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REVIEW

Enterocins of *Enterococcus faecium*, emerging natural food preservatives

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Abstract Enterococci are distinct lactic acid bacteria, and also natural inhabitants of human and animal intestinal tracts. They may enter food products during processing through direct or indirect contamination and are mostly present in fermented food products, such as cheese, sausages, olives, etc. Nowadays, they are extensively studied for the production of bacteriocins (enterocins), which prevent the growth of many food-borne and spoilage-causing pathogens, such as *Staphylococcus aureus, Listeria monocytogenes, Escherichia coli*, *Pseudomonas* spp., *Bacillus* spp. and *Clostridium* spp. Enterocins belong to class I, class IIa, class IIc, and class III of bacteriocins. Enterocins can be used in different food products in order to enhance their shelf life because they are heat stable and show activity over wide pH range. Enterocins are effective as well as safe to be used in the food system because they are "generally recognized as safe" (GRAS). *Enterococcus faecium* and *Enterococcus faecalis* are the predominant bacteriocin-producing species of *Enterococcus* in food products. The following review is focused on the bacteriocin-producing strains of *Enterococcus faecium* isolated from different traditional fermented food products. The aim of this review is to cover general features of the enterocins of *Enterococcus faecium,* the attempts made to purify them, and their potential application in different food products to improve their overall safety.

Keywords *Enterococcus faecium* · Enterocin · Bacteriocin · Food preservation

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Introduction

The genus *Enterococcus* is one of the groups of micro-organisms that comes under the category of lactic acid bacteria (LAB). The history of enterococci cannot be considered separately from that of the genus *Streptococcus.* Previously, scientists have combined all streptococci and enterococci species under the genus *Streptococcus* (Devriese and Pot 1995). But in 1984, 16S rRNA sequencing (Ludwig et al. 1985) and DNA–DNA hybridization studies (Garvie and Farrow 1981; Kilpper-Balz and Schleifer 1981; 1984; Kilpper-Balz et al. 1982; Schleifer and Kilpper-Balz 1984) have revealed that *Streptococcus faecium* and *Streptococcus faecalis* were significantly different from other streptococci. Thus, Schleifer and Kilpper-Balz (1984) proposed the transfer of the genus *Streptococcus* to the genus *Enterococcus.* The discovery of enterococci bacteria is attributed back to the nineteenth century, when Thiercelin (1899) described a new Gram-positive bacterium *Diplococcus*, which was later included in the new genus *Enterococcus* with the type species *E. proteiformis* (Thiercelin and Jouhaud 1903). However, before this, many species of *Enterococcus* were identified but not categorized separately into the *Enterococcus* genus. The genus *Enterococcus* has been reviewed a number of times (Schleifer and Kilpper-Balz 1987; Devriese and Pot 1995; Hardie and Whiley 1997).

General characteristics of enterococci

Enterococci are Gram-positive, facultative anaerobic and non-spore-forming bacteria. They show catalase negative and oxidase negative tests (Devriese and Pot 1995). The optimum temperature for the growth of enterococci is 35°C while they can also grow in a wide range of temperatures, from 10 to 45°C. They can also grow in the presence of 6.5% NaCl and at pH 9.6 (Schleifer and Kilpper-Balz 1987).

Until now, some 28 different species of *Enterococcus* have been identified, namely, *E. faecium*, *E. canis*, *E. avium*, *E. asini*, *E. gallinarum*, *E. columbae*, *E. phoeniculicola*, *E. flavescens*, *E. moraviensis*, *E. haemoperoxidus*, *E. saccharolyticus*, *E. villorum*, *E. casseliflavus*, *E. dispar*, *E. durans*, *E. faecalis*, *E. mundtii*, *E. gilvus*, *E. hirae*, *E. malodoratus*, *E. pallens*, *E. pseudoavium, E. raffinosus*, *E. cecorum*, *E. ratti*, *E. solitarius*, *E. sulfureus*, and *E. saccharominimus* (Foulquie Moreno et al. 2006). Kalina (1970) proposed a species, *E. faecium* formerly described by Orla-Jensen (1919) as *Streptococcus faecium*, and it was finally revived by Schleifer and Kilpper-Balz (1984). The cells of *E. faecium* are ovoid and occur in pairs or short chains elongated in the direction of the chain. They form smooth, circular and entire colonies. They have homogenous turbidity in broth and are non-motile (Schleifer and Kilpper-Balz 1984).

Enterococci are present everywhere in the environment. They are also natural inhabitants of the human and animal gastrointestinal tract. Among various species of the *Enterococcus* genus, *E. faecium* and *E. faecalis* are predominant (Devriese and Pot 1995). Enterococci are tolerant of extreme levels of environmental conditions and can survive under wide range of growth conditions. Enterococci can enter both raw (e.g., meat and milk) and processed foods through environmental contamination. Enterococci are the most heat resistant among non-sporulating bacteria. They are resistant to pasteurization temperatures and show growth on different substrates, a wide temperature range, extreme pH and salinity. Therefore, enterococci are also found in many fermented food products made from milk and meat, especially cheeses and sausages, respectively (Giraffa 2002). However, it has been verified that the common occurrence of *Enterococcus* spp. in many food products is not always associated with direct fecal contamination (Mundt 1986). The genus *Enterococcus* can be related to fermented dairy microflora and it seems not to be necessary to relate its source with fecal contamination (Giraffa 2003). Enterococci are "generally recognized as safe" (GRAS) lactic acid bacteria (Devriese and Pot 1995).

Description of bacteriocins of *Enteroccocus* species

The bacteriocins of lactic acid bacteria are antimicrobial peptides that are ribosomally synthesized. These are small, cationic and amphiphilic (rather hydrophobic) in nature and vary in spectrum and mode of activity. These bacteriocins also possess different molecular structure, molecular mass, thermostability, pH range of activity, and genetic determinants (McAuliffe et al. 2001; Cleveland et al. 2001; Riley and Wertz 2002).

Enterocins belong to class I, class II, class III and class IV of bacteriocins (Franz et al. 2007). Enterocins of class I are lantibiotics, i.e. small, cationic, heat-stable and hydrophobic peptides. These form pores in the membranes of target microorganisms. They also show flexible structures. An example is the two-component cytolysin from *E. faecalis* (Booth et al. 1996). Class II enterocins are small, cationic, heat-stable and hydrophobic peptides. These are not post-translationally modified, except for the cleavage of the leader peptide from the pre-bacteriocin. There are three different notable subclasses of enterocins (Ennahar et al. 2000; Cleveland et al. 2001). Enterocins that come under subclass IIa are pediocin-like bacteriocins that show strong anti-listerial activity. These *Listeria*-active peptides have aconserved N-terminal sequence, Tyr-Gly-Asn-Gly-Val, and two cysteines forming an S-S bridge in the N-terminal half of the peptide. They include Bacteriocin 31 (Tomita et al. 1996) from *E. faecalis*, Enterocin A (Aymerich et al. 1996), Enterocin CRL35 (Farias et al. 1996) and Enterocin P (Cintas et al. 1997) from *E. faecium* and Mundticin from *E. mundtii* (Bennik et al. 1998). Bacteriocins that belong to subclass IIb are composed of two polypeptide chains. Both peptides are required for full biological activity and their primary amino acid sequences are also different. This subclass includes many bacteriocins that lack the YGNGVXC motif and are synthesized as leaderless peptides which require dedicated export systems (Franz et al. 2007). Enterocin RJ-11 (Yamamoto et al. 2003), Enterocin EJ97 (Galvez et al. 1998) from *E. faecalis* and Enterocin Q, Enterocin L50A and Enterocin L50B (Cintas et al. 1998) from *E. faecium* (Cintas et al. 2000) belong to this group. The enterocins which cannot be included in the other subclasses are grouped in subclass IIc (Moll et al. 1999), e.g., Enterocin B (Casaus et al. 1997), and Enterocin 1071A and Enterocin 1071B from *E. faecalis* (Balla et al. 2000). It was proposed by Franz et al. (2007) that the enterococci that produce cyclic antimicrobial peptides should be included in class III enterocins within the enteroccal bacteriocin classification scheme, like Enterocin AS-48 from *E. faecalis* (Galvez et al. 1989). Class IV enterocins are large molecular weight and heat labile proteins, e.g., Enterolysin A produced by *E. faecalis* LMG 2333 and DPC5280 (Hickey et al. 2003, Nilsen et al. 2003).

The primary target of enterocins is the cytoplasmic membrane. The pores are formed in the cell membrane that depletes the trans-membrane potential and the pH gradient. Due to this, the indispensable intracellular molecules are leaked out from cells (Cleveland et al. 2001).

Efficiency of enterocins against food pathogenic and spoilage-causing bacteria (*Listeria* spp., *Staphylococcus* spp., *Bacillus* spp., etc.) is well demonstrated in a variety of food systems (Aymerich et al. 2000). Enterocin AS-48 produced by *E. faecalis* S-48 was the first enterocin purified to homogeneity and was defined as a cyclic peptide antibiotic (Martinez-Bueno et al. 1994). Since then, several new enterocins have been characterized. However, many of identified enterocins have not been purified to homogeneity.

Food applications of enterococci

Enterococci show some valuable biotechnological characteristics, such as the production of anti microbial enzymes with anti-listeria activity. An important feature for the application of enterococci in food technology is the production of bacteriocins (Foulquie Moreno et al. 2006). Bacteriocin production by *Enterococcus* species of the food system has been known for many years (Olasupo et al. 1994). Bacteriocins produced by enterococci are called enterocins (De Vuyst and Vandamme 1994). The first bacteriocin-like substance was reported in 1955 within the group D streptococci (Kjems 1955). Subsequently, scientists studied several enterocins. Kramer and Brandis (1975) reported Enterocin E1A produced by *E. faecium* E1 that showed anti-Listeria activity. The well-known ability of enterococci to inhibit *Listeria* spp. may be due to the close phylogenetic relationship of enterococci and listeria (Stackebrandt and Teuber 1988; Devriese and Pot 1995).

It has been noticed that the enterococci also play a role in the development of organoleptic properties of traditional fermented food products of different regions. (Foulquie Moreno et al. 2006). *Enterococcus faecium* is mostly found in raw milk (Wessels et al. 1988) and many fermented milk products (Saavedra et al. 2003). *Enterococcus faecium* also occurs in certain types of processed foods (Herranz et al. 2001). Mundt (1976) isolated *E. faecium*-like strains from plants and frozen or dried foods. *Enterococcus faecium* has also been isolated from Spanish-style green olive fermentations (Floriano et al. 1998).

Several review articles have been written on the genus *Enterococcus* but no one has yet focused on any particular species in this genus. The present review is focused on the bacteriocin-producing strains of *E. faecium* isolated from different traditional fermented food products (Abriouel et al. 2005; Ennahar et al. 2001; Foulquie Moreno et al. 2003a, b; Leroy et al. 2003; O'Keeffe et al. 1999).

Bacteriocin production from Enterococcus faecium and its general characteristics

A bacteriocin-producing strain *E. faecium* CRL 35 was isolated from regional Argentinian cheese (Tafi cheese) by Farias et al. (1994). It was noted that it produced a bacteriocin which showed activity against food-borne pathogens like *S. aureus* and *L. monocytogenes*. The bacteriocin was named Enterocin CRL 35. They observed that activity of Enterocin CRL 35 was not lost at extreme pHs, heat treatment, and storage in different conditions, but that it showed sensitivity to protease enzymes. The bacteriocin producer, *E. faecium* CTC 492, was isolated from fermented Spanish sausages by Aymerich et al. (1996). It produced a bacteriocin that was termed Enterocin A. The anti-bacterial activity of *E. faecium* CTC 492 was checked against a number of pathogenic Gram-positive bacteria. It was observed that Enterocin A inhibited six strains of *Listeria* spp., three strains of *E. faecalis* and two strains of *Pediococcus* spp. Franz et al. (1996) isolated *E. faecium* BFE 900 from black olives and observed that it produced a bacteriocin which showed activity against *Lactobacillus sakei, Clostridium butyricum, Clostridium perfringens* and *Listeria monocytogenes*. They named the bacteriocin Enterocin 900. It was also noted that Enterocin 900 was inactivated by trypsin, proteinase K, α-chymotrypsin and pepsin but not by α-amylase, catalase or other non-proteolytic enzymes tested. The enterocin was heat-stable and retained its activity at sterilization temperature (121°C for 15 min) and at a wide pH range. It showed maximum activity at pH 6.0. It was also noted that no plasmids were isolated from *E. faecium* BFE 900 which indicated that the gene for anti-bacterial activity was present on the chromosome. Table 1 summarizes the enterocins of *E. faecium* that have been purified and named.

Cintas et al. (1997) observed that *E. faecium* P13, isolated from a Spanish dry-fermented sausages, produces a bacteriocin which showed activity against several food-borne and spoilage-causing Gram-positive bacteria. The bacteriocin was named Enterocin P. It was observed that Enterocin P showed activity against *Listeria monocytogenes, Staphylococcus aureus, Clostridium perfringens, Clostridium botulinum, E. faecalis, Staphylococcus carnosus, Clostridium sporogenes, Clostridium tyrobutyricum,* and *Propionibacterium* spp. They noted that Enterocin P retain its activity when heated for 15 min at 121°C and also at extreme pH. The anti-bacterial spectrum of Enterocin P was also not affected during freeze-thawing, lyophilization, and long-term storage at −20 and 4°C. Floriano et al. (1998) isolated a bacteriocin-producing strain *E. faecium* 6T1a from Spanish-style fermented green olives. It was noted that it produced a bacterocin, which was termed Enterocin I. This bacteriocin showed activity against a number of food-borne and spoilage-causing Gram-positive bacteria of olives. The antimicrobial activity was observed against strains of *E. faecalis, Bacillus* spp., *Clostridium* spp., *Listeria* spp., *Pediococcus* spp. and *Propionibacterium* spp. It was observed that Enterocin I retained its activity when heated

Table 1 Summary of purified enterocins from *Enterococcus faecium*

Producer strains	Enterocins	Reference
CRL 35	Enterocin CRL 35	Farias et al. (1996)
CTC 492	Enterocin A	Aymerich et al. (1996)
BFE 900	Enterocin 900	Franz et al. (1996)
P13	Enterocin P	Cintas et al. (1997)
6T1a	Enterocin I	Floriano et al. (1998)
WHE 81	Enterocin 81	Ennahar et al. (1998)
AA13	Enterocin AA13	Herranz et al. (1999)
G16	Enterocin G16	Herranz et al. (1999)
L50	Enterocin Q	Cintas et al. (2000)
EFM01	Enterocin EFM01	Ennahar and Deschamps (2000)
P21	Enterocin P21	Herranz et al. (2001)
N15	Enterocin N15	Losteinkit et al. (2001)
B1	Enterocin B1	Moreno et al. (2002)
B2	Enterocin B2	Moreno et al. (2002)
F58	Enterocin F-58	Achemchem et al. (2005)
MMT21	Enterocin A	Ghrairi et al. (2008)
MMT21	Enterocin B	Ghrairi et al. (2008)
ST5Ha	Bacteriocin ST5Ha	Todorov et al. (2010)

for 5 min at 100°C but was partially inactivated by autoclaving. When the bacteriocin was treated with enzymes, it was observed that it became ineffective after treatment with proteinase K, α-chymotrypsin, thermolysin, trypsin subtilopeptidase A and pronase E, but lysozyme, catalase, α-amylase, RNase A and ficin did not affect its activity. The bacteriocin-producing strain *E. faecium* WHE 81 was isolated from Munster cheese. It was observed that the bacteriocin showed a narrow spectrum of activity. It inhibited the growth of *Listeria innocua*, *Listeria seeligerii* and *L. monocytogenes*. It was noted that the activity of the enterocin of *E. faecium* WHE 81 was completely diminished by proteolytic enzymes but not affected by catalse, α-amylase and lipase enzymes. The anti-bacterial activity was not affected at pH values from 4.0 to 8.0. The bacteriocin was named Enterocin 81 (Ennahar et al. 1998).

Herranz et al. (1999) isolated two bacteriocinogenic *E. faecium* strains. AA13 and G16. from chorizo, a typical Spanish dry-fermented sausage manufactured with no added starter cultures. They noted that cell-free supernatants of *E. faecium* AA13 and G16 showed antimicrobial activity against a number of *L. monocytogenes*, *S. aureus*, *C. perfringens* and *C. botulinum* strains. It was also found that the antimicrobial spectrum and activity of the *E. faecium* AA13 strain was greater than those of the G16 strain. The antimicrobial activities of both isolates were not lost after heat treatment at 121°C for 20 min and also remained unaffected by exposure to pH values between 2 and 11. It was also noted that proteolytic enzymes destroyed the antimicrobial activity, but α-amylase and lipase-I treatment did not affect the bacteriocidal activity of either strain.

It was observed by Ennahar and Deschamps (2000) that *Enterococcus faecium* EFM01 isolated from cheese produced Enterocin A. They found that Enterocin A showed activity against many species of *Listeria* including *L. monocytogenes*, *L. innocua* and *L. seeligeri*. It was also shown that the activity of Enterocin A was not notably affected by pH, retaining its activity at pH 4.0–9.0, while showing maximum activity at pH 6.5. *Enterococcus faecium* P21 has been isolated from Spanish dry-fermented sausages by Herranz et al. (2001). They observed that the bacteriocin was effective against food-borne and spoilage-causing Gram-positive bacteria including *S. aureus, C. botulinum, C. perfringens,* and *L. monocytogenes.* They also noted that the enterocin became ineffective after protease treatment (pepsin, trypsin, papain and protease II), though the activity was not affected by lipolytic or amylolytic enzymes such as lipase VII or α-amylase, respectively. It was also observed that the activity of the enterocin was not affected after heat treatment for 20 min at 80 and 100°C, or at pH values between 2 and 11 for 24 h at 4 and 32°C. They also noted that the bactericidal activity was not lost by lyophilization, freezing, thawing and long-term storage at –20°C for 12 months.

Losteinkit et al. (2001) isolated *E. faecium* N15 from nuka (Japanese rice-bran paste), which was used as starter for the fermentation of vegetables. They observed that it produced a bacteriocin which was effective against *L. monocytogenes* and against closely related *Enterococcus* and *Lactobacillus* bacteria. It was also noted that the activity of the enterocin was stable over a wide range of pH from 2 to 10. The activity was lost after treatment with proteolytic enzymes and α-amylase, but it was resistant to lipase. The activity remained stable after heat treatment at 100°C for 2 h. After characterization of the bacteriocin, it was implied that the bacteriocin produced by *E. faecium* NI5 was Enterocin A. *Enterococcus faecium* B1 and *Enterococcus faecium* B2 were isolated from the Malaysian mold-fermented product tempeh by Moreno et al (2002). They observed that both *E. faecium* B1 and B2 produced bacteriocins which were named Enterocin B1 and Enterocin B2, respectively. These enterocins were active against *Carnobacterium divergens, E. faecalis*, *Lactobacillus brevis*, *Clostridium piscicola*, *Lactobacillus pentosus* and *Paralactobacillus selangorensis*. Both strains also inhibited all tested strains of *L. monocytogenes*, in addition to strains of *Bacillus pumilus, Micrococcus luteus* and *L. innocua*. The activity of both enterocins were lost after treatment with α-amylase, proteinase K, α-chymotrypsin, trypsin and pepsin but were not affected by catalase, lysozyme and lipase. They observed that the antimicrobial activity of the

enterocins was lost when they were heated at 121°C for 20 min at alkaline pH. They also noted that anti-bacterial activity of Enterocin B1 was less than that of Enterocin B2. Table 2 summarizes the *E. faecium* strains studied and their spectrum of inhibition towards most sensitive bacteria.

Achemchem et al. (2005) isolated bacteriocin-producing strain *E. faecium* F58 from a soft farmhouse goat's cheese, Jben, which was made without adding starter cultures. They observed that the bacteriocin was active against several food-borne pathogenic and spoilage-causing Gram-positive bacteria which include *L. innocua, L. monocytogenes, S. aureus, B. subtilis, B. cereus, C. tyrobutyricum, C. perfringens* and *Brochothrix*. The bacteriocin was named Enterocin F-58. It was noted that the bacteriocin retained its activity at pH values from 4 to 8 and when heated to 100°C for 5 min. The bacteriocin activity was also not affected after treatment with lysozyme and lipase but it was totally lost by treatments with protease, trypsin and α-chymotrypsin. Ghrairi et al. (2008) isolated a bacteriocin-producing strain of *E. faecium* MMT21 from Tunisian rigouta cheese. They observed that the bacteriocin showed activity against a number of pathogenic and spoilage-causing bacteria of the food system which included *L. monocytogenes, S. aureus, E. faecalis* and *B. cereus*. They noted that when the bacteriocin was treated with proteinase K, pronase E and trypsin enzymes, its activity was diminished. However, the activity of the enterocin remained unaffected when heated for 15 min at 100°C and after incubation at pH values ranging from 2 to 10. In Pakistan, *E. faecium* IJ-31 was isolated from a butter sample. It was found that it produced a bacteriocin which inhibited *L. monocytogenes*, *B. subtilis* and *B. cereus*. The enterocin retained its antibacterial activity even after heating at 121°C for 15 min. The enterocin also remained stable to pH values ranging from 4 to 10, but its stability was lost after proteinase K treatment (Javed et al. 2010).

Purification of Enterococcus faecium bacteriocins

Enterocin CRL 35 was purified by precipitation with $(NH_4)_2SO_4$, gel filtration, ion exchange and reverse phase chromatography (Farias et al. 1996). They also subjected the purified Enterocin CRL 35 to Edman degradation. Only 16 residues out of 21 were identified after the sequencing from the amino terminal of Enterocin CRL 35. It was confirmed by the partial N-terminal sequence that Enterocin CRL 35 belongs to the pediocin-like (class IIa) bacteriocins. Enterocin A produced by *E. faecium* CTC 492 was the first bacteriocin of *E. faecium* which was purified and characterized at the amino acid and DNA sequence levels by Aymerich et al. (1996). Enterocin A was purified to

Table 2 Antimicrobial activity spectrum of enterocins obtained from *Enterococcus faecium*

Producer strains	Inhibitory spectrum[a]	Reference
CTC 492	*Listeria* spp., *Enterococcus faecalis*	Aymerich et al. (1996)
CRL 35	*S. aureus*, *L. monocytogenes*	Farias et al. (1996)
BFE 900	*Lb. sakei, C. butyricum, C. perfringens*, *L. monocytogenes*	Franz et al. (1996)
P13	*L. monocytogenes, S. aureus, C. perfringens, C. botulinum, E. faecalis, S. carnosus, C. sporogenes, C. tyrobutyricum, Propionibacterium* spp.	Cintas et al. (1997)
6T1a	*E. faecalis, Bacillus* spp., *Clostridium* spp., *Listeria* spp., *Pediococcus* spp., *Propionibacterium* spp.	Floriano et al. (1998)
WHE 81	*L. innocua*, *L. seeligerii, L. monocytogenes*	Ennahar et al. (1998)
AA13	*L. monocytogenes*, *S. aureus*, *C. perfringens*, *C. botulinum*	Herranz et al. (1999)
G16		Herranz et al. (1999)
L50	*Lb. sakei, Pediococcus* spp.	Cintas et al. (2000)
EFM01	*L. monocytogenes, L. innocua, L. seeligeri*	Ennahar and Deschamps (2000)
N15	*L. monocytogenes* and closely related *Enterococcus* spp. and *Lactobacillus* spp.	Losteinkit et al. (2001)
P21	*S. aureus, C. botulinum, C. perfringens, L. monocytogenes.*	Herranz et al. (2001)
B1 & B2	*Carnobacterium divergens, E. faecalis*, *Lb. brevis*, *Carnobacterium piscicola*, *Lb. pentosus* and *Paralactobacillus selangorensis*, *L. monocytogenes*, *B. pumilus, Micrococcus luteus*, *L. innocua*	Moreno et al. (2002)
F58	Genera *Listeria, Staphylococcus, Clostridium, Brochothrix*, *Bacillus*	Achemchem et al. (2005)
MMT21	*L. monocytogenes, S. aureus* and closely related LAB	Ghrairi et al. (2008)
ST5Ha	*Escherichia coli, Enterobacter cloacae, E. faecalis, Klebsiella pneumoniae , L. ivanovii, L. monocytogenes, L. innocua, Pseudomonas* spp., *S. aureus* and closely related LAB	Todorov et al. (2010)

[a] *B. Bacillus, C. Clostridium, E. Enterococcus, S. Staphylococcus, L. Listeria, Lb. Lactobacillus*, *LAB* lactic acid bacteria

homogeneity, which showed that it consists of 47 amino acid residues. The molecular weight of enterocin was equal to 4,829 Da. It was also noted that Enterocin A shared significant homology with a group of bacteriocins that belong to the genera *Lactobacillus, Pediococcus, Leuconostoc,* and *Carnobacterium.* Enterocin 900 was purified by hydrophobic interaction chromatography with Amberlite XAD8, concentrated to 100 ml by rotary evaporation, and cation-exchange chromatography with SP Sepharose by (Franz et al. 1999a, b). Following cation-exchange chromatography, the bacteriocin fraction was desalted with a Sep Pak C_{18} reverse-phase column and freeze-dried. The freeze-dried protein was further purified by high-pressure liquid chromatography (HPLC). They also observed by mass spectrometric analyses that the average molecular mass of Enterocin 900 is 5,463.0±1 Da. which was almost the same as Enterocin B produced by *E. faecium* T136 (Casaus et al. 1997). The following 53-amino-acid sequence was shown by analysis of the N-terminal amino acid sequence of the purified enterocin: GAAHAMPAGLAAPAALSLGG ALXGAAIA-GGLPGIPLGPLATAAGLAAVTS (Leu/Lys) X (Leu/Asn). They noted that the amino acids 23 and 52 were cysteines. The nucleotide sequence also showed that amino acids at positions 51 and 53 were mistakenly identified as leucine and that they were lysine and asparagine, which are indicated above in parentheses.

Ammonium sulfate precipitation, gel filtration, cation-exchange, hydrophobic-interaction, and reverse-phase liquid chromatography were used for the purification of the Enterocin P to homogeneity (Cintas et al. (1997). The theoretical molecular weight of Enterocin P was calculated to be 4,493 Da. The first 43 residues of Enterocin P were determined by Edman degradation which revealed the following sequence: ATRSYGNGVYCNNSKCWVNW GEAK-ENIAGIVISGWASGLAGMG. It was noted by partial sequencing that Enterocin P show amino acid sequence YGNGV in positions 5–9, the two conserved cysteine residues in positions 11 and 16, and the conserved valine residue in position 18. It was confirmed that Enterocin P had the consensus sequence found in the pediocin-like bacteriocins, but the identity of Enterocin P with other bacteriocins of genus *Enterococcus* was not so strong. Thus, Enterocin P was put under subclass IIa of the bacteriocins. It has been suggested that Enterocin P and other broad-spectrum bacteriocins with different modes of action can be used against spoilage and food-borne pathogenic bacteria. Enterocin I was purified to homogeneity by ammonium sulfate precipitation, binding to an SP-Sepharose fast-flow column, and phenyl-Sepharose CL-4B and C_2/C_{18} reverse-phase chromatography by Floriano et al. (1998). After the second reverse-phase chromatographic step, a final yield of 95.4% of the initial activity and a 170,000-fold increase in the specific activity of Enterocin I was obtained. SDS-PAGE analysis showed an electrophoretically pure protein with an apparent molecular size of about 5 kDa. Amino acid and nucleotide sequencing was determined by the primary structure of the enterocin. They found that Enterocin I consists of 44 amino acids and lacks the leader peptide at the N-terminal region of the gene product. It was also observed that a second open reading frame, ORF2, is located downstream of *ent I,* which encodes a putative protein that is 72.7% identical to Enterocin I. The protein encoded by ORF2 is like an immunity protein. On the basis of the homology of ORF2 to Enterocin I, it was thought that this protein might bind to the putative receptors for Enterocin I that was present on the surface of *E. faecium* 6T1a cells. Thus, it prevents the binding and provides immunity to Enterocin I. So, it has been concluded that the gene sequence is not similar with any other previously described bacteriocin. However, subsequently, it was noted that Enterocin I was similar to Enterocin L50B which has been described by Cintas et al. (1998).

Herranz et al. (1999) purified the bacteriocins of *E. faecium* AA13 and *E. faecium* G16 to homogeneity by ammonium sulfate precipitation, gel filtration, cationic exchange, hydrophobic interaction, and reverse-phase liquid chromatography. They got two peptide inhibitory fractions from each strain, denominated A and B for *E. faecium* AA13, and C and D for *E. faecium* G16. The amino acid sequence of the purified peptide fractions was also obtained by Edman degradation. Fraction B was blocked for amino acid sequencing. It was confirmed by the amino acid sequences of fractions A, C, and D that the YGNGV motif was present and contained in positions 5–9, the same as in pediocin-like bacteriocins, and the ATRS sequence in positions 1–4, a sequence already described by Cintas et al. (1997) in Enterocin P produced by *E. faecium* P13. It was confirmed by the amino acid sequences of the purified peptides that the antibacterial activity of *E. faecium* AA13 and *E. faecium* G16 was due to Enterocin P. Herranz et al. (2001) used ammonium sulphate precipitation, gel filtration, and cation-exchange, hydrophobic interaction and reverse-phase chromatographies for the purification of Enterocin A and Enterocin B. The wide bactericidal range of *E. faecium* P21 was observed which is certainly due to two peptide bacteriocins. They also determined the partial amino acid sequences of fractions A and B by Edman degradation. It revealed that the first 30 amino acid residues of the N-terminus of fraction A included 9 unidentified positions and the pediocin-like bacteriocin consensus amino acid sequence YGNGV (Aymerich et al. 1996, Ennahar et al. 2000) in positions 8–12. It has been confirmed by partial amino acid sequencing that fraction A shows a high homology with Enterocin A. The presence of 13 unidentified residues and the lack of the pediocin-like consensus

sequence have been observed when the partial amino acid sequence of the first 49 residues of fraction B were determined. So it has been confirmed that fraction B shows strong homology with Enterocin B.

Ennahar et al. (2001) observed that when Enterocin 81 was purified by using a method which included ammonium sulfate precipitation, de-salting on a reverse-phase column, and purification through cation exchange and C_2/C_{18} reverse-phase chromatographies, purified fractions containing antibacterial activity were obtained from a culture of *E. faecium* WHE 81. They thought that *E. faecium* WHE 81 produced multiple antimicrobial peptides. The analysis of the purified fractions B81 and D81 had been done by mass spectrometry and they found that the molecular mass of the isolated enterocins were 4,833.0 and 5,462.2 Da, respectively. They also determined the N-terminal amino acid sequence of the fraction B81, which is achieved for the first 24 amino acid residues. According to the measured molecular mass of the peptide, it represented about a half of the whole sequence. The partial amino acid sequence which was obtained is as follows: (T) (T/E) (H/G) SGKYYGNGVYX$_{aa}$ TKNKX$_{aa}$ TVD-(W/D) A… i.e., amino acid residues that could not be determined with certainty were inside parentheses, residues in large capitals could be replaced with residues in small capitals, and those that could not be identified were represented by X. It clearly appeared that the bacteriocin contains the YGNGV N-terminal motif. Therefore, it was confirmed that this enterocin belong to class IIa of the bacteriocins of lactic acid bacteria. The amino acid sequencing of the fraction D81 was also determined by Edman degradation. It revealed a sequence of 53 residues which was as follows: (E/Q) N D H R M P N E L N R P N - N L S K G G A K C G A A I A G G LFGIPKGPLAWAAGLANV YSKCN. They noted that this enterocin was in fact class-II bacteriocin, but it did not contain the YGNGV motif. So they confirmed that this enterocin belongs to subclass IIc of bacteriocins. It was confirmed by amino acid sequencing that these two bacteriocins were Enterocin A and Enterocin B, respectively. So it has been suggested that the anti-bacterial activity of *E. faecium* WHE 81 was due to Enterocin A and Enterocin B.

Moreno et al. (2002) partially purified enterocins by tricine-SDS-PAGE and noted that the molecular mass of Enterocin B1 was 3.4 kDa. But *E. faecium* B2 showed two inhibition zones at 5.8 kDa and 3.4 kDa, respectively, which indicated the presence of two enterocins. So they concluded that Enterocin B1 might be similar to Enterocin P as previously described by Cintas et al. (1997). On the other hand, *E. faecium* B2 produced two types of bacteriocins that showed similarity with Enterocin P (Cintas et al. 1997) and Enterocin L50 (Cintas et al. 1998). Cation exchange and hydrophobic interaction on C-18 and RP-HPLC have been used for the purification of the Enterocin F-58 to homogeneity (Achemchem et al. 2005). Two fractions of bacteriocin were revealed. The molecular mass of these fractions were 5,234.3 and 5,210.5 Da, respectively. Automated Edman degradation was used for N-terminal amino acid sequencing of both fractions which showed the following sequences, MGAIAKLVAKFGWPIVKKYYK and MGAIAKLV(A) KFG (a residue that could not be determined with any certainty is shown in parentheses) were obtained, respectively. These partial sequences were compared with other recognized bacteriocins present in protein databases. It was confirmed that fractions I and II, related to Ent F58B and F58A, respectively, were similar to enterocins L50 (B and A) (Cintas et al. 1998) and Ent I (I and J) (Floriano et al. 1998), with the only exception of the residue shown inparentheses. It was noted by PCR-amplification of total genomic DNA of *E. faecium* F58 that it also holds the structural gene for Enterocin P. An identical sequence was observed by alignment of DNA sequences of the amplified fragment from strain F58 and Ent L50. So it was confirmed that Enterocin L50 (A and B) were produced by Ent F58. Ghrairi et al. (2008) observed by RP-HPLC purification of the anti-bacterial enzyme that *E. faecium* MMT21 produced two different bacteriocins. It was confirmed by mass spectrometry analysis that these two anti-microbial enzymes were Enterocin A and Enterocin B having molecular weights 4,828.67 Da and 5,463.8 Da, respectively. This result was further confirmed by PCR amplification of enterocins A and B genes.

Mode of action of Enterococcus faecium bacteriocin

The effect of Enterocin 900 on *Lb. sakei* indicator culture was checked by Franz et al. (1996). They noted that the number of indicator cells decreased from an initial log 6.0 CFU/ml to log 4.0 CFU/ml after 1 h of incubation at 30°C. It was confirmed that Enterocin 900 had a bactericidal mode of action. The bactericidal effect of Enterocin P on growing cells of *L. monocytogenes* Scott A was observed by Cintas et al. (1997). They observed that within 45 min after addition of Enterocin P, the viable colony counts dropped rapidly to approximately 20% and after 4 h the viable count represented only 2% of the initial viable count. Ennahar et al. (1998) observed that the anti-*L. monocytogenes* effect of Enterocin 81 was very rapid. It was found that a rapid drop (about 3 log10 units) of the viable counts, initially 4.0×10^6 CFU/ml, occurred within only 30 min after exposure of indicator cells to Enterocin 81. It was confirmed by electron microscopy that Enterocin 81 did not induce cell lysis but it did exert a bactericidal mode of action.

The activity of Enterocin N15 against the growth of *Lb. sakei* JCM1157 was tested by Losteinkit et al. (2001). It was noted that, after the addition of Enterocin N15, the optical

density of the indicator culture stopped increasing after 5 h of incubation and remained constant thereafter. It was suggested that the activity of Enterocin N15 on *Lb. sakei* JCMl157 was bactericidal without concomitant cell lysis. Moreno et al. (2002) noted that Enterocin B1 and Enterocin B2 displayed a bacteriostatic mode of action against *L. innocua* LMG 13568. They also suggested that Enterocin B1 and B2 can inhibit Gram-negative pathogenic bacteria if Gram-negative cells were injured because their outer membrane layer prevents bacteriocins from reaching their target. When *L. ivanovii* ATCC 19119 and *E. faecalis* ATCC 19433 were treated with Bacteriocin ST5Ha, it resulted in the leakage of β-galactosidase from the cells which indicated the destabilization of the cell membrane permeability (Todorov et al. 2010).

Applications of Enterococcus faecium enterocins

Lactic Acid Bacteria (LAB) and *Bifidobacteria* are the leading bacterial groups that represent the majority of the probiotic supplements (Sanders 1998). Enterococci, though generally considered as normal inhabitant of gastrointestinal tract, are concomitantly the second to third most common agent of nosocomial infections (Foulquie Moreno et al. 2006). Considering this, it is important to exclude pathogenic enterococci from the consortium of microbes which are candidates for probiotics. Many researchers have approved that the probiotic properties of *E. faecium* strains have also been stipulated (Linaje et al. 2004; Saavedra et al. 2003).

Enterococcus faecium M-74 and *E. faecium* SF68 are two commercially available probiotic strains. It has been noted that *E. faecium* SF68 plays an effective role in reducing the recovery period of acute diarrhea, and in decreasing blood cholesterol levels (Benyacoub et al. 2003). It was observed by Callewaert et al. (2000) that two *Enterococcus* strains, *E. faecium* CCM 4231 and *E. faecium* RZS C13, were partially competitive during meat fermentation, strongly inhibited the growth of *Listeria*, and improved the organoleptic properties of mature dry-fermented sausages. So the authors have suggested that these strains of *E. faecium* are suitable for adding as starter cultures in dry-fermented sausages. Saavedra et al. (2003) also suggested that *E. faecium* strains, isolated from Tafi cheese, might be used as nontraditional starter cultures. They observed that the absence of any pathogenic factor in enterococci that are present in artisan cheeses would guarantee the safety of this kind of food product. *Enterococcus faecium* WHE 81 was investigated for its anti-listerial properties in the rind of Munster cheese, a red surface ripened soft cheese. Cell suspensions of the enterocin producing strain (approximately 10^5 CFU/ml) and *L. monocytogenes* (approximately 10^2 CFU/ml) were prepared in brine, and sprayed on the cheese surface along with commercial ripening culture on days 3, 5 and 7 of ripening. At the end of the ripening period, the pathogen was undetectable in the samples sprayed with *E. faecium* WHE 81 strain, with no effect on the ripening flora or pigmented bacteria which was in sharp contrast to negative control samples in which the listerial counts increased to about 10^5 CFU/g after 20 days (Izquierdo et al. 2009). Similarly, *E. faecium* 7 C5 (Giraffa and Carminati 1997) and *E. faecium* F58 (Achemchem et al. 2006) also indicated a reduction of listerial counts in Taleggio (Italian soft smear cheese) and Jben (Moroccan fresh cheese), respectively. These results show the potential of using bacteriocin-producing *E. faecium* strains as a culture adjunct to inhibit *L. monocytogenes* during cheese manufacturing.

The above review clearly demonstrates the importance of enterocins of *Enterococcus faecium*. The enterocins can be added either directly in foods or incorporated into edible or non-edible antimicrobial films. However, the use of purified or semi-purified preparations of bacteriocins as food preservatives has legal implications. Despite being produced by LAB, the enterocins intended to be used as food preservatives are considered as additives and need prior approval by the regulatory authorities, requiring detailed safety information supported by toxicological data, proof of efficacy in foods, description of the manufacturing process, and the safe maximum levels (Cleveland et al. 2001). So far, nisin is the only bacteriocin that has been approved for use as a food preservative in over 50 countries including USA, European Union, Australia and New Zealand (Delves-Broughton 2005). In addition, the processes described in the literature for production, purification and recovery of enterocins may be suitable for laboratory experiments, but they need to be optimized for commercial exploitation at economical costs. Moreover, the synergistic effect of enterocins along with physical treatments such as heat and high pressure has shown an improvement in the antimicrobial properties of the enterocins. These studies, therefore, highlight the potential of these important enterocins to be produced on a large scale and for their use as food preservatives in commercial food products.

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