* EF37. In particular, these profiles, were characterized by an higher expression during the exponential phase followed by a decrease after 8 h of incubation.

Many authors reported the widespread ability of enterococci to produce both tyramine and 2-phenylethylamine (Beutling and Walter, 2002; Bonetta *et al.*, 2008; Aymerich *et al.*, 2006). This characteristic was found also in some lactobacilli (Landete *et al.*, 2007), even if in other case a highly tyrosine selective tyrDC was described (Moreno-Arribas and Lonvaud-Funel, 2001). Marcobal *et al*. (2006a) proved that *tyrDC* gene in *E. faecium* encoded for a functional and dual decarboxylase resulting in tyrosine and phenylalanine decarboxylation. Also Landete *et al*. (2007) demonstrated that tyrDC present in LAB allowed the production of 2-phenylethylamine. Pessione *et al*. (2009) carried out a comparative proteomic investigation on *E. faecalis*, which demonstrated a membrane bound tyrDC highly overexpressed during the production of both tyramine and 2-phenylethylamine. According to these authors, a yield of 100% was observed for the conversion of tyrosine, which takes place since the exponential phase. On the other hand, the yield for 2-phenylethylamine was lower (about 10%) and its production occurred in the stationary phase when tyrosine was exhausted. Also other authors observed that phenylalanine is decarboxylated with a reduced efficiency and only when the tyrosine become a limiting substrate (Joonsten, 1988; Latorre-Moratalla *et al.*, 2014). Regarding the different amount of tyramine accumulated under the same conditions, it has been demonstrated that the presence of high amounts of tyrosine in the medium can reduce the tyramine production by some LAB (Fernández *et al.*, 2007a,b). In other words, the

increase in the availability of the precursor did not necessarily coincide with an increase in the decarboxylation.

The maximum yield for tyrosine conversion is 75.7%; then, in this study a stoichiometric conversion of the aminoacid added should produce a maximum theoretical level of 609 g/l of tyramine. Taking into account that BHI without precursor supports the production of 70-90 mg/l of tyramine, the maximum accumulation of this BA showed by *E. faecalis* EF37 in the presence of tyrosine is lower than this theoretical limit (about 150 mg/l below). However, in these conditions a higher production of 2-phenylethylamine (150 mg/l above the yield in not supplemented BHI) occurred. The overproduction of this BA was observed also in *E. faecium* FC12; however, in this case the results were reached in the presence of lower tyramine concentration. The same trend was also observed in BAM, in which *E. faecalis* EF37 seems to operate an almost complete conversion of the aromatic aminoacids added or naturally present in the medium.

4.1.5. Conclusions

The presence of *Enterococcus* strains that can decarboxylate tyrosine and 2-phenylalanine is a serious concern in fermented food for consumer's health. Indeed, even if these activities are common among enterococci, this study underlines the extremely variable decarboxylating potential of strains belonging to the same species, suggesting strain-dependent implications in food safety. In spite of the fact that all the strains tested here had the *tyrDC* gene, the amounts of tyramine (and 2-phenylethylamine) produced was strictly dependent on the amount of its transcription, which was extremely different among the strains. The composition of the media also affected and modulated the amount and ratio of these BAs by tyraminogenic strains, indicating the need of preventive measures to control BAs accumulation in foods. Future researches will be planned to a deep knowledge on the conditions which can favour the production of BAs by enterococci and on the reasons which determine the important differences among the transcripts of the same gene. This could be ascribed to different activity and specificity of tyrDC enzyme or to different regulation mechanisms. In this regards, further studies have to be performed to better explain the genetic and functional basis, and the environmental factors affecting the different decarboxylating potential of the strains.

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4.2. The capability of tyramine production and correlation between phenotypic and genetic characteristics of *Enterococcus faecium* and *Enterococcus faecalis* strains.

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Running title: Heterogeneity in tyramine production among enterococci

ABSTRACT

The aim of this study was to investigate the diversity of tyramine production capability of four *Enterococcus* strains in buffered systems in relation to their genetic characteristics and environmental conditions. Cells of the strains *Enterococcus faecalis* EF37 and ATCC 29212, and *Enterococcus faecium* FC12 and FC643 were re-suspended in phosphate/citrate buffers with different pH, NaCl concentration and incubation temperature. At intervals, cell viability and tyramine production were assessed by plate counting and HPLC analysis, respectively. The activity of a purified tyrosine decarboxylase (TDC) was determined under the same conditions, as a reference.

Reduced loss in cell viability was observed in all the tested conditions, except for pH 4 after 24 h. The TDC activity was greatly heterogeneous within the enterococci: EF37 and FC12 produced the higher tyramine concentrations, ATCC 29212 showed a reduced decarboxylase activity, while EF643 did not accumulate detectable amounts of tyramine in all the conditions assayed. Among the considerate variables, temperature was the most influencing factor on tyramine accumulation for enterococcal cells.

To further correlate the phenotypic and genetic characteristics of the enterococci, the TDC operon region carrying the genes tyrosine decarboxylase (*tyrDC*), tyrosine/tyramine permease (*tyrP*), and Na^+/H^+ antiporter (*nhaC-2*) was amplified and sequenced. The genetic organization and nucleotide sequence of this operon region were highly conserved in the enterococcal strains of the same species. The heterogeneity in tyramine production found between the two *E. faecalis* strains could be ascribed to different regulation mechanisms not yet elucidated. On the contrary, a codon stop was identified in the translated *tyrDC* sequence of *E. faecium* FC643, supporting its inability to accumulate tyramine in the tested conditions. In addition, the presence of an additional putative tyrosine decarboxylase with different substrate specificity and genetic organization was noticed for the first time.

Concluding, the high TDC activity heterogeneity within enterococci determined different accumulation of tyramine, depending on different genetic determinants, regulation mechanisms and environmental factors.

The present research contributes to elucidate the genetic characteristics of enterococcal strains and correlate specific mutations to their different strain-dependent tyraminogenic activity.

Key words: *Enterococcus faecium*, *Enterococcus faecalis*, tyramine, tyrosine decarboxylase activity, gene expression.

4.2.1. Introduction

Tyramine is a biogenic amine (BA) which can have severe acute effects if ingested in excessive amounts with food consisting in peripheral vasoconstriction, increased cardiac output, accelerated respiration, elevated blood glucose and release of norepinephrine, symptoms known also as "cheese reaction" (Shalaby, 1994; McCabe-Sellers *et al*., 2006; Marcobal *et al*., 2012).

Lactic acid bacteria (LAB) are among the most efficient producers of tyrosine decarboxylase (TDC), the enzyme responsible for tyramine formation (Marcobal *et al*., 2012). Among LAB, species belonging to the genus *Enterococcus* are recognized as the most efficient tyramine producers (Leuschner *et al.,* 1999; Suzzi and Gardini, 2003, Ladero *et al*., 2012; Marcobal *et al*., 2012). BA formation provides metabolic energy and⁄or resistance against acidic stress (Molenaar *et al*., 1993; Fernández and Zúñiga, 2006; Pereira *et al*., 2009).

Enterococci occur in many different habitats and they are often contaminant in food of animal origin (Franz *et al*., 2011). Due to their salt and pH tolerance and to their ability to grow over a wide temperature range, these LAB are particularly competitive in harsh environmental conditions, and can be a relevant component of the ripening microbiota of cheeses and sausages (Franz *et al*., 1999; Giraffa, 2003; Franz *et al*., 2011). In addition, some strains showed probiotic features, and produce bacteriocins able to limit the growth of pathogenic and degradative microorganisms (Beshkova and Frengova, 2012; Fontana *et al*., 2015). On the other hand, enterococci are among the most common nosocomial opportunistic pathogens because of their antibiotic resistance often carried on mobile genetic elements transferable to other microorganisms (Giraffa, 2002; Klein, 2003; Rossi *et al*., 2014). Moreover, several enterococcal virulence factors have been described, such as cytolysins, aggregation substances, gelatinase extracellular surface proteins (Foulquié Moreno *et al*., 2006; Hollenbeck and Rice, 2012). A further matter of concern with respect to the safety of enterococci is their tyraminogenic capacity (Suzzi and Gardini, 2003; Foulquié Moreno *et al*., 2006; Kompdra *et al*., 2008b; EFSA, 2011). In fact, the ability to produce tyramine is considered a species characteristic of *Enterococcus faecalis* and it is extremely widespread among strains of *Enterococcus faecium* and *Enterococcus durans* (Ladero *et al*., 2012).

TDC is a membrane located enzyme with large hydrophobic regions, which can efficiently work in a wide range of conditions also outside of the cells, as demonstrated in *Lactobacillus brevis* (Moreno-Arribas and Lonvaud-Funel, 2001) and in *E. faecium* and *E. faecalis* (Liu *et al*., 2014a). In any case, tyramine is often accumulated by enterococci in higher amount already during the late exponential growth, before stationary phase, suggesting that this decarboxylation activity is not necessarily a response to starvation or nutrient depletion, and no competition between sugar catabolism and amino acid decarboxylation was observed (Pessione *et al*., 2009, Bargossi *et al*.,

2015b). The tyramine formed inside microbial cells through the action of TDC, is successively excreted in the environment by the cells in exchange with tyrosine through the action of the antiporter tyrosine/tyramine permease (Marcobal *et al*., 2012).

The proteins involved in the tyramine pathway are encoded by the TDC gene cluster, which has been described in detail in various enterococcal species, such as *E. faecalis* JH2-2 (Connil *et al*., 2002), *E. faecium* RM58 (Marcobal *et al*., 2006a), and *E. durans* IPLA 655 (Ladero *et al*., 2013), and it has also been annotated in the genome sequence of other enterococci. All the tyramine biosynthetic loci revealed a high similarity either in gene sequence and organization (Marcobal *et al*., 2012). This locus usually contains the genes encoding a tyrosyl tRNA synthetase (*tyrS*), the tyrosine decarboxylase (*tyrDC*), a tyrosine/tyramine permease (*tyrP*), and a Na+/H+ antiporter (*nhaC-2*) (Linares *et al*., 2011). However, reverse transcription-PCR analyses demonstrated that different strains can have different transcriptional organizations of the TDC gene cluster and *tyrS* is often transcribed independently and not included in the catabolic operon (Perez *et al*., 2015).

The relationships between the presence of enterococci and the accumulation of tyramine has been demonstrated in several fermented food, such as fermented sausages (Gardini *et al*., 2008), cheeses (Linares *et al*., 2011) and wine (Pérez-Martin *et al*., 2014). However, not all the strains able to decarboxylate tyrosine were characterized by the same phenotypic potential in relation to the kinetics of tyramine accumulation (Bargossi *et al*., 2015b).

While the mechanisms of action and the role of TDC in LAB are well elucidated (Wolken *et al*., 2006; Pereira *et al*., 2009; Pessione *et al*., 2009), the effects on the potential decarboxylase activity of enterococcal cells in relation the main environmental factors need to be further investigated. The production of tyramine observed during the growth in laboratory media of tyraminogenic *E. faecalis* and *E. faecium* strains has been modeled in relation to environmental factors such as NaCl and tyrosine concentration, pH, pyrodoxal-5-phospate supplementation and temperature (Gardini *et al*., 2001; Marcobal *et al*., 2006a; Gardini *et al*., 2008). The effects of carbon source, tyrosine and tyramine concentration, and pH on tyramine accumulation during the growth of *E. durans* were described by Fernández *et al*. (2007a). In addition, Liu *et al*. (2014a) characterized the TDC activity of two strains of *E. faecalis* and *E. faecium*, heterologously expressed in *Escherichia coli* and purified in relation to temperature, NaCl concentration and pH.

The aim of this paper was to investigate the diversity of tyramine production capability of four *Enterococcus* strains in buffered systems in relation to their genetic characteristics and environmental conditions. The strains *E. faecalis* EF37 and ATCC 29212, and *E. faecium* FC12 and FC643 were chosen for their different behaviour in tyramine accumulation during the growth in culture media (Bargossi *et al*., 2015b). In detail, we evaluated the functionality of the TDC pathway

in stationary phase cells re-suspended in buffers with different pH, NaCl concentration or incubation temperature. The activity of a purified TDC was also assessed under the same conditions, as a reference. Finally, the nucleotide sequence of the TDC operon region carrying the genes *tyrDC*, *tyrP* and *nhaC-2* was determined.

4.2.2. Materials and methods

4.2.2.1. Enterococcal strains and evaluation of TDC activity in phosphate/citrate buffer

The strains *E. faecalis* EF37 and ATCC 29212, *E. faecium* FC12 and FC643 were stored in 20% (w/v) glycerol at -80°C and pre-cultivated twice for 24 h at 37°C in BHI Broth (Oxoid, Basingstoke, UK) added with 4.4 mM tyrosine (Sigma-Aldrich, Gallarate, Italy).
After 24 h of pre-cultivation, the cells were collected by centrifugation at 8000×g for 10 min and washed twice with physiological solution (0.9% w/v NaCl). The strains were resuspended in 20 mL of the same solution and inoculated at a concentration of approximately 8.2-8.5 log cfu/ml in phosphate/citrate buffer (obtained by mixing citric acid 0.3 M and Na_2HPO_4 0.6 M solutions) added with tyrosine 4.4 mM and incubated at 37°C for 48 h. The determination of the effect of pH on TDC activity was performed in the phosphate/citrate buffer at pH values of 7, 6, 5 and 4 and incubated at 37°C. The effect of NaCl was determined at 37°C in buffer at pH 5 adding 0, 5, 10 and 15% (w/v) of NaCl while the influence of temperature was monitored by incubation at 20, 30, 37 and 45°C in buffer at pH 5 and with no NaCl added. At defined times (0, 2, 8, 24 and 48 h) the cell viability was assessed by plate counting in BHI Agar (Oxoid, Basingstoke, UK) incubated for 48 h at 37°C. In addition, the number of enterococci was determined after 48 h with Burker counting chamber to assess the proportion of undamaged (not lysed) cells. After 2 and 24 hours of incubation, tyramine accumulation was determined.

4.2.2.2. Purified TDC enzyme activity

At the same conditions described above, also the activity of a purified TDC (Sigma-Aldrich, Gallarate, Italy) was monitored. The pure enzyme, obtained from *E. faecalis* according to the producer, was added at 0.15 U/100 mL of phosphate/citrate buffer in the different conditions. At defined times (2, 4, 8, 24 and 48 h) the tyramine accumulation was assessed by the HPLC method described below.

4.2.2.3. Biogenic amine determination

One ml of each culture obtained according to the condition described in paragraph 2.1 was centrifuged at 10000 rpm for 10 min at 10°C; pellet and supernatant were collected for further

analysis. The supernatants were used for BAs determination by HPLC after derivatization with dansyl-chloride (Sigma-Aldrich, Gallarate, Italy) according to Tabanelli *et al.* (2012). The tyramine content was analyzed using a PU-2089 Intelligent HPLC quaternary pump, Intelligent UV–VIS multiwavelength detector UV 2070 Plus (Jasco Corporation, Tokio, Japan) and a manual Rheodyne injector equipped with a 20 µl loop (Rheodyne, Rohnert Park, CA). The quantification of tyramine was performed as follows: gradient elution 0–5 min phosphate buffer (pH 7.0)/acetonitrile 35:65, 5–6 min water/acetonitrile 20/80, 6–15 min water/acetonitrile 10/90, 15–25 min phosphate buffer (pH 7.0)/acetonitrile 35:65 with flow rate 0.8 mL/min. The amount of tyramine was expressed as mM by reference to a calibration curve obtained with standard solutions.

4.2.2.4. Analysis of the TDC operon region

Total genomic DNA was extracted from cell pellets using the Wizard Genomic DNA purification system (Promega Corporation, Madison, WI), according to the manufacturer's instructions. The TDC operon fragments were obtained for each strain by PCR amplification with the partially degenerate primers reported in Table 1.

Primer code	**Sequence (5'-3')**	**Amplicon (pb)**	
		E. faecalis	***E. faecium***
TyrS-F1 Tdc-R1	GGA GCT ATA AGT ATT AAC GGT GA GAT TT(A/G) ATG TT(A/G) CG(G/C) GCA TAC CA	957	943
Tdc-F2 Tdc-R2	CAA ATG GAA GAA GAA GT(A/T) GGA CC(A/G/T) GCA CG(G/T) T(C/T)C CAT TCT TC	1287	1340
Tdc-F3 TyrP-R3	CCA GA(C/T) TAT GGC AA(C/T) AGC CCA CCT AAA GTA GAA GC(A/G) ACC AT	819	784
TyrP-F4 TyrP-R4	TGG GTG CAA ATG TTC CCA GG ACC (A/G)AT TCG (A/G)TA AGG ACG	839	940
TyrP-F5 NhaC-R5	(A/T)CT GCT TGG GT(A/T) ACT GGA CC CAT (C/T)GC AT(C/T) (A/G)T(C/T) GAA TCC AAG	1098	1056
NhaC-F6 NhaC-R6	GTG TCT TAG TTG CT(A/T) C(A/T)T GGA T CCA TAA TGA A(G/T)G T(A/G)C C(A/G)C T(A/G)A CT	1017	1017

Table 1: Newly designed primers used in this study and expected size of the amplicons.

PCR mixture was composed of 1× PCR buffer, 1,5 mM MgCl2, 200 nM dNTPs, 0,5 µM each primer and 50 ng DNA. Amplification program comprised: 95°C for 5 min, 35 cycles at 94°C, 30 sec; 56°C, 45 sec; 72°C, 1 min and final extension at 72°C, 10 min. Amplicons were purified with

the Wizard SV gel and PCR clean-up system (Promega, Italy), and cloned with the cloning kit pGEMT-easy vector system (Promega, Madison, USA). Recombinant plasmids were sequenced at the GATC Biotech Ltd (Koln, Germany). Promoters prediction was carried out by BPROM, a bacterial sigma70 promoter recognition program (http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb; Solovyev and Salamov, 2011). Putative Rho–independent transcription terminators were predicted by the Arnold Finding Terminators (http://rna.igmors.u-psud.fr/toolbox/arnold/index.php). The search procedure uses two complementary programs, Erpin (Gautheret and Lambert, 2001) and RNAmotif (Macke *et al*., 2001).

Similarity searches were performed with the Basic Local Alignment Search Tool (BLAST) available at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov). Sequence alignments were carried out with the ClustalW2 analysis Tool Web Services from the EMBL-EBI (McWilliam *et al*., 2013).

4.2.2.5. Statistical analysis

Biogenic amine values and enterococci counts for each strains and for each condition are the mean of three different samples. The presence of significant differences was tested with ANOVA, using the Tukey HSD test carried out with Statistica 6.1 (StatSoft Italy srl, Vigonza, Italy).

4.2.3. Results and discussion

4.2.3.1. Purified TDC enzyme activity

The amount of tyramine produced in phosphate/citrate buffer containing the purified TDC (0.15 U/100 ml) under different conditions is reported in Table 2.

Hours	Tyramine (mmol/l)											
	37°C, 0% NaCl				pH 5, 37°C				pH 5, 0% NaCl			
	pH 4	pH 5	pH 6	pH7	0% NaCl	5% NaCl	10% NaCl	15% NaCl	20°C	30°C	37°C	45°C
0	-	-	-	-	-	-	-	-	-	-	-	-
2	0.02 (±0.01)	0.14 (±0.02)	0.14 (±0.01)	0.08 (±0.01)	0.14 (±0.01)	0.14 (±0.00)	0.11 (±0.01)	0.10 (±0.01)	0.11 (±0.00)	0.14 (±0.01)	0.14 (±0.01)	0.13 (±0.00)
4	0.03 (±0.01)	0.19 (±0.03)	0.19 (±0.01)	0.11 (±0.02)	0.19 (±0.02)	0.18 (±0.01)	0.16 (±0.01)	0.13 (±0.00)	0.15 (±0.01)	0.19 (±0.02)	0.19 (±0.01)	0.18 (±0.01)
8	0.04 (±0.01)	0.28 (±0.03)	0.28 (±0.02)	0.16 (±0.03)	0.28 (±0.01)	0.24 (±0.02)	0.21 (±0.02)	0.18 (±0.01)	0.20 (±0.01)	0.27 (±0.02)	0.28 (±0.02)	0.27 (±0.01)
24	0.02 (±0.01)	0.60 (±0.04)	0.59 (±0.04)	0.26 (±0.02)	0.60 (±0.02)	0.50 (±0.03)	0.42 (±0.02)	0.33 (±0.02)	0.39 (±0.01)	0.55 (±0.04)	0.60 (±0.03)	0.43 (±0.02)
48	0.03 (±0.01)	0.88 (±0.06)	0.95 (±0.06)	0.37 (±0.03)	0.88 (±0.03)	0.81 (±0.02)	0.70 (±0.04)	0.54 (±0.03)	0.56 (±0.02)	0.92 (±0.05)	0.88 (±0.04)	0.64 (±0.03)

Table 2: Tyramine produced in phosphate/citrate buffer containing the purified commercial TDC (0.15U/100ml) under different conditions. Standard devistion is reported within brakets.

Similar amounts of tyramine (ranging from 0.60 and 0.59 mM) were detected at pH 5 and 6 after 24 h of incubation, suggesting that the optimum pH for TDC activity was comprised between these values. At pH 7, the amine production was drastically reduced (0.26 mM), while at pH 4 it was negligible (less than 0.02 mM). Shorter or longer incubation periods decreased and increased, respectively, the tyramine detected without significantly changing the proportion of BA produced.

The increase of NaCl concentration determined a progressive and constant decrease of the efficacy of the enzymatic activity, throughout all the incubation period. However, the purified TDC maintained a high effectiveness even at 15% NaCl (about 62% of the tyramine produced in the absence of salt).

Regarding the effect of temperature, tyramine was produced in higher amount, and with higher rate, at 30 and 37°C (without significant differences between these two temperatures) and, after 24 h incubation, 0.55 and 0.60 mM of tyramine were detected, respectively. Slower decarboxylation kinetics were observed at 45 and 20°C. However, the final amount of tyramine was rather high, about the 65% of those observed under optimal conditions, indicating a good enzymatic activity also at the minimum and maximum temperature considered in these trials.

Liu *et al.* (2014a) studied the effect of pH, temperature and salt concentration on TDC activity from two strains of *E. faecalis* and *E. faecium* and heterologously expressed in *Escherichia coli*. They found an optimum pH for tyrosine decarboxylation at 5.5 for the enzyme from *E. faecalis* and 6.0 for the enzyme from *E. faecium*. By contrast, the optimum temperature coincided for the two

enzymes and was lower (25°C) than that found here. The same authors observed no effect on TDC activity of NaCl concentration up to 4.5%. Moreno-Arribas and Lonvaud-Funel (1999) demonstrated a maximum tyrosine decarboxylase activity at pH 5.5 in cell free extracts of two *L. brevis* strains. In addition, the TDC activity of cell free extract was always higher if compared with the activity of whole cells in relation not only to pH, but also to citrate, lactate, ethanol and tyramine concentration in the medium.

4.2.3.2. Cell viability

The viability of the cells was checked at different times (2, 8 and 24 h incubation) and Table 3 reports the diminution of the log cfu/ml with respect to the initial inoculum (approx. 8.2-8.5 log cfu/ml).

Hours	**Cell load reduction**											
	37°C, 0% NaCl				**pH 5, 37°C**				**pH 5, 0% NaCl**			
	pH 4	**pH 5**	**pH 6**	**pH 7**	**0% NaCl**	**5% NaCl**	**10% NaCl**	**15% NaCl**	**20°C**	**30°C**	**37°C**	**45°C**
						EF37						
2	0.13	0.04	0.18	0.11	0.04	0.01	0.16	0.13	0.16	0.03	0.04	0.03
8	0.26	0.03	0.32	0.17	0.03	0.14	0.14	0.25	0.16	0.10	0.03	0.09
24	1.64	0.09	0.65	0.60	0.09	0.18	0.17	0.43	0.17	0.12	0.09	0.01
						ATCC 29212						
2	0.11	0.06	0.19	0.21	0.06	0.17	0.06	0.28	0.06	0.11	0.06	0.03
8	1.12	0.07	0.31	0.34	0.07	0.28	0.41	0.31	0.32	0.46	0.07	0.43
24	3.45	0.29	0.65	0.59	0.29	0.48	0.29	1.37	0.29	0.65	0.29	0.66
						FC12						
2	0.04	0.03	0.07	0.14	0.03	0.03	0.11	0.03	0.11	0.05	0.03	0.01
8	0.81	0.09	0.47	0.62	0.09	0.16	0.12	0.02	0.32	0.53	0.09	0.46
24	2.57	0.30	0.57	0.69	0.30	0.51	0.24	1.57	0.14	0.52	0.30	0.43
						FC643						
2	0.09	0.01	0.01	0.08	0.01	0.11	0.13	0.25	0.34	0.13	0.01	0.16
8	0.31	0.13	0.28	0.35	0.13	0.21	0.08	0.34	0.27	0.33	0.13	0.54
24	5.29	0.31	0.66	0.58	0.31	0.77	0.64	0.60	0.20	0.20	0.31	2.17

Table 3: Viability loss of the strains inoculated in the different buffers after 2, 8 and 24 h of incubation, expressed as diminution of the log cfu/ml with respect to the initial inoculum.

Enterococcus faecalis EF37 was characterized by a reduced loss of viability after 2 and 8 h, while after 24 h a marked reduction was observed at pH 4 (1.64 log cfu/ml). In all the other cases, the viability loss was always below 0.65 log unit and the strain showed the higher level of survivors in the presence of salt if compared with the other strains.

Also *E. faecalis* ATCC 29212 presented a reduced diminution in viable counts after 2 h (less than 0.30 log cfu/ml). After 8 h, the diminution was always lower of 0.5 log unit, with the exception of pH 4 at which the decrease was higher than 1 log unit. After 24 h of incubation high viability losses were observed at pH 4 (3.45 log cfu/ml) and in the presence of 15% NaCl (1.37 log cfu/ml).

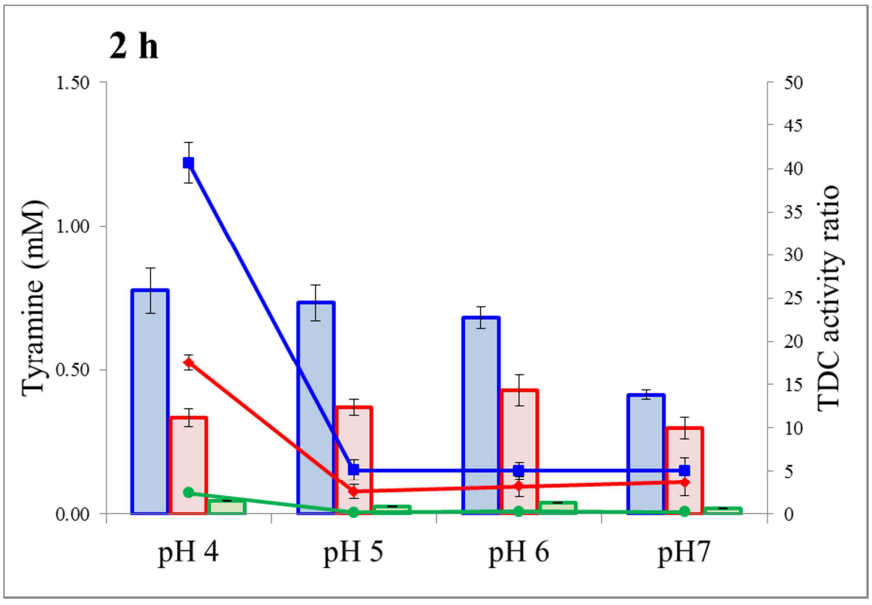
An analogous behaviour was shown by *E. faecium* FC12, with the higher level of cell death found after 24 h at pH 4 (2.57 log cfu/ml) and at 15% NaCl (1.57 log cfu/ml).

Finally, also *E. faecium* FC643 was characterized by a dramatic loss of viability after 24 h especially at 45°C (2.17 log cfu/ml) and at pH 4 (5.29 log cfu/ml).

Independently on the results of plate counting, after 24 h, the number of whole enterococcal cells was evaluated with a Burker chamber. No significant differences were found with the initial inoculum (data not shown). Thus, this suggests that, at least within 24 h, the enterococcal loss of viability was not associated to cell lysis, with a consequent release of cell decarboxylase in the buffer.

4.2.3.3. Tyramine production

Tyramine concentrations detected after 2 and 24 h for the tested strains in relation to pH, NaCl concentration and incubation temperatures are reported in Figures 1, 2 and 3, respectively.



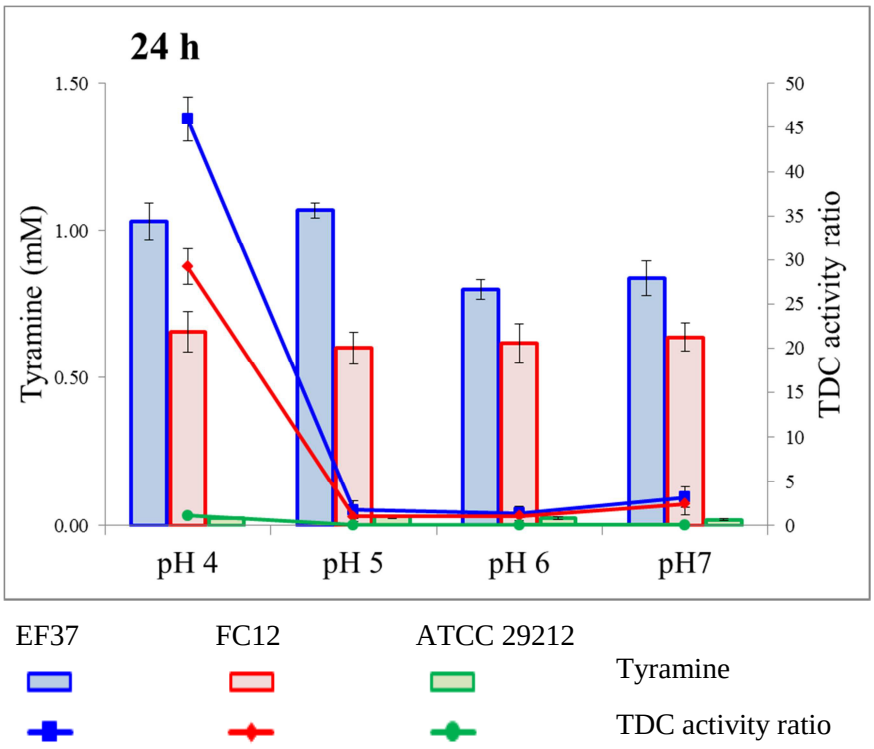
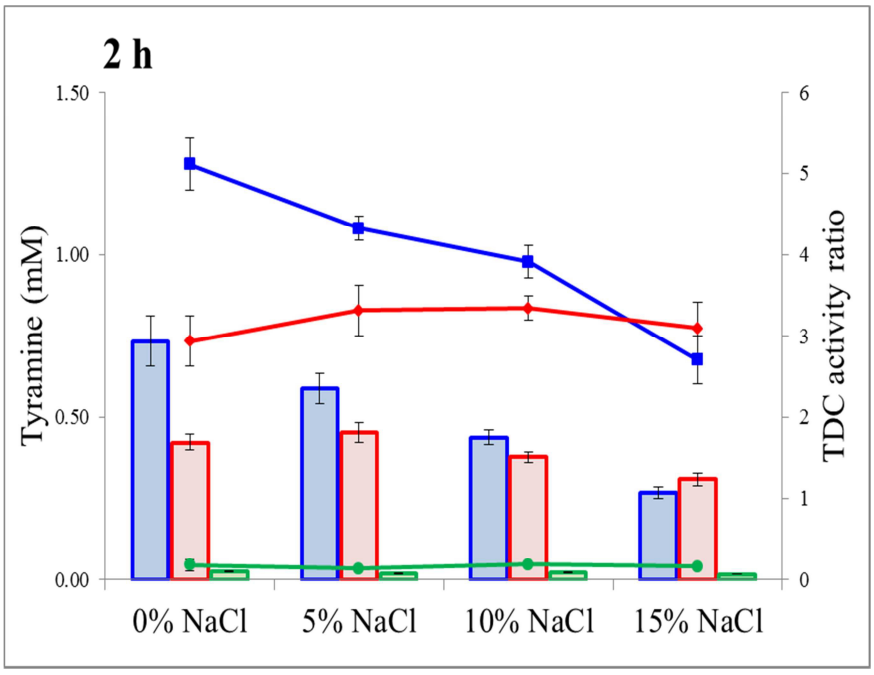


Figure 1: Tyramine produced by enterococcal strains in phosphate/citrate buffer having different pH values after 2 and 24 hours of incubation at 37°C. When ANOVA was significant ($P \leq 0.05$) lower-case letters are reported. For the same strain, values with the same letter are not statistically different ($P > 0.05$) according to the post-hoc comparisons of the ANOVA. In the same graphs also the TDC activity ratio is shown, i.e. the ratio of mM of tyramine accumulated by cells and mM of tyramine produced by commercial TDC pure enzyme (0.15 U/100 ml) in the same conditions.



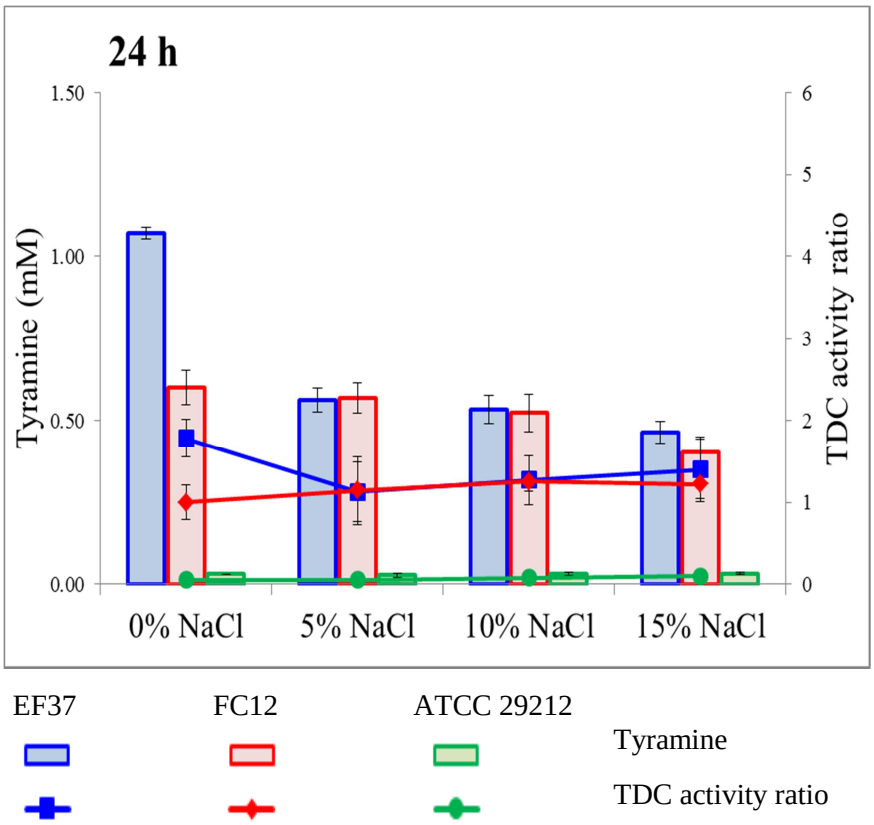
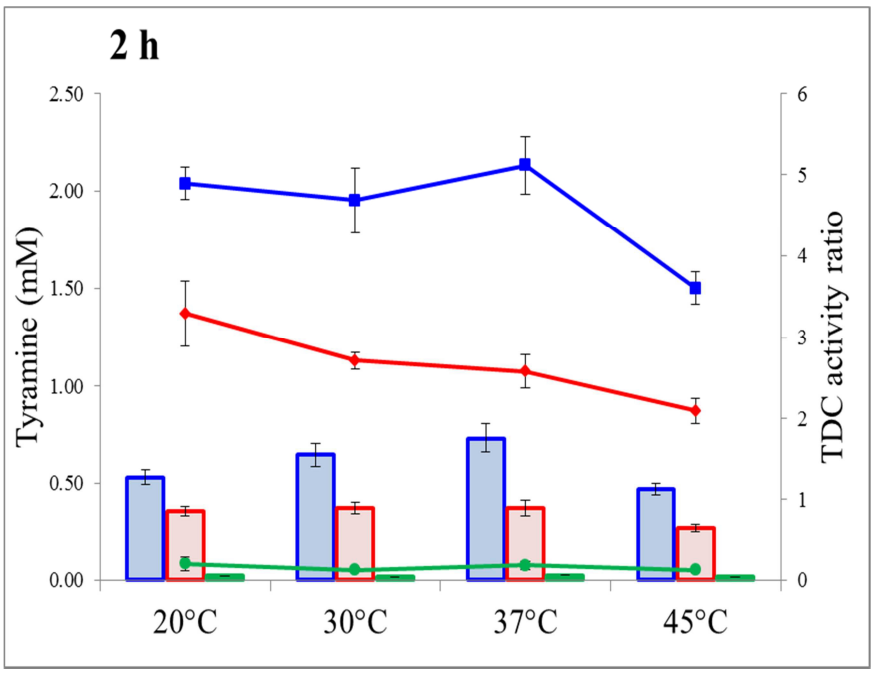


Figure 2: Tyramine produced by enterococcal strains in phosphate/citrate buffer added with different amounts of NaCl after 2 and 24 hours of incubation at 37°C. When ANOVA was significant ($P \leq 0.05$) lower-case letters are reported. For the same strain, values with the same letter are not statistically different ($P > 0.05$) according to the post-hoc comparisons of the ANOVA. In the same graphs also the TDC activity ratio is shown, i.e. the ratio of mM of tyramine accumulated by cells and mM of tyramine produced by commercial TDC pure enzyme (0.15 U/100 ml) in the same conditions.



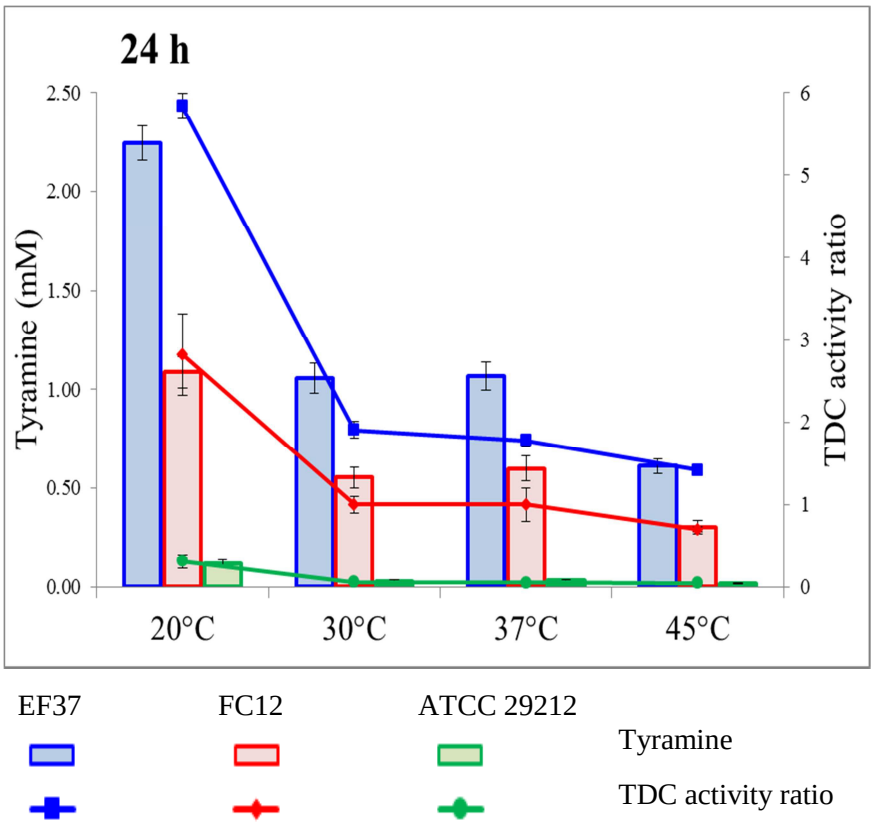


Figure 3: Tyramine produced by enterococcal strains in phosphate/citrate buffer incubated at different temperatures after 2 and 24 hours of incubation at 37°C. When ANOVA was significant ($P \leq 0.05$) lower-case letters are reported. For the same strain, values with the same letter are not statistically different ($P > 0.05$) according to the post-hoc comparisons of the ANOVA. In the same graphs also the TDC activity ratio is shown, i.e. the ratio of mM of tyramine accumulated by cells and mM of tyramine produced by commercial TDC pure enzyme (0.15 U/100 ml) in the same conditions.

All the strains were pre-cultured in presence of the precursor to activate the transcription of the TDC gene cluster (Bargossi *et al*., 2015b). The pre-adaptation in media containing tyrosine also allowed a rapid beginning of decarboxylase activity, with a detectable tyramine concentration since 2 h of incubation.

Under the adopted conditions, the strains EF37, ATCC 29212 and FC12 were able to decarboxylate amounts of the tyrosine supplied far from the maximum theoretical yield (4.4 mM). In particular, EF37 and FC12 produced the higher tyramine concentrations, while ATCC 29212 showed a reduced decarboxylase activity in all the conditions assayed (Figure 1, 2 and 3). By contrast, *E. faecium* EF643 was not able to accumulate detectable amounts of tyramine in any of the tested conditions in 24 h and, for this reason, it is not present in the Figures. These different aptitudes of the tested strains confirm the trends of tyramine accumulation during growth in more complex systems, i.e. BHI and Bover-Cid and Holzapfel medium (Bargossi *et al*., 2015b). In fact, in these media *E. faecalis* EF37 and *E. faecium* FC12 have shown an early tyramine accumulation (since the

exponential phase) and the maximum BA concentration was reached at the beginning of the stationary phase. By contrast, the accumulation of tyramine by *E. faecalis* ATCC 29212 and *E. faecium* FC643 has been delayed and characterized by a slower rate while the amine has been accumulated in relevant amounts only during the stationary phase.

The strain *E. faecalis* EF37 generally showed the highest ability to decarboxylate tyrosine in citrate/phosphate buffer while *E. faecium* FC12 accumulated slightly lower amounts of tyramine. On the other hand, *E. faecalis* ATCC 29212 showed under all the conditions tested an extremely low aptitude to produce this BA. In fact, in all the samples, both after 2 and 24 h of incubation, the amount was always below 0.15 mM.

Regarding pH (Figure 1), *E. faecalis* EF37 accumulated higher amounts of tyramine at the lower pH values, with more marked differences after 24 h. Tyramine production of *E. faecium* FC12 was weakly affected by pH, and the amounts detected varied between 0.34 and 0.43 mM after 2 h and 0.60 and 0.66 mM after 24 h of incubation.

The tyrosine decarboxylation pathway is reported to contribute to an acid response mechanism in *E. faecium* because it gives to the strain a competitive advantage in nutrient-depleted conditions, as well as in harsh acidic environments (Pereira *et al*., 2009). The same role in the maintenance of pH homeostasis in acidic environment has been described also in *E. durans* (Linares *et al*., 2009), *E. faecium* (Marcobal *et al*., 2006a) and *E. faecalis* (Perez *et al*., 2015). These latter authors have reported that tyrosine decarboxylation pathway improves survival under acidic conditions. This could explain the higher cell viability loss at pH 4 of *E. faecium* FC643, which did not produce tyramine, and of *E. faecalis* ATCC 29212, which accumulated low amounts of amine. However, in this perspective, the behavior of *E. faecium* FC12, which did not present significant differences in relation to pH, appears to be surprising and needs further investigations. On the other hand, the purified TDC showed a low activity at pH 4 (Table 2) and the environmental pH influenced the overall cell metabolism rather than the specific activity of TDC inside the cytoplasm. In other words, the heterogeneity found in the enterococcal strains could be related to the general physiological state of the cells which, in turn, influenced the TDC activity.

Figure 2 reports the tyramine accumulation in relation to NaCl concentrations. The addition of increasing amounts of salt caused a progressive diminution in tyrosine decarboxylation by *E. faecalis* EF37 after 2 h. This strain accumulated 0.73 mM of tyramine in the absence of NaCl and tyramine decreased to 0.59 mM in the presence of 5% NaCl and 0.27 mM with 15% NaCl. After 24 h, small differences in tyramine content (ranging from 0.46 mM and 0.57 mM) were observed in the presence of the different NaCl concentrations, while this amount was about doubled in the control sample without salt (1.07 mM). The decarboxylating activity of FC12 was not particularly affected

by increasing NaCl concentrations, except for a partial reduction at 15% NaCl, both after 2 and 24 h of incubation. The tyramine production by *E. faecalis* ATCC 29212 was negligible, without differences in relation to salt modulation.

A general reduction of tyramine accumulation by enterococci due to NaCl concentration has already been described both in vitro (Liu *et al.*, 2014b) and in fermented foods (Gardini *et al.*, 2008). In addition, the same trend was observed using the purified TDC, as evidenced in Table 2.

Bunkova *et al*. (2011) showed that the presence of the higher NaCl concentration tested (2%) favoured the accumulation of tyramine by *Lactococcus lactis* strains that started during the active growth phase of the cells.

For *E. faecalis* EF37, the effect of the temperature was noteworthy. In fact, after 2 h, the higher tyramine accumulation was observed at 37°C (0.73 mM), which is the optimal temperature for enterococci, while for the purified enzyme no differences were observed within 30 and 37°C. Lower tyramine concentrations were found at 20 and 45°C (0.53 and 0.47 mM, respectively). By contrast, after 24 h of incubation, the highest content of tyramine were found at 20°C (2.25 mM), while in the samples incubated at 30 and 37°C this concentration was halved. In the trial at 45°C the concentration remained quite stable (0.60 mM) with respect to sample collected after 2 h.

For *E. faecium* FC12, after 2 h of incubation, no significant differences were found between 20 and 37°C, while the BA concentration was reduced at 45°C. This lower production at the higher temperature tested was found also after 24 h of incubation. In addition, at the lowest temperature (20°C) the highest tyramine accumulation was observed as already found for *E. faecalis* EF37. This behaviour was confirmed also for *E. faecalis* ATCC 29212, which accumulated the highest amount of amine in the sample incubated at 20°C for 24 h (0.12 mM). In general, the effect of temperature has been tested in relation to the growth of the cells and, under these conditions, lower incubation temperature are associated with lower BA accumulation (Masson *et al*., 1996; Gardini *et al*., 2001). Under the conditions applied to the cells suspended in the buffer, the higher tyramine levels were detected at the lower temperature, suggesting the possibility that the tyrosine decarboxylase activity can be highly activated under not favourable temperature.

The lines drawn in Figures 1, 2 and 3 represent the ratio between the tyramine produced by the cells in the buffer and the tyramine accumulated under the same conditions by the purified enzyme (reported in Table 2). These values were added with the aim to highlight the different performances of TDC when it worked inside or outside the cells.

First of all, this index reflects the inability of *E. faecalis* ATCC 29212 to decarboxylate tyrosine under the adopted conditions. In the other two strains, the decarboxylase activity after 2 h of incubation is always higher than in samples with the purified enzyme and, consequently, the ratio

was higher than 1. By contrast, after 24 h, the amine produced by the purified enzyme and the enterococcal strains was comparable with ratio close to 1. A noteworthy exception to this trend was represented by the samples inoculated with *E. faecalis* EF37 and *E. faecium* FC12 incubated at 20°C and at pH 4. In these cases, the decarboxylase activity was extremely higher in the presence of cells and the ratios were comprised between 3 and 6 at 20°C and 30 and 45 at pH 4.

In other words, the decarboxylating activity inside the cells is influenced by the chemico-physical factors through two different mechanisms; on one side the environmental factors directly affect the enzyme activity while, on the other side, they regulate the overall cell metabolism and, in turn, the rate of exchange between inside and outside. The interaction between these two aspects can explain the different responses of tyramine accumulation observed using the purified enzyme and the viable cells. In this perspective, it is noteworthy the cell response at 20°C of the strains EF37 and FC12 in which the production of tyramine seems to be an important strategy for the enterococcal growth at this unfavourable temperature.

Therefore, the final results of the decarboxylase activity, i.e. the amount of BA accumulated, depends on the activity of TDC which works inside the cell, but also by the ability of the cells to transport the precursor in the cytoplasm and to excrete the final product (tyramine) outside. This antiport, driven by the tyrP activity (Marcobal *et al*., 2012), could be affected by the environmental conditions.

4.2.3.4. Analysis of the TDC operon region

To characterize the TDC pathway of the four enterococcal strains, we amplified and sequenced the region carrying the genes *tyrDC*, *tyrP* and *nhaC-2*. The six sets of newly designed primers amplified overlapping fragments of the expected size (Table 1) for all the strains including *E. faecalis* ATCC 29212 which was used as a control, since its complete genome sequence was already available (gb|CP008816.1|; Minogue *et al*., 2014). The TDC cluster sequence of all the strains shares the same genetic organization, which comprises, downstream the gene *tyrS*, the three predictable complete open reading frames (ORF) corresponding to the genes *tyrDC*, *tyrP* and *nhaC-2*. They are oriented in the same direction and encode polypeptides larger than 300 amino acids (Figure 4).

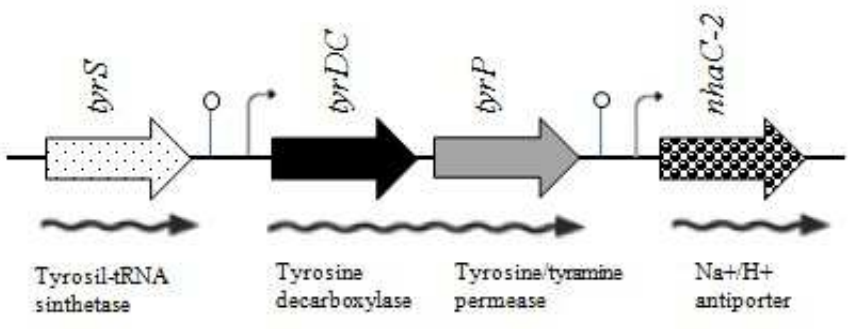


Figure 4: Genetic organization of the TDC region containing the genes *tyrS*, *tyrDC*, *tyrP*, and *nhaC-2* in the enterococcal strains studied. ORFs are represented by arrows, putative promoters by broken arrows, transcription terminator regions by lollipops. Expected mRNA and enzymatic function are also indicated.

Sequence analysis performed by the software BPROM and Arnold did not revealed the presence of putative promoters and terminators between the genes *tyrDC* and *tyrP*, indicating that they could be co-transcribed. Putative promoters and terminators were conversely found upstream the start codon of the genes *tyrDC* and *nhaC-2* (Figure 4). This suggests that the expression of *tyrDC* and *tyrP* is probably independent from that of the flanking genes *tyrS* and *nhaC-2*. Different polycistronic mRNA transcripts have been described in enterococcal strains, such as *tyrS-tyrDC-tyrP* (Connil *et al*., 2002), *tyrDC-tyrP* (Linares *et al*., 2009), *tyrDC-tyrP-nhaC-2* and *tyrP-nhaC-2* (Perez *et al*., 2015). So *tyrDC* and *tyrP* can be transcribed from different manner and future Reverse-Transcription-PCR experiments are needed to clarify the transcriptional organization of the TDC gene cluster in the examined strains.

BLASTN analysis of the 5259 bp nucleotide sequence of *E. faecalis* EF37 TDC operon region showed an overall identity of 99% (5231/5259 bp) with that of *E. faecalis* ATCC 29212, and 100% identity (5259/5259 bp) with that of another completely sequenced strain of the same species, *E. faecalis* D32 (gb|CP003726.1|).

BLASTX analysis and comparison of the deduced amino acid sequences of the two *E. faecalis* strains was also carried out. These analyses revealed only two substitutions in amino acid sequences of EF37 and ATCC 29212. The first one (leucine to methionine) was found at the beginning of the ORF coding for the amino acid permease and the second one (isoleucine to valine) was located in the ORF corresponding to the Na^+/H^+ antiporter (position 18). These amino acid substitutions are conservative, and thus probably have no effect on the enzymatic activities.

Nucleotide sequence analysis of the *E. faecium* FC12 showed the highest identity (5293/5294 bp, 99%) with the operon of the strain *E. faecium* NRRL B-2354 (complete genome NC_020207),

while the identity decrease to a value of 97% (5128/5296 bp) with the other *E. faecium* strain studied in this research.

As regards amino acid residues, BLASTX analysis of the TDC locus of FC12 showed a 100% of identity with TDCs (frame +3, 625 aa), amino acid permeases (frame +2, 456 aa) and Na+/H+ antiporters (frame + 3, 414 aa) in the database. Amino acid sequences corresponding to the genes *tyrP* and *nhaC-2* of *E. faecium* FC643 were characterized by a 100% of identity with known amino acid permeases (frame +3, 456 aa) and Na+/H+ antiporters (391 aa) proteins, respectively. The TDC region translated sequence of FC643 showed an identity of 99% (624/625) with the amino acid sequences of almost all *E. faecium* strains present in database. However, a premature codon stop, introduced by a non-sense mutation (TGG/TAG) was found at position 40 of the protein. This mutation probably produces an abnormally shortened protein, supporting the inability of the strain FC643 to accumulate tyramine in the tested conditions at 24 h.

Nevertheless, Bargossi *et al*. (2015b) highlighted the capacity of FC643 to accumulate lower level of tyramine in stationary phase of growth in complex media if compared with *E. faecium* FC12. Moreover, this strain did not accumulated 2-phenylethylamine in the same conditions. The decarboxylase activity of FC643, even if slow and reduced, in the presence of the premature codon stop in TDC region, could be ascribed to the presence of an additional gene coding for a decarboxylase enzyme involved in tyramine production. Indeed, comparison analysis of the *tyrDC* sequence to databanks allowed the identification of another gene coding for a putative tyrosine decarboxylase in all the publicly available whole genome sequences of *E. faecium*, *i.e. E. faecium* strains Aus0085 (AGS74230.1), NRRL B-2354 (AGE29157.1), DO (AFK57968.1), Aus0004 (AFC62424.1), and T110 (AII38451.1). This gene was not detected in the genome of *E. faecalis*. The additional enzyme has a nucleotide and amino acid identity score of 67-68% with the first known tyrosine decarboxylase, but it maintains catalytic residues involved in enzyme activity, the consensus pattern for pyridoxal phosphate-dependent decarboxylases where lysine (K) is the attachment site for the cofactor and the conserved LHVDAAY motif (Sandmeier, 1994) (Figure 5).

Figure 5: Comparison of TDC protein sequences found in the genome of *E. faecium* Aus0085. The HVDAAY conserved motif and PLP binding site characteristics of the group II PLP-dependent decarboxylase family are indicated in bold and highlighted in gray.

Nowadays no information are available about this additional enzyme that in the complete genomes of *E. faecium* is always followed by two amino acid permease coding genes. To elucidate the role of these proteins in *E. faecium* strains amino acid metabolism further investigations are needed.

4.2.4. Conclusion

The tyramine production by cells re-suspended in buffered systems highlighted the heterogeneity of TDC activity within enterococci. The study of the genetic characteristics of the *E. faecium* strains allowed to correlate specific mutations in the *tyrDC* gene sequence to their different tyraminogenic activity, and suggested the involvement of another gene annotated as putative tyrosine decarboxylase in the complete genome of *E. faecium.* To our knowledge, the potential role of an additional decarboxylase enzyme with different substrate specificity and genetic organization was here noticed for the first time. The two *E. faecalis* strains showed highly conserved TDC operon region, thus their phenotypic behaviour could be ascribed to different regulation mechanisms not yet elucidated, and affected by environmental factors or by the overall cell metabolism. In fact, the higher tyramine concentration produced by the enterococcal strains was found in the less favourable conditions for the purified TDC (at 20°C and pH 4). Further investigations have to be performed for better understanding the genetic determinants and mechanisms involved in tyramine production under different chemico-physical conditions by the application of “omic” approaches.

4.3. Growth, biogenic amine production and *tyrDC* transcription of enterococci in synthetic medium containing defined amino acid concentration

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4.3.1. Introduction

Tyramine is a biogenic amine (BA) produced by decarboxylation of tyrosine. The adverse effects due to large amount of tyramine ingestion with diet are known as “cheese reaction” and consist in migraine, hypertension and vasoconstriction, increased cardiac output, increased respiration, elevated blood glucose, and release of norepinephrine (EFSA, 2011; Marcobal *et al*., 2012; Shalaby, 1996).

Even if many microorganisms can accumulate biogenic amines in foods, the presence of the enzyme tyrosine decarboxylase (tyrDC) is particularly widespread among all lactic acid bacteria LAB species (Marcobal *et al*., 2012). In this framework, LAB belonging to the genus *Enterococcus* are recognized as the most efficient tyramine producers (Suzzi and Gardini, 2003; Özogul and Özogul, 2007; Capozzi *et al*., 2011; Kuley and Özogul, 2011; Ladero *et al*., 2012).

Enterococci occur in many different habitats and, due to their association with the gastrointestinal tract, they are often contaminant in food of animal origin (Franz *et al*., 2003; Franz *et al*., 2011). When present in the raw material, enterococci can survive to the fermentation process and can be found in fermented foods such as sausages and cheeses in which they can have a relevant role during ripening (Franz *et al*., 2011; Giraffa, 2003). In fact, due to their salt and low pH tolerance, as well as their ability to grow over a wide temperature range, these LAB are particularly competitive especially when the environmental conditions become harsher. The presence of excessive tyramine content in cheese and fermented meat is often attributed to these microorganisms (Foulquié Moreno *et al*., 2006; Joosten and Northolt, 1989; Suzzi and Gardini, 2003; Komprda *et al*., 2008a,b).

The presence of a gene coding for the tyrosine decarboxylase (*tyrDC*) is considered a species characteristic in *E. faecalis*, while it is widely diffused in *E. faecium* (Ladero *et al*., 2012). This gene has been found also in *E. durans* (Linares *et al*., 2009) and, recently, tyramine production has been studied in *E. mundtii* (Gatto *et al*., submitted).

The tyrosine decarboxylase cluster of enterococci usually contains four genes. In addition to *tyrDC* (responsible for the decarboxylation), also *tyrS* (aminoacyl transfer RNA (tRNA) synthetase-like gene), *tyrP* (encoding for a tyrosine/tyramine antiporter) and often *nhaC-2* (encoding an Na^+/H^+ antiporter) are present (Marcobal *et al*., 2012, Connil *et al*., 2002, Coton *et al*., 2011). Tyrosine is a substrate for which tyrDC has a great affinity. However, it has been demonstrated that enterococci can decarboxylate also phenylalanine with the same enzyme even if with a lower efficiency and when tyrosine is almost completely depleted (Pessione *et al*., 2009).

Decarboxylation of tyrosine can be advantageous in microorganisms because it i) is a metabolic response against acidic stresses and ii) constitutes a mechanism through which proton motive force is generated (Connil *et al*., 2002, Marcobal *et al*., 2012, Pessione *et al*., 2009; Pereira *et al*., 2009).

In spite of the several works relating the tyramine content to enterococci activity in many fermented foods, scarce information is still available about the regulation and the physiological role of tyramine production in LAB and especially in enterococci. Some authors investigated also the possible role of tyrosine decarboxylation in GIT resistance, immunomodulation and adhesion of pathogen to enterocytes (de Palencia *et al*., 2011; Lyte, 2004; Pereira *et al*. 2009).

Recently, Perez *et al*. (2015) demonstrated that the activation of the tyrDC favored the survival of *E. faecalis* when subjected to gastrointestinal stress and low pH and substrate availability induced the expression of the *tyrDC* (and *tyrP* and 2*nhaC-2*, which are cotranscribed). Further, tyrosine enhanced *tyrDC* expression, while it repressed *tyrS*, as already observed in *E. durans* (Linares *et al*. 2012a) and in *Sporolactobacillus* sp. (Coton *et al*., 2011). Also in *L. brevis* the expression of *tyrDC* was increased by substrate availability while less evident was the correlation between tyramine production and survival at low pH in wine (Arena *et al*., 2011).

In spite of the extremely wide diffusion of tyrDC among enterococci, different behavior in the production rate and in the growth phase in which tyramine is accumulated have been evidenced by several authors. Tyramine is often produced by some enterococci in higher amount already during the late exponential growth suggesting that this decarboxylation activity is not necessarily a response to starvation or nutrient depletion (Pessione *et al*., 2009). This trend was observed also by Bargossi *et al*. (2015a) in the strain *E. faecalis* EF37 and *E. faecium* FC12; however, in the same experiment, the strains *E. faecalis* ATCC 29212 and *E. faecium* FC643 produced tyramine only after the reaching of the stationary phase. These differences were partially explained by different transcription levels of *tyrDC.* In a successive work, the unusual scarce activity of *E. faecium* FC643, was explained by a premature codon stop, introduced by a non-sense mutation (TGG/TAG) of *tyrDCA* (Bargossi *et al*., 2015b).

In this work, a defined medium containing precise amounts of tyrosine and phenylalanine was used for the growth of two strains of *E. faecalis*. The strains used were inoculated after their pre-grown with or without pre-induction in media containing or not tyrosine. Growth was monitored through optical density increase at 600 nm (OD_{600}) and cell concentration was periodically monitored together with the amounts of tyramine and 2-phenylethylamine produced. Then, the level of transcription of *tyrDC* in not induced cells was measured in buffered media at pH 5.5 and 7.0 containing different amounts of tyrosine, with the aim to better understand the mechanisms, which allow different behaviour in tyramine (and 2-phenylethylamine) accumulation.

4.3.2. Materials and methods

4.3.2.1. Enterococcal strains and evaluation of tyrDC activity in synthetic media

The strains *E. faecalis* EF37 and ATCC 29212, stored in 20% (w/v) glycerol at -80°C, were pre-cultivated twice for 24 h at 37°C in synthetic medium, with full defined composition (adapted from Jensen and Hammer, 1993 and reported in Table 1), added or not with 4.4 mM tyrosine (Sigma-Aldrich, Gallarate, Italy) to evaluate the effect of pre-adaptation.

Components	Concentration	Components	Concentration
Macro component (mM)		**Amino acid (mM)**	
NaCl	42.78	L-alanine	3.40
Glucose	55.51	L-arginine	1.10
$MgSO_4 \cdot 7H_2O$	0.81	L-asparagine	0.80
$MnSO_4 \cdot 4H_2O$	0.22	L-cysteine	0.80
$FeSO_4 \cdot 7H_2O$	0.14	L-glutamate	2.10
Di-ammonium hydrogen citrate	8.84	L-glutamine	0.70
K_2HPO_4	11.48	Glycine	2.70
$CaCO_3$	1.00	L-histidine	0.30
Piridoxal-5-phosphate monohydrate	0.19	l-isoleucine	0.80
Tween 80	1 g/l	L-leucine	0.80
		L-lysine HCl	1.40
Vitamin (µM)		L-methionine	0.70
Biotin	0.40	L-phenylalanine	2.20-4.40
Folic acid	2.30	L-proline	2.60
Riboflavin	2.60	L-serine	2.90
Niacinamide	8.00	L-theonine	1.70
Thiamine HCl	3.00	L-tryptophan	0.50
Pantothenate	2.00	L-tyrosine	2.20-4.40
		L-valine	0.90
Micro nutrient (µM)			
$(HH_4)_6Mo_7O_{24}$	0.003		
H_3BO_3	0.400		
$CoCl_2$	0.030		
$CuSO_4$	0.010		
$ZnSO_4$	0.010		

Table 1: Synthetic media composition (adapted from Jensen and Hammer, 1993).

After 24 h of pre-cultivation, the culture were inoculated at a concentration of approximately 5.3-5.5 log cfu/ml in synthetic medium with different combination of tyrosine (tyr) and phenylalanine (phe) concentration: 0 mM tyr-0 mM phe; 0 mM tyr-4.4 mM phe; 4.4 mM tyr-0 mM phe; 2.2 mM tyr-2.2 mM phe and 4.4 mM tyr-4.4 mM phe. The samples were incubated at 20°C, 30°C and 40°C for 200h.

4.3.2.2. Growth parameters

The evaluation of enterococcal growth in the different media was performed by measuring the OD_{600} with a UV-VIS spectrophotometer (Jenway, 6705UV-Vis, Staffordshire, UK). The OD_{600} data were fitted with the Gompertz equation as modified by Zwietering *et al*. (1990).

$$y = Ae^{-e^{\left[\left(\frac{\mu_{\max} e}{A}\right)(\lambda - t)+1\right]}}$$

where y is the OD_{600} at time t, A represent the maximum OD_{600} value reached, μ_{max} is the maximum OD_{600} increase rate in exponential phase and λ is the lag time.

4.3.2.3.Biogenic amine determination

One ml of each culture obtained according to the condition described in paragraph 4.3.2.1. was centrifuged at 6000 rpm for 10 min at 6°C, then the supernatant were collected for BAs determination by HPLC after derivatization with dansyl-chloride (Sigma-Aldrich, Gallarate, Italy) according to Tabanelli *et al*. (2012). Tyramine and 2-phenylathylamine contents were analyzed using a PU-2089 Intelligent HPLC quaternary pump, Intelligent UV–VIS multiwavelength detector UV 2070 Plus (Jasco Corporation, Tokio, Japan) and a manual Rheodyne injector equipped with a 20 µl loop (Rheodyne, Rohnert Park, CA). The quantification of the amines was performed as follows: gradient elution 0-5 min phosphate buffer (pH 7.0)/acetonitrile 35:65, 5-6 min water/acetonitrile 20/80, 6-15 min water/acetonitrile 10/90, 15-25 min phosphate buffer (pH 7.0)/acetonitrile 35:65 with flow rate 0.8 mL/min. The amount of tyramine and 2-phenylethylamine were expressed as mM by reference to a calibration curve obtained with standard solutions.

4.3.2.4. RNA extraction and Real Time PCR

The strains *E. faecalis* EF37 and ATCC 29212 were pre-cultivated at 37°C in synthetic medium without tyrosine and phenylalanine. After 24 h the cells were collected by centrifugation at 6000 rpm for 10 min and washed twice with physiological solution (0.9% w/v NaCl). The strains were resuspended in the same solution and inoculated at a concentration of approximately 8.2-8.5 log cfu/ml in phosphate/citrate buffer (obtained by mixing citric acid 0.3 M and Na_2HPO_4 0.6 M solutions) with two different pH, 5.5 and 7, and two different tyrosine concentration, 0.28 and 4.4 mM, and incubated at 30°C for 1 h. The determination RNA extraction was performed after 15, 30 and 60 min of incubation.

Two ml of each culture were centrifuged at 10000 rpm to collect the pellet. Cells were washed twice with 500 µl of sterile diethyl pyrocarbonate (DEPC) treated water, then five cycle of bead beater (Mini-BeadBeater, BioSpec Products, Bartlesville, Okla) of 1 min, interspersed with 1 min in ice, were performed with 500 µl of a LETS solution (200 mM LiCl, 20 mM EDTA, 20 mM Tris, 0,4% SDS, 0,1% DEPC), 250 mg of 450 µm diameter glass beads (Sigma-Aldrich, Gallarate, Italy), 500 µl of a phenol-chloroform-isoamyl alcohol solution (25:24:1 v/v; Sigma-Aldrich, Gallarate,

Italy). After centrifugation (4°C, 13000 rpm, 10 min), the supernatant was treated with 600 µl of a chloroform-isoamyl alcohol solution (24:1 v/v; Sigma-Aldrich, Gallarate, Italy). The supernatant was collected after centrifugation and were added 60 µl of 3 M potassium acetate and 1 ml of ice-cold absolute ethanol and left for 1 h at -80 °C. Total RNA was pelleted by centrifugation at 13000 rpm for 15 min at 4°C, washed with 500 µl of ethanol 70%, and dissolved in 35 µl of diethyl pyrocarbonate (DEPC) treated water.

DNA elimination was performed using 50 U of RNase-free DNase I recombinant (Roche Diagnostic, Germany) in 50 µl of DNAse reaction buffer (40 mM Tris-HCl, 10 mM NaCl, 6 mM MgCl2, 1 mM CaCl2, pH 7.9) for 70 min at 25°C. A PCR assay was carried out to check for any contaminating DNA, and, when necessary, the DNase treatment was repeated.

RNA integrity, concentration, and purity were checked by electrophoresis on a 1,5% (wt/vol) agarose gel and by measurement with the NanoDropTM Lite Spectrophotometer (Thermo Fisher Scientific Inc. MA, USA). DNA-free RNA samples were stored at -80°C until use.

A tyrDC fragment of about 336 bp was amplified using the primers DEC5 (5'-CGT TGT TGG TGT TGT TGG CAC NAC NGA RGA RG-3') and DEC3 (5'-CCG CCA GCA GAA TAT GGA AYR TAN CCC AT-3'), following the PCR conditions described by Torriani et al., 2008. PCR product was visualized on a 1.5% agarose gel.

Total cDNA was synthesized from 1 µg of RNA using the ImProm-IITM Reverse Transcriptase kit (Promega, USA), following the manufacturer's recommendations.

The expression level of the *tyrDC* gene was analyzed by a RT-qPCR assay with primers TYR3f (5'-CGT ACA CAT TCA GTT GCA TGG CAT-3') and TYR4r (5'-ATG TCC TAC TTC TTC TTC CAT TTG-3'); thermo cycler, reaction mixture, and amplification program were described in Torriani et al. (2008), as well as the procedure of the absolute quantification of the tyrDC copies number.

4.3.2.5. Statistical analysis

The growth model was fitted using the statistical package Statistica for Windows 6.1 (Statsoft Italia, Vigonza, Italy).

4.3.3. Results

4.3.3.1. Growth kinetics of *Enterococcus faecalis* strains in defined medium

The strains *E. faecalis* EF37 and *E. faecalis* ATCC 29212 were pre-grown in the presence or in the absence of the *tyrDC* inducer (tyrosine) and then inoculated in a synthetic medium containing different amounts of tyrosine and phenylalanine. The growth was indirectly monitored by

evaluating the OD_{600} over time. The data obtained were modelled using the Gompertz equation as modified by Zwietering *et al*. (1990) and the estimates of the resulting parameters (A, μ_{max} and λ) for both the strains are shown in Figure 1.

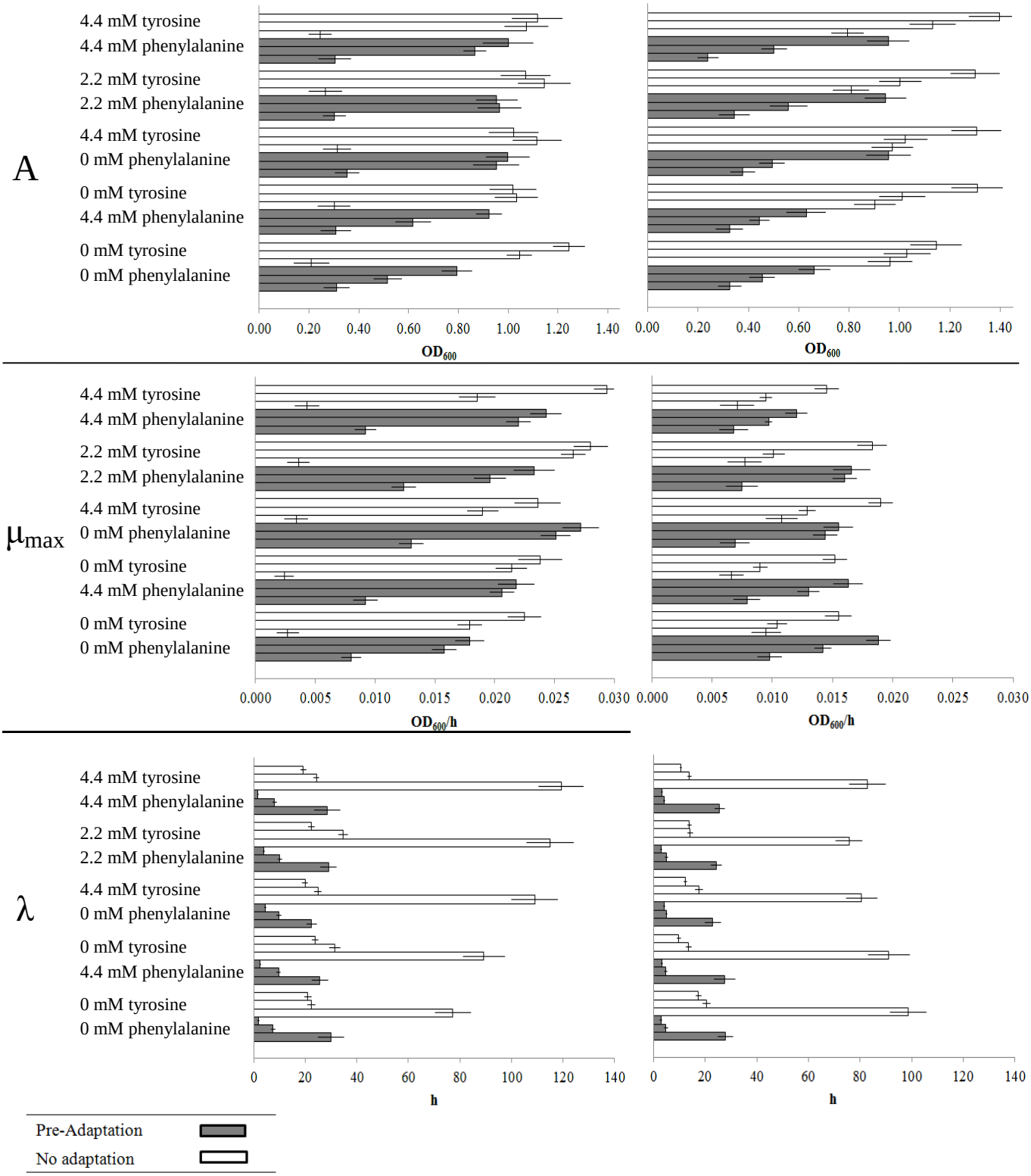


Figure 1: Gompertz equation parameters for enterococcal growth measured as OD_{600} in the five synthetic media. Each group of white or grey bar represent the sample at 40°C, 30°C and 20°C from top to bottom.

Independently on the pre-grown conditions, all the parameters were affected by the temperature of incubation and, as expected, while *A* and μ_{max} increased with temperature, λ showed an opposite

trend. Regarding the strain *E. faecalis* EF37, the *A* parameter was scarcely affected by the pre-adaptation of cells, at least in the presence of tyrosine, while, where no precursors were not added, the pre-adapted cells were characterized by lower *A* values. By contrast, in all the conditions tested lower *A* estimates were observed using pre-adapted *E. faecalis* ATCC 29212 with respect to the not adapted cells.

The main differences regarding μ_{max} in *E. faecalis* EF37 concerned the samples incubated at 20°C, which had μ_{max} markedly higher when pre-adapted. Less relevant differences were found among the samples incubated at 30 and 40°C. The adaptation in *E. faecalis* ATCC 29212 did not determined significant differences in the μ_{max} values at 20°C, while this parameter was always higher in the pre-adapted samples at 30°C. In samples containing tyrosine incubated at 40°C, μ_{max} values were higher in not pre-adapted cells.

The major differences related to the pre-adaptation concerned the λ estimates. In fact, in *E. faecalis* EF37 incubated at 20°C the absence of adaptation markedly prolonged λ (three times and more), even in the samples in which neither tyrosine or phenylalanine were added. Remarkable differences were observed also at 30 and 40°C. An analogous behavior was recorded also for *E. faecalis* ATCC 29212.

4.3.3.2. Tyramine and 2-phenyethylamine accumulation

The media containing different amounts of tyrosine and phenylalanine were periodically analysed to determine the quantity of tyramine and 2-phenylethylamine produced during the incubation at different temperatures. The results obtained for tyramine are shown in Figure 2 and 3.

For the strain *E. faecalis* EF37, the induction of tyrDC by pre-growing the strain in the presence of precursor determined a faster tyramine accumulation rate. When tyrosine was added at 2.2 and 4.4 mM the maximum biogenic amine concentration was reached after about 16, 24 and 48 h of incubation at 40, 30 and 20°C, respectively. In the sample added with 2.2 mM of tyrosine the yield of tyrosine conversion ranged between 1.85 and 1.99 mM, very close to the maximum theoretical yield. In the samples added with tyrosine 4.4 mM, tyramine was accumulated at concentration comprised between 2.70 and 3.06 mM. In the absence of tyrosine added (0 mM tyrosine-0 mM phenylalanine, 0 mM tyrosine-4.4 mM phenylalanine), a small tyramine accumulation (about 0.15 mM) was evidenced only in the pre-adapted enterococci. This is presumably due to the intracellular turnover and synthesis of amino acids, indicating a decarboxylase activity even in the absence of an external precursor induced by the presence of the precursor in the cytoplasm. When the cells were not adapted, the tyramine accumulation was extremely slowed down. In the presence of tyrosine added at 2.2 and 4.4. mM, the rate of tyramine production was again related to the temperature

(40°C>30°C>20°C) but the biogenic amine accumulation started after about 24 h at 40°C, 48 h at 30°C and 150 h at 20°C. Moreover, with the exception of the samples at 40°C, which reached maximum BA concentrations comparable with the adapted cells, the rate of tyramine accumulation was slower and its amount at the end of incubation (192 h) was markedly lower if compared with the respective condition using adapted cells. Using not adapted cells the tyramine accumulation was negligible in the absence of both tyrosine and phenylalanine, while small amount (about 0.1 mM) were detected at 0 mM tyrosine and 4.4 mM phenylalanine, even if the amounts were lower than those observed under the same conditions using adapted cells.

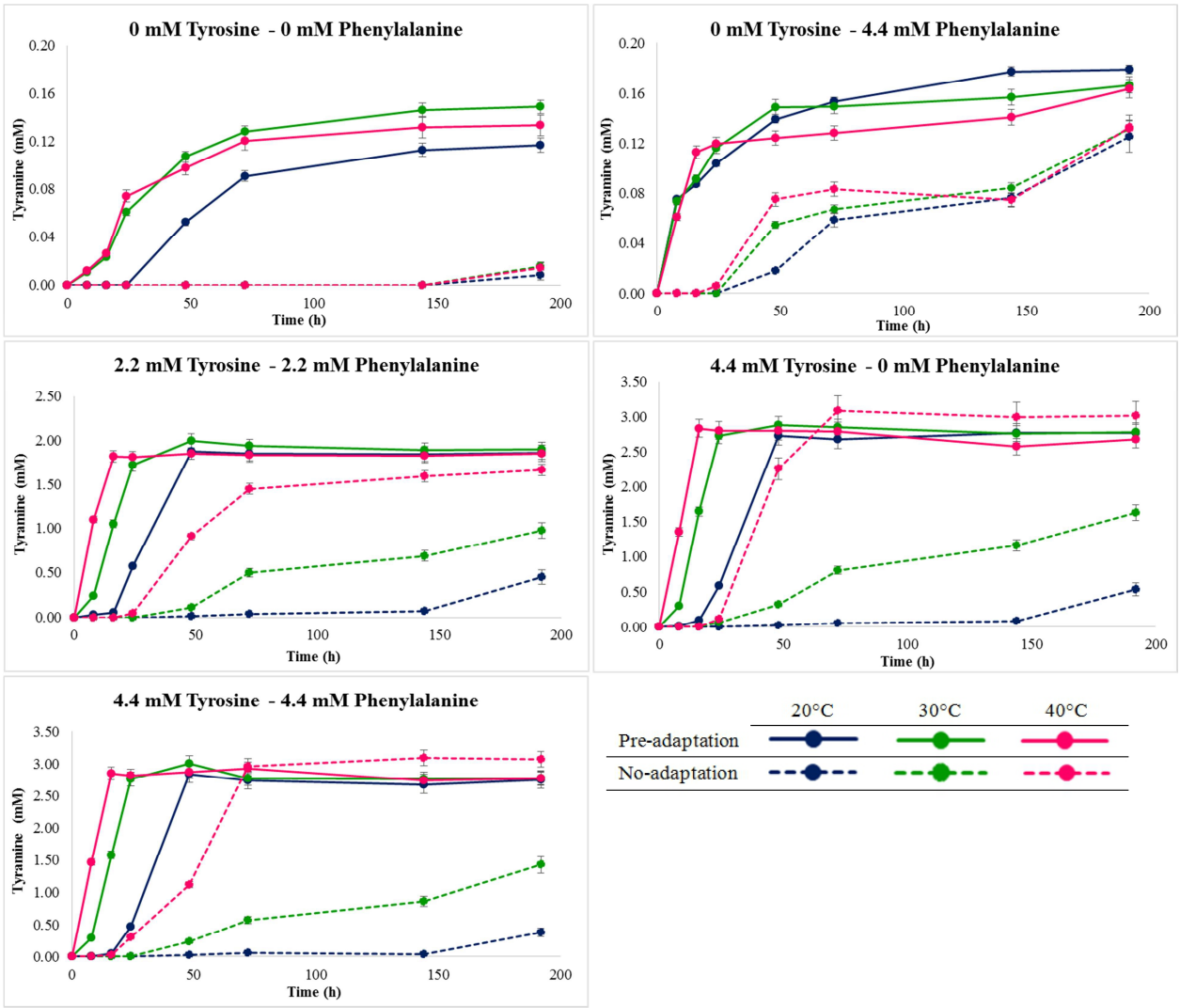


Figure 2: Amounts of tyramine produced in the five synthetic media by *E. faecalis* EF37 with or without pre-adaptation during the incubation at different temperatures.

The strain *E. faecalis* ATCC 29212 showed a quite different behavior (Figure 3). In all the conditions, the tyramine accumulation was lower than that observed using the strain EF37. In addition, regarding the samples containing tyrosine, surprisingly tyramine accumulation was faster and higher in the samples inoculated with not adapted cells at 40 and 30°C, while negligible

differences were observed at 20°C. In all the cases, the final tyramine amount (after 192 h) was strongly dependent on the temperature and it never exceeded 1.2 mM, with the exception of the samples at 40°C inoculated with not adapted cells. In addition, the final tyramine content was always lower than 1.7 mM, independently on the initial precursor concentration.

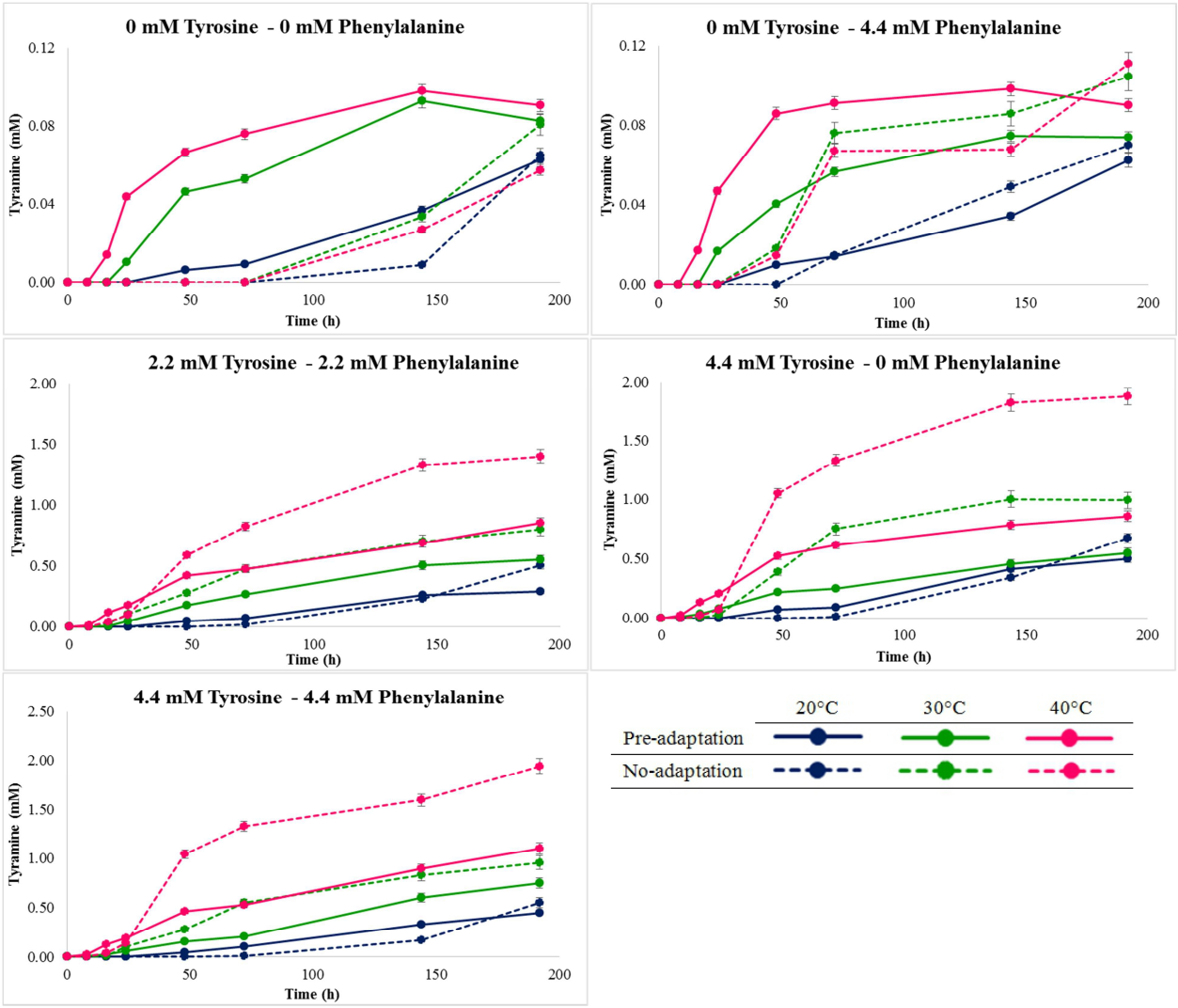


Figure 3: Amounts of tyramine produced in the five synthetic media by *E. faecalis* ATCC 29212 with or without pre-adaptation during the incubation at different temperatures.

Regarding 2-phenylethylamine production, this amine was detected in very low amount (<0.04 mM) in all the samples inoculated with *E. faecalis* ATCC 29212, independently on the induction of the cells (data not shown). Similar low levels of the amine were found when the medium was inoculated with not adapted *E. faecalis* EF37 cells and with adapted cells of the same strain in the conditions 0 mM tyrosine-0 mM phenylalanine and 4.4 mM tyrosine-0 mM phenylalanine. Hovewer, in the presence of phenylalanine, pre-induced cells were able to accumulate 2-phenylethylammine. This conversion was characterized by a lower yield if compared with tyramine. In fact, in the more favorable conditions, *E. faecalis* EF37 accumulated 0.8-0.9 mM in the presence

of phenylalanine 4.4 mM and about 0.45 mM in the presence of phenylalanine 2.2 mM (Figure 4). At 40°C the amine was accumulated more rapidly than at 30°C in the first 24 h of incubation; however, in the successive sampling times the amount were similar. In the samples incubated at 20°C the accumulation was slower and started after 48 h.

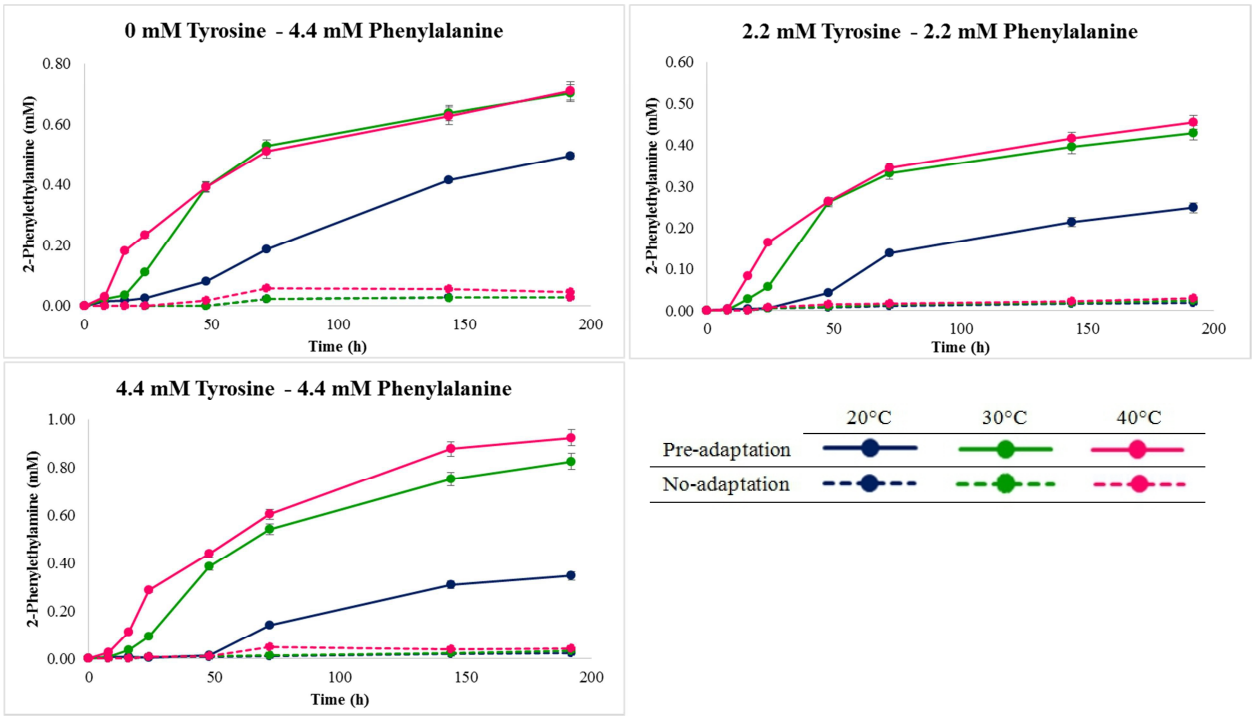


Figure 4: Amounts of 2-phenylethylammine produced by *E. faecalis* EF37 with or without pre-adaptation during the incubation at different temperatures in the media where phenylalanine was added.

4.3.3.3. Relationships between cell concentration and biogenic amine accumulation

In Table 2 the growth level of the strains after 72 h is reported. The data are expressed as percentage of the maximum OD_{600} (according to the Gompertz parameter *A* reported in Figure 1) reached after this time. For the adapted cells of both the strains this values was very high, indicating that the maximum cell concentration was almost reached. In the samples inoculated with *E. faecalis* EF37 this value was higher than 96% at 30 and 40°C; only the samples incubated at 20°C had lower percentages, which were, in any case, higher than 86%. A similar behaviour was evidenced also for *E. faecalis* ATCC 29212, with slightly lower values, especially at 20°C.

Completely different was the situation of the not pre adapted cells. The growth at 20°C after 72 h was very low (always inferior to 4%) for both the strains. *Enterococcus faecalis* EF37 reached after 72 h an OD_{600} ranging between 51.5 and 77.2% of the maximum estimated at 30°C, while *E. faecalis* ATCC 29212 had performances comprised between 48.7 and 65.6%. Higher growth

percentage were reached by the strains at 40°C (between 86.7 and 93.9% for *E. faecalis* EF37, and between 61.6 and 83.8% for *E. faecalis* ATCC 29212).

Synthetic media	**Temperature**	***E. faecalis* EF37**		***E. faecalis* ATCC 29212**	
		Adapted cells	Not adapted cells	Adapted cells	Not adapted cells
Tyrosine 0 mM Phenylalanine 0 mM	20	86.7	3.8	93.0	0.4
	30	98.7	51.5	99.1	51.7
	40	96.4	86.7	98.7	69.6
Tyrosine 0 mM Phenylalanine 4.4 mM	20	93.8	1.9	86.8	1.9
	30	99.0	75.8	98.7	51.7
	40	96.9	88.1	97.9	68.3
Tyrosine 4.4 mM Phenylalanine 0 mM	20	98.1	< 0.1	79.1	3.0
	30	96.9	73.5	98.7	65.6
	40	98.2	90.1	92.2	83.8
Tyrosine 2.2 mM Phenylalanine 2.2 mM	20	91.5	< 0.1	85.2	4.0
	30	97.8	77.2	98.5	57.3
	40	97.1	92.3	90.4	79.9
Tyrosine 4.4 mM Phenylalanine 4.4 mM	20	92.7	< 0.1	92.8	1.1
	30	96.7	74.8	92.7	48.7
	40	97.4	93.9	92.2	61.6

Table 2: Growth level of the strains *E. faecalis* EF37 and *E. faecalis* ATCC 29212 at the different conditions after 72 h. The data are expressed as percentage of the maximum OD_{600} (the A parameter of the Gompertz equation) reached after this time.

For the samples incubated with not pre adapted cells, in which the growth rates were significantly slower the adapted ones, also the precursor, i.e. tyrosine and phenylalanine, were quantified after 72 h, in addition to the BAs (Table 3). As already observed, in the media inoculated with *E. faecalis* EF37, after 72 h tyramine had already reached its maximum concentration in all the samples with pre-adapted cells (Figure 2). The pre adapted cells of *E. faecalis* ATCC 29212 after 72 h produced tyramine amounts below those obtained at the end of the incubation period (Figure 3). In addition, the pre adapted cells of the strain EF37 after 72 produced also 2-phenylethylamine (about the half of the final amount).

In the samples at 20°C (characterized by a lower growth extent after 72 h) the production of BAs was negligible. However, the amount of the precursors was slightly reduced in *E. faecalis* EF 37; this trend was particularly evident in the media containing 4 mM of tyrosine and/or phenylalanine. A similar behaviour was recorded also for *E faecalis* ATCC 29212, which, at the same temperature,

showed a more intense phenyalanine consumption (up to 1 mM) in the absence of 2-phenylethylamine production.

The media containing *E. faecalis* EF37 incubated at 30°C were characterized by a more intense amino acid consumption. Tyrosine was depleted more intensively than phenylalanine, and only partially decarboxylated to tyramine. Similar tyramine concentration were recorded in *E. faecalis* ATCC 29212 which also consumed higher phenylalanine concentration without producing 2-phenylalanine. An almost complete consumption of tyrosine was observed in the sample incubated at 40°C inoculated with *E. faecalis* EF37 even if the concentration of tyramine was far from the maximum theoretical concentration (about 2.9 mM in the sample containing tyrosine 4.4 mM and 1.4 mM with tyrosine 2.2 mM). Phenylalanine concentration further decreased if compared with the sample at 30°C. At the same temperature, the presence of *E. faecalis* ATCC 29212 caused a less marked diminution of tyrosine content (with a consequent lower amounts of tyramine), while the concentrations of phenylalanine were comparable with the values observed for *E. faecalis* EF37.

Synthetic media	Biogenic amine/precursor	*E. faecalis* EF37			*E. faecalis* ATCC 29212		
		20°C	30°C	40°C	20°C	30°C	40°C
Tyrosine 0 mM Phenylalanine 0 mM	Tyramine	-*	-	-	-	-	-
	Tyrosine	-	0.06 (0.04)	0.03 (0.02)	-	0.07 (0.04)	0.06 (0.03)
	2-Phenylethylamine	-	-	-	-	-	-
	Phenylalanine	-	0.14 (0.06)	-	-	0.15 (0.06)	0.10 (0.05)
Tyrosine 0 mM Phenylalanine 4.4 mM	Tyramine	0.08 (0.05)	0.07 (0.03)	0.08 (0.04)	0.01 (0.02)	0.08 (0.04)	0.07 (0.02)
	Tyrosine	0.01 (0.02)	-	-	-	0.01 (0.03)	0.10 (0.02)
	2-Phenylethylamine	0.01 (0.01)	0.02 (0.03)	0.06 (0.04)	-	-	-
	Phenylalanine	4.13 (0.35)	3.58 (0.19)	3.03 (0.25)	3.60 (0.12)	3.05 (0.18)	2.85 (0.16)
Tyrosine 4.4 mM Phenylalanine 0 mM	Tyramine	0.06 (0.03)	0.80 (0.10)	2.91 (0.10)	0.01 (0.01)	0.75 (0.08)	1.33 (0.05)
	Tyrosine	3.94 (0.29)	2.91 (0.16)	0.08 (0.05)	4.19 (0.27)	3.27 (0.20)	2.50 (0.13)
	2-Phenylethylamine	-	-	0.01 (0.02)	-	-	-
	Phenylalanine	-	0.19 (0.02)	-	-	0.16 (0.04)	0.13 (0.03)
Tyrosine 2.2 mM Phenylalanine 2.2 mM	Tyramine	0.04 (0.02)	0.50 (0.04)	1.45 (0.08)	0.02 (0.01)	0.48 (0.06)	0.82 (0.09)
	Tyrosine	2.14 (0.12)	1.48 (0.07)	0.04 (0.01)	2.12 (0.08)	1.58 (0.14)	1.46 (0.12)
	2-Phenylethylamine	0.02 (0.01)	0.02 (0.01)	0.02 (0.02)	-	0.02 (0.01)	0.02 (0.02)
	Phenylalanine	2.17 (0.10)	1.90 (0.12)	1.35 (0.06)	1.65 (0.07)	1.46 (0.08)	1.32 (0.15)
Tyrosine 4.4 mM Phenylalanine 4.4 mM	Tyramine	0.06 (0.00)	0.57 (0.06)	2.96 (0.12)	0.01 (0.01)	0.55 (0.08)	1.33 (0.10)
	Tyrosine	4.25 (0.24)	3.43 (0.17)	0.14 (0.03)	4.19 (0.22)	3.55 (0.16)	2.65 (0.11)
	2-Phenylethylamine	0.02 (0.01)	0.02 (0.01)	0.04 (0.03)	-	0.02 (0.02)	0.02 (0.03)
	Phenylalanine	3.93 (0.16)	3.47 (0.22)	2.94 (0.19)	3.46 (0.15)	3.05 (0.10)	2.90 (0.07)

Table 3: Biogenic amines and amino acids precursor quantification after 72 h of incubation of *E. faecalis* EF37 and *E. faecalis* ATCC 29212 in the different media. The data are expressed in mM and the standard deviation is reported within the brackets.

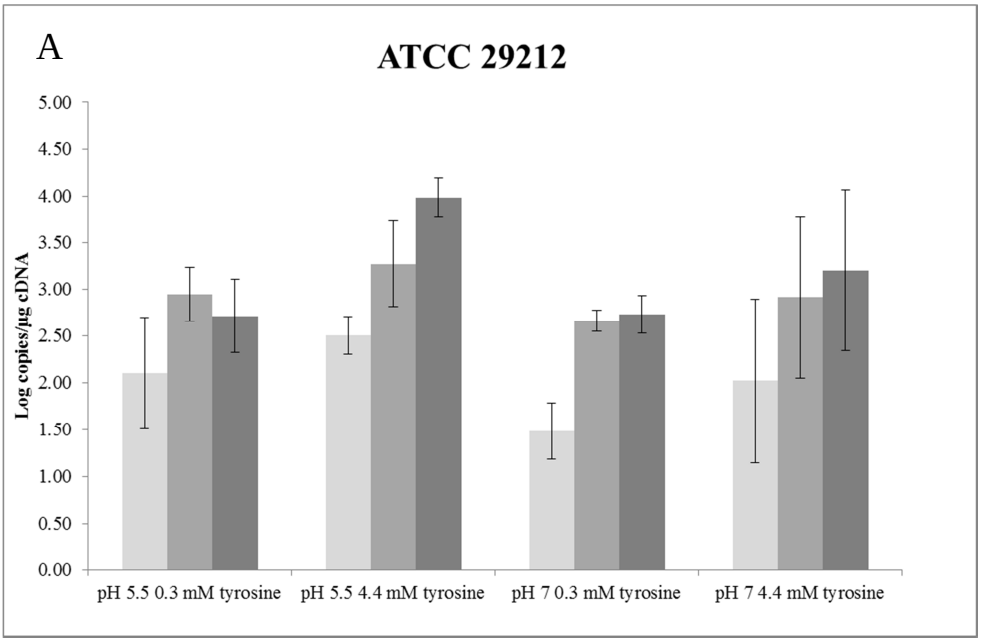
*: Under the detection limit

4.3.3.4. Expression of the gene *tyrDC* in synthetic media

A previous investigation has demonstrated that the gene *tyrDC* of the strains *E. faecalis* ATCC 29212 and EF37 showed different transcriptional behaviour during growth in BHI medium (Bargossi *et al*., 2015a). These results were in accordance with the ability of these strains to accumulate different amounts of tyramine. Furthermore, it was observed that the mRNA level was high after 2 h incubation for both the strains in BHI added or not with tyrosine. Gene transcription could probably be affected by the tyrosine present in the pre-cultivation medium or could be modulated just after the exposure to the substrate. Thus, in order to deeply investigate the expression of the gene *tyrDC* in these enterococcal strains, further analysis were carried out in short time distances after inoculation of cells pre-cultivated in absence of precursor into a synthetic medium with different amounts of tyrosine (0.3 and 4.4 mM) and pH (5.5 and 7.0).

As shown in Figure 4a, the expression levels of the gene *tyrDC* in the strain ATCC 29212, calculated by the absolute quantification method previously described (Torriani *et al*., 2008), increased gradually in the condition with 4.4 mM tyrosine, while the expression level reached high values [up to 4 log (copies/µg cDNA)] after 30 min at the lower substrate concentration. Acidic pH had a moderate effect on the *tyrDC* transcription in presence of high amounts of tyrosine.

As regard the strain EF37 (Figure 4b), a similar expression trend was observed in presence of 4.4 mM tyrosine, i.e. a gradual augment, but more appreciable at low pH. Differently from the strain ATCC 29212 the maximum gene expression [up to 3.5 log (copies/µg cDNA)] was reached only after 15 min of incubation both at pH 5.5 and 7.0.



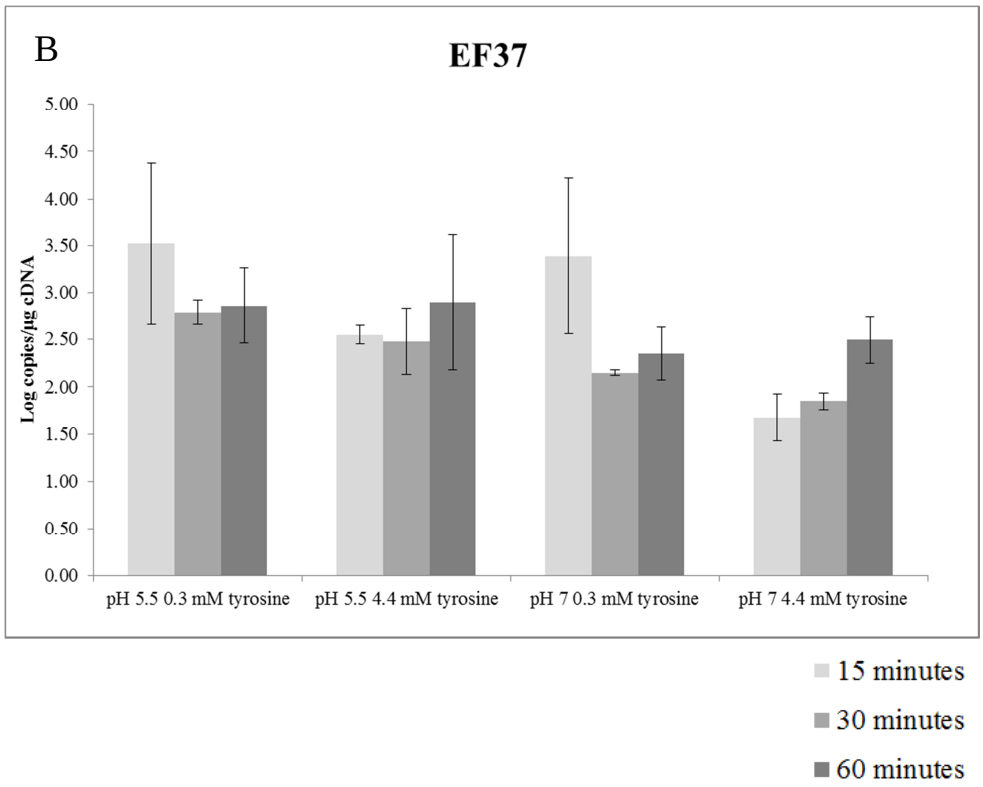


Figure 4: Expression of the gene tyrDC in the strains *Enterococcus faecalis* EF37 and ATCC 29212 after inoculation into a synthetic medium up to 60 min.

Table 4 reports the fold-changes of the *tyrDC* transcript for each strain in all the assayed conditions, where the condition with the lowest level of expression was selected as the calibrator for all experiments. These data underline more evidently the different short-term transcriptional response of the two strains. Indeed, the presence of 0.3 mM tyrosine induced the *tyrDC* transcription in both the strains, but EF37 showed the highest response in the shortest time. Otherwise, the strain ATCC 29212 reached high concentration of mRNA only after 1 h in presence of 4.4 mM tyrosine. Also, pH had a strong impact on the transcription levels, since the highest values were observed at pH 5.5.

Strain	Time (min)	mRNA relative levels			
		pH 5.5 0.3 mM tyrosine	pH 5.5 4.4 mM tyrosine	pH 7 0.3 mM tyrosine	pH 7 4.4 mM tyrosine
ATCC 29212	15	4.14	9.11	1.00	8.73
	30	28.22	71.95	12.59	64.84
	60	17.24	274.43	15.84	119.76
EF37	15	177.79	6.81	133.21	1.00
	30	12.15	7.13	2.68	1.34
	60	6.21	3.83	2.66	6.21

Table 4: Relative *tyrDC* transcript amount under different substrate and pH conditions.

4.3.4. Discussion

The pre-induction obtained by incubating the pre-cultures in the presence of tyrosine favoured the growth performances in the synthetic media independently on the presence of tyrosine or phenylalanine added. This fact was not necessarily correlated with a higher and/or faster tyramine and 2-phenylethylamine accumulation in the strain *E. faecalis* ATCC 29212. In fact, in the strain *E. faecalis* ATCC 29212, in spite of the pre-adaptation, tyramine accumulation was higher and faster in not pre-adapted cells. However, *E. faecalis* ATCC 29212 was characterized by a production of tyramine which took place only after the beginning of the stationary phase and was absent in the presence of an active primary metabolism (i.e. sugar fermentation). This delayed production seems to determine the ineffectiveness of the preliminar pre-adaptation. In pre-induced cells, tyramine accumulation was close to the maximum theoretical yield only in the media containing tyrosine 2.2 mM, while in the samples containing tyrosine 4.4 mM the yield was lowered, indicating a possible inhibition of tyrDC when the concentration of amine increased up to a threshold level of about 3.0 mM. In any case, tyrosine was almost completely depleted in the media.

By contrast, the pre-induction was essential for the rapid accumulation of tyramine by *E. faecalis* EF37 and the use of cell in which the decarboxysative pathway was activated resulted essential to reach the maximum tyramine accumulation within the first 24 h (at 30 and 40°C) and 48 h (at 20°C) of incubation.

2-phenylethylamine was produced in relevant amount only by the strain EF37. In any case its accumulation started only when tyramine was accumulated at its maximum concentration and the yield was far from its theoretical potential, at least in the incubation period considered here.

Especially in pre-induced cells, tyramine was accumulated, though in very low amounts, also when tyrosine (and phenylalanine) was added in the medium, indicating that the decarboxylation activity is stimulated inside the cell even by the turnover of cytoplasmatic amino acids.

Tyrosine decarboxylation is a metabolic pathway widely diffused among enterococci (Marcobal *et al*., 2012). However, the resulting tyramine accumulation can be extremely heterogeneous within the genus and the species both quantitatively and quantitatively. This heterogeneity has been recently evidenced in two strains of *E. faecalis* (EF37 and ATCC 29212) and two of *E. faecium* (Bargossi *et al*., 2015a) grown in nutritionally rich or poor media added or not with tyrosine. *E. faecalis* EF37 and *E. faecium* FC12 produced tyramine in high amount since the exponential growth phase while *E. faecium* FC12 and *E. faecalis* ATCC 29212 showed a slower decarboxylase activity, which took place mainly in the stationary phase (Bargossi *et al*., 2015a). The strains differed also for their ability to produce 2-phenylethylamine, which, between *E. faecalis*, was more enhanced in EF37 strain.

The same strains were characterized by different behaviour when transferred in buffered systems containing tyrosine incubated under different conditions (temperature, NaCl concentration and pH) (Bargossi *et al*., 2015b). The work confirmed that the genetic organization and nucleotide sequence of the tyrosine decarboxylase cluster was highly conserved in the enterococcal strains of these species. In particular, the cluster sequence of *E. faecalis* EF37 and ATCC 29212 shares the same genetic organization, which comprises the four predictable complete open reading frames (ORF) corresponding to the genes *tyrS*, *tyrDC*, *tyrP* and *nhaC*. They are oriented in the same direction and encode polypeptides larger than 300 amino acids. In particular, BLASTN analysis of the 5259 bp nucleotide sequence of *E. faecalis* EF37 TDC operon region showed an overall identity of 99% (5231/5259 bp) with that of *E. faecalis* ATCC 29212, and 100% identity (5259/5259 bp) with that of another completely sequenced strain of the same species, *E. faecalis* D32 (gb|CP003726.1|). Two conservative aminoacid substitution were found (one in the amino acid permease and one in the Na^+/H^+ antiporter), which probably have no effect on the enzymatic activities (Bargossi *et al*., 2015b).

The use of the synthetic medium used in this work allowed to strictly control the amounts of precursors (tyrosine and phenylalanine) and to evaluate the effects of pre-adaptation on the metabolic potential of cells. The first evidences of these trials confirmed results obtained for these two strains in complex media (Bargossi *et al*., 2015a), according to which *E. faecalis* EF37 accumulated tyramine since the exponential phase and the maximum biogenic amine concentration was rapidly reached. By contrast *E. faecalis* ATCC 29212 delayed the tyramine production to the stationary phase. The production of tyramine during the exponential phase has been already

observed in *E. faecalis* (Pessione *et al*., 2009), while other LAB, such as *Carnobacterium divergens*, preferentially accumulated this amine in the stationary phase (Masson *et al*., 1999).
In the samples in which tyrosine was added at 2.2 mM, the pre-adapted cells of the strain *E. faecalis* EF37 showed a high final yield of tyramine, ranging between 84 and 90% with respect to the precursor added, while the same yield was considerably reduced in the presence of tyrosine 4.4 mM (from 61 to 70%). A possible inhibiting effect of increasing tyramine concentration on decarboxylase activity has already been observed in *E. durans* (Fernández *et al*., 2007a), *L. brevis* (Moreno-Arribas and Lonvaud-Funel, 1999). The higher decarboxylating activity of the tyrDC of *E. faecalis* EF37 was evident also in the accumulation of 2-phenylethylamine. The ability to decarboxylate phenylalanine by the same tyrDC responsible for tyramine production was firstly demontrated by Marcobal *et al*. (2006a,b) in *E. faecium* and it is rather common among enterococci, as observed by Bonetta *et al*. (2008) in *E. faecalis*. A similar behaviour was found in *L. curvatus* strains isolated from sausages, many of which were able to decarboxylate both the amino acids (Aymerich *et al*., 2006); the same aptitudes was less diffused among *L. brevis* strains from wine (Landete *et al*., 2007). In any case, usually phenylalanine is decarboxylate only when tyrosine is depleted and with a minor efficiency (Gardini *et al*., 2008; Marcobal *et al*., 2012; Bargossi *et al*., 2015a). In this work, under the adopted conditions only EF37 was able to accumulate significant amount of 2-phenylethylamine in the presence of the precursor, confirming a trend observed by Bargossi *et al*. (2015a). These trends were also confirmed by the transcriptional response of the two strains. The transcription of tyrDC was more rapid and efficient in *E. faecalis* EF37 and the pH at which it resulted higher was 5.5. These findings suggest a relevant effect of the growth medium composition on the gene *tyrDC* that is probably regulated by different mechanisms in the two studied strains. Indeed the two strains tested showed great differences in the mRNA levels of the gene involved in tyramine production. These transcriptomic differences were observed from the beginning of growth and can account for the different production of tyramine detected for these strains.

4.3.5. Conclusion

Significant results, coming from the present investigation, underline differences between enterococcal strains both at transcriptional and metabolic level.
An interesting result of this work was the evidence that pre-adapted cells (pre-grown on tyrosine) of both the strains grew faster than not pre-adapted ones, independently on the addition of tyrosine and phenylalanine. The better growth performances of pre-adapted cells were mainly linked to a drastic reduction of the λ parameter of the OD_{600} growth curves, especially in the samples incubated at

20°C. The improvement of growth parameters was not necessarily associated to the tyrDC activity, as demonstrated by the faster growth curve in the absence of tyrosine and phenylalanine, and by the growth parameters estimated for *E. faecalis* ATCC 29212 for which the faster growth was not associated by a concomitant increase of tyramine. Indeed, Pessione *et al*. (2009) in their proteomic study on the tyrDC activity on phenylalanine and tyrosine, demonstrated that the pre-induction of *E. faecalis* cells grown in the presence of tyrosine determined marked increases of the expression of *tyrDC*, but also many other important gene shown an increased expression. In particular, the pre-adaptation increase the transcription of genes of the glycolytic pathway (such as mannose-6-p-isomerase, phosphoglycerate mutase, enolase and pyruvate kinase), pyruvate metabolism (lactate dehydrogenase, pyruvate dehydrogenase, formate acetyltransferase) as well as enzymes involved in purine and pyrimidine metabolism, ABC trasporters as well as some stress proteins (such as DNAj and gls 24). In other words, the pre-adaptation in the presence of tyrosine stimulated not only the transcription of the tyrosine decarboxylase cluster; other genes which can confer competitive advantages to the cells were transcribed in higher proportion by enterococci in the presence of tyrosine.

4.4. Tyrosine decarboxylase activity of *Enterococcus mundtii*: new insights on phenotypic and genetic aspects.

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Running title: Tyramine production by *Enterococcus mundtii*

ABSTRACT

The capability to accumulate tyramine and 2-phenylethylamine by two strains of *Enterococcus mundtii* was evaluated in a cultural medium added or not with 1 g/l tyrosine. Both the strains possessed a tyramine decarboxylase which determined tyramine accumulation from the beginning of exponential phase of growth, independently on the tyrosine addition. The strains accumulated also 2-phenylethylamine, although with lower efficiency and in greater extent when tyrosine was not added to the medium. Accordingly, the tyrosine decarboxylase (*tyrDC*) gene expression level increased during the exponential phase with tyrosine added, while it remained constant and high without precursor. The genetic organization as well as sequence identity levels of *tyrDC* and tyrosine permease (*tyrP*) genes indicated that the tyramine-forming pathway in *E. mundtii* is similar to those in phylogenetically closer enterococcal species, such as *E. faecium*, *E. hirae*, and *E. durans*; however the gene Na^+/H^+ antiporter (*nhaC*), that usually follow *tyrP* is missing. In addition, analysis of available genomic data of *E. mundtii* QU 25 and other *Enterococcus* strains revealed an unexpectedly presence of additional genes encoding for decarboxylase and permease. It is speculated the occurrence of a duplication event and the acquisition of different specificity for these enzymes that deserves further investigations.

Key words: Enterococcus mundtii, tyramine, 2-phenylethylamine, *tyrDC* gene expression, TDC operon

4.4.1. Introduction

Tyramine is a biogenic amine (BA) deriving from tyrosine decarboxylation and can have severe acute effects if ingested in excessive amounts with food, consisting in peripheral vasoconstriction, increased cardiac output, accelerated respiration, elevated blood glucose and release of norepinephrine, symptoms known also as "cheese reaction" (Shalaby, 1994; McCabe-Sellers *et al.*, 2006; Marcobal *et al.*, 2012). Tyrosine decarboxylase, the enzyme responsible for tyramine production, can use as substrate also phenylalanine, producing 2-phenylethylamine, whose adverse effects are similar to tyramine (Marcobal *et al.*, 2006a).

In general, the amino acid decarboxylation leading to BA formation provides metabolic energy and⁄or resistance against acid stress (Molenaar *et al.*, 1993; Fernández and Zúñiga, 2006; Pereira *et al*., 2009). The microorganisms responsible for tyramine accumulation in foods belong mainly to the group of lactic acid bacteria (LAB) (Marcobal *et al.*, 2012). Among LAB, species belonging to the genus *Enterococcus* are recognized as the most frequent and intensive tyramine producers (Leuschner *et al*., 1999; Suzzi and Gardini, 2003, Ladero *et al*., 2012).

Due to their salt and pH tolerance, and to their ability to grow over a wide temperature range, enterococci are isolated from different habitats and are often contaminants in food of animal origin, such as cheese and sausages (Franz *et al*., 2011; Giraffa, 2003). In spite of their homolactic metabolism, their potential role in cheese ripening and their ability to produce bacteriocins (Fontana *et al.*, 2015; Beshkova and Frengova, 2012), enterococci have a controversial status and they are often considered at the crossroad of food safety (Franz *et al*., 1999). In fact, this group is considered as indicator of the hygienic quality of raw material and food, as well as marker of fecal contamination (Leclerc *et al*., 1996). In addition, virulence factors can be present (Foulquié-Moreno *et al*., 2006; Hollenbeck and Rice, 2012) and they can act as opportunistic human pathogens frequently associated with nosocomial infections due to their antibiotic resistance with a high capacity to disseminate this resistance to other microorganisms (Giraffa, 2002; Klein, 2003; Rossi *et al*., 2014). Furthermore, they are strong tyramine producers and this ability has been deeply exploited in *Enterococcus faecalis* (in which tyramine production is considered a species trait), *Enterococcus faecium* and *Enterococcus durans* (Linares *et al*., 2009; Ladero *et al*., 2012; Bargossi *et al*., 2015a; Bargossi *et al*., 2015b). For this reasons, the presence of enterococci has been put in relation with the presence of tyramine in several fermented foods, such as fermented sausages (Gardini *et al*., 2008), cheeses (Linares *et al*., 2011) and wine (Pérez-Martin *et al*., 2014).

The enterococcal species most frequently isolated from fermented foods are *E. faecalis* and *E. faecium*, but also *E. durans, Enterococcus gallinarum, Enterococcus casseliflavus, Enterococcus hirae* can be found in food matrices (Franz *et al.*, 2003; Giraffa, 2003; Foulquié Moreno *et al*.,

2006; Komprda *et al*., 2008b, Corsetti *et al*., 2007). Recently, also *E. mundtii* has been isolated from the food chain; it is a non-motile, yellow-pigmented enterococcus infrequently associated to human infection (Collins and Farrow, 1986; Higashide *et al*., 2005). Strains of *E. mundtii* have been isolated from soy and cereals (Todorov *et al*., 2005; Corsetti *et al*., 2007), water (Moore *et al*., 2008; Graves and Weaver, 2010; Furtula *et al*., 2013), soil (Collins *et al.*, 1986; Bigwood *et al*., 2012) and forage grass or silage, in which this species is often the predominant among enterococci (Muller *et al*., 2001; Ni *et al*., 2015). It has also been isolated from animals (Espeche *et al*., 2014; Collins *et al*., 1986) and from food (Schöbitz *et al*., 2014; Vera Pingitore *et al*., 2012).

This species has been deeply studied in relation to the bacteriocin produced, among which mundticine (de Kwaadsteniet *et al*., 2004; Todorov *et al*., 2005; Corsetti *et al*., 2007; Feng *et al*., 2009; Vera Pingitore *et al*., 2012; Espeche *et al*., 2014).

Recently, the genome of *E. mundtii* QU 25 isolated from ovine faeces has been completely sequenced (Shiwa *et al*., 2014) and comparative analysis of the genetic content of this species with respect to other representative enterococcal species of diverse origins was conducted (Repizo *et al*., 2014). Despite to those recent acquisitions, scarce information is available about *E. mundtii* tyraminogenic potential. Trivedi *et al*. (2009) carried out a study testing the ability to decarboxylate tyrosine in several enterococci isolated from different foodstuff. Regarding *E. mundtii*, four of five strains isolated from meat products and six of 12 isolated from vegetables and fruits possessed this ability. Also Kalhotka *et al*. (2012) found an *E. mundtii* strain able to produce tyramine and agmatine. This latter amine derives from the decarboxylation of arginine and can be transformed in putrescine by a specific deiminase (Linares *et al*., 2015).

In this research, the tyramine and 2-phenylethylamine accumulation by two *E. mundtii* strains isolated from grass silage was studied during their growth in a rich medium. In addition, information on the genetic basis of the tyraminogenic potential of *E. mundtii* were obtained analysing the expression of the tyrosine decarboxylase (*tyrDC*) gene, the sequence of *tyrDC* and tyrosine permease (*tyrP*) genes, and the genetic organization of the TDC operon region.

4.4.2. Results and discussion

4.4.2.1. Tyramine-positive enterococci

In the first part of the research, 35 isolates of coccal LAB, originating from different matrices and positive for the production of tyramine according to the method of Bover-Cid and Holzapfel medium (Bover-Cid and Holzapfel, 1999) were considered. These isolates were presumptively identified as enterococci based on their physiological and morphological characteristics (von Wright and Axelsson, 2012). They were cocci, Gram-positive, catalase-negative, non-spore-forming,

occurring both as single cells and in chains. They were able to growth at 10°C and 45°C, at pH 4.4 and 9.6, and in the presence of 6.5% of NaCl.

To confirm the decarboxylase activity revealed by the Bover-Cid and Holzapfel medium, the occurrence of the gene *tyrDC*, coding for tyrosine decarboxylase (TDC), was examined. A *tyrDC* gene fragment was amplified according to Torriani *et al*. (2008). For all the 35 isolates the 336 bp amplicon was obtained, confirming their tyraminogenic potential.

Successively, RAPD-PCR fingerprinting technique with the primer 1254 (Table 1) was applied to investigate the genetic diversity of the strains.

PCR type	Target	Primer code	Sequence (5'-3')	Amplicon (pb)	Reference
RAPD-PCR	Arbitrary DNA sequences	1254	CCG CAG CCA A	Variable	Akopyanz *et al.*, 1992
RT-qPCR	*tyrDC*	TYR3f	CGT ACA CAT TCA GTT GCA TGG CAT	171	Torriani *et al*., 2008
		TYR4r	ATG TCC TAC TTC TTC TTC CAT TTG		
	tyrDC	DEC5	CGT TGT TGG TGT TGT TGG CAC NAC NGA RGA RG	350	
		DEC3	CCG CCA GCA GAA TAT GGA AYR TAN CCC AT		
Conventional	*pheS*	pheS-21-F	CAY CCN GCH SGY GAY ATG C	455	Naser *et al*., 2005
		pheS-22-R	CCW ARV CCR AAR GCA AAR CC		
	tyrS/ tyrDC	TyrS-F1	GGA GCT ATA AGT ATT AAC GGT GA	940	
		Tdc-R1	GAT TT(A/G) ATG TT(A/G) CG(G/C) GCA TAC CA		
	tyrDC	Tdc-F2	CAA ATG GAA GAA GAA GT(A/T) GGA	1340	Bargossi *et al*., 2015a
		Tdc-R2	CC(A/G/T) GCA CG(G/T) T(C/T)C CAT TCT TC		
	tyrDC/ tyrP	Tdc-F3	CCA GA(C/T) TAT GGC AA(C/T) AGC CCA	788	
		TyrP-R3	CCT AAA GTA GAA GC(A/G) ACC AT		
	tyrP	TyrP-F4	TGG GTG CAA ATG TTC CCA GG	940	
		TyrP-R4	ACC (A/G)AT TCG (A/G)TA AGG ACG		
	tyrP/ nhaC-2	TyrP-F5	(A/T)CT GCT TGG GT(A/T) ACT GGA CC	na*	
		NhaC-R5	CAT (C/T)GC AT(C/T) (A/G)T(C/T) GAA TCC AAG		

Table 1: Primers used in this study in RAPD-PCR, RT-qPCR and conventional PCR reactions and expected amplicon size.

* na: no amplicon

This molecular typing method has proved to be reliable, discriminative and suitable for the study of a large number of strains in short time (Vancanneyt *et al*., 2002). The primer 1254 generated reproducible RAPD-PCR fingerprints thanks to an accurate standardization of all the PCR and electrophoresis conditions. The reproducibility of PCR assays and running conditions was higher than 90%. Figure 1 shows the resulting dendrogram from cluster analysis of the RAPD-PCR fingerprints.

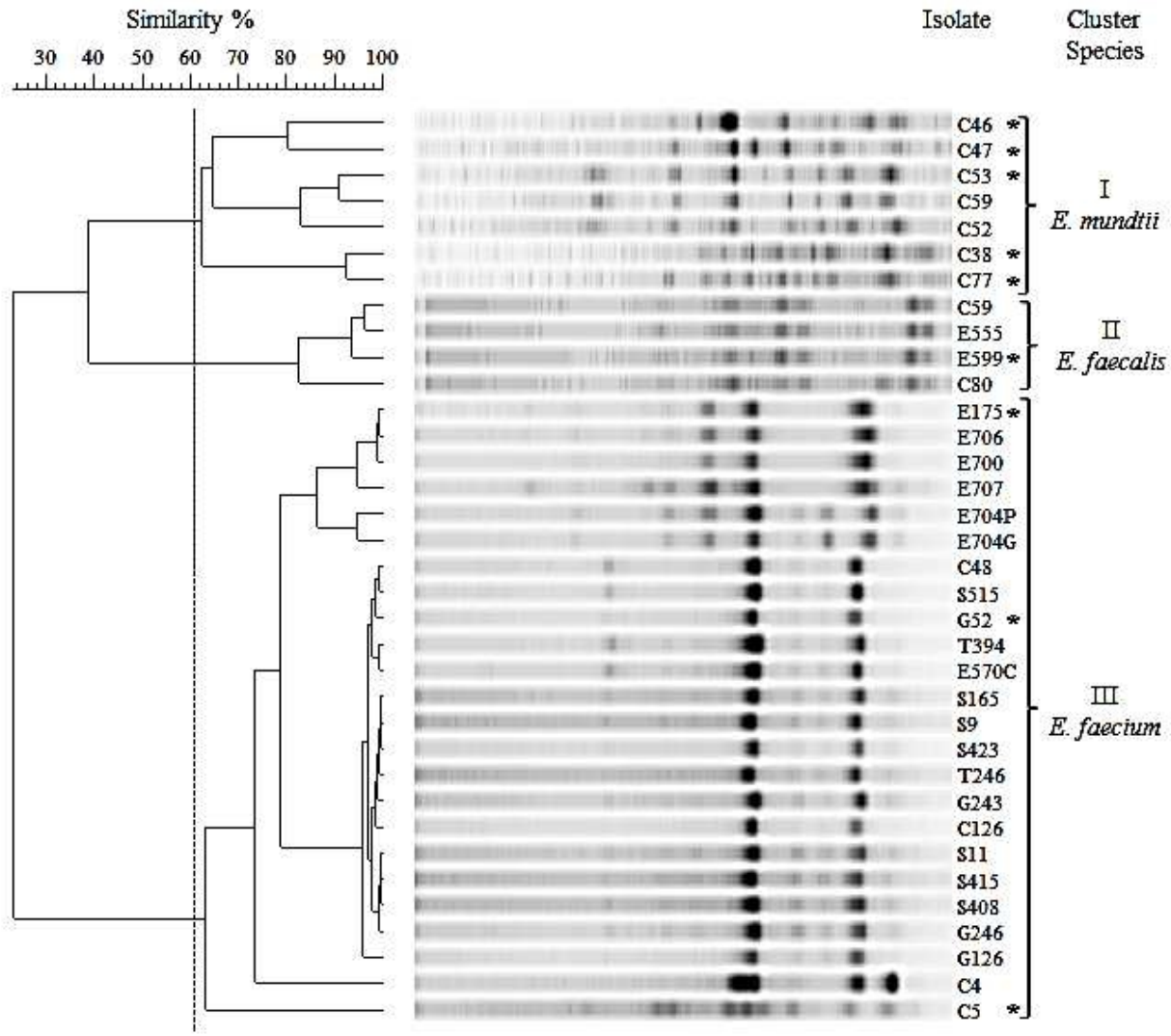

Figure 1: UPGMA dendogram derived from RAPD-PCR-fingerprinting patterns of all the 35 isolates using the primer 1254. The codes of the isolates are indicated on the right-hand side of the figure. The vertical dotted line indicates the 60% similarity level that delineates the species *E. mundtii* (cluster I), *E. faecalis* (cluster II) and *E. faecium* (cluster III). Isolates marked with * were identified by phenylalanyl-tRNA synthase α-subunit (pheS) gene sequence analysis.

Using a profile similarity of about 60% as a threshold, the 35 isolates were subdivided into three clusters. Seven strains were grouped in the first cluster, four strains belonged to the second cluster

and, finally, 24 strains were clustered in the third group. For each cluster, some representative isolates were chosen to proceed with their identification at the species level by the *pheS* gene analysis. Indeed, this gene is considered a reliable genomic marker for differentiating the species within the genus *Enterococcus*, and it was demonstrated to be much more discriminatory than 16S rRNA (Naser *et al*., 2005). The *pheS* gene has a high degree of homogeneity among strains of the same enterococcal species (at least 97% sequence similarity), whereas, distinct species reveal at maximum 86% gene sequence similarity. The *pheS* partial gene sequence data obtained indicated that the strains C38, C46, C47, C53 and C77, grouped in the cluster I, can be assigned to the species *E. mundtii* (99-100% identity), the strain E599 (cluster II) to *E. faecalis* (100% identity), while the strains E175, G52 and C5 (cluster 3) to *E. faecium* (100% identity).

These results confirmed the tyrosine decarboxylase potential of *E. faecalis* and *E. faecium*, the stronger tyramine producers (Aymerich *et al*., 2006; Gardini *et al*., 2008; Bonetta *et al*., 2008; Ladero *et al*., 2012; Marcobal *et al.*, 2012). On the other hand, tyramine production is considered a species characteristic of *E. faecalis* (Ladero *et al*., 2012). In addition, the tyraminogenic potential of *E. durans* has been deeply studied (Fernández *et al*., 2007; Linares *et al*., 2009).

Regarding *E. mundtii*, scarce are the studies regarding their capability to accumulate tyramine and the genetic aspects involved in its accumulations. Kalhotka *et al*. (2012) investigated the decarboxylase activity of enterococci isolated from goat milk and found that all of the tested strains, identified as *E. mundtii, E. faecium* and *E. durans*, showed significant tyrosine and arginine decarboxylase activity, in relation to temperature and duration of cultivation. In addition, Trivedi *et al*. (2009) studied the ability to decarboxylate tyrosine in many enterococcal strains isolated from different foodstuffs and found that more than 90% of isolates showed the presence of the gene *tyrDC*. In particular, these authors found that 10 of 17 *E. mundtii* strains were tyramine producers. These preliminary studies indicated the occurrence of tyramine producing *E. mundtii* strains, but did not highlight the tyraminogenic potential of this species. Moreover, the molecular aspects involved in the tyramine biosynthesis have not yet studied in depth. For this reason, two of the *E. mundtii* strains considered here were chosen as targets for investigating their tyramine accumulation capability and tyrosine metabolism. In particular, the strains *E. mundtii* C53 and C46, isolated from grass silage and belonging to two different RAPD-PCR subclusters, were considered.

4.4.2.2. Growth parameters and tyramine production of *Enterococcus mundtii* strains

The growth of the strains *E. mundtii* C46 and C53 was monitored by measuring the OD_{600} increase in BHI medium added or not with tyrosine. The OD_{600} changes were modelled with the Gompertz equation (Zwietering *et al*., 1990) and the estimates of the parameters are reported in Table 2.

Strain	Cultural medium	Gompertz equation parameters[a] (Standard error)			R^2	Maximum cell concentration (log CFU/ml)
		A	μ_{max}	λ		
C46	BHI + tyr[b]	1.153 (0.029)	0.635 (0.079)	1.771 (0.119)	0.994	9.09 (±0.04)
	BHI	1.269 (0.036)	0.615 (0.077)	2.556 (0.132)	0.994	9.06 (±0.01)
C53	BHI + tyr	1.113 (0.037)	0.594 (0.101)	2.024 (0.177)	0.990	9.01 (±0.02)
	BHI	1.215 (0.028)	0.563 (0.060)	2.345 (0.121)	0.996	8.97 (±0.05)

Table 2: Gompertz equation parameters for enterococcal growth measured as OD_{600}. R^2 is given as diagnostics of the regression. The maximum cell concentrations (expressed as log CFU/ml) at the beginning of the stationary phase is also reported.

[a]*A*: maximum OD_{600} value reached; μ_{max}: maximum OD_{600} increase rate in exponential phase (OD_{600}/h); λ: lag phase duration (h)

[b]BHI Broth plus 1 g/l tyrosine

All the parameters were characterized by a high significance ($p \leq 0.05$). Both the strains reached the maximum value of OD_{600} (*A*), ranging between 1.11 and 1.27, after 6-8 h incubation at 37°C. The curves presented a very short lag phase (λ), followed by a sharp increase of OD_{600}. As far as *A* and λ, no marked differences were found among the two strains, while *E. mundtii* C53 presented a lower maximum OD_{600} increase rate in exponential phase (μ_{max}). Moreover, the addition of tyrosine generally determined lower values of *A*, higher values of μ_{max} and a shorter lag phase. Table 2 reports also the cell counts detected at beginning of the stationary phase. The models obtained are graphically represented in Figure 2, which reports the growth curves in the first 24 h of incubation.

As a reference, in the same figure also the growth curves obtained under the same conditions by Bargossi *et al*. (2015b) for *E. faecalis* EF37, a strong tyramine producer (Gardini *et al*., 2008), which showed analogous behaviours.

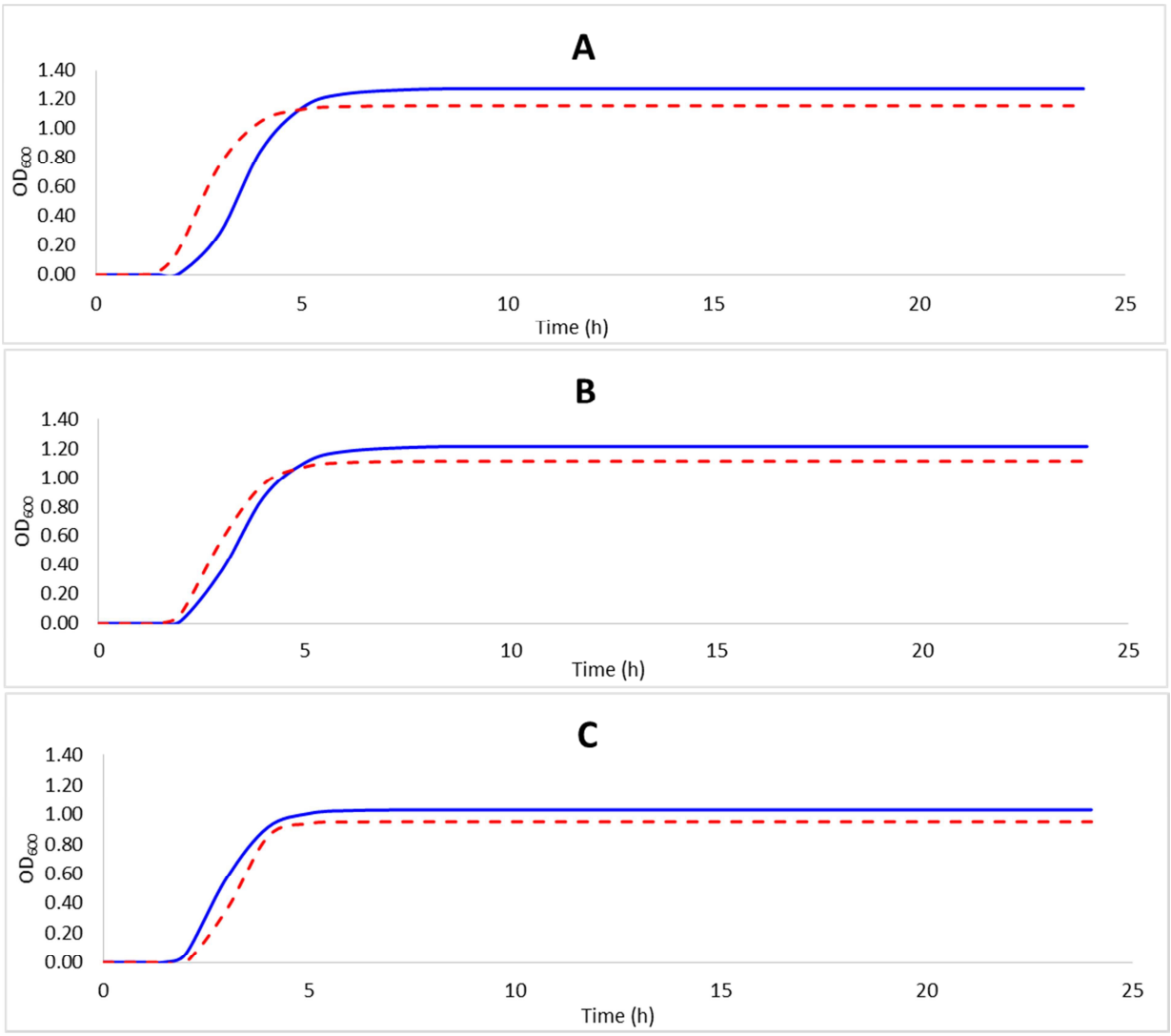

Figure 2: Growth curves of *E. mundtii* C46 (A) and *E. mundtii* C53 (B) obtained according to the Gompertz parameters reported in Table 2. The growth was obtained in BHI not added (blue solid line) or added (red dotted line) with tyrosine. As a comparison, also the growth curves obtained under the same conditions for the strain *E. faecalis* EF37 (C) are reports according to the data of Bargossi *et al*. (2015b).

The production of tyramine by *E. mundtii* C46 and C53 during their growth in BHI, added or not with the precursor, is shown in Table 3, which reports also the accumulation of 2-phenylethylamine. Also in this case, the data already available for *E. faecalis* EF37 (Bargossi *et al*., 2015b) are reported. It is well known that enterococci can decarboxylate phenylalanine producing 2-phenylethylamine through the activity of the same decarboxylase. The characteristics of this BA are very similar to tyramine, but it is produced with a lower efficiency (Marcobal *et al*., 2006a).

Time (h)	*E. mundtii* C53						*E. mundtii* C46						*E. faecalis* EF37[1]					
	BHI			BHI + 0.1% tyrosine			BHI			BHI + 0.1% tyrosine			BHI			BHI + 0.1% tyrosine		
	OD_{600}[2]	TYR (mg/l)	2-PHE (mg/l)	OD_{600}	TYR (mg/l)	2-PHE (mg/l)	OD_{600}	TYR (mg/l)	2-PHE (mg/l)	OD_{600}	TYR (mg/l)	2-PHE (mg/l)	OD_{600}	TYR (mg/l)	2-PHE (mg/l)	OD_{600}	TYR (mg/l)	2-PHE (mg/l)
2	0.000	8.35 (±0.41)	-[3]	0.000	20.31 (±0.32)	-	0.004	7.14 (±0.19)	-	0.167	15.66 (±0.65)	-	0.059	n.d.[4]	n.d.	0.000	n.d.	n.d.
3	0.367	21.30 (±1.12)	-	0.575	42.18 (±1.05)	-	0.279	21.56 (±0.72)	-	0.748	72.89 (±2.04)	-	0.575	n.d.	n.d.	0.359	n.d.	n.d.
4	0.865	32.16 (±1.84)	-	0.953	64.88 (±1.54)	-	0.846	36.59 (±0.08)	-	1.047	130.34 (±2.56)	-	0.913	n.d.	n.d.	0.851	n.d.	n.d.
5	1.103	46.29 (±1.70)	-	1.073	93.59 (±2.32)	-	1.139	61.37 (±1.81)	-	1.128	189.87 (±3.63)	-	1.004	n.d.	n.d.	0.936	n.d.	n.d.
8	1.212	72.25 (±2.31)	-	1.112	221.25 (±5.48)	-	1.267	97.55 (±2.50)	4.80 (±0.06)	1.153	396.36 (±5.68)	-	1.029	11.65 (±1.75)	39.67 (±1.71)	0.947	503.75 (±6.16)	85.21 (±2.12)
24	1.215	101.71 (±3.44)	11.77 (±0.48)	1.113	508.88 (±8.93)	4.07 (±0.80)	1.269	112.33 (±6.32)	33.24 (±1.24)	1.153	630.09 (±6.75)	6.72 (±0.74)	1.029	90.97 (±6.71)	177.10 (±5.46)	0.947	536.16 (±4.32)	295.61 (±5.75)
48	1.215	116.73 (±6.78)	32.52 (±0.87)	1.113	691.44 (±8.49)	6.91 (±0.22)	1.269	121.42 (±4.96)	63.21 (±3.09)	1.153	770.35 (±7.06)	14.84 (±0.95)	1.029	69.64 (±2.93)	213.79 (±7.25)	0.947	551.40 (±4.43)	405.80 (±6.17)
72	1.215	129.12 (±4.09)	56.26 (±0.94)	1.113	757.43 (±9.69)	24.59 (±0.65)	1.269	127.57 (±5.24)	91.00 (±2.16)	1.153	781.50 (±5.83)	43.46 (±1.92)	1.029	68.30 (±4.88)	262.45 (±6.87)	0.947	513.94 (±5.65)	428.50 (±4.91)
96	1.215	134.15 (±5.11)	75.63 (±1.68)	1.113	766.57 (±9.91)	20.55 (±0.71)	1.269	129.46 (±4.68)	108.56 (±3.82)	1.153	797.28 (±11.05)	44.94 (±2.16)	1.029	n.d.	n.d.	0.947	n.d.	n.d.

Table 3: OD_{600} and tyramine (TYR) and 2-phenylethylamine (2-PHE) production by *E. mundtii* C53 and C46 during their growth in BHI, added or not with tyrosine. It is also reported the production of TYR and 2-PHE of *E. faecalis* strain (rielaborated from Bargossi *et al*., 2015b). the standard deviations are reported within brackets.

[1]: rielaborated from Bargossi *et al*. (2015b); [2]: optical density at the different sampling time as predicted by the Gompetz model (Table 2); [3]: under the detection limit (0.5 mg/L); [4]: not determined.

In all the tested conditions, the two *E. mundtii* strains were able to accumulate tyramine independently on the addition of tyrosine. In fact, the decarboxylase activity was detected also in the medium not supplemented with tyrosine, because BHI contains amino acid sources (proteins and peptides) among which precursors for TDC. This observation was previously reported by Bargossi *et al*. (2015b) for *E. faecalis* and *E. faecium* grown in the media BHI and Bover-Cid and Holzapfel.

The data showed that the two *E. mundtii* strains began to produce tyramine after 2 h from the inoculum, both in the presence and in the absence of the precursor, and they continued to gradually accumulate tyramine during their stationary phase. In all the conditions, the maximum tyramine concentration was reached after 48 h for the strain C46 and after 72 h for the strain C53. However, the final amount of tyramine was similar for both the strains. In fact, it not exceeded 135 mg/l in BHI medium, while, in presence of tyrosine added, the final amount of tyramine was about 767 mg/l and 797 mg/l for the strains C53 and C46, respectively. As reported in Table 3, Bargossi *et al*. (2015b) found that *E. faecalis* EF37 under the same conditions after 8 h reached the maximum tyramine concentration in the presence of tyramine added. The E. *mundtii* strains showed a slower tyramine production kinetics, but the final amount was significantly higher than *E. faecalis* EF37 (approx. 500 mg/l). In the absence of tyrosine added, the strain *E. mundti* C46 was characterized by a faster tyramine accumulation in BHI. The major differences between *E. faecalis* EF37 and the *E. mundtii* strains were in the ability to accumulate 2-phenylethylamine, which was dramatically higher in *E. faecalis*. These amounts were higher than those reported by Liu *et al.* (2013) who, testing the tyraminogenic potential of *E. faecalis* strains from water-boiled salted duck, found concentrations of tyramine lower than 330 mg/l in MRS broth added with 0.1% tyrosine.

The two *E. mundtii* strains were also able to decarboxylate phenylalanine leading to the production of 2-phenylethylamine (Table 3). This BA was accumulated only after 24 h of growth for the strain C53, while C46 began to produce this compound already after 8 h in absence of tyrosine added.

The 2-phenylethylamine accumulation increased during subsequent incubation and reached its maximum level after 72 h with amended tyrosine and after 96 h without this amino acid. Moreover, the production of 2-phenylethylamine was higher when tyrosine was not added to the growth

medium. Indeed, in this case, concentrations of about 76 mg/l and 109 mg/l for *E. mundtii* C53 and C46, respectively, were reached, compared with concentrations lower than 45 mg/l in BHI when tyrosine was added to the medium. Interestingly, however, the accumulation of this BA became relevant when the tyramine concentration reached its maximum level (independently on the addition of the precursor). In any case, the amount of this BA was lower than that accumulated by *E. faecium* FC12 and *E. faecalis* EF37 (more than 400 mg/L) grown in the same medium (Bargossi *et al*., 2015b). These findings could reflect the lower efficiency of the *E. mundtii* TDC for phenylalanine decarboxylation and could indicate that these amounts of tyramine can lower or inhibit further decarboxylase activities in the tested strains.

The continue tyramine accumulation until late stationary growth phase observed in this research could represent an advantage for the microorganism against acidification during the fermentation process and growth. In fact, the decarboxylation of amino acids has been indicated as a mechanism through which LAB and human pathogenic bacteria can resist acidic conditions (Lund *et al*., 2014; Romano *et al*., 2014) and this protective effect seems to be mediated via the maintenance of intracellular pH (Perez *et al*., 2015). The same role in the maintenance of pH homeostasis in acidic environment has been also described in *E. durans* (Linares *et al*., 2009) and *E. faecium* (Marcobal *et al*., 2006a).

4.4.2.3. Time course of *tyrDC* gene expression

The two *E. mundtii* strains analysed in this study showed similar trends in the accumulation of tyramine and phenylethylamine, and produced comparable final levels of these BAs in the different tested conditions. Thus, only the strain C46 was selected for transcriptional analysis of the gene *tyrDC*. Table 4 reports the *tyrDC* expression data obtained by RT-qPCR during 72 h growth in BHI supplemented or not with tyrosine.

Cultural medium	Log (copies/µg cDNA) at time (h)							
	2	3	4	5	8	24	48	72
BHI	3.4 (±0.06)	3.0 (±0.03)	2.5 (±0.03)	2.7 (±0.30)	3.1 (±0.004)	2.9 (±0.14)	3.0 (±0.13)	2.3 (±0.83)
BHI + tyr	2.9 (±0.002)	3.5 (±0.06)	4.6 (±0.05)	4.1 (±0.13)	2.5 (±0.03)	3.1 (±0.11)	1.6 (±0.04)	1.6 (±0.13)

Table 4: Tyrosine decarboxylase (*tyrDC*) gene expression data for *E. mundtii* C46 grown in BHI added or not with 0.1% tyrosine during 72 h, as determined by RT-qPCR.

In the medium without tyrosine added, a high value of transcript (3 log copies/µg cDNA) was already observed after 2 h (early exponential phase), probably due to the strong residual effect of the precursor present in the pre-cultivation medium. The amount of *tyrDC* transcript remained rather stable throughout all the period monitored. The addition of the precursor affected considerably the *tyrDC* expression level depending on the growth phase. Indeed, the expression of *tyrDC* increased rapidly, peaked (about 4.6 log copies/µg cDNA) at 4 h during the exponential phase of growth, when the highest number of cells for ml was reached. After 8 h, the gene expression decreased progressively until the end of the 72 h period monitored.

As notice above, this *E. mundtii* strain was able to accumulate greater amounts of BAs than that of other previously studied enterococcal strains *E. faecalis* EF37 and *E. faecium* FC12 under the same conditions (Bargossi *et al*., 2015b). However, the maximum *tyrDC* gene copies number of *E. mundtii* C46, obtained after 4 h growth in BHI added with tyrosine, did not reach the value found for *E. faecalis* EF37 (6.1 log copies/µg cDNA) in the same conditions. The expression trend of *E. mundtii* C46 in BHI without tyrosine was more similar to that of *E. faecium* FC12 which presented a rather constant *tyrDC* transcript level during the entire incubation period. However, in BHI added with tyrosine the expression profile differed between *E. mundtii* C46 and *E. faecium* FC12 because the *tyrDC* gene transcript reached the maximum level in the exponential (4 h) and in the stationary phase (24 h), respectively, when the highest cell number of 9 log CFU/mL was detected for both the strains.

4.4.2.4. Analysis of the TDC operon region

The characteristics of the TDC operon region involved in tyramine production have been described in several tyraminogenic bacterial strains, including enterococci (Connil *et al*., 2002, Lucas *et al*., 2003, Fernandez *et al*., 2004, Coton *et al*., 2004, Marcobal *et al*., 2012; Bargossi *et al.*, 2015a). However, the molecular knowledge of this region for *E. mundtii* is extremely scarce. Therefore, it was carried out an investigation to determine the DNA and amino acid sequences of the *E. mundtii* C46 tyramine production-associated genes and the genetic organization of the TDC operon region, considering also the available genome sequencing data. In particular, the region downstream the gene *tyrS* including the genes *tyrDC* and *tyrP*, which encode for the tyrosine decarboxylase and the tyrosine/tyramine permease, respectively, was amplified and sequenced. Indeed, the gene Na^+/H^+ antiporter (*nhaC*), that usually follow *tyrP* in the TDC operon of several tyramine-producing LAB, such as *E. faecalis*, *E. faecium* and *L. brevis* (Marcobal *et al*., 2012; Bargossi *et al*., 2015a) was not recognized by PCR performed with the primers covering the intergenic region between *tyrP* and *nhaC*. Such gene organization was found also in the fully sequenced and assembled genome of *E.*

mundtii QU 25 (Shiwa *et al*., 2014) (GCA_000504125.1), that shows a lacI family transcriptional regulator gene downstream *tyrP* (Figure 2a).

BLASTN analysis of the 3677 bp nucleotide sequence of the *E. mundtii* C46 TDC operon region showed the best overall identity of 99% (3673/3677 nt) with that of *E. mundtii* QU 25. High levels of DNA sequence identity (> 80%) were also found for several strains belonging to other enterococcal species: *E. hirae* ATCC 9790 (1884/2282, 83%), *E. durans* KLDS 6.0930 and KLDS 6.0933 (1876/2285, 82%), and *E. faecium* Aus0085, NRRL B-2354, Aus0004, DO, and T110 (1877/2286, 82%). On the contrary, lower sequence identity (76%) was achieved for strains belonging to the species *E. faecalis* (e.g. ATCC 29212, and V583). Putative promoter and terminator were found upstream the start codon of the genes *tyrDC* (Figure 2a), but not in the short intergenic sequence before the gene *tyrP*, suggesting that these two genes are probably co-transcribed, as already showed for other species, such as *E. faecalis* and *L. brevis* (Marcobal *et al*., 2012).

Surprisingly, BLASTN analysis discovered in the genome of *E. mundtii* QU 25 (Shiwa *et al*., 2014) the presence of another region constituted by two genes similar to *tyrDC* and *tyrP*. These genes showed lower sequence identity values, 69% and 64%, respectively, with those present in the TDC operon. The genetic organization of the genomic segment that includes these two genes is shown in Figure 2b. This additional portion was also recovered in the genome of other enterococcal strains, such as *E. hirae* ATCC 9790, *E. faecium* NRRL B-2354, *E. durans* KLDS6.0930 and KLDS6.0930. However, in these strains a further putative amino acid permease was annotated between the tyrosine permease and the cation transporter E1-E2 family ATPase. The presence of a gene associated to a transposase after the ATPase encoding gene in *E. mundtii* QU 25 (Shiwa *et al*., 2014) is of particular interest, as it could be involved in spontaneous events of gene duplication or horizontal transfer.

BLASTX analysis and comparison of the deduced amino acid sequences of *E. mundtii* C46 TDC operon region were also carried out. The translated nucleotide sequence generated two proteins in the frame +1 and +2, respectively. The first one showed the highest identity with a tyrosine decarboxylase (BAO05941.1) of *E. mundtii* QU 25 (624/624 nt, 100%) and *E. mundtii* CRL35 (616/624 nt, 99%) and decreasing identity (90-71%) with decarboxylases from other species of the genus *Enterococcus*. On the contrary, lower similarity (61-59%) was found with the additional PLP-dependent decarboxylase detected with BLASTN analysis. The second protein presented a putative conserved domain associate to a putative glutamate/gamma-aminobutyrate antiporter (TIGR03813). This sequence showed 100% identity with the amino acid permease family protein of *E. mundtii* QU 25 and *E. mundtii* ATCC 883, and decreasing identity with the amino acid

permeases of other species of the genus *Enterococcus*. Also in this case, lower identity (58-60%) was found with the additional amino acid permease detected with BLASTN analysis.

These sequence analysis results taken together indicated the presence in the *E. muntii* genome of a TDC operon with a classical genetic organization (i.e. *tyrS*, *tyrDC* and *tyrP*) and provided evidences for a new additional copy consisting of three ORF. According to Lynch and Conery (2000) duplications of a genome segments have been thought to be a primary source of material for the origin of evolutionary novelties, including new gene functions and expression patterns. Therefore, the additional copy may acquire a novel, beneficial function and become preserved by natural selection, with the other copy retaining the original function. Recently, Bargossi *et al*. (2015a) described the compromised tyrosine decarboxylase activity of the strain *E. faecium* FC643 due to a codon stop in the translated *tyrDC* sequence. However, this strain showed a slow and reduced production of tyramine, and not 2-phenylethylamine, probably due to the presence of the additional enzyme with different substrate specificity and regulation mechanism respect to the decarboxylase encoded by the gene *tyrDC* of the TDC operon.

As regards *E. mundtii*, it can be supposed that all the genes in the two operon regions detected are expressed and produce functional products. However, the role of the additional proteins in the context of biogenic amine production needs further deep investigation.

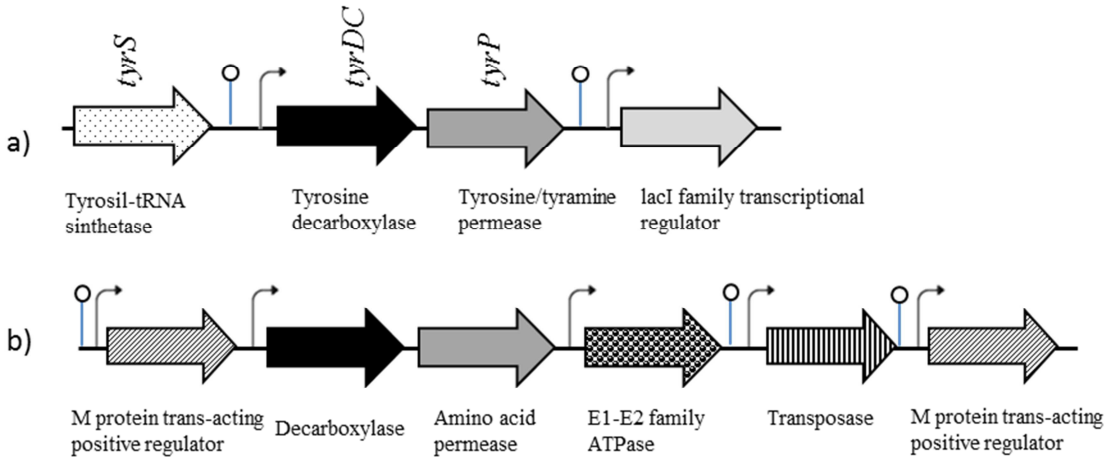

Figure 2: a) Organization of the TDC operon in the strain *E. mundtii* QU 25 (GCA_000504125.1); b) genome fragment encoding for an additional PLP-dependent decarboxylase, an APC family amino acid transporter and a cation transporter E1-E2 family ATPase; upstream it is recognized a M protein trans-acting positive regulator and downstream an ISEfa11 (ISL3 family) transposase, followed by an additional M trans-acting positive regulator gene.

4.4.3. Conclusions

In this study the capability of *E. mundtii* strains to accumulate tyrosine and 2-phenylalanine in cultural media was assessed, and more information on the genetic basis of their tyraminogenic potential were obtained for the first time. The two strains considered here produced relevant amounts of tyramine, greater than those accumulated by other strains belonging to *E. faecium* and *E. faecalis* previously studied in the same conditions (Bargossi *et al.*, 2015b). By contrast, their ability to decarboxylate phenylalanine was less enhanced if compared with the same strains. Likewise the other enterococcal strains, the expression analysis of the gene *tyrDC* showed that an excess of the precursor tyrosine affected the amount of the transcript during the exponential phase of growth, and that the amino acids fraction present in the medium also modulated the level of the transcript. The genetic organization as well as sequence identity levels of the genes *tyrDC* and *tyrP* indicated that the tyramine-forming pathway in *E. mundtii* is similar to those in phylogenetically closer enterococcal species, such as *E. faecium*, *E. hirae*, and *E. durans*, however the gene Na^+/H^+ antiporter (*nhaC*), that usually follow *tyrP* is missing. Analysis of the available data on genome content and organization of *E. mundtii* QU 25 (Shiwa et al., 2014) and other *Enterococcus* strains revealed an unexpectedly presence of another region that includes two genes encoding for an additional PLP-dependent decarboxylase and an amino acid permease. It is tempting to speculate that a duplication event occurred and the evolution of this redundant copy induced the acquisition of different specificity leading to the maintenance of both the functional copies. Thus, this discovery uncovers another level of complexity in the enterococcal biogenic amines regulatory network. Further studies have to be performed to better explain the genetic and functional characteristics of these further enzymes and their correlation with tyrosine decarboxylating potential of enterococci.

4.4.4. Experimental procedures

4.4.4.1. Characterization of the strains and screening procedure for tyramine production

Thirty-five isolates of cocci LAB from the collection of the Biotechnology Department of the Verona University were tested for morphological characteristics, Gram test, catalase test, growth in the presence of 6.5% NaCl, growth at 15°C and 45°C and at pH 4.4 and 9.6, as well as for their homo or heterolactic fermentation.

The tyrosine decarboxylase activity of the isolates was evaluated using the screening plate method described by Bover-Cid and Holzapfel (1999).

4.4.4.2. *TyrDC* gene detection

Genomic DNA of tyramine-positive isolates was obtained from 1 ml of overnight culture by using the Wizard Genomic DNA purification system (Promega Corporation, Madison, WI), following the manufacturer's instructions. Isolates were assayed for the presence of the gene *tyrDC* by PCR analysis with the primers DEC5 and DEC3 (Table 1), following the conditions described previously (Torriani *et al*., 2008). PCR product was visualized on a 2% agarose gel.

4.4.4.3. Randomly amplified polymorphic DNA (RAPD) analysis and identification of tyramine-positive cocci

In order to genetically typify the 35 tyramine-positive coccal strains, a preliminary RAPD-PCR analysis was performed with the primer 1254 (Table 1). Species identification was carry out by phenylalanyl-tRNA synthase α-subunit (*pheS*) gene sequence analysis (Naser *et al*., 2005). The *pheS* partial gene amplification was obtained with the primers pheS-21-F and pheS-22-R (Table 1). PCR conditions were set according to Naser *et al.* (2005) with exception that annealing temperature was 50°C. The expected amplicon (455 bp) was purified with the Wizard SV gel and PCR clean-up system (Promega Corporation) and cloned with the cloning kit pGEMT-easy vector system (Promega Corporation). Recombinant plasmids were sequenced at the GATC Biotech Ltd (Koln, Germany). Data were analyzed with the Basic Local Alignment Search Tool (BLAST) provided by National Center for Biotechnology Information (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

4.4.4.4. Growth parameters of two *Enterococcus mundtii* strains and tyramine production

Two strains (C46 and C53), isolated from grass silage and identified as *Enterococcus mundtii*, were used for deeper investigations.

The two considered *E. mundtii* strains were stored in 20% (w/v) glycerol at -80°C and pre-cultivated for 24 h at 37°C in BHI Broth (Oxoid, Basingstoke, UK) added with 1000 mg/l of tyrosine (Sigma-Aldrich, Gallarate, Italy). After 24 h of pre-cultivation, the microorganisms were inoculated, at a concentration of approximately 7 log CFU/ml, in BHI Broth, added or not with 1 g/l of tyrosine and incubated at 37°C for 72 h. The evaluation of the strain growth in BHI was performed by measuring the OD_{600} with a UV-VIS spectrophotometer (Cary 60 UV-Vis, Agilent Technologies, Santa Clara, CA) with plastic cuvettes (1.5 ml) at defined times (1, 2, 3, 4, 5, 6, 7, 8, 24, 48, 72 and 96 h). The OD_{600} data were fitted with the Gompertz equation as modified by Zwietering *et al*. (1990).

$$y = k + Ae^{-e^{\left[\left(\frac{\mu_{max}e}{A}\right)(\lambda - t)+1\right]}}$$

where y is the OD_{600} at time t, A represent the maximum OD_{600} value reached, μ_{max} is the maximum OD_{600} increase rate in exponential phase and λ is the lag time.

The maximum cell concentration reached was determined at the beginning of the stationary phase by plate counting enterococci onto BHI agar.

The BAs were determined after 2, 3, 4, 5, 8, 24, 48, 72 and 96 h of incubation. The cultures were centrifuged at 10000 rpm for 10 min at 10°C, and the supernatants were used for BAs determination by HPLC after derivatization with dansyl-chloride (Sigma-Aldrich, Gallarate, Italy) according to Bargossi *et al*. (2015b). The quantification was performed according to Tabanelli *et al*. (2012) and the amount of tyramine and 2-phenylethylamine was expressed as mg/ml by reference to a calibration curve obtained with standard solutions. The trials were always analyzed in triplicate.

4.4.4.5. RNA isolation, cDNA synthesis and RT-qPCR assay

Two ml aliquots of *E. mundtii* cultures were centrifuged at 3000 rpm for 10 min and the obtained cell pellets were frozen at -80°C until the time of RNA extraction. Total RNA was isolated from cell pellets according to Bargossi *et al*. (2015b). Total cDNA was synthesized from 1 µg of RNA using the ImProm-IITM Reverse Transcriptase kit (Promega Corporation), following the manufacturer's recommendations.

The expression level of the gene *tyrDC* was analyzed by a reverse transcription-quantitative real time PCR (RT-qPCR) assay with the primers TYR3f and TYR4r (Table 1); thermo cycler, reaction mixture, and amplification program were previously described in Torriani *et al*. (2008), as well as the procedure of the absolute quantification of the *tyrDC* copies number.

4.4.4.6. Analysis of the TDC operon region

The TDC operon fragments were obtained for *E. mundtii* C46 by PCR amplification with the partially degenerate primers reported in Table 1. PCR mixture was composed of 1× PCR buffer, 1.5 mM $MgCl_2$, 200 nM dNTPs, 0.5 µM each primer and 50 ng DNA. Amplification program comprised: 95°C for 5 min, 35 cycles at 94°C, 30 sec; 56°C, 45 sec; 72°C, 1 min, and final extension at 72°C, 10 min. Amplicons were purified, cloned and sequenced as reported in the paragraph 2.3.

Promoters prediction was carried out by BPROM, a bacterial sigma70 promoter recognition program

(http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb; Solovyev and Salamov, 2011). Putative Rho–independent transcription terminators were predicted by the Arnold Finding Terminators (http://rna.igmors.u-psud.fr/toolbox/arnold/index.php).
Similarity searches were performed with the BLAST programs available at the NCBI. Sequence alignments were carried out with the Clustal Omega analysis Tool Web Services from the EMBL-EBI (Sievers *et al*., 2011).

4.4.4.7. Statistical analysis

The growth model was fitted using the statistical package Statistica for Windows 6.1 (Statsoft Italia, Vigonza, Italy).

4.5. Control of tyramine and histamine accumulation by lactic acid bacteria using bacteriocin forming lactococci.

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ABSTRACT

The aim of this study was to evaluate the competitive effects of three bacteriocin producing strains of *L. lactis* subsp. *lactis* against two aminobiogenic lactic acid bacteria, i.e. the tyramine producing strain *Enterococcus faecalis* EF37 and the histamine producing strain *Streptococcus thermophilus* PRI60, inoculated at different initial concentration (from 2 to 6 log cfu/ml). The results showed that the three *L. lactis* subsp. *lactis* strains were able to produce bacteriocins: in particular, *L. lactis* subsp. *lactis* VR84 and EG46 produced, respectively, nisin Z and lacticin 481, while for the strains CG27 the bacteriocin has not been yet identified, even if its peptidic nature has been demonstrated. The co-culture of *E. faecalis* EF37 in combination with lactococci significantly reduced the growth potential of this aminobiogenic strain, both in terms of growth rate and maximum cell concentration, depending on the initial inoculum level of *E. faecalis*. Tyramine accumulation was strongly reduced when *E. faecalis* EF37 was inoculated at 2 log cfu/ml and, to a lesser extent, at 3 log cfu/ml, as a result of a lower cell load of the aminobiogenic strain. All the lactococci were more efficient in inhibiting streptococci in comparison with *E. faecalis* EF37; in particular, *L. lactis* subsp. *lactis* VR84 induced the death of *S. thermophilus* PRI60 and allowed the detection of histamine traces only at higher streptococci inoculum levels (5-6 log cfu/ml). The other two lactococcal strains did not show a lethal action against *S. thermophilus* PRI60, but were able to reduce its growth extent and histamine accumulation, even if *L. lactis* subsp. *lactis* EG46 was less effective when the initial streptococci concentration was 5 and 6 log cfu/ml. This preliminary study has clarified some aspects regarding the ratio between bacteriocinogenic strains and aminobiogenic strains with respect to the possibility to accumulate BA and has also showed that different bacteriocins can have different effects on BA production on the same strain. This knowledge is essentially aimed to use bacteriocinogenic lactococci as a predictable strategy against aminobiogenic bacteria present in cheese or other fermented foods.

Keywords: Bacteriocins, Competition, Biogenic amines, *Lactococcus lactis*, *Enterococcus faecalis*, *Streptococcus thermophilus.*

4.5.1. Introduction

Biopreservation refers to those processes in which the extension of food shelf life and safety improvement are obtained with the use of microorganisms, or their extracellular extracts, able to inhibit the growth of other bacteria, due to antimicrobial metabolites production (Papagianni, 2012). Such strategy is based upon the idea that some non-pathogenic bacteria can compete successfully with pathogenic and spoilage microorganisms avoiding their survival and growth in food (Stiles, 1996). This approach has been used since several centuries for the preservation of fermented food where the microbial population plays several roles, among which the inhibition of undesirable microbiota.

In recent years, particular interest has been posed on protective cultures, which are selected food-grade bacteria inoculated in food, due to their antagonistic properties rather than for their influence on the organoleptic or nutritional values. In fact, the microorganisms used as protective cultures should not affect the sensorial profile of the product (Rodgers, 2001). Lactic acid bacteria (LAB) are often used as biopreservation agents because they can produce a wide range of antimicrobial metabolites, such as organic acids, diacetyl, acetoin, hydrogen peroxide, antifungal peptides and bacteriocins without safety implications (Ghanbari *et al*., 2013).

Among the substances produced by bacteria with antimicrobial properties, bacteriocins have been deeply studied. Bacteriocins are defined as a heterologous group of ribosomally synthesized, extracellularly released bioactive peptides or proteins displaying antimicrobial activity against other bacteria (Klaenhammer, 1993; Nishie *et al*., 2012). In spite of the fact that several microbial groups (including Gram positive and Gram negative bacteria) can produce these molecules, the researchers' interest is focused mainly on LAB bacteriocins (Guinane *et al*., 2005; Parente and Ricciardi, 1999; Reis *et al*., 2012). This is due to the wide potential applications of these food-grade bacteria that open interesting perspectives for bacteriocin producing LAB (used as starter or protective cultures) or bacteriocin preparation in food (Beshkova and Frengova, 2012).

Lactococci are LAB ubiquitous in foods and they are widely present in dairy products because of their technological properties (Casalta and Montel, 2008). In fact, they are an important component of cheese microbiota, both during initial cheese-making steps, when they are often used as starter cultures, and during the ripening phase, when a complex microbiota determines transformations, which allow the obtaining of the peculiar cheese characteristics (Cogan *et al*., 2007). Moreover, they can have an important role as protective cultures in food preservation. In fact, they can exert important antimicrobial actions by synthesizing a variety of bacteriocins, such as nisins, lacticins and lactococcins (Beshkova and Frengova, 2012; Ghanbari *et al*., 2013; Leroy and de Vuyst, 2010; Stoyanova *et al*., 2012). In particular, nisin has a wide range of applications because of its broad

bactericidal spectrum and its mode of action. Since it can be easily broken down by digestive proteases and it does not disturb gut biota, it is to date the first bacteriocin approved for commercial use (Guinane *et al*., 2005).

Due to the activities of ripening microbiota during cheese making and ripening, also undesirable reactions can take place, such as the formation of biogenic amines (BAs). These substances are accumulated through the microbial decarboxylation of aminoacids. The most dangerous are histamine (produced from histidine) and tyramine (produced from tyrosine) (EFSA, 2011). Even if the selection of starter cultures is based on the absence of these features, the presence of aminobiogenic microorganisms in natural starter cultures or among ripening microbiota is often unavoidable (Linares *et al*., 2011; Novella-Rodriguez *et al*., 2002). Among LAB, many strains are endowed with high decarboxylating potential. For example, enterococci are known as the most efficient tyramine producers in fermented foods (Ladero *et al*., 2012; Marcobal *et al*., 2012). In addition, recently, the presence of efficient histaminogenic strains of *Streptococcus thermophilus* has been reported (Calles-Enriquez *et al*., 2010; Rossi *et al*., 2011; Tabanelli *et al*., 2012; Trip *et al*., 2011).

Even if some strains can produce tyramine (Buňková *et al*., 2011; de Llano *et al*., 1998), this feature in the genus *Lactococcus* is not widespread. For this reason, the selection of not tyraminogenic lactococci able to produce bacteriocins could represent an important tool to control BA accumulation in dairy products. In fact, the competition between two or more species in a habitat (such as cheese) affects both the partners in a negative way. However, it usually leads to an increase in the relative abundance of one of the interacting bacteria and to the possible exclusion or reduction of the other ones from the microbiota (Smid and Lacroix, 2013). Through this ability, selected lactococcal strains could contribute to reduce the risks of survival and multiplication of aminobiogenic microbiota during ripening and storage of fermented foods.

The aim of this research was the evaluation of the competitive effects against aminobiogenic LAB of three bacteriocin producing *Lactococcus lactis* strains isolated from raw cow milk. In particular, the tyramine producing strain *Enterococcus faecalis* EF37 (Gardini *et al*., 2001; Gardini *et al*., 2008) and the histamine producing strain *Streptococcus thermophilus* PRI60 (Rossi *et al*., 2011; Tabanelli *et al*., 2012) were considered as target microorganisms. The effects of the inhibiting potential of *L. lactis* strains were evaluated *in vitro* and both the population dynamics and BA production were assessed.

4.5.2. Material and methods

4.5.2.1. Isolation and characterization of lactococci with antimicrobial activity

A total of 25 LAB were isolated from samples of raw cow milk from different local dairies. Colonies grown on M17 (Oxoid, Basingstoke, UK) plates incubated at 15°C were randomly selected, purified and deposited in the laboratory culture collection. The isolates were subjected to a preliminary phenotypic characterization: cell morphology, Gram stain, gas production from glucose, catalase reaction, growth at 45°C and with 6.5% (w/v) NaCl. *Lactococcus lactis* subsp. *lactis* LMG 6890^T and *L. lactis* subsp. *cremoris* LMG 6897^T served as control strains.

The presumptive lactococci isolated as described above were tested for their inhibiting potential towards the tyramine producer *E. faecalis* EF37 (Gardini *et al*., 2001) and the histamine producer *S. thermophilus* PRI60 (Rossi *et al*., 2011; Tabanelli *et al*., 2012). All strains were cultivated in M17 medium at 30°C.

The antibacterial activity of lactococci was determined by using a deferred agar spot test under aerobic conditions (Schillinger and Lücke, 1989). M17 was used as bottom and upper layer medium. The production of antimicrobial substances was confirmed by well-diffusion agar assay using filter-sterilized and neutralized cell-free supernatants, as described by Aktypis *et al*. (1998). The presence of an inhibition zone greater than 5 mm around the well indicated a positive result. To evaluate the sensitivity of the inhibitory substances to proteolytic enzymes, the well-diffusion agar assay was repeated after treatment of the filter-sterilized cell-free supernatants with proteinase K (2 mg/ml), and pancreatin (1 mg/ml) at 37°C for 4 h.

4.5.2.2. Molecular identification of lactococcal isolates

The presumptive lactococcal isolates showing the greater inhibition diameter in the deferred agar spot test (CG27, VR84 and EG46) were identified by 16S rDNA sequencing following by subspecies-specific PCR. Genomic DNA was extracted from pure cultures using the Instagene matrix (Bio-Rad Laboratories, Italy) according to the manufacturer's instructions.

In order to amplify the 16S rDNA gene the primers LpigF/LpigR (5'-TACGGGAGGCAGCAGTAG-3' and 5'-CATGGTGTGACGGGCGGT-3') (Eurofins MWG Operon, Germany) and the PCR conditions described by Di Cagno *et al.* (2011) were used. The resulting amplicons (each about 600 kb long) were purified with the QIAquick PCR Purification Kit (Qiagen, USA) and sequenced at the BMR Genomics sequencing facility (Padova, Italy) using the same primers used for amplification. Sequence similarity searches were performed using the BLAST network service (http://blast.ncbi.nlm.nih.gov/).

The primers Lhis5F/Lhis6R (5'-CTTCGTTATGATTTTACA-3' and 5'-AATATCAACAATTCCATG-3') and the conditions described by Beimfohr *et al*. (1997) were used for the distinction of the subspecies *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. The

expected sizes of amplification products for *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* were 1,149 bp and 934 bp, respectively. To investigate the genetic diversity of the three lactococcal isolates, typing was carried out by randomly amplified polymorphic DNA (RAPD)-PCR with primer M13 according to Zapparoli *et al*. (1998).

4.5.2.3. Determination of the bacteriocin-encoding genes

A screening for structural genes encoding bacteriocins previously described for *L. lactis* was done using PCR of the genomic DNA from the three lactococcal strains CG27, VR84 and EG46, identified as *L. lactis* subsp. *lactis*.

PCRs were carried out with the primers listed in Table 1 and in a 20 μl reaction mixture containing 200 mM dNTPs, 1.5 mM MgCl2, 0.5 U of Taq polymerase (GoTaq, Promega, Italy) and 2.5 μM of each primer.

The amplification programs comprised an initial denaturation at 94° C for 5 min followed by 30 cycles of denaturation for 45 s at 94° C, annealing for 60 s at the appropriate temperature, and a final extension at 72° C for 60 s. All primers were used with an annealing temperature of 54°C except for the primer pair LcnQZ-F/LcnQZ-R where a 53°C annealing temperature was used. The generated PCR products were purified and sequenced with the specific primers, as previously described, and then compared with the sequences for known bacteriocins produced by *L. lactis* strains, using the BLAST network service.

4.5.2.4. Screening of biogenic amine potential of *L. lactis* EG46, VR84 and CG27

The lactococcal strains used in competition trials were tested for their ability to produce biogenic amines (tyramine, histamine, putrescine, cadaverine and 2-phenylethylamine) firstly with the method proposed by Bover-Cid and Holzapfel (1999) and then with HPLC analysis of the culture media used to study the competition, i.e. M17 broth (Oxoid) and MRS broth (Oxoid), added with 0.1% (w/v) precursors (tyrosine, histidine, ornithine, lysine and phenylalanine). The latter trials were carried out after a 48 h fermentation at 30°C with an initial inoculum of about 7 log cfu/ml of the three strains.

4.5.2.5. Competition between lactococci and the biogenic amine producing strains

Before each trials *E. faecalis* EF37 and *S. thermophilus* PRI60 were pre-cultured twice in media added with precursors (tyrosine and histidine, respectively) at 0.05% (w/v). The competition between each *Lactococcus* strain and *E. faecalis* EF37 was analysed using MRS Broth (Oxoid) as medium while the competition between lactococci and *S. thermophilus* PRI60 was studied in M17

broth. Both the media were added with 0.05% (w/v) of tyrosine or histidine as precursors. Competition was evaluated by inoculating one of the *Lactococcus* strain (at a level of about 7 log cfu/ml) in the presence of different initial inoculum level of the aminobiogenic strains (approx. 2, 3, 4, 5, 6 log cfu/ml). The co-culture samples and broths with *S. thermophilus* PRI60 and *E. faecalis* EF37 inoculated alone were incubated at 30°C (± 1) and periodically monitored for microbial counts and biogenic amine content. Also, the growth of *L. lactis* CG27, VR84 and EG46 alone was monitored in the same conditions.

4.5.2.6. Microbial counts

Plate counts were performed at regular intervals for 48 h using selective conditions for the different species. Specifically, lactococci were enumerated onto M17 incubated at 15°C for 72 h, *S. thermophilus* PRI60 and *E. faecalis* EF37 onto M17 and Slanetz and Bartley media (Oxoid), respectively, incubated at 45° C for 48 h.

4.5.2.7. Biogenic amine determination

Five-mL cultures were centrifuged at 8000 g for 10 min at 10° C, and the supernatants were used for biogenic amines (BAs) determination by HPLC after derivatization with dansyl-chloride (Sigma-Aldrich, Gallarate, Italy) according to Martuscelli *et al.* (2000). The BA content was analyzed using a PU-2089 Intelligent HPLC quaternary pump, Intelligent UV–VIS multiwavelength detector UV 2070 Plus (Jasco Corporation, Tokio, Japan) and a manual Rheodyne injector equipped with a 20 μL loop (Rheodyne, Rohnert Park, CA). The BA quantification was performed according to Tabanelli *et al*. (2012). The amount of histamine and tyramine was expressed as mg/mL by reference to a calibration curve obtained with histamine standards.

4.5.2.8. Growth modelling

Microbial growth was modelled with the Baranyi model (Baranyi and Roberts, 1994):

$$y(t) = y_0 + \mu_{max}A(t) - ln\left(1 + \frac{e^{\mu_{max}A(t)} - 1}{e^{(y_{max} - y_0)}}\right)$$

where

$$A(t) = t + \frac{1}{\mu_{max}} ln\left(e^{-\mu_{max}\,t} + e^{-h_0} - e^{(-\mu_{max}\,t - h_0)}\right)$$

$y(t)$ is the log cfu/ml of cell concentration (at time t), y_0 is the initial cell concentration and y_{max} is the maximum cell concentration (both expressed as log cfu/ml, μ_{max} is the maximum specific growth rate in the exponential phase (expressed as log (cfu/ml)/h), and h_0 is a measure of the initial physiological state of cells and expresses the work necessary for the cells to adapt to the environmental condition. In particular, $h_0=\mu_{max}\lambda$, where λ is the duration of lag phase.

The death of cell was modelled with the classical model derived from the Arrhenius equation (Stumbo, 1973):

$$LogN_t = LogN_0 - kt$$

where N_t represents the cell concentration at time t, N_0 the cell concentration at time 0, while k represents the rate of cell death with time.

The models were fitted using the non-linear regression procedure of the statistical package Statistica per Windows 6.1 (Statsoft Italia, Vigonza, Italy).

4.5.3. Results

4.5.3.1. Isolation of lactococci with antimicrobial activity

Twenty-five presumptive lactococci (Gram positive, catalase negative, chain forming homofermentative cocci able to grow at 6.5% NaCl and at 15°C, but not at 45°C) were isolated from raw cow milk and screened for their inhibition activity by an agar spot test against two LAB producing BAs, i.e. *E. faecalis* EF37 producing tyramine (Gardini *et al*., 2001), and *S. thermophilus* PRI60 producing histamine (Rossi *et al.*, 2011). On the basis of their inhibition potential showed in this preliminary test (data not shown), three lactococci were chosen for further experiments. These isolates (CG27, VR84 and EG46) were identified and all of them belonged to the species *L. lactis* subsp. *lactis*. Indeed, the 16S rDNA amplification indicated that these isolates presented 99% similarity with the 16S rDNA sequences reported for *L. lactis* subsp. *lactis* in the GenBank database. The amplification of genomic DNA with subspecies specific primers yielded a 934 bp fragment, which corresponded to the expected size for *L. lactis* subsp. *lactis* strains. The genetic polymorphism detected by RAPD-PCR with primer M13 (data not shown) permitted to clearly discriminated the three lactococcal isolates as different strains.

None of the chosen strains was able to produce histamine, tyramine, cadaverine, putrescine and 2-phenylethylamine after a 72 h growth in Bover-Cid and Holzapfel medium or in MRS and M17 broth, added with 0.1% of precursor aminoacids.

The three strains were then analysed for their ability to produce bacteriocins. Results of the well-diffusion agar assay using filter-sterilized cell-free supernatants treated with proteolytic enzymes

(proteinase K and pancreatin) indicated the proteinaceus nature of the inhibitory substances and suggested that the three strains were potential bacteriocin producers.

The amplification of genomic DNA extracted from *L. lactis* subsp. *lactis* VR84 and EG46 with primers targeting known bacteriocins generated positive results for nisin Z and lacticin 481, respectively. The sequence of these amplicons presented 100% similarity to the nisin Z gene and 97% similarity to the lacticin 481 gene. By contrast, for *L. lactis* subsp. *lactis* CG27 no amplification was recorded for the genes enconding nisin, lacticin 481, lacticin 3147, lacticin RM, lacticin Q, lacticin Z, lactococcin A, lactococcin B, lactococcin M, lactococcin G, lactococcin Q, lactococcin 513, and lactococcin 972 (Table 1), even if the proteinaceous nature of the antimicrobial substance was demonstrated.

Target	**Primer**	**Sequence (5' – 3') a**	*Product (pb)*	*Reference*
Lacticin 481	LactAF	TCTGCACTCACTTCATTAGTTA	366	Martínez *et al.* (1998)
	LactAR	AAGGTAATTACACCTCTTTTAT		
Lactococcin 513	Lcn513F	GCTCCAAAAAGCGCTAGATC	466	Villani *et al.* (2001)
	Lcn513R	GCTGGCTACGATATTGCTAG		
Lacticin RM	LactRM-F	ATCCTATCCGATACCGTCAG	644	
	LactRM-R	GTTTTCCTTGAACCATTGGG		
Nisin	NisinF	GGATAGTATCCATGTCTG	250	Li and O'Sullivan (2002)
	NisinR	CAATGATTTCGTTCGAAG		
Lactococcin	LcnFor	GAAGAGGCAATCAGTAGAG		Alegría *et al.*(2010)
A	LcnA	GTGTTCTATTTATAGCTAATG	525	
B	LcnB	CCAGGATTTTCTTTGATTTACTTC	578	
M	LcnM	GTGTACTGGTCTAGCATAAG	546	
Lactococcin 972	Lcn972F	TTGTAGCTCCTGCAGAAGGAACAT	232	
	Lcn972R	GCCTTAGCTTTGAATTCTTACCAAA		
Lacticin 3147	L3147For	TACTGGGGAAATAACGG	663	
	L3147Rev	TGGACAAGTATTGGTAC		
Lactococcin G and Q	LactGQ-F	GAAAGAATTATCAGAAAAAG	379	
	LactGQ-R	CCACTTATCTTTATTTCCCTCT		
Lacticin Q and Z	LcnQZ-F	ATGGCAGGGTTTTTAAAAGTAGT	155	This study
	LcnQZ-R	TTAATACCYAAAATTTGCTTAAT		

Table 1: Primers for genes encoding known bacteriocins used in this study.

4.5.3.2. Competition between lactococci and the tyramine producing *Enterococcus faecalis* EF37

The growth of enterococci inoculated at concentrations of about 2, 3, 4, 5 and 6 log cfu/ml in M17 medium incubated at 30°C in the presence of an initial inoculum of lactococci of about 7 log cfu/ml was monitored for 48 h by plate counting. In addition, also the growth of *E. faecalis* EF37 at the same initial concentration in the absence of lactococci was analysed. These data were modelled with the Baranyi and Roberts (1994) equation and the estimated parameters are reported in Table 2. The same samples were also analysed in order to determine the tyramine concentration.

When *E. faecalis* EF37 was grown alone, the growth rate during the exponential phase (μ_{max}) was quite constant (between 0.91-1.02 (log cfu/ml)/h) independently of the initial inoculum; also the y_{max} values (the maximum cell concentration reached) was similar in all the samples (8.80-9.03 log cfu/ml).

At the lowest initial inoculum (2 log cfu/ml), the presence of the three lactococci significantly decreased the growth potential of *E. faecalis* EF37, both reducing the μ_{max} and drastically limiting to values lower than 5 log cfu/ml the maximum cell concentration, without significant differences in relation to the *L. lactis* subsp. *lactis* strain (Table 2). The increase of the initial concentration of *E. faecalis* resulted in a correspondent raise of y_{max} (which was higher than 8 log cfu/ml with initial concentration of 5 and 6 log cfu/ml) and in an increase of μ_{max}. Only the strain *L. lactis* subsp. *lactis* CG27 was able to exert a slight inhibiting activity also at the higher initial *E. faecalis* μ_{max} value with respect to the control.

In Figure 1 the growth curves of *E. faecalis* EF37 at the different initial cell concentrations alone or in the presence of the lactococcal strains are showed together with the corresponding tyramine accumulation.

When *E. faecalis* EF37 was inoculated without the competitive cultures, at the lower enterococci initial concentrations (2 and 3 log cfu/ml), the accumulation of tyramine was detectable only after the beginning of the stationary phase, while in the other conditions tyramine was already detectable during the late exponential phase. However, independently of the initial enterococci inoculum, tyramine reached a final concentration of about 300 mg/l after 48 h. This concentration did not significantly change if the incubation was prolonged up to 96 h (data not shown).

The presence of the three lactococci decreased the rate of tyramine accumulation and its final amount depending on initial inoculum and *L. lactis* subsp. *lactis* strain. The tyramine final amount was reduced with minor efficacy at the higher *E. faecalis* EF37 initial concentration while, when EF37 was inoculated at 2 or 3 log cfu/ml, tyramine did not exceed 80 mg/l at the end of incubation period.

The lactococcal strains VR84 and EG46 were able to reduce the accumulation rate and the final amount of tyramine, also in the presence of 4 log cfu/ml *E. faecalis* EF37 initial inoculum. In fact, in this case, they limited at about 200 mg/l the accumulation of tyramine. By contrast, no difference with respect to the control was found in the presence of *L. lactis* subsp. *lactis* CG27.

At higher initial concentrations of *E. faecalis*, also the inhibition due to the strains VR84 and EG46 was less evident, even if *L. lactis* subsp. *lactis* VR84 was able to slow the rate of tyramine accumulation, but not the final tyramine amount.

Parameter/Diagnostic	*E. faecalis* EF37 initial inoculum: 2 log cfu/ml			
	EF37 without competition	*L. lactis* subsp. *lactis* strain		
		CG27	EG46	VR84
y_{min}	2.05 (± 0.10)	2.19 (± 0.14)	1.92 (± 0.16)	2.07 (± 0.13)
μ_{max}	0.98 (± 0.04)	0.52 (± 0.06)	0.32 (± 0.08)	0.47 (± 0.10)
h_0	3.02 (± 0.34)	1.35 (± 0.23)	0.22 (± 0.12)	2.45 (± 0.38)
y_{max}	8.80 (± 0.10)	4.97 (± 0.07)	4.73 (± 0.08)	4.64 (± 0.07)
RMSE	0.319	0.212	0.123	0.285
R	0.980	0.994	0.991	0.992
Parameter/Diagnostic	*E. faecalis* EF37 initial inoculum: 3 log cfu/ml			
	EF37 without competition	*L. lactis* subsp. *lactis* strain		
		CG27	EG46	VR84
y_{min}	2.99 (± 0.10)	3.13 (± 0.16)	3.10 (± 0.13)	3.07 (± 0.16)
μ_{max}	0.97 (± 0.05)	0.55 (± 0.11)	0.52 (± 0.13)	0.51 (± 0.09)
h_0	2.88 (± 0.36)	1.23 (± 0.20)	1.64 (± 0.27)	2.39 (± 0.45)
y_{max}	8.97 (± 0.09)	6.21 (± 0.08)	5.71 (± 0.09)	6.60 (± 0.08)
RMSE	0.319	0.212	0.123	0.285
R	0.996	0.995	0.993	0.994
Parameter/Diagnostic	*E. faecalis* EF37 initial inoculum: 4 log cfu/ml			
	EF37 without competition	*L. lactis* subsp. *lactis* strain		
		CG27	EG46	VR84
y_{min}	3.82 (± 0.10)	4.08 (± 0.23)	4.07 (± 0.14)	4.07 (± 0.13)
μ_{max}	0.91 (± 0.05)	0.46 (± 0.09)	0.94 (± 0.23)	0.54 (± 0.08)
h_0	2.15 (± 0.35)	0.20 (± 0.12)	3.73 (± 0.75)	2.01 (± 0.57)
y_{max}	8.99 (± 0.07)	7.64 (± 0.14)	6.81 (± 0.09)	7.33 (± 0.06)
RMSE	0.319	0.212	0.123	0.285
R	0.995	0.990	0.991	0.996
Parameter/Diagnostic	*E. faecalis* EF37 initial inoculum: 5 log cfu/ml			
	EF37 without competition	*L. lactis* subsp. *lactis* strain		
		CG27	EG46	VR84
y_{min}	4.95 (± 0.07)	5.10 (± 0.28)	5.30 (± 0.21)	4.97 (± 0.21)
μ_{max}	0.99 (± 0.06)	0.43 (± 0.15)	0.88 (± 0.17)	0.94 (± 0.20)
h_0	2.63 (± 0.33)	1.14 (± 0.45)	4.02 (± 0.58)	3.88 (± 0.82)
y_{max}	8.87 (± 0.04)	8.34 (± 0.17)	8.37 (± 0.12)	8.99 (± 0.20)
RMSE	0.319	0.212	0.123	0.285
R	0.992	0.993	0.992	0.991
Parameter/Diagnostic	*E. faecalis* EF37 initial inoculum: 6 log cfu/ml			
	EF37 without competition	*L. lactis* subsp. *lactis* strain		
		CG27	EG46	VR84
y_{min}	6.00 (± 0.06)	6.11 (± 0.24)	6.08 (± 0.15)	6.11 (± 0.13)
μ_{max}	1.02 (± 0.08)	0.68 (± 0.13)	0.79 (± 0.09)	0.84 (± 0.07)
h_0	2.79 (± 0.41)	2.39 (± 0.16)	2.40 (± 0.28)	1.29 (± 0.35)
y_{max}	9.03 (± 0.05)	8.43 (± 0.12)	8.94 (± 0.11)	9.18 (± 0.06)
RMSE	0.319	0.212	0.123	0.285
R	0.987	0.973	0.991	0.995

Table 2: Estimates of the Baranyi and Roberts model parameters describing the population dynamics of *E. faecalis* EF37, inoculated at different initial concentrations, grown alone or in competition withbacteriocinogenic *L. lactis* subsp. *lactis* strains (at a concentration of 7 log cfu/ml). The parameters are

reported with the relative standard error (within brackets), residual mean square error (RSME) and correlation coefficient (R).

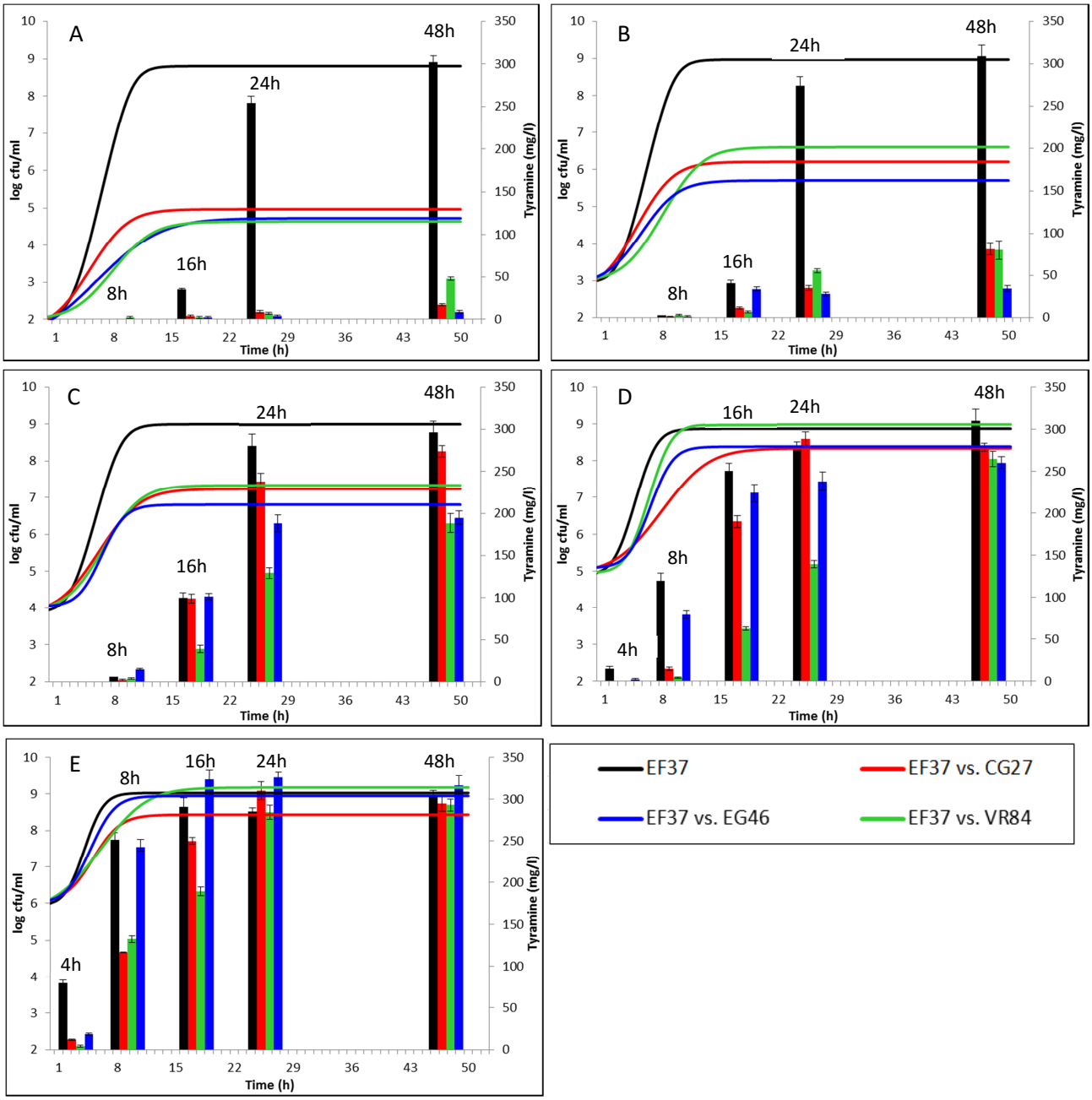

Figure 1: Growth curves (according to the parameters of Table 2) of *Enterococcus faecalis* EF37 with or without competitor cultures of bacteriocinogenic *L. lactis* subsp. *lactis* strains. In the same figure also the tyramine accumulated after 4, 8, 16, 24 and 48 h is reported. The graphs are referred to initial concentrations of 2 (A), 3 (B), 4 (C), 5 (D) and 6 (E) log cfu/ml of *E. faecalis* EF37.

4.5.3.3. Competition between lactococci and the histamine producing *Streptococcus thermophilus* PRI60.

The inhibition of *S. thermophilus* PRI60 growth and its histamine production due to the presence of the bacteriocinogenic lactococci were evaluated using the same experimental design adopted for *E. faecalis* EF37. When cultured alone, *S. thermophilus* PRI60 reached always a maximum cell concentration of about 9.2-9.4 log cfu/ml. Also in this case the microbial growth was modelled with the Baranyi and Roberts equation (Table 3). The streptococci growth alone or in co-culture with lactococci and the corresponding histamine accumulation in relation to the initial inoculum are shown in Figure 2. All the lactococci were more efficient in inhibiting *S. thermophilus* PRI60 in comparison with *E. faecalis* EF37. In particular, the nisin Z producer strain *L. lactis* subsp. *lactis* VR84 caused the death of the streptococci. For this reason, it was not possible to calculate under these conditions the parameters of Baranyi and Roberts equation, and Table 3 reports for this strain the parameters of the linear inactivation model. In this case, $logN_0$ defines the initial cell log cfu/ml while $-k$ is the rate ((log cfu/ml)/h) at which streptococci were killed as result of the competition with *L. lactis* VR84. The rate of inactivation was rather constant (about 0.5 (log cfu/ml)/h), independently on the initial cell concentration, and no survivor were detected at the end of incubation.

The other two lactococcal strains did not show a lethal action against *S. thermophilus* PRI60, but were able to reduce consistently its final amount in relation to the initial streptococci inoculum. Among the strains employed, *L. lactis* subsp. *lactis* CG27 was more efficient than EG46 in reducing the streptococci final concentration (at 3.2, 4.4 and 5.8 log cfu/ml) at the lower inoculum levels. No significant differences were observed in relation to the presence of these two lactococcal strains at initial *S. thermophilus* PRI60 inoculum of 5 and 6 log cfu/ml. However, in this case the final streptococci concentrations were comprised between 6.8 and 7.9 log cfu/ml, well below the level reached by *S. thermophilus* PRI60 grown alone under the same conditions (9.2-9.4 log cfu/ml).

The data relative to the histamine indicated that when the strain was not in competition with lactococci, the biogenic amine accumulation started at the beginning of the stationary phase (independently on the streptococci initial concentration) and rapidly reached a maximum level of about 200 mg/l after 48 h. As already observed for *E. faecalis* EF37, this value did not significantly change by prolonging the incubation time till 96 h (data not shown). The competition with lactococci dramatically decreased this accumulation (Figure 2). In the presence of *L. lactis* subsp. *lactis* VR84 only traces of histamine were detected in the samples inoculated with 5 and 6 log cfu/ml. Also *L. lactis* subsp. *lactis* CG27 drastically decreased the production of histamine. Reduced amounts (about 75 mg/l) at the end of incubation were found only when *S. thermophilus*

PRI60 was inoculated at 6 log cfu/ml. *Lactococcus lactis* subsp. *lactis* EG46 was less effective in limiting histamine production and, at the end of incubation, about 80 mg/l and 160 mg/l of this biogenic amine were found in the samples with initial streptococci inoculum of 5 and 6 log cfu/ml, respectively. However, also in this case, these amounts were lower than those produced by *S. thermophilus* PRI60 grown alone.

Parameter/Diagnostic	*S. thermophilus* PRI60 initial inoculum: 2 log cfu/ml			
	PRI60 without competition	*L. lactis* subsp. *lactis* strain		
		CG27	EG46	VR84*
y_{min}	1.86 (± 0.22)	1.88 (± 0.12)	1.82 (± 0.32)	N_0 2.03 (± 0.08)
μ_{max}	0.56 (± 0.04)	0.55 (± 0.15)	0.49 (± 0.12)	-*k* 0.51 (± 0.14)
h_0	1.32 (± 0.52)	1.56 (± 0.39)	1.68 (± 0.33)	-
y_{max}	9.46 (± 1.18)	3.22 (± 0.66)	4.69 (± 0.37)	-
RMSE	0.196	0.049	0.162	0.106
R	0.998	0.994	0.973	0.988
Parameter/Diagnostic	*S. thermophilus* PRI60 initial inoculum: 3 log cfu/ml			
	PRI60 without competition	*L. lactis* subsp. *lactis* strain		
		CG27	EG46	VR84
y_{min}	2.80 (± 0.16)	2.89 (± 0.27)	2.84 (± 0.13)	N_0 3.01 (± 0.13)
μ_{max}	0.53 (± 0.04)	1.43 (± 0.66)	1.02 (± 0.16)	-*k* 0.50 (± 0.12)
h_0	1.08 (± 0.18)	3.79 (± 0.77)	3.71 (± 0.86)	-
y_{max}	9.30 (± 0.12)	4.37 (± 0.09)	5.63 (± 0.07)	-
RMSE	0.138	0.131	0.118	0.140
R	0.998	0.934	0.995	0.978
Parameter/Diagnostic	*S. thermophilus* PRI60 initial inoculum: 4 log cfu/ml			
	PRI60 without competition	*L. lactis* subsp. *lactis* strain		
		CG27	EG46	VR84
y_{min}	3.91 (± 0.17)	3.89 (± 0.34)	3.78 (± 0.25)	N_0 3.96 (± 0.18)
μ_{max}	0.55 (± 0.05)	0.84 (± 0.27)	0.47 (± 0.10)	-*k* 0.49 (± 0.10)
h_0	1.38 (± 0.52)	3.05 (± 0.81)	1.52 (± 0.43)	-
y_{max}	9.35 (± 0.12)	5.76 (± 0.15)	6.71 (± 0.13)	-
RMSE	0.151	0.312	0.215	0.209
R	0.997	0.925	0.983	0.991
Parameter/Diagnostic	*S. thermophilus* PRI60 initial inoculum: 5 log cfu/ml			
	PRI60 without competition	*L. lactis* subsp. *lactis* strain		
		CG27	EG46	VR84
y_{min}	4.91 (± 0.19)	4.86 (± 0.20)	5.17 (± 0.24)	N_0 5.14 (± 0.10)
μ_{max}	0.73 (± 0.10)	1.77 (± 0.61)	1.64 (± 0.08)	-*k* 0.49 (± 0.08)
h_0	2.08 (± 0.74)	4.42 (± 0.93)	6.30 (± 0.93)	-
y_{max}	9.37 (± 0.11)	6.92 (± 0.07)	6.83 (± 0.18)	-
RMSE	0.163	0.170	0.262	0.202
R	0.996	0.976	0.930	0.964
Parameter/Diagnostic	*S. thermophilus* PRI60 initial inoculum: 6 log cfu/ml			
	PRI60 without competition	*L. lactis* subsp. *lactis* strain		
		CG27	EG46	VR84
y_{min}	6.05 (± 0.17)	5.90 (± 0.05)	5.83 (± 0.15)	N_0 5.96 (± 0.18)
μ_{max}	0.92 (± 0.17)	2.27 (± 0.32)	1.21 (± 0.40)	-*k* 0.53 (± 0.04)
h_0	2.95 (± 0.97)	5.86 (± 0.89)	4.34 (± 0.81)	-
y_{max}	9.27 (± 0.10)	7.47 (± 0.02)	7.89 (± 0.08)	-
RMSE	0.118	0.044	0.138	0.322
R	0.994	0.997	0.986	0.982

Table 3: Estimates of the Baranyi and Roberts model parameters describing the population dynamics of S. thermophilus PRI60 inoculated at different initial concentrations grown alone or in competition with bacteriocinogenic L. lactis subsp. lactis strains (at a concentration of 7 log cfu/ml). The parameters are

reported with the relative standard error (within brackets) and residual mean square error (RSME) and correlation coefficient (R).

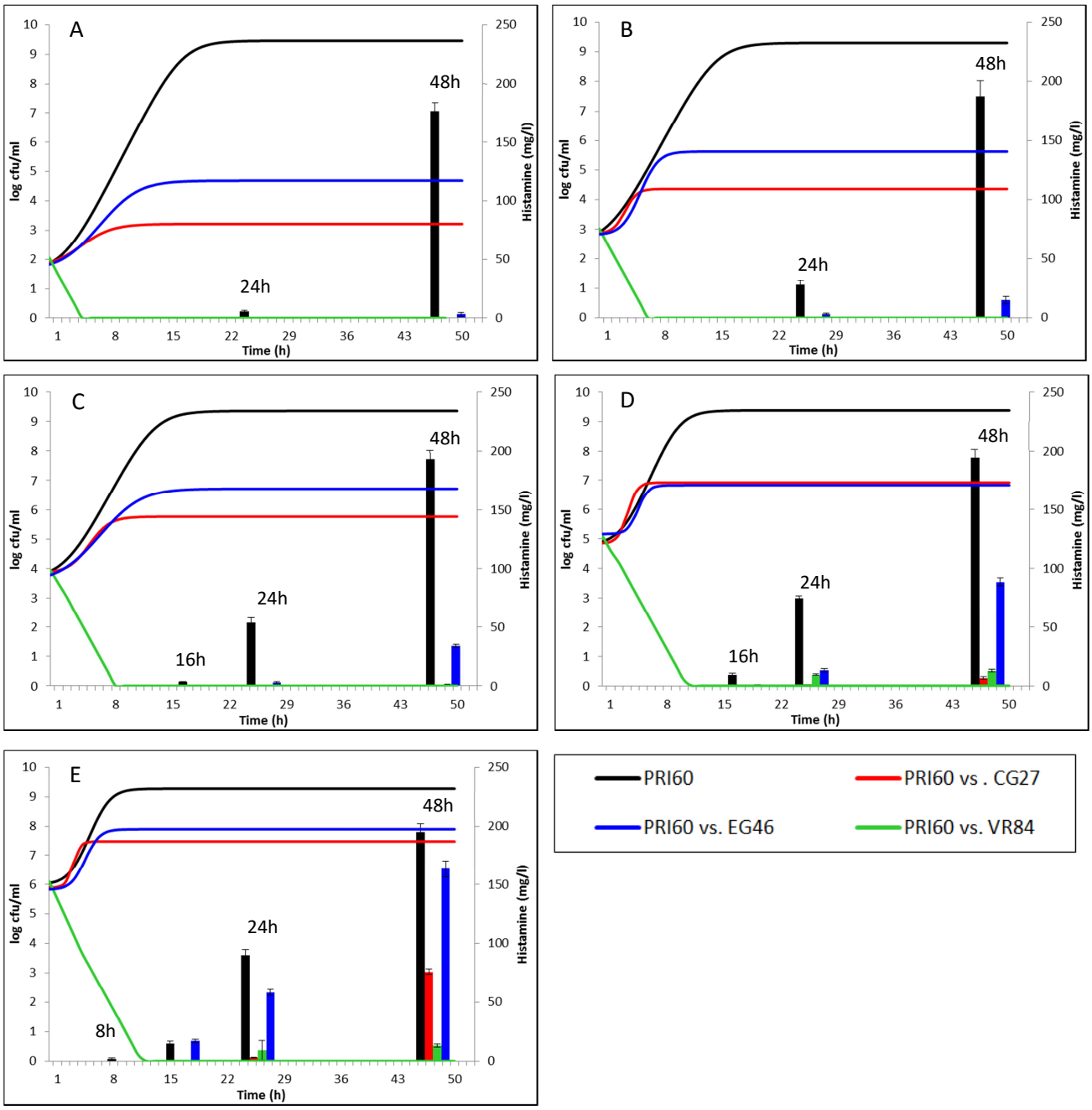

Figure 2: Growth curves (according to the parameters of Table 3) of *Streptococcus thermophilus* PRI60 with or without competitor cultures of bacteriocinogenic *L. lactis* subsp. *lactis* strains. In the same figure also the histamine accumulated after 4, 8, 16, 24 and 48 h is reported. The graphs are referred to initial concentrations of 2 (A), 3 (B), 4 (C), 5 (D) and 6 (E) log cfu/ml of *S. thermophilus* PRI60. Since the presence of *L. lactis* subsp. lactis VR84 caused the death of the strain PRI60, the curve reported is not the microbial growth modelled with Baranyi and Roberts model but the death kinetic modelled with Arrhenius equation.

4.5.4. Discussion

The reduction of BA content in food, and especially in fermented foods, is attracting increasing attention by researchers and international Agencies (EFSA, 2011). Effective strategies able to limit the accumulation of the most dangerous BAs (tyramine and histamine) can be based on the inhibition of aminobiogenic microorganism growth or of their decarboxylase activity, with the aim to improve the quality of foods. Up to date, the potential of modified atmosphere packaging, high hydrostatic or homogenization pressure, irradiation or thermal treatments have been tested with this purpose (Naila *et al*., 2010). In fermented foods a crucial role can be played by compositional and process variables (temperature, salt, pH), by raw material microbial quality and by the choice of proper starter cultures (Ancín-Azpilicueta *et al*., 2008; Linares *et al*., 2011; Suzzi and Gardini, 2003). The presence of the bacteriocin producing bacteria, like those studied here, can be a further tool to control undesirable BA accumulation. In fact, the antimicrobial potential of bacteriocins has been studied mainly with respect to pathogenic species such as *Listeria monocytogenes*, *Staphylococcus aureus* and *Clostridium* spp. (Balciunas *et al*., 2013; Guinane *et al*., 2005; Reis *et al*., 2012). In particular, the possible application of antimicrobial strategies based on bacteriocins use has been tested in dairy industry (Beshkova and Frengova, 2012; Leroy and de Vuyst, 2010) and in other food products (Chen and Hoover, 2003). In a paper of some years ago, bacteriocinogenic LAB strains were used to contrast the decarboxylation activity of a histamine producing *Lactobacillus buchneri* strain. In particular, two bacteriocinogenic *E. faecalis* strains and a nisin producer *L. lactis* strain were employed (Joonsten and Nuñez, 1996). Good results were obtained in cheese in which the initial concentration of histaminobiogenic strain was 2.28 log cfu/ml. However, usually *E. faecalis* produces tyramine, which is considered a species trait for these microorganisms (Ladero *et al.*, 2012), and the production of this BA was not considered in the paper. In addition, Özogul (2011) demonstrated possible interactions between LAB strains and foodborne pathogens with respect to BA production.

The lactococcal strains considered in this research did not produce BAs but were able to synthesize three different types of bacteriocins. One of the strains (*L. lactis* subsp. *lactis* CG27) likely produced a bacteriocin not yet identified, whose peptidic nature was, however, demonstrated. Further studies are needed to characterize the antimicrobial substance produced by this strain. By contrast, the strains *L. lactis* subsp. *lactis* VR84 and EG46 produced nisin Z and lacticin 481, respectively. Lacticin 481 is a medium spectrum lantabiotic able to inhibit other LAB and clostridia with a bacteriolytic mode of action (Guinane *et al*., 2005), while nisin is a single peptide composed of 34 amino acid residues and five natural variants are known. It displays a broad spectrum of activity against different Gram positive bacteria and inhibits outgrowth of spores of bacilli and

clostridia (Leroy and de Vuyst, 2010). The effects of these bacteriocins have already been studied in cheese manufacture for several reasons, including pathogen and spoilage microorganism control and acceleration of ripening by favouring the lysis of starter LAB cells (Beshkova and Frengova, 2012).

In the absence of competition, the accumulation of tyramine by *E. faecalis* EF37 was detectable since the late exponential phase or at the beginning of stationary phase, depending on the initial inoculum. In any case, the final amount of tyramine produced (about 300 mg/l) was close to the maximum theoretical concentration allowed by the precursor added (378 mg/l if all the tyrosine added was converted). Pessione *et al*. (2009) found that tyramine production in *E. faecalis* DISAV1022 was accumulated in large amounts during the late exponential growth and was maintained until tyrosine was depleted, suggesting that tyrosine decarboxylation was not a response to starvation or nutrient depletion typical of the stationary phase. In these trials, this observation was confirmed for *E. faecalis* EF37 only for the higher initial inoculums (5 and 6 log cfu/ml). A similar behaviour was observed in the presence of lactococcal bioprotective cultures.

All the three bacteriocin producing lactococci were able to reduce the growth performance of *E. faecalis* EF37 by limiting its final concentration, with some exceptions only when it was inoculated at 5 log cfu/ml or more. Several studies on food microorganism interactions highlighted that the relationships between the populations involve the reduction of maximum population density without showing significant effects on lag time and growth rate. Then, the minority population decelerates when the majority population reaches its maximum (Buchanan and Bagi, 1997; Carlin et., 1996; Cornu *et al*., 2011; Devlieghere *et al*., 2001). Also in this case, the most important effect of the presence of lactococci was their ability to reduce, whenever possible, the cell density of *E. faecalis* EF37.

In spite of this inhibition, enterococcal cells remained viable in the medium and could accumulate tyramine, whose quantity and rate firstly depended on the maximum cell concentration reached by enterococci and by the time of incubation. In co-cultured samples, the production of tyramine was very low during incubation only in the medium inoculated with 2 log cfu/ml and, to a lesser extent, with 3 log cfu/ml. Under the conditions adopted in these trials, tyramine accumulation was detectable only when enterococci concentration reached and exceeded a threshold of about 6 log cfu/ml or after a prolonged incubation if the concentration reached was lower (between 4 and 5 log cfu/ml). However, in these cases the tyramine was accumulated at very low concentrations (from 10 to 80 mg/l) if compared with the control.

The positive relation between extent of growth and tyramine production in *E. faecalis* EF37 in relation to pH, temperature and salt concentration has already been described (Gardini *et al*., 2001).

Nevertheless, the amount of tyramine produced was often negatively correlated with the transcription of tyrosine decarboxylase gene (*tyrDC*), suggesting a higher *tyrDC* expression under suboptimal environmental conditions (Torriani *et al*., 2008). In other words, the tyramine accumulation was the results of two interactive factors, the amount of *tyrDC* transcripte inside the cells and the number of cells in the medium. The presence of some cases of similar *Enterococcus faecalis* EF37 (see for example Figure 1C) and *S. thermophilus* PRI60 (Figure 2D) final cell concentration associated with different BA amounts suggests that the reduction of biogenic amine accumulation could be associated to a diminution of decarboxylase transcription or to intracellular conditions less favourable for enzyme activity caused by the different bacteriocin mechanism of action. However, this hypothesis needs to be supported by further experiments.

The ability of the species *S. thermophilus* to produce histamine has been recently demonstrated (Calles-Enriquéz *et al*., 2010; Gezginc *et al*., 2013; Rossi *et al*., 2011; Tabanelli *et al*., 2012; Trip *et al*., 2011). *S. thermophilus* PRI60 accumulated histamine (alone or in competition) only after the beginning of stationary phase. The maximum expression of histidine-decarboxylating gene (*hdcA*) during the stationary phase in *S. thermophilus* has been already observed, together with a reduced transcription activity during the lag and exponential growth phases (Calles-Enriquez *et al*., 2010). Also Pessione *et al*. (2005) indicated in the stationary phase the maximum histamine biosynthesis in *Lactobacillus* 30a. By contrast, Landete *et al.* (2006) found that the expression of *hdcA* gene was higher in the exponential phase in wine LAB (lactobacilli, pediococci and oenococci) and decreased in the stationary phase; however, the enzyme accumulated in the cells could express its maximum activity when the environmental conditions became harsher. In addition, histamine acted as a competitive inhibitor towards *hdcA* expression (Landete *et al*., 2006; Rollan *et al*., 1995). This fact could explain, at least in part, that the maximum histamine production by *S. thermophilus* PRI60 in the absence of competitors was well below the maximum theoretical concentration (358 mg/l). This means that about 55% of the available histidine was converted into histamine by *S. thermophilus* PRI60, while *E. faecalis* EF37 was able to decarboxylate tyrosine at 78%.

In general, *S. thermophilus* PRI60 was less competitive than *E. faecalis* EF37 when grown in co-culture with bacteriocinogenic lactococcal strains. The production of nisin Z by *L. lactis* subsp. *lactis* VR84 rapidly killed streptococcal cells. This drastic effect of nisin on this species has already been described and is responsible for cell lysis (Aslim and Alp, 2009; de Arauz *et al*., 2009; Garde *et al*., 2004) which makes the intracellular enzymes free in the medium. The aminoacid decarboxylases which produce BAs are often more active in cell free extracts than inside the viable cells (Moreno-Arribas and Lonvaud-Funel, 2001) and this ability was demonstrated for the hdcA of *S. thermophilus* PRI60 both *in vitro* and in cheese (Gardini *et al*., 2012; Tabanelli *et al*., 2012;). In

these trials, however, the possible cell lysis did not determine a histamine accumulation. This could confirm that *hdcA* gene was actively expressed only during the stationary phase, although a basal level of *hdcA* expression occurs even without histidine (Landete *et al*., 2006). The latter aspect could be the reason for the low presence of histamine in the samples containing *L. lactis* subsp. *lactis* VR84 and characterized by the higher *S. thermophilus* PRI60 inoculum. In any case, independently on the cell lysis, the bacteriocinogenic strains demonstrated the capacity to limit the accumulation of histamine even when streptococci were present at high concentration; this fact opens promising developments for the use of bioprotective cultures in dairy industry.

4.5.5. Conclusions

The presence of aminobiogenic LAB in natural starter cultures or among the ripening microbiota is a serious problem for the safety features of many fermented products. Lactococci are often used as starter cultures in many cheeses since they can keep their viability and produce bacteriocins during all the processing and ripening. In fact, the lactococci will continue to synthesize bacteriocin, avoiding the risks linked to the *ex situ* philosophy, due to the degradation of the bacteriocins by peptidases. Moreover, the use of bacteriocins *ex situ* (i.e. produced by bacteria and added as ingredients or food additives) is still limited, probably because of the generally negative attitude of consumers towards food additives. In this context, the *in situ* production of bacteriocins by microorganisms present in food constitutes an advantage, both for legal aspects (labelling) and economic costs.

This preliminary study has clarified some aspects regarding the ratio between bacteriocinogenic strains and aminobiogenic strains with respect to the possibility to accumulate BA and has also showed that different bacteriocins can have different effects on BA production on the same strain. Further investigations are needed to better elucidate the mechanism of these interactions also in real systems. This knowledge is essentially aimed to use bacteriocinogenic lactococci as a predictable strategy against aminobiogenic bacteria present in cheese or other fermented foods.

Chapter 5

Conclusions

Even if the presence of biogenic amines in foods (and the risks associated with them) is known since a long period (Gale, 1946), systematic studies regarding their presence have been carried out only in relatively recent times. The reviews of Shalaby (1996) and Silla-Santos (1996) had the merit to collect the fragmented information about this issue and were the starting point for a drastic multiplication of scientific publications regarding the presence of biogenic amine in food products and the elucidation of the metabolic and genetic drivers of their production by microrganisms. Combining the words “biogenic amine” and “food” the number of publication selected by the Web of Science passed from about 500 in the year 2000 to more than 4500 in 2015.

This increasing scientific effort allowed to obtain a deeper knowledge about the genetic and biochemical mechanisms responsible for biogenic amines production by foodborne microorganisms, but also furnished important information about the possibility to reduce their accumulation in food and the risks associated with their presence.

The possible ways to achieve this goal in food are mainly based on two strategies, which always are strictly interacting each other: the modulation of process and environmental factors including storage and distribution conditions and the control of the microbiota associated with foods.

In this perspective, the researches carried out in this PhD thesis are a contribution aimed to give a deeper insight of the factors and biological mechanisms influencing the activity of tyrosine decarboxylases in LAB belonging to the genus *Enterococcus*. In particular, these studies indicate that, even if the presence of *tyrDC* gene is, as expected, widespread among enterococci, the potential for tyramine (and 2-phenylethylamine) accumulation can be very different. These differences interested the kinetics of accumulation (both qualitatively and quantitatively), the effects of some technological variables (temperature, salt concentration and pH) on the decarboxylase activity and also the genetic of the tyrosine decarboxylase cluster as well as the transcription of the genes involved.

In addition, fundamental differences concerning the responses to environmental factors of the pure enzyme and microbial cells were highlighted, indicating that the decarboxylation activity has to be viewed in the light of the overall cell metabolism.

During this work, a particular attention has been posed on strains belonging to the species *Enterococcus faecalis* and *Enterococcus faecium*. However, a relevant part of the work has been addressed to the elucidation of the mechanisms of tyrosine decarboxylation in the species *Enterococcus mundtii*. This is the first contribution to the study of decarboxylases in this species, which can be associate with animal feed (silage) but also with fermented foods of animal origin. Finally, also the use of bioprotective cultures producing bacteriocins as antagonists against biogenic

amine producing microorganisms has been exploited and this strategy seems to be a promising tool to reduce the risks due to excessive biogenic amine accumulation in fermented foods.

A- DISSEMINATION OF THE RESULTS:

A1- Papers published in peer-reviewed journals with impact factor

1. Tabanelli, G., Montanari, C., Bargossi, E., Lanciotti, R., Gatto, V., Felis, G., Torriani, S. and Gardini, F. (2014). Control of tyramine and histamine accumulation by lactic acid bacteria using bacteriocin forming lactococci. *International Journal of Food Microbiology* 190, 14-23. doi: 10.1016/j.ijfoodmicro.2014.08.023
2. Bargossi, E., Tabanelli, G., Montanari, C., Lanciotti, R., Gatto, V., Gardini, F. and Torriani, S. (2015). Tyrosine decarboxylase activity of enterococci grown in media with different nutritional potential: tyramine and 2-phenylethylamine accumulation and *tyrDC* gene expression. *Frontiers in Microbiology* 6, 259. doi: 10.3389/fmicb.2015.00259
3. Tabanelli, G., Bargossi, E., Gardini, A., Lanciotti, R., Magnani, R., Gardini, F. and Montanari, C. (2015). Physico-chemical and microbiological characterization of Italian fermented sausages in relation to their size. *Journal of the Science of Food and Agriculture*. doi: 10.1002/jsfa.7442
4. Bargossi, E., Gardini, F., Gatto, V., Montanari, C., Torriani, S. and Tabanelli, G. (2015). The capability of tyramine production and correlation between phenotypic and genetic characteristics of *Enterococcus faecium* and *Enterococcus faecalis* strains. *Frontiers in Microbiology* 6, 1371. doi: 10.3389/fmicb.2015.01371
5. Gatto, V., Tabanelli, G., Montanari, C., Prodomi, V., Bargossi, E., Torriani, S. and Gardini, F. Tyrosine decarboxylase activity of *Enterococcus mundtii*: new insights on phenotypic and genetic aspects. (submitted).

A2- Partecipation to national or international congress

1. Bargossi, E., Tabanelli, G., Montanari, C., Torriani, S., Felis, G., Lanciotti, R. and Gardini F.. *In vitro* tyramine accumulation by different strains of lactic acid bacteria. International Congress “Spoilers in Food”, 1st-3rd July 2013, Quimper (France). Book of abstract, page 55.
2. Bargossi, E.. Tyramine production by different strains of lactic acid bacteria in relation to the cultural medium. 18th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 25-27 september 2013, Conegliano (Italy). Book of abstract, page 317.
3. Tabanelli, G., Bargossi, E., Montanari, C., Patrignani, F., Lanciotti, R., Coloretti, F., Grazia, L. and Gardini, F.. Control of tyramine production by *Enterococcus faecalis in vitro* through the use of *Lactococcus lactis* bioprotective cultures. 2nd International Conference on Microbial

diversity, MD2013, 23-25 october 2013, Turin (Italy). Book of abstract, 327. Designated by the MD2013 Scientific Committee as the recipient of the FEMS scholarship.

4. Bargossi, E.. Competition among bacteriocin producing lactococci and aminobiogenic lactic acid bacteria strains. 19th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 24-26 september 2014, Bari (Italy), page 129.
5. Montanari, C., Tabanelli, G., Bargossi, E., Lanciotti, R., Gatto, V., Torriani, S. and Gardini, F.. *In vitro* tyramine accumulation by different *Enterococcus* strains. 11th Symposium on Lactic Acid Bacteria, Egmond aan Zee, Netherlands. 31 August-4 September, 2014. Book of abstract B058.
6. Tabanelli, G., Bargossi, E., Montanari, C., Lanciotti, S., Torriani, S. and Gardini, F. Tyramine production of enterococci under different pH, NaCl concentration and incubation temperature. 11th Symposium on Lactic Acid Bacteria, Egmond aan Zee, Netherlands. 31 August-4 September, 2014. Book of abstract E045.
7. Tabanelli, G., Bargossi, E., Montanari, C., Patrignani, F., Lanciotti, R., Gatto, V., Torriani, S. and Gardini F. Control of biogenic amine production by *Enterococcus faecalis* and *Streptococcus thermophilus* in vitro through the use of *Lactococcus lactis* bioprotective cultures. 11th Symposium on Lactic Acid Bacteria, Egmond aan Zee, Netherlands. 31 August-4 September, 2014. Book of abstract E053.
8. Tabanelli, G., Montanari, C., Bargossi, E., Patrignani, F., Lanciotti, R. and Gardini, F.. Characterization of typical Italian fermented sausages in relation to chemico-physical and microbiological features. 2nd international symposium on fermented meat, 20-23 October 2014, Valencia (Spain). Book of abstract, 90.
9. Bargossi, E.. Studies of tyraminodevarboxylase activity of enterococci in different media. 20th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology, 23-25 september 2015, Perugia (Italy), page 228.

B- OTHER PUBLICATIONS

1. Montanari, C., Bargossi, E., Lanciotti, R., Chinnici, F., Gardini, F. and Tabanelli, G. (2014). Effects of two different sourdoughs on the characteristics of Pandoro, a typical Italian sweet leavened baked good. *LWT - Food Science and Technology* 59, 289-299. doi: 10.1016/j.lwt.2014.04.045
2. Siroli, L., Patrignani, F., Montanari, C., Tabanelli, G., Bargossi, E., Gardini, F. and Lanciotti, R. (2014). Characterization of Oregano (*Origanum vulgare*) essential oil and definition of its

antimicrobial activity against *Listeria monocytogenes* and *Escherichia coli in vitro* system and onto foodstuff surfaces. *African Journal of Microbiology Research* 8, 2746-2753. doi: 10.5897/ajmr2014.6677

3. Montanari, C., Bargossi, E., Gardini, A., Lanciotti, R., Magnani, R., Gardini, F. and Tabanelli, G. (2016). Correlation between volatile profiles of Italian fermented sausages and their size and starter culture. *Food Chemistry* 192, 736-744. doi: 10.1016/j.foodchem.2015.07.062.

4. Montanari, C., Tabanelli, G., Patrignani, F., Bargossi, E., Gardini, F., Vinderola, G., Reinheimer, J. and Lanciotti, R.. Effect of a sub-lethal treatment of HPH on volatilome, membrane fatty acid composition and cell outermost structures of two probiotic strains. 2nd International Conference on Microbial diversity, MD2013, 23-25 october 2013, Turin (Italy). Book of abstract, page 268.

5. Patrignani, F., Montanari, C., Siroli, L., Tabanelli, G., Vernocchi, P., Bargossi, E., Serrazanetti, I. D., Grazia, L., Gardini, F. and Lanciotti, R.. Volatile molecule profiles and sulphur compounds in Trebbiano wines obtained by different *Saccharomyces cerevisiae* strains. 2nd International conference on Microbial diversity, MD2013, 23-25 october 2013, Turin (Italy). Book of abstract, page 299.

6. Vernocchi, P., Patrignani, F., Montanari, C., Parpinello, G., Serrazanetti, D. I., Del Chierico, F., Putignani, L., Tabanelli, G., Bargossi, E., Siroli, L., Gardini, F. and Lanciotti, R.. Yeast microbial ecology, aroma profiles and safety features of wines from biological or biodynamic grapes in relation to starter addition. 2nd International Conference on Microbial diversity, MD2013, 23-25 october 2013, Turin (Italy). Book of abstract, page 255.

7. Montanari, C., Tabanelli, G., Bargossi, E., Patrignani, F., Lanciotti, R. and Gardini, F.. Volatiloma profile of traditional Italian fermented sausages in relation to diameter, fat content and starter cultures. 2nd International Symposium on Fermented Meat, 20-23 October 2014, Valencia (Spain). Book of abstract, page 89.

8. Tabanelli, G., Montanari, C., Bargossi, E., Lanciotti, R., Grazia, L. and Gardini, F. Effects of size and starter cultures on the ripening and final characteristics of fermented sausages. 3rd International Conference on Microbial diversity “The challenge of complexity”, MD2015, 27-29 October 2015, Perugia (Italy). Book of abstract, 271.

9. Montanari, C., Tabanelli, G., Bargossi, E., Pasini, F., Caboni, M. F. and Gardini, F. Spontaneous fermentation of a cheese-type vegan product: evaluation of the microbial

community. 3rd International Conference on Microbial diversity “The challenge of complexity”, MD2015, 27-29 October 2015, Perugia (Italy). Book of abstract, 271.

10. Tabanelli, G., Montanari, C., Bargossi, E., Patrignani, F., Lanciotti, R., Rossi, M., Amaretti, A., Raimondi, S., Gardini, F. Characterization of sourdough for the production of Colomba, an italian traditional sweet-leavened baked good. 3rd International conference on Microbial diversity, “The challenge of complexity”, , MD2015, 27-29 October 2015, Perugia, Italy. Book of abstract, 272.

References

Adav, S. S., Lee, D. J., Wang, A. and Ren, N. (2009). Functional consortium for hydrogen production from cellobiose: concentration-to-extinction approach. *Bioresource Technology* 100, 2546-2550.

Akopyanz, N., Bukanov, N. O., Westblom, T. U., Kresovich, S. and D. E. Berg. (1992). DNA diversity among clinical isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. *Nucleic Acid Research* 20, 5137-5142.

Aktypis, A., Kalantzopoulos, G., Huis in't Veld, J. H. J. and ten Brink, B. (1998). Purification and characterization of thermophilin T, a novel bacteriocin produced by *Streptococcus thermophilus* ACA-DC 0040. *Journal of Applied Microbiology* 84, 568-576.

Al Bulushi, I., Poole, S., Deeth, H. C. and Dykes, G. A. (2009). Biogenic amines in fish: roles in intoxication, spoilage, and nitrosamine formation - A review. *Critical Review in Food Science and Nutrition* 49, 369-377.

Alegría, A., Delgado, S., Roces, C., López, B. and Baltasar, M. (2010). Bacteriocins produced by wild *Lactococcus lactis* strains isolated from traditional, starter-free cheeses made of raw milk. *International Journal of Food Microbiology* 143, 61-66.

Allen, W. D., Linggood, M. A. and Porter, P. (1996). *Enterococcus* organisms and their use as probiotics in alleviating irritable bowel syndrome symptoms. European Patent 0508701 (B1).

Ancín-Azpilicueta, C., González-Marco, A. and Jiménez-Moreno, N. (2008). Current knowledge about the presence of amines in wine. *Critical Review in Food Science and Nutrition* 48, 257-275.

Andersson, U., Levander, F. and Radström, P. (2001). Trehalose-6-phosphate phosphorylase is part of a novel metabolic pathway for trehalose utilization in *Lactococcus lactis*. *Journal of Biological Chemistry* 46, 42707-42713.

Andersson, D. I. and Hughes, D. (2010). Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature Reviews Microbiology* 8, 260-271.

Anli, R. E., Vural, N., Demiray, S. and Mert, B. (2006). Biogenic amine content of beers consumed in Turkey and influence of storage conditions on biogenic amine formation. *Journal of the Institute of Brewing* 112, 267-274.

Ansorena, D., Montel, M. C., Rokka, M., Talon, R., Enrola, S., Rizzo, A., Raemaekers, M. and Demeyer, D. (2002). Analysis of biogenic amines in northern and southern European sausages and role of flora in amine production. *Meat Science* 61, 141-147.

Arena, M. E. and Manca de Nadra, M. C. (2001). Biogenic amine production by *Lactobacillus*. *Journal of Applied Microbiology* 90, 158-162.

Arena, M. P., Romano, A., Capozzi, V., Beneduce, L., Ghariani, M., Grieco, F., Lucas, P. and Spano, G. (2011). Expression of *Lactobacillus brevis* IOEB 9809 tyrosine decarboxylase and agmatine deiminase genes in wine correlates with substrate availability. *Letters in Applied Microbiology* 53, 395-402.

Aslim, B. and Alp, G. (2009). The effect of immobilization on some probiotic properties of *Streptococcus thermophilus* strains. *Annals of Microbiology* 59, 127-132.

Ayensa Aguirre, M. G. (1993). Estudio de la evoluci6n de1 proceso de anchoado de sardina (*Sardina pilchardus, Wulb*). *Alimentaria* 248, 71-85.

Aymerich, T., Martín, B., Garriga, M., Vidal-Carou, M. C., Bover-Cid, S. and Hugas, M. (2006). Safety properties and molecular strain typing of lactic acid bacteria from slightly fermented sausages. *Journal of Applied Microbiology* 100, 40-49.

Baixas-Noguera, S., Bover-Cid, S., Veciana-Nogués, M. T., Mariné-Font, A. and Vidal-Carou M. C. (2005). Biogenic amine index for freshness evaluation in iced Mediterranean hake (*Merluccius merluccius*). *Journal of Food Protection* 68, 2433-2438.

Balciunas, E. M., Martinez, F. A. C., Todorov, S. D., Franco, B. D. G. M., Converti, A., Oliveira and R. P. D. (2013). Novel biotechnological applications of bacteriocins: A review. *Food Control* 32, 134-142.

Bandell, M., Lhotte, M. E., Marty-Teysset, C., Veyrat, A., Prévost, H., Dartois, V., Diviès, C., Konings, W. N. and Lolkema J. S. (1998). Mechanism of the citrate transporters in carbohydrate and citrate cometabolism in *Lactococcus* and *Leuconostoc* species. *Applied and Environmental Microbiology* 64, 1594-1600.

Baranyi, J. and Roberts, T. A. (1994). A dynamic approach to predicting bacterial growth in food. *International Journal of Food Microbiology* 23, 277–294.

Bardócz, S., Duguid, T. J., Brown, D. S., Grant, G., Pusztai, A., White, A. and Ralph, A. (1995a). The importance of dietary polyamines in cell regeneration and growth. *British Journal of Nutrition* 73, 819-828.

Bardócz, S. (1995b). Polyamines in food and their consequences for food quality and human health. *Trends in Food Science and Technology* 6, 341-346.

Bargossi, E., Gardini, F., Gatto, V., Montanari, C., Torriani, S. and Tabanelli, G. (2015a). The capability of tyramine production and correlation between phenotypic and genetic characteristics of *Enterococcus faecium* and *Enterococcus faecalis* strains. *Frontiers in Microbiology* 6, 1371.

Bargossi, E., Tabanelli, G., Montanari, C., Lanciotti, R., Gatto, V., Gardini, F. and Torriani, S. (2015b). Tyrosine decarboxylase activity of enterococci grown in media with different nutritional potential: tyramine and 2-phenylethylamine accumulation and *tyrDC* gene expression. *Frontiers in Microbiology* 6, 259.

Beimfohr, C., Ludwig, W. and Schleifer, K. H. (1997). Rapid genotypic differentiation of *Lactococcus lactis* subspecies and biovar. *Systematic and Applied Microbiology* 20, 216-221.

Bellomo, G., Mangiagle, A., Nicastro, L. and Frigerio, G. (1980). A controlled double-blind study of SF68 strain as a new biological preparation for the treatment of diarrhoea in pediatrics. *Current Therapeutic Research* 28, 927-936.

Ben Omar, N., Castro, A., Lucas, R., Abriouel, H., Yousif, N. M. K., Franz, C. M. A. P., Holzapfel, W. H., Pérez-Pulido, R., Martínez-Cañamero, M. and Gálvez, A. (2004). Functional and safety aspects of enterococci isolated from different Spanish foods. *Systematic and Applied Microbiology* 27, 118-130.

Beneduce, L., Romano, A., Capozzi, V., Lucas, P., Barnavon, L., Bach, B., Vuchot, P., Grieco, F. and Spano, G. (2010). Biogenic amines in regional wines. *Annals of Microbiology* 60, 573-578.

Ben-Gigirey, B., de Sousa, J. M. V. B., Villa, T. G. and Barros-Velazquez, J. (2000). Characterization of biogenic amine-producing *Stenotrophomonas maltophilia* strains isolated from white muscle of fresh and frozen albacore tuna. *International Journal of Food Microbiology* 57, 19-31.

Beshkova, D. and Frengova, G. (2012). Bacteriocins from lactic acid bacteria: microorganisms of potential biotechnological importance for the dairy industry. *Engineering in Life Science* 12, 419-432.

Beutling, D. M. and Walter, D. (2002). 2-phenylethylamine formation by enterococci in vitro. *European Food Research and Technology* 215, 240-242.

Bigwood, T., Hudson, J. A., Cooney, J., McIntyre, L., Billington, C., Heinemann, J. A. and Wall, F. (2012). Inhibition of *Listeria monocytogenes* by *Enterococcus mundtii* isolated from soil. *Food Microbiology* 32, 354-360.

Bizzini, A., Zhao, C., Budin-Verneuil, A., Sauvageot, N., Giard, J. C., Auffay, Y. and Hartke, A. (2009). Glycerol is metabolized in a complex and strain-dependent manner in *Enterococcus faecalis*. *Journal of Bacteriology* 192, 779-785.

Bøhle, L. A., Fargestad, E. M., Veiseth-Kent, E., Steinmoen, H., Nes, I. F., Eijsink, V. G. H. and Mathiesen, G. (2010). Identification of proteins related to the stress response in *Enterococcus faecalis* V583 caused by bovine bile. *Proteome Science* 8, 37-48.

Bonetta, S., Bonetta, S., Carraro, E., Coïsson, J. D., Travaglia, F. and Arlorio, M. (2008). Detection of biogenic amine producer bacteria in a typical italian goat cheese. *Journal of Food Protection*, 1, 205-209.

Bottone, E., Allerhand, J. and Pisano, A. (1971). Characteristics of a bacteriocin derived from *Streptococcus faecalis* var. *zymogenes* antagonistic to *Diplococcus pneumoniae*. *Applied Microbiology* 22, 200-204.

Bover-Cid, S. and Holzapfel, W. (1999). Improved screening procedure for biogenic amine production by lactic acid bacteria. *International Journal of Food Microbiology* 53, 33-41.

Bover-Cid, S., Hugas, M., Izquierdo-Pulido, M. and Vidal-Carou, M. C. (2000). Reduction of biogenic amine formation using a negative amino acid-decarboxylase starter culture for fermentation of fuet sausage. *Journal of Food Protection* 63, 237-243.

Bover-Cid, S., Hugas, M., Izquierdo-Pulido, M. and Vidal-Carou, M. C. (2001b). Amino acid-decarboxylase activity of bacteria isolated from fermented pork sausages. *International Journal of Food Microbiology* 66, 185-189.

Bover-Cid, S., Miguélez-Arrizado, J. and Vidal-Carou, M. C. (2001c). Biogenic amine accumulation in ripened sausages affected by the addition of sodium sulphite. *International Journal of Food Microbiology* 46, 95-104.

Bover-Cid, S., Torriani, S., Gatto, V., Tofalo, R., Suzzi, G., Belletti, N. and Gardini, F. (2009). Relationships between microbial population dynamics and putrescine and cadaverine accumulation during dry fermented sausage ripening. *Journal of Applied Microbiology* 106, 1397-1407.

Brabban, A. D., Hite, E. and Callaway, T. R. (2005). Evolution of foodborne pathogens via temperate bacteriophage-mediated gene transfer. *Foodborne Pathogens and Disease* 4, 287-303.

Bravo, F., Burdaspal, P., Suhez, J.A. and Iiiigo, B. (1983). Histaminogenesis en vines. III. Fermentaciones de mosto de uva por asociaciones binarias levadura-bacteria. *Alimentaria* 148, 31-34.

Buchanan, R. L. and Bagi, L. K. (1997). Microbial competition: effect of culture conditions on the suppression of *Listeria monocytogenes* Scott A by *Carnobacterium piscicola*. *Journal of Food Protection* 60, 254-261.

Buňka, F., Taťáková, M., Ŝtajnochová, K., Purevdorj, K. and Buňková, L. (2015). Effects of temperature, pH and NaCl content on in vitro putrescine and cadaverine production through the growth of *Serratia marcescens* CCM 303. *Journal of Environmental Science and Health, Part B: Pesticides, Food Contaminants, and Agricultural Wastes* 50, 797-808.

Buňková, L., Buňka, F., Hlobilová, M., Vakátková, Z.,·Nováková, D. and Dráb, V. (2009). Tyramine production of technological important strains of *Lactobacillus, Lactococcus* and *Streptococcus*. *European Food Research and Technology* 229, 533-538.

Buňková, L., Buňka F., Mantlová, G., Cablová, Sedlácec, I., Svec, P., Pachlová, V. and Krácmar, S. (2010). The effect of ripening and storage conditions on the distribution of tyramine, putrescine and cadaverine in Edam-cheese. *Food Microbiology* 27, 880-888.

Buňková, L., Buňka, F., Pollaková, E., Podešvová, T. and Dráb, V. (2011). The effect of lactose, NaCl and an aero/anaerobic environment on the tyrosine decarboxylase activity of *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis*. *International Journal of Food Microbiology* 147, 112-119.

Calles-Enríquez, M., Eriksen, B. H., Andersen, P. S., Rattray, F. P., Johansen, A. H., Fernández, M., Ladero, V. and Alvarez, M. A. (2010). Sequencing and transcriptional analysis of the *Streptococcus thermophilus* histamine biosynthesis gene cluster: factors that affect differential hdcA expression. *Applied and Environmental Microbiology* 76, 6231-6238.

Cantoni, C., Bersani, C., Damenis, L. and Comi, G. (1994). Ammine biogene negli insaccati crudi stagionati. *Industrie Alimentari* 23, 1239-1243.

Capozzi, V., Ladero, V., Beneduce, L., Fernández, M., Alvarez, M. A., Benoit, B., Laurent, B., Grieco, F. and Spano, G. (2011). Isolation and characterization of tyramine-producing *Enterococcus faecium* strains from red wine. *Food Microbiology* 28, 434-439.

Cariolato, D., Andrighetto, C. and Lombardi, A. (2008). Occurrence of virulence factors and antibiotic resistances in *Enterococcus faecalis* and *Enterococcus faecium* collected from dairy and human samples in North Italy. *Food Control* 19, 886-892.

Carlin, F., Nguyen The, C. and Morris, C. E. (1996). Influence of background microflora on *Listeria monocytogenes* on minimally processed fresh broad-leaved endive (*Cichorium endivia* var. *latifolia*). *Journal of Food Protection* 59, 698-703.

Carrasco de Mendoza, M., Meinardi, C. A., Meinardi, S. A. and Arturo, C. (1989). Actividad caseinolitica exocelular de enterococos para starters lácticos. *Revista Argentina de Lactología* 2, 27-37.

Carrasco de Mendoza, M. S., Scarinci, M. S., Huerto-Garat, H. E. and Simonetta, A. C. (1992). Technological properties of enterococci in lactic starters: acidifying and lipolytic activities. *Microbiologie, Aliments, Nutrition* 10, 289-293.

Casalta, E. and Montel, M. C. (2008). Safety assessment of dairy microorganisms: the *Lactococcus* genus. *International Journal of Food Microbiology* 126, 271-273.

Chander, H., Batish, V. H., Babu, S. and Singh, R. S. (1989). Factors affecting amine production by a selected strain of *Lactobacillus bulgaricus*. *Journal of Food Science* 54, 940-942.

Chassard, C., Delmas, E., Robert, C. and Bernalier-Donadille, A. (2010). The cellulose-degrading microbial community of the human gut varies according to the presence or absence of methanogens. *FEMS Microbiology Ecology* 74, 205-213.

Chen, H. and Hoover, D. G. (2003). Bateriocins and their food applications. *Comprehensive Reviews in Food Science and Food Safety* 2, 82-100.

Chu, C. H. and Bjeldanes, L. F. (1981). Effect of diamines, polyamines and tuna fish extracts on the binding of histamine to mucin in vitro. *Journal of Food Science* 47, 79-80.

Cleveland, J., Montville, T. J., Nes, I. F. and Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *International Journal of Food Microbiology* 71, 1-20.

Clewell, D.B. (1990). Movable genetic elements and antibiotic resistance in enterococci. *European Journal of Clinical Microbiology & Infectious Diseases* 9, 90-102.

Clewell, D. B. (1993). Bacterial sex pheromone-induced plasmid transfer. *Cell* 73, 9-12.

Cocconcelli, P. S., Cattivelli, D. and Gazzola, S. (2003). Gene transfer of vancomycin and tetracycline resistances among *Enterococcus faecalis* during cheese and sausage fermentations. *International Journal of Food Microbiology* 88, 315-323.

Cogan, T. M., Beresford, T. P., Steele, J., Broadbent, J., Shah, N. P. and Ustunol, Z. (2007). Advances in starter cultures and cultured foods. *Journal of Dairy Science* 90, 4005-4021.

Collins, M. D., Jones, D., Farrow, J. A. E., Kilpper-Balz, R. and Schleifer, K. H. (1984). *Enterococcus avium* nom. rev., comb. nov.; *E. casseliflavus* nom. rev., comb. nov.; *E. durans* nom. rev., comb. nov.; *E. gallinarum* comb. nov.; and *E. malodoratus* sp. nov. *International Journal of Systematic Bacteriology* 34, 220-223.

Collins, M.D., Farrow, J. A. E. and Jones, D. (1986). *Enterococcus mundtii* sp. nov. *International Journal of Systematic Bacteriology* 36, 8-12.

Commission Regulation (EC) No 2073/2005 of 15 November 2005 on Microbiological criteria for foodstuffs. *EUR-Lex*.

Connil, N., Le Breton, Y., Dousset, X., Auffray, Y., Rincé, A. and Prévost, H. (2002). Identification of the *Enterococcus faecalis* tyrosine decarboxylase operon involved in tyramine production. *Applied and Environmental Microbiology* 68, 3537-3544.

Cornu, M., Billoir, E., Bergis, H., Beaufort, A. and Zuliani, V. (2011). Modeling microbial competition in food: application to the behavior of *Listeria monocytogenes* and lactic acid flora in pork meat products. *Food Microbiology* 28, 639-647.

Corsetti, A., Settanni, L., Chaves López, C., Felis, G. E., Mastrangelo, M. and Suzzi, G. (2007). A taxonomic survey of lactic acid bacteria isolated from wheat (*Triticum durum*) kernels and non-conventional flours. *Systematic and Applied Microbiology* 30, 561-571.

Coton, M., Coton, E., Lucas, P., and Lonvaud-Funel, A. (2004) Identification of the gene encoding a putative tyrosine decarboxylase of *Carnobacterium divergens* 508. Development of molecular tools for the detection of tyramine-producing bacteria. *Food Microbiology* 21, 125-130.

Coton, E., Mulder, N., Coton, M., Pochet, S., Trip, H. and Lolkema, J. S. (2010). Origin of the putrescine producing ability of the coagulase-negative bacterium *Staphylococcus epidermidis* 2015B. *Applied and Environmental Microbiology* 76, 5570-5576.

Coton, M., Fernàndez, M., Trip, H., Ladero, V., Mulder, N. L., Lolkema, J. S., Alverez, M. A. and Coton, E. (2011). Characterization of the tyramine-producing pathway in *Sporolactobacillus* sp. *Microbiology* 157, 1841-1849.

Cox, C. R., Coburn, P. S. and Gilmore, M. S. (2005). Enterococcal cytolisin: a novel two component peptide system that serves as a bacterial defence against eukaryotic and prokaryotic cells. *Current Protein and Peptide Science* 6, 77-84.

Coyette, J. and Hancock, L. E. (2002). Enterococcal cell wall. In: Enterococci: pathogenesis, molecular biology, and antibiotic resistance. M. S. Gilmore *et al*. (Eds.), The AMS Press, Washington, DC.

Dalgaard, P., Emborg, J., Kjolby, A., Sorensen, N. and Ballin, N. (2008). Histamine and biogenic amines - formation and importance in seafood. In: Improving seafood products for the consumer. Borrensen T, (Eds.), Woodhead Publishing Ltd., Cambridge, UK.

de Arauz, L. J., Jozala, A. F., Mazzola, P. G. and Penna, T. C. V. (2009). Nisin biotechnological production and application: a review. *Trends in Food Science & Technology* 20, 146-154.

De Fátima Silva Lopes, M., Simões, A. P., Tenreiro, R., Figueiredo Marques, J. J. and Barreto Crespo, M. T. (2006). Activity and expression of a virulence factor, gelatinase, in dairy enterococci. *International Journal of Food Microbiology* 112, 208-214.

De Kwaadsteniet, M., Todorov, S. D., Knoetze, H. and Dicks, L. M. T. (2005). Characterization of a 3944 Da bacteriocin, produced by *Enterococcus mundtii* ST15, with activity against Gram-positive and Gram-negative bacteria *International Journal of Food Microbiology* 105, 433-444.

de las Rivas, B., Marcobal, A., Carrascosa, A. and Muñoz, R. (2006). PCR detection of foodborne bacteria producing the biogenic amines histamine, tyramine, putrescine, and cadaverine. *Journal of Food Protection* 10, 2320-2566.

de las Rivas, B., Marcobal, A. and Munõz, R. (2007). Gene organization of the ornithine decarboxylase-encoding region in *Morganella morganii*. *Journal of Applied Microbiology* 102, 1551-1560.

de las Rivas, B., Ruiz-Capillas, C., Carrascosa, A. V., Curiel, J. A., Jiménez-Colmenero, F. and Muñoz, R. (2008). Biogenic amine production by Gram-positive bacteria isolated from Spanish dry cured "chorizo" sausage treated with high pressure and kept in chilled storage. *Meat Science* 80, 272-277.

de Llano, G. D., Cuesta, P. and Rodríguez, A. (1998). Biogenic amine production by wild lactococcal and *Leuconostoc* strains. *Letters in Applied Microbiology* 26, 270-274.

de Palencia, F. P., Fernández, M., Mohedano, M. L., Ladero, V., Quevedo, C., Alvarez, M. A. and López, P. (2011). The role of tyramine synthesis by food-borne *Enterococcus durans* in the adaptation to the gastrointestinal tract environment. *Applied and Environmental Microbiology* 77, 699-702.

Devlieghere, F., Geeraerd, A. H., Versyck, K. J., Vandewaetere, B., Van Impe, J. and Debevere, J. (2001). Growth of *Listeria monocytogenes* in modified atmosphere packed cooked meat products: a predictive model. *Food Microbiology* 18, 53-66.

Devriese, L. A., Pot, B. and Collins, M. D. (1993). Phenotypic identification of the genus *Enterococcus* and differentiation of phylogenetically distinct enterococcal species and species groups. *Journal of Applied Bacteriology* 75, 399-408.

Di Cagno, R., De Angelis, M., De Pasquale, I., Ndagijimana, M., Vernocchi, P., Ricciuti, P., Gagliardi, F., Laghi, L., Crecchio, C., Guerzoni, M. E., Gobetti, M. and Francavilla, R. (2011). Duodenal and faecal

microbiota of celiac children: molecular, phenotype and metabolome characterization. *BMC Microbiology* 11, 1-21.

Dovat, A. M., Reinbold, G. W., Hammond, E. G. and Vedamuthu, E.R. (1970). Lipolytic and proteolytic activity of enterococci and lactic group streptococci isolated from young Cheddar cheese. *Journal of Milk and Food Technology* 33, 365-372.

Dumont, E., De Geeter, H. and Huyghebaert, A. (1992). Presence and formation of biogenic amines in local Belgian beers. *Med. Fat. Landbauww. Univ. Gent*. 57, 423-427.

Eerola, S., HinnkkaInenn, R., Lindfors, E. and Hirvi, T. (1993). Liquid chromatographic determination of biogenic amines in dry sausages. *International Journal of AOAC* 76, 575-580.

Eerola, S., Sagues, A. X. R., Lilleberg, L. and Aalto, H. (1997). Biogenic amines in dry sausages during shelf-life storage. *Zeitung Lebensmittel For Untersuchung und Forschung A* 205, 351-355.

Eerola, S., Maijala, R., Roig-Sague´s, A. X., Salminen, M. and Hirvi, T. K. (1998). Biogenic amines in dry sausages as affected by starter culture and contaminant amine-positive *Lactobacillus*. *Journal of Food Science* 61, 1243-1246.

Eaton, T. J. and Gasson, M. J. (2001). Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Applied and Environmental Microbiology* 67, 1628-1635.

Eaton, T. J. and Gasson, M. J. (2002). A variant enterococcal surface protein Espfm in *Enterococcus faecium*; distribution among food, commensal, medical, and environmental isolates. *FEMS Microbiology Letters* 216, 269-275.

EFSA (2011). Scientific opinion on risk based control of biogenic amine formation in fermented foods. *European Food Safety Authority Journal* 9, 2393-2486.

Emborg, J., Dalgaard, P. and Ahrens, P. (2006). *Morganella psychrotolerans* sp. nov., a histamine producing bacterium isolated from various seafoods. *International Journal of Systematic and Evolutionary Microbiology* 56, 2473-2479.

Ennahar, S., Sashihara, T., Sonomoto, K. and Ishizaki, A. (2000). Class IIa bacteriocins: biosynthesis, structure and activity. *FEMS Microbiology Reviews* 24, 85-106.

Espeche, M. C., Juárez Tomás, M. S., Wiese, B., Bru, E. and Nader-Macías, F. M. E. (2014). Physicochemical factors differentially affect the biomass and bacteriocin production by bovine *Enterococcus mundtii* CRL1656. *Journal of Dairy Science* 97, 789-797.

Feng, G., Guron, G. K P., Churey, J. J. and Worobo, R .W. (2009). Characterization of Mundticin L, a class IIa anti-listeria bacteriocin from *Enterococcus mundtii* CUGF08. *Applied and Environmental Microbiology* 75, 5708-5713.

Fernández, M., Linares, D. M. and Alvarez, M. A. (2004). Sequencing of the tyrosine decarboxylase cluster of *Lactococcus lactis* IPLA 655 and the development of a PCR method for detecting tyrosine decarboxylating lactic acid bacteria. *Journal of Food Protection* 67, 2521-2529.

Fernández, M. and Zúñiga, M. (2006). Amino acid catabolic pathways of lactic acid bacteria. *Critical Review in Microbiology* 32, 155-183.

Fernández, M., Linares, D. M., Rodríguez, A. and Alvarez, M. A. (2007a). Factors affecting tyramine production in *Enterococcus durans* IPLA 655. *Applied Microbiology and Biotechnology* 73, 1400-1406.

Fernández, M., Linares, D. M., del Río, B., Ladero, V. and Alvarez, M. A. (2007b). HPLC quantification of biogenic amines in cheeses: correlation with PCR-detection of tyramine-producing microorganisms. *Journal of Dairy Research* 74, 276-282.

Ferreira, I. M. and Pinho, O. (2006). Biogenic amines in Portuguese traditional foods and wines. *Journal of Food Protection* 69, 2293-2303.

Ferreira, V., Barbosa, J., Vendeiro, S., Mota, A., Silva, F., Monteiro, M., Hogg, T., Gibbs, P. and Teixeira, P. (2006). Chemical and microbiological characterization of alheira: A typical Portuguese fermented sausage with particular reference to factors relating to food safety. *Meat Science* 73, 570-575.

Ferreira, V., Barbosa, J., Silva, J., Vendeiro, S., Mota, A., Silva, F., Monteiro, M. J., Hohh, T., Gibs, P. and Teixeira, P. (2007). Chemical and microbiological characterization of ‘‘Salpicão de Vinhais” and ‘‘Chouriça de Vinhais”: Traditional dry sausages produced in the North of Portugal. *Food Microbiology* 24, 618-623.

Fisher, K. and Phillips, C. (2009). The ecology, epidemiology and virulence of *Enterococcu*s. *Microbiology+* 155, 1749-1757.

Fontana, C., Cocconcelli, P. S., Vignolo, G. and Saavedra, L. (2015). Occurrence of antilisterial structural bacteriocins genes in meat borne lactic acid bacteria. *Food Control* 47, 53-59.

Foulquié Moreno, M. R., Sarantinopoulos, P., Tsakalidou, E. and De Vuyst, L. (2006). The role and application of enterococci in food and health. *International Journal of Food Microbiology* 106, 1-24.

Franz, C. M. A. P., Holzapfel, W. H. and Stiles, M. E. (1999). Enterococci at the crossroads of food safety? *International Journal of Food Microbiology* 47, 1-24.

Franz, C. M. A. P., Muscholl-Silberhorn, A. B., Yousif, N. M. K., Vancanneyt, M., Swings, J. and Holzapfel, W. (2001). Incidence of virulence factors and antibiotic resistance among enterococci isolated from food. *Applied and Environmental Microbiology* 67, 4385-4389.

Franz, C. M. A. P., Stiles, M. E., Schleifer, K. H. and Holzapfel, W. H. (2003). Enterococci in foods-a conundrum for food safety. *International Journal of Food Microbiology* 88, 105-122.

Franz, C. M. A. P., Huch, M., Abriouel, H., Holzapfel, W. and Gálvez, A. (2011). Enterococci as probiotics and their implications in food safety. *International Journal of Food Microbiology* 151, 125-140.

Furtula, V., Jackson, C. R., Farrell, E. G., Barrett, J. B., Hiott, L.M. and Chambers, P.A. (2013). Antimicrobial resistance in *Enterococcus* spp. isolated from environmental samples in an area of intensive poultry production. *International Journal of Environmental Research and Public Health* 10, 1020-1036.

Gale, E. F. (1946). The bacterial amino acid decarboxylase. *Advances in Enzymology* 6, 1-32.

Garcia, M. T., Martinez Canamero, M., Lucas, R., Ben Omar, N., Perez Pulido, R. and Galvez, A. (2004). Inhibition of *Listeria monocytogenes* by enterocin EJ97 produced by *Enterococcus faecalis* EJ97. *International Journal of Food Microbiology* 90, 161-170.

Garde, S., Ávila, M., Medina, M. and Nuñez, M. (2004). Fast induction of nisin resistance in *Streptococcus thermophilus* INIA 463 during growth in milk. *International Journal of Food Microbiology* 96, 165-172.

Gardini, F., Martuscelli, M., Caruso, M. C., Galgano, F., Crudele, M. A., Favati, F., Guerzoni, M. E. and Suzzi, G. (2001). Effects of pH, temperature and NaCl concentration on the growth kinetics, proteolytic activity and biogenic amine production of *Enterococcus faecalis*. *International Journal of Food Microbiology* 64, 105-117.

Gardini, F., Bover-Cid, S., Tofalo, R., Belletti, N., Gatto, V., Suzzi, G. and Torriani, S. (2008). Modelling the aminogenic potential of *Enterococcus faecalis* EF37 in dry fermented sausages through chemical and molecular approaches. *Applied and Environmental Microbiology* 74, 2740-2750.

Gardini, F., Rossi, F., Rizzotti, L., Torriani, S., Grazia, L., Chiavari, C., Coloretti, F. and Tabanelli, G. (2012). Role of *Streptococcus thermophilus* PRI60 in histamine accumulation in cheese. *International Dairy Journal* 27, 71-76.

Garrett, R. H. and Grisham, C. M. (2012). *Biochemistry*. Brooks/Cole Pub Co (Edt).

Gatto, V., Tabanelli, G., Montanari, C., Prodomi, V., Bargossi, E., Torriani, S. and Gardini, F. (submitted). Tyrosine decarboxylase activity of *Enterococcus mundtii*: new insights on phenotypic and genetic aspects. Submitted to *Microbial Biotechnology*.

Gautheret, D. and Lambert, A. (2001). Direct RNA motif definition and identification from multiple sequence alignments using secondary structure profiles. *Journal of Molecular Biol*ogy 313, 1003-1011.

Gazzola, S., Fontana, C., Bassi, D. and Cocconcelli, P. S. (2012). Assessment of tetracycline and erythromycin resistance transfer during sausage fermentation by culture dependent and independent methods. *Food Microbiology* 30, 348-354.

Gentry Weeks, C. R., Karkhoff Schweizer, R., Pikis, A., Estay, M. and Keith, J. M. (1999). Survival of *Enterococcus faecalis* in mouse peritoneal macrophages. *Infection and Immunity* 67, 2160-2165.

Gezginc, Y., Akyol, I., Kuley, E. and Özogul F. (2013). Biogenic amines formation in *Streptococcus thermophilus* isolated from home-made natural yogurt. *Food Chemistry* 138, 655-662.

Ghanbari, M., Jami, M., Domig, K. J. and Kneifel, W. (2013). Seafood biopreservation by lactic acid bacteria - A review. *LWT - Food Science and Technology* 54, 315-324.

Giard, J. C., Rincé, A., Benachour, A., Hartke, A., Laplace, J. M., Le Breton, Y., Pichereau, V., Verneuil, N. and Auffray, Y. (2003). The response to environmental stresses in *Enterococcus faecalis*. *Recent Research Developments in Microbiology* 7, 325-339.

Gilmore, M. S., Segarra, R. A., Booth, M. C., Bogie, C. P., Hall, L. R. and Clewell, D. B. (1994). Genetic structure of *Enterococcus faecalis* plasmid pAD1- encoded cytolytic toxin system and its relationship to lantibiotic determinants. *Journal of Bacteriology* 176, 7335-7344.

Gilmore, M. S., Clewell, D. B., Ike, Y. and Shankar, N. (2014). *Enterococci. From commensals to leading causes of drug resistant infection*. Massachusetts Eye and Ear Infirmary, Boston.

Giraffa, G. (2002). Enterococci from foods. *FEMS Microbiology Reviews* 26, 163-171.

Giraffa, G. (2003). Functionality of enterococci in dairy products. *International Journal of Food Microbiology* 88, 215-222.

Goldenfeld, N. and Woese, C. (2007). Biology's next revolution. *Nature* 445, 369.

González-Tenorio, R., Fonseca, B., Caro, I., Fernández-Diez, A., Kuri, V., Soto, S. and Mateo, J. (2013). Changes in biogenic amine levels during storage of Mexican-style soft and Spanish-style dry-ripened sausages with different aw values under modified atmosphere. *Meat Science* 94, 369-375.

Granato, P.A. and Jackson, R.W. (1969). Bicomponent nature of lysin from *Streptococcus zymogenes*. *Journal of Bacteriology* 100, 856-868.

Granato, P. A. and Jackson, R. W. (1971a). Characterization of the a component of *Streptococcus zymogenes* lysin. *Journal of Bacteriology* 107, 551-556.

Granato, P. A. and Jackson, R. W. (1971b). Purification and characterization of the L-component of *Streptococcus zymogenes* lysin. *Journal of Bacteriology* 108, 804-808.

Graves, A. K. and Weaver, R. W. (2010). Characterization of enterococci populations collected from a subsurface flow constructed wetland. *Journal of Applied Microbiology* 108, 1226-1234.

Guinane, C. M., Cotter, P. D., Hill, C. and Ross, R. P. (2005). Microbial solutions to microbial problems; lactococcal bacteriocins for the control of undesirable biota in food. *Journal of Applied Microbiology* 98, 1316-1325.

Guirard, B. M. and Snel, E. E. (1987). Purification and properties of Pyridoxal-5'-Phosphate-dependent histidine decarboxylases from *Klebsiella planticola* and *Enterobacter aerogenes*. *Journal of Bacteriology* 169, 3963-3968.

Halász, A., Baráth, A., Simon-Sarkadi, L. and Holzapfel, W. (1994). Biogenic amines and their production by micro-organisms in food. *Trends in Food Science and Technology* 5, 42-49.

Hernandez-Jover, T., Izquierdo-Pulido, M., Veciana-Nogue´s, M. T., Marine´-Font, A. and Vidal-Carou, M. C. (1997). Biogenic amine and polyamine contents in meat and meat products. *Journal of Agricultural and Food Chemistry* 45, 2098-2102.

Hernandez-Herrero, M. M., Roig-Sagues, A. X., Rodriguez-Jerez, J. J. and Mora-Ventura M. T. (1999). Halotolerant and halophilic histamine-forming bacteria isolated during the ripening of salted anchovies. *Journal of Food Protection* 62, 509-514.

Higashide, T., Takahashi, M., Kobayashi, A., Ohkubo, S., Sakurai, M., Shirao, Y., Tamura, T. and Sugiyama, K. (2005). Endophthalmitis caused by *Enterococcus mundtii*. *Journal of Clinical Microbiology* 43, 1475-1476.

Hollenbeck, B. L. and Rice, L. B. (2012). Intrinsic and acquired resistance mechanisms in *Enterococcus*. *Virulence* 3, 421-433.

Hugas, M., Garriga, M. and Aymerich, M. T. (2003). Functionality of enterococci in meat products. *International Journal of Food Microbiology* 88, 223-233.

Hugenholtz, J. (1993). Citrate metabolism in lactic acid bacteria. *FEMS Microbiology Reviews* 12, 165-178.

Hui, J. Y. and Taylor, S. L. (1985). Inhibition of in vivo histamine metabolism in rats by foodborne and pharmacologic inhibitors of diamine oxidase, histamine N-methyltransferase, and monoamine oxidase. *Toxicology and Applied Pharmacology* 81, 241-249.

Hungerford, J. M. (2010). Scombroid poisoning: A review. *Toxicon* 56, 231-243.

Ike, Y., Clewell, D. B., Segarra, R. A. and Gilmore, M. S. (1990). Genetic analysis of the pAD1 hemolysin/bacteriocin determinant in *Enterococcus faecalis*: Tn917 insertional mutagenesis and cloning. *Journal of Bacteriology* 172, 155-163.

Innocente, N. and D`Agostin, P. (2002). Formation of biogenic amines in a typical semi hard Italian cheese. *Journal of Food Protection* 65, 9, 1498-1501.

Jakubovics, N. S. and Jenkinson, H. F. (2001). Out of the iron age: new insights into the critical role of manganese homeostasis in bacteria. *Microbiology* 147, 1709-1718.

Jarisch, R. (2004). *Histamin-Intoleranz, Histamin und Seekrankheit*. 2nd ed. Thieme, Stuttgart.

Jensen, P. R. and Hammer, K. (1993). Minimal requirements for exponential growth of *Lactococcus lactis*. Applied and Environmental Microbiology 59, 4363-4366.

Joosten, H. M. L. J. (1988). Conditions allowing the formation of biogenic amine in cheese: 4. Factors influencing the amounts formed. *Netherlands Milk and Dairy Journal* 41, 329-357.

Joosten, H. M. L. J. and Northold, M. D. (1989). Detection, growth and amine-producing capacity of lactobacilli in cheese. *Applied and Environmental Microbiology* 55, 2356-2359.

Joosten, H. M. L. J. and Nuñez, M. (1996). Prevention of histamine formation in cheese by bacteriocin-producing lactic acid bacteria. *Applied and Environmental Microbiology* 62, 1178-1181.

Kalač, P. and Krízek, M. (2003). A review of biogenic amines and polyamines in beer. *Journal of the Institute of Brewing* 109, 123-128.

Kalač, P. and Krausová, P. (2005). A review of dietary polyamines: Formation, implications for growth and health and occurrence in foods. *Food Chemistry* 90, 219-230.

Kalhotka, L., Manga, I., Přichystalová, J., Hůlová, M., Vyletělová, M. and Šustová, K. (2012). Decarboxylase activity test of the genus *Enterococcus* isolated from goat milk and cheese. *Acta Veterinaria Brno* 81, 145-151.

Kamio, Y., Hannu, P., Toerawakit, Y. and Paulin, L. (1986). Cadaverine covalently linked to a peptidoglycan is an essential constituent of the peptidoglycan necessary for the normal growth in *Selenomonas ruminantium*. *Journal of Biological Chemistry* 261, 6585-6589.

Kamio, Y. and Nakamura, K. (1987). Putrescine and cadaverine are constituents of peptidoglycan in *Veillonella alcalescens* and *Veillonella parvula*. *Journal of Bacteriology* 169, 2881-2884.

Kandler, O. (1983). Carbohydrate metabolism in lactic acid bacteria. *Antonie van Leeuwenhoek* 49, 209-224.

Kanki, M., Yoda, T., Ishibashi, M. and Tsukamoto, T. (2004). *Photobacterium phosphoreum* caused a histamine fish poisoning incident. *International Journal of Food Microbiology* 92, 79-87.

Kanki, M., Yoda, T., Tsukamoto, T. and Baba, E. (2007). Histidine decarboxylases and their role in accumulation of histamine in tuna and dried saury. *Applied and Environmental Microbiology* 73, 1467-1473.

Karmas, E. (1981). Biogenic amines as indicators of seafood freshness. LWT Lebensmittel-Wissenschaft & Technologie 14, 273-275.

Kato, T., Matsuda, T., Yoneyama, Y., Kato, H. and Nakamura, R. (1993). Isolation of *Enterococcus faecium* with antibacterial activity and characterization of its bacteriocin. *Bioscience, Biotechnology and Biochemistry* 57, 551-556.

Kawano, M., Abuki, R., Igarashi, K. and Kakinuma, Y. (2001). Potassium uptake with low affinity and high rate in *Enterococcus hirae* at alkaline pH. *Archives of Microbiology* 175, 41-45.

Kayaoglu, G. and Orstavik, D. (2004). Virulence factors of *Enterococcus faecalis*: relationship to endodontic disease. *Critical Reviews in Oral Biology & Medicine* 15, 308-320.

Kennes, C. Dubourguier, H. C., Albagnac, G. and Nyns, E. J. (1991). Citrate metabolism by *Lactobacillus plantarum* isolated from orange juice. *Journal of Applied Bacteriology* 70, 380-384.

Kim, M. and Whitesides, G. M. (1988). L-lactate dehydrogenase: substrate specificity and use as a catalyst in the synthesis of homochiral 2-hydroxy acids. *Journal of the American Chemical Society* 110, 2959-2964.

Kimura, B., Konagaya, Y. and Fujii T. (2001). Histamine formation by *Tetragenococcus muriaticus*, a halophilic lactic acid bacterium isolated from fish sauce. *International Journal of Food Microbiology* 70, 71-77.

Klaenhammer, T. R. (1993). Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiology Reviews* 12, 39-86.

Klare, I., Werner, G. and Witte, W. (2001). Enterococci. Habitats, infections, virulence factors, resistances to antibiotics, transfer of resistance determinants. *Contributions to Microbiology* 8, 108-122.

Klein, G. (2003). Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. *International Journal of Food Microbiol*ogy 88, 123-131.

Knope, K. E., Sloan-Gardner, T. S. and Stafford, R. J. (2014). Histamine fish poisoning in Australia, 2001 to 2013. *Communicable Diseases Intelligence Q Rep.* 38, 285-293.

Komprda, T., Neznalova`, J., Standara, S. and Bover-Cid, S. (2001). Effect of starter culture and storage temperature on the content of biogenic amines in dry fermented sausages poličan. *Meat Science* 59, 267-276.

Komprda, T., Burdychová, R., Dohnal, V., Cwiková, O., Sládková, P. and Dvorácková, H. (2008a). Tyramine production in Dutch-type semi-hard cheese from two different producers. *Food Microbiology* 25, 219-227.

Komprda, T., Burdychová, R., Dohnal, V., Cwiková, O. and Sládková, P. (2008b). Some factors influencing biogenic amines and polyamines content in Dutch-type semi-hard cheese. *European Food Research and Technology* 227, 29-36.

Krämer, J. and Brandis, H. (1975). Mode of action of two *Streptococcus faecium* bacteriocins. *Antimicrobial Agents and Chemotherapy* 7, 117-120.

Krämer, J., Keness, J. and Brandis, H. (1983). Transfer of miniplasmid determining bacteriocin production and bacteriocin immunity in *Streptococcus faecium*. *FEMS Microbiology Letters* 20, 385- 389.

Kühnen, E., Sahl, H. G. and Brandis, H. (1985). Purification and properties of LIQ 4, an antibacterial substance produced by *Streptococcus faecalis* var. *liquefaciens* K4. *Journal of General and Applied Microbiology* 131, 1925-1932.

Kuley, E. and Özogul, F. (2011). Synergistic and antagonistic effect of lactic acid bacteria on tyramine production by food-borne pathogenic bacteria in tyrosine decarboxylase broth. *Food Chemistry* 127, 1163-1168.

La Gioia, F., Rizzotti, L., Rossi, F., Gardini, F., Tabanelli, G. and Torrisni, S. (2011). Identification of a tyrosine decarboxylase gene (*tdcA*) in *Streptococcus thermphilus* 1TT45 and analysis of its expression and tyramine production in milk. *Applied and Environmental Microbiology* 77, 1140-1144.

Ladero, V., Fernández, M., Cuesta, I. and Alvarez, M. A. (2010). Quantitative detection and identification of tyramine-producing enterococci and lactobacilli in cheese by multiplex qPCR. *Food Microbiology* 27, 933-939.

Ladero, V., Sánchez-Llana, E., Fernández, M. and Alvarez, M. A. (2011). Survival of biogenic amine-producing dairy LAB strains at pasteurisation conditions. *International Journal of Food Science and Technology* 46, 516-521.

Ladero, V., Fernández, M., Calles-Enríquez, M., Sánchez-Llana, E., Cañedo, E., Martín, M. C. and Alvarez, M. A. (2012). Is the production of the biogenic amines tyramine and putrescine a species-level trait in enterococci? *Food Microbiology* 30, 132-138.

Ladero, V., Linares, D. M, del Rio, B., Fernandez, M., Martin, M. C. and Alvarez, M. A. (2013). Draft genome sequence of the tyramine producer *Enterococcus durans* strain IPLA 655. *Genome Announcement*s 3, e00265-13.

Landete, J. M., Pardo, I. and Ferrer, S. (2006). Histamine, histidine, and growth phase mediated regulation of the histidine decarboxylase gene in lactic acid bacteria isolated from wine. *FEMS Microbiology Letters* 260, 84-90.

Landete, J. M., Pardo, I. and Ferrer, S. (2007). Tyramine and phenylethylamine production among lactic acid bacteria isolated from wine. *International Journal of Food Microbiology* 115, 364-368.

Landete, J. M., de las Rivas, B., Marcobal, A. and Muñoz, R. (2008). Updated molecular knowledge about histamine biosynthesis by bacteria. *Critical Review in Food Science and Nutrition* 48, 697-714

Latorre-Moratalla, M. L., Bosch-Fusté, J., Bover-Cid, S., Aymerich, T. and Vidal-Carou, M. C. (2011). Contribution of enterococci to the volatile profile of slightly-fermented sausages. *Food science and Technology* 44, 145-152.

Latorre-Moratalla, M. L., Bover-Cid, S., Bosch-Fusté, J., Veciana-Nogués, M. T. and Vidal-Carou, C. (2014). Amino acid availability as an influential factor on the biogenic amine formation in dry fermented sausages. *Food Control* 36, 76-81.

Lau, K. L., Barbano, M. and Rasmussen, R. R. (1991). Influence of pasteurization of milk on protein break down in Cheddar cheese during aging. *Journal of Dairy Science* 74, 727-740.

Lebiedzinska, A., Lamparczyk, H., Ganowiak, Z. and Eller, K. I. (1991). Differences in biogenic amine patterns in fish obtained from commercial sources. *Zeitschrift fur Lebensmittel Untersuchung Und Forschung A – Food Research and Techonology* 192, 240-243.

Leclerc, H., Devriese, L. A. and Mossel, D. A. A. (1996). Taxonomical changes in intestinal (faecal) enterococci and streptococci: consequences on their use as indicators of faecal contamination in drinking water. *Journal of Applied Bacteriology* 81, 459-466.

Lee, Y. H., Kim, B. H., Kim, J. H., Yoon, W. S., Bang, S. H. and Park, Y. K. (2007). CadC has a global translational effect during acid adaptation in *Salmonella enterica* serovar *Typhimurium*. *Journal of Bacteriology* 189, 2417-2425.

Lehane, L. and Olley, J. (2000). Histamine fish poisoning revisited. *International Journal of Food Microbiology* 58, 1-37.

Leroy, F. and de Vuyst, L. (2010). Bacteriocins of lactic acid bacteria to combat undesirable bacteria in dairy products. *Australian Journal of Dairy Technology* 65, 143-149.

Leuschner, R. G., Heidel, M. and Hammes, W. P. (1998). Histamine and tyramine degradation by food fermenting microorganisms. *International Journal of Food Microbiology* 39, 1-10.

Leuschner, R. G. K., Kurihara, R. and Hammes, W. P. (1999). Formation of biogenic amines by proteolytic enterococci during ripening. *Journal of the Science of Food and Agriculture* 79, 1141-1144.

Li, H. P. and O'Sullivan, D. J. (2002). Heterologous expression of the *Lactococcus lactis* bacteriocin, nisin, in a dairy *Enterococcus* strain. *Applied and Environmental Microbiology* 68, 3392-3400.

Linares, D. M., Fernández, M., Martín, M. C. and Alvarez, M.A. (2009). Tyramine biosynthesis in *Enterococcus durans* is transcriptionally regulated by the extracellular pH and tyrosine concentration. *Microbial Biotechnology* 2, 625-633.

Linares, D. M., Martín, M. C., Ladero, V., Alvarez, M. A. and Fernández, M. (2011). Biogenic amines in dairy products. *Critical Reviews in Food Science and Nutrition* 51, 691-703.

Linares, D. M., Fernández, M., Del-Rio, B., Ladero, V., Martin, M. C. and Alvarez, M. A. (2012a). The *tyrosyl-tRNA synthetase* like gene located in the tyramine biosynthesis cluster of *Enterococcus durans* is transcriptionally regulated by tyrosine concentration and extracellular pH. *BMC Microbiology* 12, 23-34.

Linares, D. M., del Río,B., Ladero, V., Martínez, N., Fernández, M., Martín M. C. and Álvarez, M. A. (2012b). Factors influencing biogenic amines accumulation in dairy products. *Frontiers in Microbiology* 3, 180.

Linares, D. M., del Rio, B., Bedruello, B., Ladero, V., Cruz, M., de Jong, A., Kuipers, O. P., Fernández, M. and Alvarez, M. A. (2015). AguR, a transmembrane transcription activator of the putrescine biosynthesis operon in *Lactococcus lactis*, acts in response to the agmatine concentration. *Applied and Environmental Microbiology* 81, 6145-6157.

Lisiecki, P. and Mikucki, J. (2006). Iron supply of enterococci by 2-oxoacids and hydroxyacids. *Polish Journal of Microbiology* 55, 195-202.

Lisiecki, P., Wysocki, P. and Mikucki, J. (2000). Occurrence of siderophores in enterococci. *Zentralblatt Bakteriologie* 289, 807-815.

Liu, F., Du, L., Xu, W., Wang, D., Zhang, M., Zhu, Y. and Xu, W. (2013). Production of tyramine by *Enterococcus faecalis* strains in water-boiled salted duck. *Journal of Food Protection* 76, 854-859.

Liu, F., Xu, W., Du, L., Wang, D., Zhu, Y., Geng, Z., Zhang, M. and Xu, W. (2014a). Heterologous expression and characterization of tyrosine decarboxylase from *Enterococcus faecalis* R612Z1 and *Enterococcus faecium* R615Z1. *Journal of Food Protection* 77, 592-598.

Liu, F., Du, L., Wu, H., Wang, D., Zhu, Y., Geng, Z., Zhang, M. and Xu, W. (2014b). Effects of storage temperature on tyramine production by *Enterococcus faecalis* R612Z1 in water-boiled salted ducks. *Journal of Food Protection* 77, 1804-1808.

Lopez-Caballero, M. E., Sanchez-Fernandez, J. A. and Moral, A. (2001). Growth and metabolic activity of *Shewanella putrefaciens* maintained under different CO_2 and O_2 concentrations. *International Journal of Food Microbiology* 64, 277-287.

Lorenzo, J. M., Cachaldora, A., Fonseca, S., Gómez, M., Franco, I. and Carballo, J. (2010). Production of biogenic amines “in vitro” in relation to the growth phase by *Enterobacteriaceae* species isolated from traditional sausages. *Meat Science* 86, 684-691.

Loret, S., Deloyer, P. and Dandrifosse, G. (2005). Levels of biogenic amines as a measure of the quality of the beer fermentation process: data from Belgian samples. *Food Chemistry* 89, 519-525.

Lucas, P., Landete J., Coton, M., Coton, E. and Lonvaud-Funel, A. (2003). The tyrosine decarboxylase operon of *Lactobacillus brevis* IOEB 9809: characterization and conservation in tyramine-producing bacteria. *FEMS Microbiology Letters* 229, 65-71.

Lund, B. M. (1965). A comparison by the use of gel electrophoresis of soluble protein components and esterase enzymes of some group D streptococci. *Journal of General Microbiology* 40, 413-419.

Lund, P., Tramonti, A. and De Biase, D. (2014). Coping with low pH: molecular strategies in neutralophilic bacteria. *FEMS Microbiology Review* 38, 1-35.

Lynch, M. and Conery, J. S. (2000). The evolutionary fate and consequences of duplicate genes. *Science* 290, 1151-1155.

Lyon, W. J., Olson, D. G. and Murano, E. A. (1995). Isolation and purification of enterocin EL1, a bacteriocin produced by a strain of *Enterococcus faecium*. *Journal of Food Protection* 58, 890-898.

Lyte, M. (2004). The biogenic amine tyramine modulates the adherence of *Escherichia coli* O157:H7 to intestinal mucosa. *Journal of Food Protection* 67, 878-883.

Macke, T. J., Ecker, D. J., Gautheret, D., Case, D. A. and Sampath, R. (2001). RNAMotif, an RNA secondary structure definition and search algorithm. *Nucleic Acids Research* 29,4724-4735.

Magnus, C. A., Ingledew, W. M. and McCurdy, A. R. (1986). Thermal resistance of streptococci isolated from pasteurized ham. *Canadian Institute of Food Science and Technology Journal* 19, 62-67.

Magnus, C. A., McCurdy, A. R. and Ingledew, W. M. (1988). Further studies on the thermal resistance of *Streptococcus faecium* and *Streptococcus faecalis* in pasteurized ham. *Canadian Institute of Food Science and Technology Journal* 21, 209-212.

Maijala, R. (1993). Formation of histamine and tyramine by some lactic acid bacteria in MRS-broth and modified decarboxylation agar. *Letters in Applied Microbiology* 17, 40-43.

Maijala, R., Eerola, S., Lievonen, S., Hill, P. and Hirvi, T. (1995). Formation of biogenic amines during ripening of dry sausages as affected by starter cultures and thawing time of raw materials. *Journal of Food Science* 60, 1187-1190.

Maintz, L. and Novak, N. (2007). Histamine and histamine intolerance. *American Journal of Clinical Nutrition* 85, 1185-1196.

Mannu, L., Paba, A., Daga, E., Comunian, R., Zanetti, S., Duprè, I. and Sechi, L. A. (2003). Comparison of the incidence of virulence determinants and antibiotic resistance between *Enterococcus faecium* strains of dairy, animal and clinical origin. *International Journal of Food Microbiology* 88, 291-304.

Manolopoulou, E., Sarantinopoulos, P., Zoidou, E., Aktypis, A., Moschopoulou, E., Kandarakis, I. G. and Anifantakis, E. M. (2003). Evolution of microbial populations during traditional Feta cheese manufacture and ripening. *International Journal of Food Microbiology* 82, 153-161.

Marcobal, A., de las Rivas, B., and Muñoz, R. (2006a). First genetic characterization of a bacterial β-phenylethylamine biosynthetic enzyme in *Enterococcus faecium* RM58. *FEMS Microbiology Letters* 258, 144-149.

Marcobal, A., Martín-Álvarez, P. J., Moreno-Arribas, M. V. and Muñoz, R. (2006b). A multifactorial design for studying factors influencing growth and tyramine production of the lactic acid bacteria *Lactobacillus brevis* CECT 4669 and *Enterococcus faecium* BIFI-58. *Research in Microbiology* 157, 417-424.

Marcobal, A., de Las Rivas, B., Landete, J. M., Tabera, L. and Muñoz, R. (2012). Tyramine and phenylethylamine biosynthesis by food bacteria. *Critical Review in Food Science and Nutrition* 52, 448-467.

Marino, M., Maifreni, M., Bartolomeoli, I. and Rondinini, G. (2008). Evaluation of amino acid-decarboxylative microbiota throughout the ripening of an Italian PDO cheese produced using different manufacturing practices. *Journal of Applied Microbiology* 105, 540-549.

Martínez-Murcia, A. J. and Collins, M. D. (1991). *Enterococcus sulfureus*, a new yellow-pigmented *Enterococcus* species. *FEMS Microbiology Letters* 64, 69-74.

Martínez, M. I., Rodríguez, E., Medina, M., Hernández, P. E. and Rodríguez, J. M. (1998). Detection of specific bacteriocin-producing lactic acid bacteria by colony hybridization. *Journal of Applied Microbiology* 84, 1099-1103.

Martuscelli, M., Crudele, M. A., Gardini, F. and Suzzi, G. (2000). Biogenic amine formation and oxidation by *Staphylococcus xylosus* strains from artisanal fermented sausages. *Letters in Applied Microbiology* 31, 228-232.

Masson, F., Talon, R. and Montel, M. C. (1996). Histamine and tyramine production by bacteria from meat products. *International Journal of Food Microbiol*ogy 32, 199-207.

Masson, F., Johansson, G. and Montel M. C. (1999). Tyramine production by a strain of *Carnobacterium divergens* inoculated in meat–fat mixture. *Meat Science* 52, 65-69.

Maxa, E. and Brandes, W. (1993). Biogene Amine in Fruchtsaften. *Mitteilungen Klosterneuburg* 43, 101-106.

McAuliffe, O., Ross, R. P. and Hill, C. (2001). Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiology Reviews* 25, 285-308.

McCabe-Sellers, B., Staggs, C. G. and Bogle, M. L. (2006). Tyramine in foods and monoamine oxidase inhibitor drugs: a crossroad where medicine, nutrition, pharmacy, and food industry converge. *Journal of Food Composition and Analysis* 19, S58-S65.

McWilliam, H., Li, W., Uludag, M., Squizzato, S., Park, Y. M., Buso, N., Cowley, A. P. and Lopez, R. (2013). Analysis Tool Web Services from the EMBL-EBI. *Nucleic Acids Re*search 41, W597-W600.

Middlebrooks, B. L., Toom, P. M., Douglas, W. L., Harrison, R. E. and McDowell, S. (1988). Effects of storage time and temperature on the microflora and amine development in Spanish mackerel (*Scomberomorus macularus*). *Journal of Food Science* 53, 1024-1029

Mokhtari, A., Blancato, V. S., Repizo, G. D., Henry, C., Pikis, A., Bourand, A., de Fátima Álvarez, M., Immel, S., Mechakra-Maza, A., Hartke, A., Thompson, J., Magni, C. and Deutscher, J. (2013). *Enterococcus faecalis* utilizes maltose by connecting two incompatible metabolic routes via a novel maltose 6′-phosphate phosphatase (MapP). *Molecular Microbiology* 88, 234-253.

Molenaar, D., Bosscher, J. S., ten Brink, B., Driessen, A. J. and Konings, W. N. (1993). Generation of a proton motive force by histidine decarboxylation and electrogenic histidine/histamine antiport in *Lactobacillus buchneri*. *Journal of Bacteriology* 175, 2864-2870.

Moore, D. F., Guzman, J. A. and McGee, C. (2008). Species distribution and antimicrobial resistance of enterococci isolated from surface and ocean water. *Journal of Applied Microbiology* 105, 1017-1025.

Moreno-Arribas, V. and Lonvaud-Funel, A. (1999). Tyrosine decarboxylase activity of *Lactobacillus brevis* IOEB 9809 isolated from wine and *L. brevis* ATCC 367. *FEMS Microbiology Letters* 180, 55-60.

Moreno-Arribas, V. and Lonvaud-Funel, A. (2001). Purification and characterization of tyrosine decarboxylase of *Lactobacillus brevis* IOEB 9809 isolated from wine. *FEMS Microbiology Letters* 195, 103-107.

Moret, S. and Conte, L. S. (1996). High-performance liquid chromatographic evaluation of biogenic amines in foods. An analysis of different methods of sample preparation in relation to food characteristics. *Journal of Chromatography* 729, 363-369.

Muller, T., Ulrich, A., Ott, E. M. and Muller, M. (2001). Identification of plant-associated enterococci. *Journal of Applied Microbiology* 91, 268-278.

Mundt, O. J. (1986). *Enterococci*. In: Bergey's Manual of Systematic Bacteriology, vol. 2. Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G. (Eds.), Williams and Wilkins, Baltimore, 1063-1065.

Naila, A., Flint, S., Fletcher, G., Bremer, P. and Meerdink, G. (2010). Control of biogenic amines in food-existing and emerging approaches. *Journal of Food Science* 75, R139-R150.

Nakagawa, S. (1979). Studies on bacteriocins produced by group D streptococci: I. Characterization of several bacteriocins. *Hiroshima Journal of Medical Sciences* 27, 809-825.

Nakagawa, S. and Matsuo, Y. (1981). Bacteriocin and hemolysin from *Streptococcus faecium*. *Antimicrobial Agents and Chemotherapy* 20, 542-544.

Naser, S. M., Thompson, F. L., Hoste, B., Gevers, D., Dawyndt, P., Vancanneyt, M. and Swings, J. (2005). Application of multilocus sequence analysis (MLSA) for rapid identification of *Enterococcus* species based on rpoA and pheS genes. *Microbiology* 151, 2141-2150.

Nawaz, M., Wang, J., Zhou, A., Ma, C., Wu, X., Moore, J. E., Millar, B. C. and Xu, J. (2011). Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. *Current Microbiology* 62, 1081-1089.

Nes, I. F., Diep, D. B., Havarstein, L. S., Brurberg, M. B., Eijsink, V. and Holo, H. (1996). Biosynthesis of bacteriocins in lactic acid bacteria. *Antonie van Leeuwenhoek* 70, 113-128.

Ni, K., Wang, Y., Li, D., Cai, Y. and Pang, H. (2015). Characterization, identification and application o lactic acid bacteria isolated from forage paddy. *PLoS One* 10, e0121967.

Nishie, M., Nagao, J. and Sonomoto, K. (2012). Antibacterial peptides "bacteriocins": an overview of their diverse characteristics and applications. *Biocontrol Science* 17, 1-16.

Nout, M. J. R. (1994). Fermented foods and food safety. *Food Research International* 27, 291-298.

Novella-Rodríguez, S., Veciana-Nogúes, M. T., Roig-Sagúes, A. X., Trujillo-Mesa, A. J., Vidal-Carou, M. C. (2002). Influence of starter and nonstarter bacteria on the formation of biogenic amines in goat cheese during ripening. *Journal of Dairy Science* 85, 2471-2478.

Novella-Rodríguez, S., Veciana-Nogués, M. T., Izquierdo-Pulido, M. and Vidal-Carou M. C. (2003). Distribution of biogenic amines and polyamines in cheese. *Journal of Food Science* 68, 750-755.

Novella-Rodríguez, S., Veciana-Nogués, M. T., Roig-Sagués, A. X., Trujillo-Mesa, A. J. and Vidal-Carou, M. C. (2004). Evaluation of biogenic amines and microbial counts throughout the ripening of goat cheeses from pasteurized and raw milk. *Journal of Dairy Research* 71, 245-252.

Olasupo, N. A., Schillinger, U., Franz, C. M. A. P. and Holzapfel, W. H. (1994). Bacteriocin production by *Enterococcus faecium* NA01 from Fwara - a fermented skimmed cow milk product from West Africa. *Letters in Applied Microbiology* 19, 438-441.

Önal, A. (2007). A review: Current analytical methods for the determination of biogenic amines in foods. *Food Chemistry* 103, 1475-1486.

Özogul, F. (2011). Effects of specific lactic acid bacteria species on biogenic amine production by food-borne pathogen. *International Journal of Food Science and Technology* 46, 478-484.

Özogul, F. and Özogul, Y. (2006). Biogenic amine content and biogenic amine quality indices of sardines (*Sardina pilchardus*) stored in modified atmosphere packaging and vacuum packaging. *Food Chemistry* 99, 574-578.

Özogul, F. and Özogul, Y. (2007). The ability of biogenic amines and ammonia production by single bacterial cultures. *European Food Research and Technology* 225, 385-394.

Papagianni, M. (2012). *Food Fermentation and production of biopreservatives*. In: Handbook of animal-based fermented food and beverage technology. Hui, Y. H. and Özgül E. (Eds.), CRC Press, Boca Raton, 109-124.

Parente, E. and Ricciardi, A. (1999). Production, recovery and purification of bacteriocins from lactic acid bacteria. *Journal of Applied Microbiology and Biotechnology* 52, 628-638.

Paulsen, P. and Bauer, F. (1997). Biogenic amines in fermented sausages: 2. Factors influencing the formation of biogenic amines in fermented sausages. *Fleischwirtschaft* 77, 32-34.

Pereira, C. I., Barreto Crespo, M. T. and San Romão, M. V. (2001). Evidence for proteolytic activity and biogenic amines production in *Lactobacillus curvatus* and *L. homohiochii*. *International Journal of Food Microbiology* 68, 211-216.

Pereira, C. I., Matos, D., San Romaõ, M. V. and Barreto Crespo, M. T. (2009). Dual role for the tyrosine decarboxylation pathway in *Enterococcus faecium* E17: response to an acid challenge. *Applied and Environmental Microbiology* 75, 345-352.

Pérez, M., Calles-Enríquez, M., Nes, I., Martin, M. C., Fernandez, M., Ladero, V. and Alvarez, M. A. (2015). Tyramine biosynthesis is transcriptionally induced at low pH and improves the fitness of *Enterococcus faecalis* in acidic environments. *Applied Microbiology and Biotechnology* 99, 3547-3558.

Pérez-Martín, F., Seseña, S., Miguel Izquierdo, P. and Palop, M. L. (2014). Are *Enterococcus* populations present during malolactic fermentation of red wine safe? *Food Microbiology* 42, 95-101.

Pessione, E., Mazzoli, R., Giuffrida, M. G., Lamberti, C., Garcia-Moruno, E., Barello, C., Conti, A. and Giunta, C. (2005). A proteomic approach to studying biogenic amine producing lactic acid bacteria. *Proteomics* 5, 687-698.

Pessione, E., Pessione, A., Lamberti, C., Coïsson, D. J., Riedel, K., Mazzoli, R., Bonetta, S.,Eberl, L. and Giunta, C. (2009). First evidence of a membrane-bound, tyramine and β-phenylethylamine producing, tyrosine decarboxylase in *Enterococcus faecalis*: a two-dimensional electrophoresis proteomic study. *Proteomics*, 9, 2695-2710.

Peykov, S. Z., Aleksandrova, V. D. and Dimov, S. G. (2012). Rapid identification of *Enterococcus faecalis* by species-specific primers based on the genes involved in the Entner-Doudoroff pathway. *Molecular Biology Reports* 39, 7025-7030.

Pircher, A., Bauer, F. and Paulsen, P. (2007). Formation of cadaverine, histamine, putrescine and tyramine by bacteria isolated from meat, fermented sausages and cheeses. *European Food Research and Technology* 226, 225-231.

Polidori, M., Nuccorini, A., Tascini, C., Gemigani, G., Iapoce, R., Leonildi, A., Tagliaferri, E. and Menichetti, F. (2011). Vancomycin-resistant *Enterococcus faecium* (VRE) bacteremia in infective endocarditis successfully treated with combination daptomycin and tigecycline. *Journal of Chemotherapy* 23, 240-241.

Pritchard, G. G. and Wimpenny, J. W. (1978). Cytochrome formation, oxygen-induced proton extrusion and respiratory activity in *Streptococcus faecalis* var. *zymogenes* grown in the presence of haematin. *Journal of General Microbiology* 104, 15-22.

Rabie, M. A., Siliha, H., el-Saidy, S., el-Badawy, A. A. and Malcata, F. X. (2011). Reduced biogenic amine contents in sauerkraut via addition of selected lactic acid bacteria. *Food Chemistry* 129, 1778-1782.

Ramesh, A. and Venugopalan, V. K. (1986). Densities and characteristics of histamine-forming luminous bacteria of marine fish. *Food Microbiology* 3, 103-105.

Randazzo, C. L., Restuccia, C., Romano, A. D. and Caggia, C. (2004). *Lactobacillus casei*, dominant species in naturally fermented Sicilian green olives. *International Journal of Food Microbiology* 90, 9-14.

Razavi, A., Gmür, R., Imfeld, T. and Zehnder, M. (2007). Recovery of *Enterococcus faecalis* from cheese in the oral cavity of healthy subjects. *Oral Microbiology and Immunology* 22, 248-251.

Rea, M. C., and Cogan, T. M. (2003a). Catabolite repression in *Enterococcus faecalis*. *Systematic and Applied Microbiology* 26, 159-164.

Rea, M. C., and Cogan, T. M. (2003b). Glucose prevents citrate metabolism by enterococci. *International Journal of Food Microbiology* 88, 201-206.

Recsei, P. A. and Snell, E. E. (1984). Pyruvoyl Enzymes. *Annual Review of Biochemistry* 53, 357-387.

Reichelt, T., Kennes, J. and Krämer, J. (1984). Co-transfer of two plasmids determining bacteriocin production and sucrose utilization in *Streptococcus faecium*. *FEMS Microbiology Letters* 23, 147-150.

Reis, J. A., Paula, A. T., Casarotti, S. N. and Penna, A. L. B. (2012). Lactic acid bacteria antimicrobial compounds: characteristics and application. *Food Engineering Reviews* 4, 124-140.

Repizo, G. D., Espariz, M., Blancato, V. S., Suárez, C. A., Esteban, L. and Magni, C. (2014). Genomic comparative analysis of the environmental *Enterococcus mundtii* against enterococcal representative species. *BMC Genomics* 15, 489.

Rhee, J. E., Rhee, J. H., Ryu, P. Y. and Choi, S. H. (2002). Identification of the cadBA operon from *Vibrio vulnificus* and its influence on survival to acid stress. *FEMS Microbiology Letters* 208, 245-251.

Riley, M. A. and Wertz, J. E. (2002). Bacteriocin diversity: ecological and evolutionary perspectives. *Biochimie* 84, 357-364.

Rince, A., Giard, J. C., Pichereau, V., Flahaut, S. and Auffray, Y. (2001). Identification and characterization of gsp65, an organic hydroperoxide resistance (ohr) gene encoding a general stress protein in *Enterococcus faecalis*. *Journal of Bacteriology* 183, 1482-1488.

Ritchey, T. W. and Seeley Jun, H. W. (1974). Cytochromes in *Streptococcus faecalis* var. *zymogenes* grown in a haematincontaining medium. *Journal of General Microbiology* 85, 220-228.

Rodgers, S. (2001). Preserving non-fermented refrigerated foods with microbial cultures-a review. *Trends in Food Science & Technology* 12, 276-284.

Rodriguez-Jerez, J. J., Mora-Ventura, M. T., Lopez-Sabater, E. I. and Hernandez-Herrero, M. (1994). Histidine, lysine and ornithine decarboxylase bacteria in Spanish salted semi-preserved anchovies. *Journal of Food Protection* 57, 784-787.

Rollan, G. C., Coton, E. and Lonvaud-Funel, A. (1995). Histidine decarboxylase activity of *Leuconostoc oenos* 9204. *Food Microbiology* 12, 455-461.

Romano, A., Ladero, V., Alvarez, M. A. and Lucas, P. M. (2014). Putrescine production via the ornithine decarboxylation pathway improves the acid stress survival of *Lactobacillus brevis* and is part of a horizontally transferred acid resistance locus. *International Journal of Food Microbiology* 175, 14-19.

Romano, A., Trip, H., Lonvaud-Funel, A., Lolkema, J. S., and Lucas, P. M. (2012). Evidence of two functionally distinct ornithine decarboxylation systems in lactic acid bacteria. *Applied and Environmental Microbiology* 78, 1953-1961.

Rossi, E. A., Vendramini, R. C., Carlos, I. Z., Pei, Y. C. and de Valdez, G. F. (1999). Development of a novel fermented soymilk product with potential probiotic properties. *European Food Research and Technology* 209, 305-307.

Rossi, F., Gardini, F., Rizzotti, L., La Gioia, F., Tabanelli, G. and Torriani, S. (2011). Quantitative analysis of histidine decarboxylase gene (*hdcA*) transcription and histamine production by *Streptococcus thermophilus* PRI60 under conditions relevant to cheese making. *Applied and Environmental Microbiology* 77, 2817-2822.

Rossi, F., Rizzotti, L., Felis, G. E. and Torriani, S. (2014). Horizontal gene transfer among microorganisms in food: current knowledge and future perspective. *Food Microbiology* 42, 232-243.

Ruiz-Capillas, C. and Jiménez-Colmenero, F. (2004). Biogenic amines in meat and meat products. *Critical Reviews in Food Science and Nutrition* 44, 489-499.

Salminen, S., Isolauri, E. and Salminen, E. (1996). Clinical uses of probiotics for stabilising gut mucosal barrier: successful strains and future challenges. *Antonie van Leeuwenhoek* 70, 347-358.

Sandmeier, E. (1994). Multiple evolutionary origin of pyridoxal-5'-phosphate-dependent amino acid decarboxylases. *European Journal of Biochem*istry 221, 997-1002.

Sarantinopoulos, P., Andrighetto, C., Georgalaki, M. D., Rea, M. C., Lombardi, A., Cogan, T. M., Kalantzopoulos, G. and Tsakalidou, E. (2001a). Biochemical properties of enterococci relevant to their technological performance. *International Dairy Journal* 11, 621-647.

Sarantinopoulos, P., Kalantzopoulos, G. and Tsakalidou, E. (2001b). Citrate metabolism by *Enterococcus faecalis* FAIR-E 229. *Applied and Environmental Microbiology* 67, 5482-5487.

Saris, P. E. J., Immonen, T., Reis, M. and Sahl, H. G. (1996). Immunity to lantibiotics. *Antonie van Leeuwenhoek* 69, 151-159.

Schillinger, U. and Lücke, F. K. (1989). Antibacterial activity of *Lactobacillus sake* isolated from meat. *Applied and Environmental Microbiology* 55, 1901-1906.

Schneller, R., Good, P. and Jenny, M. (1997). Influence of pasteurised milk, raw milk and different ripening cultures on biogenic amine concentrations in semi-soft cheeses during ripening. *Zeitschrift fur Lebensmittel – Untersuchung Und-Forschung A – Food Research and Technology* 204, 265-272.

Schöbitz, R., González, C., Villarreal, K., Horzella, M., Nahuelquín, Y. and Fuentes, R. (2014). A biocontroller to eliminate *Listeria monocytogenes* from the food processing environment. *Food Control* 36, 217-223.

Sedewitz, B., Schleifer, K. H. and Götz, F. (1984). Physiological role of pyruvate oxidase in the aerobic metabolism of *Lactobacillus plantarum*. *Journal of Bacteriology* 160, 462-465.

Semedo, T., Santos, M. A., Lopes, M. F. S., Marques, J. J. F., Crespo, M. T. B. and Tenreiro, R. (2003a). Virulence factors in food, clinical and reference enterococci: A common trait in the genus? *Systematic and Applied Microbiology* 26, 13-22.

Semedo, T., Santos, M. A., Martins, P., Lopes, M. F. S., Marques, J. J. F., Tenreiro, R., and Barreto Crespo, M. T. (2003b). Comparative study using type strains and clinical and food isolates to examine hemolytic activity and occurrence of the cyl operon in enterococci. *Journal of Clinical Microbiology* 41, 2569-2576.

Shalaby, A. R. (1996). Significance of biogenic amines to food safety and human health. *Food Research International* 29, 675-690.

Shiwa, Y., Yanase, H., Hirose, Y., Satomi, S., Araya-Kojima, T., Watanabe, S., Zendo, T., Chibazakura, T., Shimizu-Kadota, M., Yoshikawa, H. and Sonomoto, K. (2014). Complete genome sequence of *Enterococcus mundtii* QU 25, an efficient L-(1)-lactic acid-producing bacterium. *Dna Research* 21, 369-377.

Sievers, F., Wilm, A., Dineen, D. G., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J. D. and Higgins, D. G. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology* 7, 539.

Silla-Santos, M. H. (1996). Biogenic amines: Their importance in foods. *International Journal of Food Microbiology* 29, 213-231.

Smid, E. J. and Lacroix, C. (2013). Microbe–microbe interactions in mixed culture food fermentations. *Current Opinion in Biotechnology* 24, 148-154.

Solovyev, V. and Salamov, A. (2011). *"Automatic annotation of microbial genomes and metagenomic sequences"*. In: Metagenomics and its applications in agriculture, biomedicine and environmental studies. R. W. Li (Eds.). Hauppauge, NY: Nova Science Publishers, 61-78.

Somkuti, G. A. and Babel, F. J. (1969). Hydrolytic breakdown of casein by a proteinase of *Streptococcus faecalis* var. *liquefaciens*. *Journal of Dairy Science* 52, 1186-1191.

Spano, G., Russo, P., Lonvaud-Funel, A., Lucas, P., Alexandre, H., Grandvalet, C., Coton, E., Coton, M., Barnavon, L., Bach, B., Rattray, F., Bunte, A., Magni, C., Ladero, V., Alvarez, M., Fernández, M., Lopez, P., de Palencia, P. F., Corbi, A., Trip, H. and Lolkema, J. S. (2010). Biogenic amines in fermented foods. *European Journal of Clinical Nutrition* 64, S95-S100.

Stiles, M. E., Ramji, N. W. and Paradis, D. C. (1978). Incidence and relationship of group D streptococci with other indicator organisms in meats. *Canadian Journal of Microbiology* 24, 1502-1508.

Stiles, M. E. (1996). Biopreservation by lactic acid bacteria. *Antonie Van Leeuwenhoek* 70, 331-345.

Stoyanova, L. G., Ustyugova, E. A. and Netrusov, A. I. (2012). Antibacterial metabolites of lactic acid bacteria: their diversity and properties. *Applied Biochemistry and Microbiology* 48, 229-243.

Stratton, J. E., Hutkin, R. W. and Taylor, S. L. (1991). Biogenic amines in cheese and other fermented foods: a review. *Journal of Food Protection* 54, 460-470.

Stumbo, C.R. (1973). *Thermobacteriology in Food Processing*. Academic Press, New York.

Suzzi, G., Caruso, M., Gardini, F., Lombardi, A., Vannini, L., Guerzoni, M. E., Andrighetto, C. and Lanorte, M. T. (2000). A survey of the enterococci isolated from an artisanal Italian goat's cheese (Semicotto Caprino). *Journal of Applied Microbiology* 89, 267-274.

Suzzi, G. and Gardini, F. (2003). Biogenic amines in dry fermented sausages: a review. *International Journal of Food Microbiology* 88, 41-54.

Süβmuth, S. D., Muscholl-Siberhorn, A., Wirth, R., Susa, M., Marre, R. and Rozdzinski, E. (2000). Aggregation substance promotes adherence, phagocytosis, and intracellular survival of *Enterococcus faecalis* within human macrophages and suppresses respiratory burst. *Infection and Immunology* 68, 4900-4906.

Svec, P., Devriese, L. A., Sedlacek, I., Baele, M., Vancanneyt, M., Haesebrouck, F., Swings, J. and Doskar, J. (2001). *Enterococcus haemoperoxidus* sp. nov. and *Enterococcus moraviensis* sp. nov., isolated from water. *International Journal of Systematic and Evolutionary Microbiology* 51, 1567-1574.

Tabanelli, G., Torriani, S., Rossi, F., Rizzotti, L. and Gardini, F. (2012). Effect of chemico-physical parameters on the histidine decarboxylase (HdcA) enzymatic activity in *Streptococcus thermophilus* PRI60. *Journal of Food Science* 77, M231-M237.

Tabanelli, G., Montanari, C., Grazia, L., Lanciotti, R. and Gardini, F. (2013). Effect of a_w packaging time and atmosphere composition on aroma profile, biogenic amine content and microbiological features of dry fermented sausage. *Meat Science* 94, 177-186.

Tabanelli, G., Bargossi, E., Gardini, A., Lanciotti, R., Magnani, R., Gardini, F. and Montanari, C. (2015). Physico-chemical and microbiological characterisation of Italian fermented sausages in relation to their size. *Journal of the Science of Food and Agriculture*.

Tanaka, Y., Kimura, B., Takahashi, H., Watanabe, T., Obata, H., Kai, A., Morozumi S. and Fujii T. (2008). Lysine decarboxylase of *Vibrio parahaemolyticus*: kinetics of transcription and role in acid resistance. *Journal of Applied Microbiology* 104, 1283-1293.

Taylor, L. and Woychik, N. A. (1982). Simple medium for assessing quantitative production of histamine by *Enterobacteriaceae*, *Journal of Food Protection* 45, 747-751.

Taylor, S. L. and Speckard, M. (1984). Inhibition of bacterial histamine production by sorbate and other antimicrobial agents. *Journal of Food Protection* 47, 508-511.

ten Brink, B., Damink, C., Joosten, H. M. L. J. and Huis in't Veld, J. H. J. (1990). Occurrence and formation of biologically active amines in foods. *International Journal of Food Microbiology* 11, 73-84.

Thomas, T. D., Ellwood, D. C. and Longyear, V. M. C. (1979). Change from homo to heterolactic fermentation by *Streptococcus lactis* resulting from glucose limitation in anaerobic chemostat cultures. *Journal of Bacteriology* 138, 109-117.

Thurlow, L. R., Thomas, V. C., Narayanan, S., Olson, S., Fleming, S. and Hancock, L. E. (2010). Gelatinase contributes to the pathogenesis of endocarditis caused by *Enterococcus faecalis*. *Infection and Immunity* 78, 4936-4943.

Tkachenko, A., Nesterova, L. and Pshenichnov, M. (2001). The role of the natural polyamine putrescine in defense against oxidative stress in *Escherichia coli*. *Archives Microbiology* 176, 155-157.

Todorov, S.D., Wachsman, M.B., Knoetze, H., Meincken, M. and Dicks, L.M.T. (2005). An antibacterial and antiviral peptide produced by *Enterococcus mundtii* ST4V isolated from soya beans. *International Journal of Antimicrobial Agents* 25, 508-513.

Torri Tarelli, G., Carminati, D. and Giraffa, G. (1994). Production of bacteriocins active against *Listeria monocytogenes* and *Listeria innocua* from dairy enterococci. *Food Microbiology* 11, 243-252.

Torriani, S., Gatto, V., Sembeni, S., Tofalo, R., Suzzi, G., Belletti, N., Gardini, F. and Bover-Cid, S. (2008). Rapid detection and quantification of tyrosine decarboxylase gene (*tdc*) and its expression in Gram-positive bacteria associated with fermented foods using PCR-based method. *Journal of Food Protection 7*1, 93-101.

Trip, H., Mulder, N. L., Rattray, F. P. and Lolkema, J. S. (2011). HdcB, a novel enzyme catalysing maturation of pyruvoyl-dependent histidine decarboxylase. *Molecular Microbiology* 79, 861-871.

Trivedi, K., Borkovcová, I. and Karpíšková, R. (2009). Tyramine production by enterococci from various foodstuffs: a threat to the consumers. *Czech Journal of Food Science* 27, S357-S360.

Valenzuela, A. S., Omar, N. B., Abriouel, H., López, R. L., Ortega, E., Cañamero, M. M. and Gálvez, A. (2008). Risk factors in enterococci isolated from foods in Morocco: determination of antimicrobial resistance and incidence of virulence traits. *Food and Chemical Toxicology* 46, 2648-2652.

Valsamaki, K., Michaelidou, A. and Polychroniadou, A. (2000). Biogenic amine production in Feta cheese. *Food Chemistry* 71, 259-266.

Van der Auwera, G. A., Timmery, S., Hoton, F. and Mahillon, J. (2007). Plasmid exchanges among members of the *Bacillus* cereus group in foodstuffs. *International Journal of Food Microbiology* 113, 164-172.

Vancanneyt, M., Lombardi, A., Andrighetto, C., Knijff, E., Torriani, S., Björkroth, K. J., Franz, C. M., Foulquié Moreno, M. R., Revets, H., De Vuyst, L., Swing, J., Kersters, K., Dellaglio, F. and Holzapfel, W. H. (2002). Intraspecies genomic groups in *Enterococcus faecium* and their correlation with origin and pathogenicity. *Applied and Environmental Microbiology* 68, 1381-1391.

Vancanneyt, M., Zamfir, M., Devriese, L. A., Lefebvre, K., Engelbeen, K., Vandemeulebroecke, K., Amar, M., De Vuyst, L., Haesebrouck, F. and Swings, J. (2004). *Enterococcus saccharominimus* sp. nov., from dairy products. *International Journal of Systematic and Evolutionary Microbiology* 54, 2175-2179.

Vaningelgem, F., Ghijsels, V., Tsakalidou E. and De Vuyst L. (2006). Cometabolism of citrate and glucose by *Enterococcus faecium* FAIR-E 198 in the absence of cellular growth. *Applied and Environmental Microbiology* 72, 319-326.

Vera Pingitore, E., Todorov, S. D., Sesma, F. and Franco, B. D. (2012). Application of bacteriocinogenic *Enterococcus mundtii* CRL35 and *Enterococcus faecium* ST88Ch in the control of *Listeria monocytogenes* in fresh Minas cheese. *Food Microbiology* 32, 38-47.

Verraes, C., Van Boxstael, S., Van Meervenne, E., Van Coillie, E., Butaye, P., Catry, B., de Schaetzen, M. A., Van Huffel, X., Imberechts, H., Dierick, K., Daube, G., Saegerman, C., De Block, J., Dewulf, J. and Herman, L. (2013). Antimicrobial resistance in the food chain: a review. *International Journal of Environmental Research and Public Health* 10, 2643-2669.

Vido, K. Le Bars, D., Mistou, M. Y., Anglade, P., Gruss, A. and Gaudu, P. (2004). Proteome analyses of heme-dependent respiration in *Lactococcus lactis*: involvement of the proteolytic system. *Journal of Bacteriology* 186, 1648-1657.

Vignaroli, C., Zandri, G., Aquilanti, L., Pasquaroli, S. and Biavasco, F. (2011). Multidrug resistant enterococci in animal meat and faeces and co-transfer of resistance from an *Enterococcus durans* to a human *Enterococcus faecium*. *Current Microbiology* 62, 1438-1447.

Villani, F., Salzano, G., Sorrentino, E., Pepe, O., Marino, P. and Coppola, S. (1993). Enterocin 226NWC, a bacteriocin produced by *Enterococcus faecalis* 226, active against *Listeria monocytogenes*. *Journal of Applied Bacteriology* 74, 380-387.

Villani, F., Aponte, M., Blaiotta, G., Mauriello, G., Pepe, O. and Moschetti, G. (2001). Detection and characterization of a bacteriocin, garviecin L1-5, produced by *Lactococcus garvieae* isolated from raw cow's milk. *Journal of Applied Microbiology* 90, 430-439.

Vinci, G. and Antonelli, M. L. (2002). Biogenic amine quality index of freshness in red and white meat. *Food Control* 13, 519-524.

Von Wright, A. and Axelsson, L. (2011). *Lactic acid bacteria: an introduction*. In: Lactic acid bacteria, microbial and functional aspects (4th ed.). S. Lahtinen, A. C. Ouwehand, S. Salminen and A. von Wright (Eds.), Boca Raton: CRC Press, 1-16.

Wallace, D. L. and Harmon, L. G. (1970). Intracellular protease from *Streptococcus durans*. *Journal of Dairy Science* 53, 394-402.

Wang, A., Gao, L., Ren, N., Xu, J. and Liu, C. (2009). Bio-hydrogen production from cellulose by sequential co-culture of cellulosic hydrogen bacteria of *Enterococcus gallinarum* G1 and *Ethanoigenens harbinense* B49. *Biotechnology Letters* 31, 1321-1326.

Wessels, D., Jooste, P. J. and Mostert, J. F. (1990). Technologically important characteristics of *Enterococcus* isolates from milk and dairy products. *International Journal of Food Microbiology* 10, 349-352.

Wolken, W. A. M., Lucas, P., Lonvaud-Funel, A. and Lolkema, J. (2006). The mechanism of the tyrosine transporter TyrP supports a proton motive tyrosine decarboxylation pathway in *Lactobacillus brevis. Journal of Bacteriol*ogy 188, 2198-2206.

Wunderlich, P. F., Braun, L., Fumagalli, I., D'Apuzzo, V., Heim, F., Karly, M., Lodi, R., Politta, G., Vonbank, F. and Ja Zeltner, L. (1989). Double-blind report on the efficacy of lactic acid-producing *Enterococcus* SF68 in the prevention of antibiotic-associated diarrhoea and in the treatment of acute diarrhoea. *Journal of International Medical Research* 17, 333-338.

Wunderlichová, L., Buňková, L., Koutnỳ, M., Jaňcová, P. and Buňka, F. (2014). Formation, degradation, and detoxification of putrescine by foodborne bacteria: a review. *Comprehensive Reviews in Food Science and Food Safety* 13, 1012-1033.

Xanthopoulos, V., Polychroniadou, A., Litopoulou-Tzanetaki, E. and Tzanetakis, N. (2000). Characteristics of Anevato cheese made from raw or heat-treated goat milk inoculated with a lactic starter. *Lebensmittel-Wissenschaft und- Technologie* 33, 483-488.

Yamazaki, A., Watanabe, K., Nishimura, Y. and Kamihara, T. (1976). Mutants of *Streptococcus faecalis* concerning pyruvate dehydrogenation. *FEBS Letters* 64, 364-368.

Yankah, V. V., Ohshima, T. and Koizumi, C. (1993). Effects of processing and storage on some chemical characteristics and lipid composition of a Ghanaian fermented fish product. *Journal of the Science of Food and Agriculture* 63, 227-235.

Zapparoli, G., Torriani, S. and Dellaglio, F. (1998). Differentiation of *Lactobacillus sanfrancisciensis* strains by randomly amplified polymorphic DNA and pulsed-field gel electrophoresis. *FEMS Microbiology Letters* 166, 325-332.

Zhang, X., Vrijenhoek, J. E., Bonten, M. J., Willems, R. J. and van Schaik, W. A (2011). A genetic element present on megaplasmids allows *Enterococcus faecium* to use raffinose as carbon source. *Environmental Microbiology* 13, 518-528.

Zhang, K. and Ni, Y. (2014). Tyrosine decarboxylase from *Lactobacillus brevis*: soluble expression and characterization. *Protein Expression and Purification* 94, 33-39.

Zwietering, M. H., Jongenburger, I., Rombouts, F. M. and van't Riet, K. (1990). Modelling of the bacterial growth curve. *Applied and Environmental Microbiology* 56, 1875-1881.

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