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1 **Enterocin C, a Class IIb Bacteriocin Produced by *E. faecalis* C901, a Strain**
2 **Isolated from Human Colostrum**

3

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14 **Running title:** Enterocin C from *E. faecalis* C901

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1 **ABSTRACT**

2

3 Enterocin C (EntC), a class IIb bacteriocin was purified from culture supernatants of
4 *Enterococcus faecalis* C901, a strain isolated from human colostrum. Enterocin C consists of
5 two distinct peptides, named EntC1 and EntC2, whose complementary action is required for
6 full antimicrobial activity. The structural genes *entC1* and *entC2* encoding enterocins EntC1
7 and EntC2, respetively, and that encoding the putative immunity protein (*EntCI*) are located in
8 the 9-kb plasmid pEntC, harboured by *E. faecalis* C901. The N-terminal sequence of both
9 antimicrobial peptides revealed that EntC1 is identical to Ent1071A, one of the two peptides
10 that form enterocin 1071 (Ent1071), a bacteriocin produced by *E. faecalis* BFE 1071. In
11 contrast, EntC2 presents the non-polar alanine residue at position 17 ($Ala_{17}$) instead of the polar
12 threonine residue ($Thr_{17}$) in Ent1071B, the second peptide constituting Ent1071. In spite of
13 peptide similarities, EntC differs from Ent1071 in major aspects, including the complementary
14 activity among its constitutive peptides and its wider inhibitory spectrum of activity. Different
15 amphiphilic $\alpha$-helical conformations between EntC2 and Ent1071B could explain both,
16 acquired complementary activity and increased antimicrobial spectrum.

1 **INTRODUCTION**

2

3 Colostrum and breast milk protect the newborn against infectious diseases, being this
4 effect due to the combined action of a variety of protective factors present in these biological
5 fluids, such as inmunoglobulins, inmunocompetent cells, antimicrobial fatty acids, polyamines,
6 fucoylated oligosaccharides, lysozyme, lactoferrin, and antimicrobial peptides, which inactivate
7 pathogens individually, additively, and synergistically (27). In addition, breast milk is an
8 excellent and continuous source of commensal and potentially probiotic bacteria to the infant
9 gut, including staphylococci, streptococci, and other lactic acid bacteria (LAB) (23, 33, 35).
10 Some of the LAB strains isolated from this biological fluid have the ability to inhibit the
11 growth of a wide spectrum of pathogenic bacteria by competitive exclusion and/or through the
12 production of antimicrobial compounds, such as organic acids, hydrogen peroxide or the
13 bacteriocin nisin, a lactococcal bacteriocin (6, 34, 43).
14 Bacteriocins are antimicrobial compounds of proteinaceous nature with inhibitory
15 activity against microorganisms that usually are closely related to the producing bacteria. In
16 recent years, bacteriocins from LAB have been extensively studied due to their potential as
17 food preservatives. The ability to produce such antimicrobial peptides is wide spread among
18 enterococci (40), a property that makes them attractive to the food industry; in fact, several
19 enterococcal strains with histories of safe use are included as components of starter,
20 bioprotective or probiotic cultures (20).
21 Enterococci form an essential part of the indigenous human microbiota soon after birth,
22 being *Enterococcus faecium* and *Enterococcus faecalis* the most common species in human
23 mucosal surfaces (16, 26, 46). On the other hand, some enterococcal strains can behave as
24 opportunistic pathogens and cause nosocomial infections especially in patients with underlying

1 diseases (29). Moreover, enterococci are noted for their capacity to exchange genetic
2 information, including antibiotic resistance genes, by conjugation (12, 15).
3 In this study, a two-peptide bacteriocin (enterocin C) produced by *E. faecalis* C901, a
4 strain originally isolated from human colostrum provided by a healthy woman, was
5 biochemical and genetically characterized. In addition, this strain was screened for the presence
6 of potential virulence determinants and for its sensitivity to several clinically-relevant
7 antibiotics.
8
9 **MATERIALS AND METHODS**

10 **Bacterial strains and media.** *E. faecalis* C901 and all the bacterial strains used as
11 indicators in this study (Tables 1 and 2) were grown routinely in MRS medium (Oxoid,
12 Basingstoke, Hampshire, England) at 37ºC, with the exception of *Actinomyces* sp.,
13 *Enterococcus gallinarum*, *E. saccharolyticus*, *Escherichia coli*, *Staphylococcus* sp. and
14 *Streptococcus* sp., which were grown in Brain Heart Infusion (BHI) medium (Oxoid). All the
15 strains from human sources (Table 1) belonged to our own collection while those from food
16 origin were obtained from different bacterial collections (Table 2). They were maintained as
17 frozen stocks at –80ºC in MRS or BHI (Oxoid) plus 20% (vol/vol) glycerol.
18
19 **Bacteriocin assays.** Bacteriocin activity in cell-free supernatants (CFSs) from
20 exponential and stationary-phase broth cultures (8 and 16 h, respectively) of *E. faecalis* C901
21 was assayed by using the agar drop diffusion test as described previously (31), using the strains
22 listed in Tables 1 and 2 as indicator microorganisms. Subsequently, *E. faecalis* L1443 was used
23 as the indicator strain throughout the whole process of enterocin C purification and, also, to
24 investigate the complementary activity of the two peptides (EntC1 and EntC2) that integrate
25 enterocin C. For the late purpose, 5-µl aliquots of purified EntC1 were mixed with equal

1 volumes of purified EntC2, and the mixes were assayed for antimicrobial activity by the agar
2 drop diffusion test. Five-μl aliquots of each purified peptide were separately assayed in order to
3 compare the results. Quantification of complementary activity among peptides EntC1 and
4 EntC2 of enterocin C was carried out by the microtiter plate assay system (21). The inhibitory
5 activity was expressed as bacteriocin units per millilitre (BU/ml) as described previously (31).
6
7 **Bacteriocin purification.** All the purification steps were carried out at room
8 temperature, and all of the chromatographic equipment and media were purchased from
9 Amersham Biosciences Europe GmbH (Freiburg, Germany). Peptides EntC1 and EntC2 were
10 purified from a 2-L culture of *E. faecalis* C901. After 24 h at 37ºC without shaking, cells were
11 removed by centrifugation at 10,000 × *g* for 10 min at 4ºC and, then, the bacteriocin was
12 purified from the CFS using the procedure described by Maldonado *et al*. (30). Briefly, the CFS
13 was precipitated with ammonium sulfate, desalted, and consecutively applied to cation-
14 exchange and hydrophobic-interaction columns. Finally, samples were subjected to $C_2/C_{18}$
15 reverse-phase chromatography in FPLC (RPC-FPLC). Fractions showing inhibitory activity
16 after the $C_2/C_{18}$ reverse-phase column were pooled and subjected to several runs until both
17 EntC1 and EntC2 peptides were purified to homogeneity.
18
19 **SDS-PAGE.** During the purification process, the $C_2/C_{18}$ reverse-phase column fractions
20 were analyzed in duplicate by SDS-PAGE using an 18% acrylamide resolving gel. After
21 electrophoresis at 100 mV for 2 h, one gel was silver stained (52) while the other was used to
22 detect inhibitory activity in an overlay assay with *E. faecalis* L1443 as the indicator strain (8).
23
24 **N-terminal amino acid sequence and mass spectrometry.** The N-terminal amino acid
25 sequences of purified EntC1 and EntC2 peptides were determined by automated Edman

1 degradation with a Beckman LF3000 sequencer/phenylthiohydantoin amino acid analyzer
2 (System Gold, Beckman, Fullerton, CA). Molecular mass of the peptides was determined by
3 Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-
4 TOF). These analysis were performed by Dr. Silvia Bronsoms (Servei de Proteòmica i
5 Bioinformàtica, Universitat Autònoma, Barcelona, Spain).
6
7 **PCR sequencing and location of the enterocin C structural and immunity genes.**
8 The primer pair 1071A-for (5'- ATGAAGCAATATAAAGTATTGAATG -3') and 1071I-rev
9 (5'- TTACTTAATTAAATAGTTCAGTACA -3') were designed on the basis of the genes
10 encoding Ent1071A (*ent1071A*) and Ent1071B (*ent1071B*) and their immunity protein (*ent*I)
11 (GenBank accession number: AF458698). DNA was amplified in 25-µl reaction mixtures
12 containing 2.5 mM Mg $Cl_2$, 1× reaction buffer, 200 µM concentrations of each of the
13 deoxynucleotides triphosphates (dNTPs), 1 µM of each of the primers, and 1,25 U of *Taq* DNA
14 polymerase (Ecotaq; Ecogen, Barcelona, Spain). Amplification included denaturation at 94ºC
15 for 4 min, followed by 30 cycles of denaturation at 94ºC for 30 s, annealing at 56ºC for 1 min,
16 polymerization at 72ºC for 1 min, and a final polymerization step at 72ºC for 5 min.
17 The amplified fragment was excised from a 0.7% agarose gel, purified using the
18 Nucleospin® Extract II kit (Macherey-Nagel, Düren, Germany), and both strands were
19 sequenced using primers 1071A-for and 1071I-rev at the Genomics Unit of the Universidad
20 Complutense (Madrid, Spain).
21 To locate the genes encoding EntC1 and EntC2 peptides, PCR assays were performed as
22 described above, using plasmid or chromosomal DNA from *E. faecalis* C901 as templates.
23 Plasmid DNA was extracted as described by Anderson and McKay (2) while total genomic
24 DNA was isolated using the QIAmp tissue kit as recommended by the manufacturer.
25

1 **PCR screening of virulence and vancomycin-resistance (*van*) genes in *E. faecalis*
2 **C901.** A novel multiplex PCR assay was designed to detect the presence of the potential
3 virulence determinants *gelE*, *cylA*, $efaA_{fs}$, $eps_{fs}$, $agg_2$, *ccf*, *cpd*, *cad,* and *cob* in *E. faecalis* C901
4 using the primer pairs previously proposed by Eaton and Gasson (15). PCR amplifications were
5 performed in 25-µl reaction mixtures containing 3 mM Mg $Cl_2$, 1.5× reaction buffer, 400 µM
6 of each deoxynucleotide triphosphate (dNTP), 0.2 µM of each primer, and 2,5 U of *Taq* DNA
7 polymerase (Ecotaq). A colony suspension in deionized water (5 µl) was used as DNA
8 template. PCR conditions included initial denaturation at 94°C for 5 min, followed by 30 cycles
9 of denaturation at 94°C for 1 min, annealing at 51°C for 30 s, elongation at 72°C for 1 min 30
10 s, and a final extension at 72°C for 5 min. *E. faecalis* P229 (15) was used as a positive control.
11 PCR for detection of *vanA* and *vanB* genes were carried out according to Dutka-Malen
12 *et al*. (14) and Ramos-Trujillo *et al*. (49), respectively. *E. faecium* BM4147 ($VanA^+$) and *E.*
13 *faecalis* V583 ($VanB^+$) were used as positive controls.
14
15 **Computer analysis of DNA sequences.** The Clone Manager Professional Suite
16 software (version 6.00) was used for DNA analysis. The WinPep program (available at
17 http://www.ipw.agrl.ethz.ch/~lhennig/winpep.html) was used for physico-chemical analysis of
18 peptides (isoelectric point, molecular weight and hydropathy plot) (24). Edmunson α-helical
19 wheel (51) was plotted with the Helical Wheel Applet program
20 (http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html).
21
22 **Nucleotide sequence accession number.** The DNA sequence presented in this article
23 has been deposited in the GenBank database under the accession number EU862242.
24

1 **Hemolytic activity and susceptibility to antibiotics.** Hemolytic activity of *E. faecalis*
2 C901 was determined on Columbia agar plates supplemented with 5% horse blood (COH,
3 BioMerieux) and incubated for 72 h at 37°C. Minimal inhibitory concentrations (MICs) for
4 several antibiotics commonly used against enterococcal infections were determined by a
5 microdilution method using the Sensititre plates Staenc1F (Trek Diagnostic Systems,
6 Cleveland, OH) following the manufacturer's instructions. Briefly, colonies from solid media
7 were suspended in saline solution to reach a 0,5 McFarland turbidity. Fifty µL of this
8 suspension were transferred to a 10 ml tube of Mueller–Hinton broth (bacterial concentration of
9 ca. $10^6$ cfu/ml) and, finally, 100 µl of this suspension were inoculated into each plate well.
10 Antibiotics analyzed were amoxycillin/clavulanic acid (AUG), ampicillin (AMP),
11 chloramphenicol (CHL), ciprofloxacin (CIP), clindamycin (CLI), erythromycin (ERY),
12 fosfomycin (FOS), gentamicin (GEN), imipenem (IMI), mupirocin (MUP), nitrofurantoine
13 (NIT), linezolid (LNZ), oxacillin (OXA), penicillin (PEN), quinupristin/dalfopriscin (Q/D),
14 rifampin (RIF), streptomycin (STR), teicoplanin (TEI), trimethoprim/sufamethoxazole (SXT),
15 tetracycline (TET) and vancomycin (VAN). When available, the National Committee for
16 Clinical Laboratory Standards (NCCLS; 2002) were used for determination of
17 sensitivity/resistance (38).
18
19 ## RESULTS
20 **Antimicrobial activity of *E. faecalis* C901.** CFSs obtained from exponential and
21 stationary-phase *E. faecalis* C901 cultures showed antimicrobial activity against many indicator
22 strains used in this study, including species both related and non-related to the producing strain,
23 such as *A. neuii*, *E. faecalis*, *E. faecium*, *F. hominis*, *L. lactis*, *L. paracasei*, *L. mesenteroides*,
24 *P. acnes*, *S. caprae*, *S. epidermidis*, *S. anginosus* and *S. intermedius* (Tables 1 and 2). It is

noteworthy that all but one *E. faecium* and *E. faecalis* strains tested as indicators were inhibited, including other bacteriocin-producing strains.

**Purification of EntC1 and EntC2 peptides.** Similarly to many LAB bacteriocins, the compound responsible for the antimicrobial activity produced by *E. faecalis* C901 precipitated in the presence of ammonium sulfate and showed a cationic and highly hydrophobic nature. Evidence that two peptides were involved in enterocin C activity was obtained after the first run in the $C_2/C_{18}$ reverse-phase column, when the agar drop diffusion test revealed that maximal antimicrobial activity was coincident with fractions in which two distinct absorbance peaks overlapped (not shown). Several additional runs were necessary to obtain fractions containing pure peptides, which were named EntC1 and EntC2, according to the order they eluted from the column. Fractions containing EntC1 showed little or no inhibitory activity in contrast to that displayed by fractions containing EntC2. SDS-PAGE gels showed that both peptides had been purified to homogeneity and displayed similar molecular weight (ca. 2.5 kDa; Fig. 1A). When SDS-PAGE gels were assayed for antimicrobial activity, both peptides were active against the indicator strain *E. faecalis* L1443, being the activity displayed by EntC2 notably higher (Fig. 1A). In addition, a small inhibition halo was observed in the zone between the bands corresponding to EntC1 and EntC2, which resulted from diffusion and mixing of both peptides, suggesting again their complementary nature (Fig. 1A). Complementary assays revealed that the bacteriocin activity of the two-peptide mix was greater than that observed for each separated peptide (Fig. 1B). More specifically, when bacteriocin activity of eluted fractions containing pure peptides was titrated, EntC1 showed no or just residual activity, meanwhile EntC2 showed 200 BU/ml. However, when EntC1 and EntC2 were combined in the same proportion, bacteriocin activity was 25600 BU/ml, ca. 128 times more than EntC2

1 assayed alone. These results confirmed the existence of complementary activity between the
2 peptides EntC1 and EntC2.
3
4 **Partial amino acid sequence and mass spectrometry of EntC1 and EntC2.** Partial
5 amino acid sequencing of EntC1 and EntC2 showed that the 10 N-terminal amino acids shared
6 100% homology with the first 10 N-terminal amino acids of mature enterocin 1071A
7 (Ent1071A) and 1071B (Ent1071B), respectively, from *E. faecalis* BFE 1071. Mass
8 spectrometry analysis (MALDI-TOF) rendered molecular masses of 4,284 Da for EntC1 and
9 3,867 Da for EntC2 (Fig. 2). The MW of mature Ent1071A and Ent1071B were 4,285 and
10 3,899 respectively (4). These results suggested that the amino acid sequence of peptide EntC1
11 could be identical to that of Ent1071A while the differences in the molecular masses between
12 EntC2 and Ent1071B could be attributable to a variation in their amino acid sequence.
13
14 **Genetic analysis and DNA sequencing of enterocin C structural genes**. In order to
15 analyse the DNA sequence encoding enterocin C, a 786-bp DNA fragment was amplified with
16 primers 1071A-for/1071I-rev using DNA from pENTC, a 9-kb size plasmid extracted from *E.*
17 *faecalis* C901, as template. Sequencing of this DNA fragment revealed the presence of three
18 open reading frames (ORFs) which shared high homology with the structural genes encoding
19 enterocins 1071A (*ent*1071A) and 1071B (*ent*1071B) and their immunity protein 1071I
20 (*ent1071I*). Detailed analysis of this sequence showed that *ent*C1 and *ent*C2 encoded two
21 peptides of 57 and 62 amino acids, respectively. Both peptides contain leader sequences of the
22 double-glycine type that, upon processing, give rise to mature peptides of 39 and 35 amino
23 acids, respectively, whose deduced molecular masses (4,284 and 3,899 Da respectively) were
24 coincident with those obtained experimentally for EntC1 and EntC2 peptides through mass
25 spectrometry (Fig. 3). As expected, the deduced amino acid sequence of mature peptide EntC1

1 was identical to mature enterocin 1071A. However, the amino acid sequence deduced for
2 mature EntC2, differed in one amino acid with that of mature enterocin 1071B (Fig. 3). More
3 specifically, EntC2 contains an alanine residue in position 17 ($A_{17}$) instead of the threonine
4 residue of the peptide Ent1071B at the same position ($T_{17}$), due to the substitution of a single
5 nucleotide (GCA in *entC2*, while ACA in *ent1071B*).The molecular mass predicted for the
6 EntC2 peptide corresponded exactly with that obtained by MALDI-TOF, confirming the $A_{17}$ of
7 enterocin C901. The double-glycine leader sequences of both the EntC1 and EntC2 peptides
8 were identical to those described for Ent1071A and Ent1071B, respectively.

9 Downstream of *entC2*, a third ORF named *entCI* was found. This ORF encodes a
10 putative protein of 125 amino acid residues with a theoretical pI of 9.4, and a MW of 14,866
11 Da, which shares 100% homology with 1071I, the protein that putatively confers immunity to
12 enterocin 1071A and 1071B. Finally, we found that the whole operon encoding enterocin C
13 production and immunity is harboured by a 9-kb plasmid in *E. faecalis* C901, which was
14 designated pENTC.

15

16 **Virulence determinants, *van* genes and sensivity to antibiotics of *E. faecalis* C901.**
17 The genes *gelE*, *efaA$_{fs}$*, *eps*$_{\mathrm{fs}}$, *agg$_2$*, *ccf*, *cpd*, *cad* and *cob* were present in *E. faecalis* C901 (Fig.
18 4) but *cylA* was not detected. Haemolysis was not observed on COH plates. On the other hand,
19 genes *van*A and *van*B, conferring resistance to vancomycin, were absent in this strain. MICs of
20 selected antibiotics are shown in table 3. In summary, *E. faecalis* C901 was sensitive to most of
21 the antibiotics tested with the exceptions of tetracycline and quinupristin-dalfopriscin.

22

23 **DISCUSSION**

24 To our knowledge, this is the first report describing the isolation of a bacteriocin-
25 producing LAB from human colostrum. Our results clearly indicate that enterocin C belongs to

1 the class IIb bacteriocins (39) and, therefore, consists of two different peptides, EntC1 and
2 EntC2, whose combination is necessary to obtain full bacteriocin activity.

3 Enterocin C is nearly identical to enterocin 1071, a bacteriocin previously described
4 which has been purified from two independent enterococcal strains: *E. faecalis* BFE1071 and
5 *E. faecalis* FAIR E-309 (4, 19). In fact, the amino acid sequence of peptide EntC1 is identical
6 to that of Ent1071A, while EntC2 differed only in one amino acid from enterocin 1071B ($A_{17}$
7 and $T_{17}$, respectively). In contrast with the results obtained in this work with peptides EntC1
8 and EntC2, whose activity is clearly complementary, it has been described that enterocin
9 1071A acts independently from enterocin 1071B (5). In addition, it has been highlighted that
10 the absence of activity against lactococci is a typical feature of enterocins 1071A and 1071B (4,
11 5, 19). However, enterocin C shows inhibitory activity against *L. lactis* strains including *L.*
12 *lactis* IL1403 (Tables 1 and 2), a strain resistant to enterocin 1071 (4). In the same manner,
13 enterocin C was active against *L. sakei* NCFB 2714 but not against *L. salivarius* NCFB 2747,
14 two strains which were resistant and sensitive, respectively, to enterocin 1071 (4). Therefore,
15 the inhibitory spectrum of enterocin C appears to be quite distinct to that of enterocin 1071.

16 A single amino acid change in one of the peptides which compose class IIb bacteriocins
17 could be responsible for determining the specificity of the target strain. Lactococcin G
18 (composed by LcnGα plus LcnGβ) and lactococcin Q (composed by LcnQα plus LcnQβ) are
19 two homologous (88% identity) two-peptide bacteriocins whose inhibitory activity is limited to
20 lactococcal strains (42, 55). The peptide LcnGα differs in six amino acids with LcnQα while
21 peptide LcnGβ differs in only three amino acids with LcnQβ. Both bacteriocins are also similar
22 to enterocin 1071 (57 and 59 % identities to lactococcins G and Q, respectively) (44). Zendo *et*
23 *al*. (55) suggested that the different amino acid residues between LcnGβ and LcnQβ might be
24 involved in the intensity of the antibacterial activity rather than in determining the specificity
25 for target cells. On the other hand, since enterocin 1071 was not active against lactococcal

1 strains, they proposed that the amino acid residues that differ between lactococcins and
2 enterocin 1071 might be involved in the specific recognition of target cells. Taking into account
3 these observations, it could be possible that the amino acid residue at position 17 (Ala) in the
4 peptide EntC2 of enterocin C is located in a zone involved in specificity determination.

5 In a recent study, Oppegård *et al.* (45) analysed bacteriocins lactococcin G and
6 enterocin 1071 by site-directed mutagenesis. Their results suggested that the β peptide of each
7 bacteriocin (LcnGβ and Ent1071B) is important at determining target cell specificity, specially
8 the N-terminal residues. Besides, the C-terminal residues might be involved in specific
9 interaction with the cognate α peptide (LcnGα and Ent1071A, respectively). Thus, EntC2
10 represents an Ent1071B natural variant whose study in combination with EntC1 and/or other
11 similar two-peptide bacteriocins, such as lactococcins G and Q, could serve to elucidate the
12 mode of action of these antimicrobial peptides as well as determine how target cell specificity
13 is achieved. Actually, one single amino acid difference between EntC2 and Ent1071B ($Ala_{17}$
14 and $Thr_{17}$, respectively) appears to be responsible for the high inhibitory activity of enterocin C
15 against lactococci compared with the lack of activity of enterocin 1071. This amino acid
16 change could also be responsible for the considerable enhancement in antimicrobial activity
17 due to complementarity observed in enterocin C, as this change is not neutral, for a polar amino
18 acid (Thr in Ent1071B) has been substituted by a non-polar one (Ala in EntC2). On the other
19 hand, when Ent1071A and Ent1071B peptides were obtained after site-directed mutagenesis
20 from LcnGα and LcnGβ, respectively, and heterologously expressed in *L. sake* Lb706, the
21 mixture was active against lactococci (45). This result could indicate that the actual way by
22 which a specific bacteriocin producer strain synthesizes a bacteriocin plays an extremely
23 important role in its final power and spectrum of activity. Apart from particular amino acid
24 changes that could determine important conformational modifications, facts such as the relative
25 amounts produced of each complementary peptide by a specific producer strain could explain

1 why very similar two-peptide bacteriocins, such as enterocin C and enterocin 1071, can differ
2 in their activity spectrum and complementary effect between their constitutive peptides.
3 Amphiphilic α-helical conformations are found in several pore-forming antimicrobial peptides
4 (48). Class IIb bacteriocins, as lactococcin G, are thought to form pores due to the amphiphilic
5 character or their constituting peptides, which can adopt an α-helix conformation (22, 45).
6 Attending to their amino acid sequence, amphiphilic α-helical motifs of enterocin C and 1071
7 are represented as an Edmunson α-helical wheel (53) in figure 5. As it is shown in this figure,
8 the non-polar $Ala_{17}$ in EntC2 is in the place of the polar $Thr_{17}$ in Ent1071B just in the
9 hydrophilic side of the α-helix. This change in the polarity of that region could facilitate
10 dimerization with EntC1 or interaction with the membrane of target cells. Thus, it could ease
11 pore formation in the membranes of sensitive strains and/or increase the sensitivity of these. It
12 is noticeable that the amphiphilic regions of lactococcins Gβ and Qβ are highly similar to that
13 of EntC2 when represented as an α-helical wheel (Fig. 5), despite the differences in their
14 aminoacid sequences (Fig. 3).

15 *E. faecalis* C901, isolated from human colostrum, produces a bacteriocin that is almost
16 identical to enterocin 1071 produced by *E. faecalis* BFE 1071 and Fair-E 309, two strains that
17 were isolated from mini pig faeces (4) and cheese (19), respectively. Enterocin C, as as it is
18 with enterocin 1071 (5), is encoded by a plasmid that we have named pENTC and which is
19 currently under sequencing. The extremely efficient plasmid conjugation system of *E. faecalis*
20 (25) can explain the widespread of these highly homologous enterocins among different strains.
21 In fact, *E. faecalis* C901 has shown to possess the genes *cpd, cob, ccf, cad* encoding sex
22 pheromones which could facilitate conjugation with other *E. faecalis* strains. In addition, this
23 strain harbours the *gelE*, $efaA_{fs}$, $eps_{fs}$, and $agg_2$ genes but it lacked the *cylA* gene. The presence
24 of the cited genes seems to be widespread among *E. faecalis* strains, including those with a
25 long history of safe use in the food industry (15, 18). Despite the apparently "dualistic" nature

of enterococci, the incidence of virulence determinants and/or any other factor of clinical significance, such as the antibiotic resistance pattern or the gene transfer potential, appear to be strain specific among isolates studied so far. Vancanneyt *et al*. (54) compared the genotypes of strains of the related specie *E. faecium* from human, animal and food origin and found that all human isolates involved in clinical infection fell into a well defined subgroup, suggesting that there could be a common genetic basis for strains associated with human disease. Pillai *et al*. (47) also suggested that virulent subpopulations of *E. faecalis* may exist. Therefore, the safety of any enterococcal strain should be individually evaluated.

Enterococci are among the predominant bacteria in human milk of healthy women (23, 33, 35) and in faeces of healthy breastfed neonates (1, 51), which suggests that they may play important biological functions. The roles of enterococci in human hosts, both in health and disease, are far from clear, but the inclusion of enterococal strains among the microorganisms that will be studied in the frame of the human microbiome project may provide new clues in the next years.

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18

1 **LEGENDS OF THE FIGURES**

2

3 **Legend of Figure 1.** A) Left panel, silver stained SDS-PAGE corresponding to EntC1 and
4 EntC2 purified peptides as indicated; right panel, bioassay of the same EntC1 and EntC2
5 samples using *E. faecalis* L1443 as the sensitive strain, showing the corresponding inhibition
6 zones; the arrow indicates the inhibition halo produced by the complementary action of both
7 peptides; on the left of the panels, molecular weight markers. B) Spot-on-lawn test showing the
8 inhibitory activity of EntC1 and EntC2 purified peptides and a mixture of them to demonstrate
9 their complementary action; *E. faecalis* L1443 was used as the sensitive strain; the bar
10 represents 10 mm.

11

12 **Legend of Figure 2.** MALDI-TOF mass spectra analysis corresponding to EntC1 and EntC2
13 peptides. a.i., arbitrary intensity. Estimated molecular weights are indicated on top of the
14 corresponding mass spectra peaks.

15

16 **Legend of Figure 3.** Amino acid sequence of EntC1 and EntC2 mature peptides deduced from
17 their coding DNA sequences and alignment with homologous two-peptide bacteriocins
18 Enterocin 1071 (Ent1071A + Ent1071B), Lactococcin G (LcnGα + LcnGβ) and Lactococcin Q
19 (LcnQα + LcnQβ). Identical amino acid residues are boxed. For LcnQ, only those amino acid
20 residues which are different from LcnG are indicated; among these, residues in italics are those
21 identical to EntC1. Amino acid residues which are different in EntC2 and 1071B are
22 underlined. Theoretical (t) and experimental (e) molecular weights are indicated.

23

24 **Legend of Figure 4.** Agarose gel electrophoresis of multiplex PCR analysis of virulence
25 determinants. Lanes: M, molecular weigh marker; the actual sizes are indicated on the left;

1 C901, result using total DNA from *E. faecalis* C901 as the template; P229, result using total
2 DNA from *E. faecalis* P229 as the template, used as the positive control in the PCR reactions.
3 Virulence genes corresponding to each specific PCR-amplified DNA band are indicated at the
4 right side.
5
6 **Legend of Figure 5.** Edmundson α-helical wheel representation of the amphiphilic regions in
7 enterocins EntC2 and Ent1071B, and lactococcins LcnGβ and LcnQβ. In all peptides the
8 amphiphilic region starts at the amino acid residue 8 and ends with the amino acid residue 25.
9 Polar and nonpolar amino acid residues are shown as white or shaded circles, respectively.
10 Residues which are different in enterocin EntC2 with respect to enterocin Ent1071B, and in
11 lactococcin LcnQβ to lactococcin LcnGβ are marked with boxes.
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**Table 1.** Inhibitory spectrum of cell-free supernatants from *Enterococcus faecalis* C901 against bacterial strains from human origin.

| **Bacterial species** | **Strain**[a] | **Sensitivity**[b] |
|---|---|---|
| *Actynomyces neuii* | FR1543 | ++ |
| | P1543 | + |
| *Bifidobacterium longum* | H1542 | - |
| *Enterococcus faecalis* | C301, L1443 | ++++ |
| | EV1444, FR1441, FR1542 H1441, HK223, LAM43 MA006, SC1541 | +++ |
| | C1002, EV1542, L1543 LA1442, LV123, M1441 M1541, P1441a, SC1442 | ++ |
| *Enterococcus faecium* | C656 | +++ |
| *Enterococcus gallinarum* | HU521 | - |
| *Enterococcus sacharolyticus* | HU522 | - |
| *Escherichia coli* | FR1545 | - |
| *Facklamia hominis* | EV1443 | ++ |
| *Lactobacillus fermentum* | Lc40 | - |
| *Lactobacillus gasseri* | EV1461, LA2441, Lc9, Lc23 | - |
| *Lactobacillus paracasei* | C1351, C1352 | ++++ |
| *Lactobacillus reuteri* | EV1763, LA1746 | - |
| *Lactobacillus rhamnosus* | FR1762 | - |
| *Lactobacillus salivarius* | HN6 | - |
| *Leuconostoc mesenteroides* | C1353 | ++++ |
| *Propionibacterium acnes* | P1544, SC1441, SC1544b | +++ |
| *Propionibacterium avium* | H1544b | - |
| *Propionibacterium granulosum* | C1441 | - |
| *Staphylococcus caprae* | FR1541a | + |
| *Staphylococcus epidermidis* | EV1541 | - |
| | C1541 EV1441 FR1444 | - |
| | FR1541b L1442 L1544 | - |
| | L1546 M1564 P1441b | - |
| | P1541 SC1444b | - |
| *Streptococcus anginosus* | EV1442 | +++ |
| | FR1442, L1441, LA1441 | - |
| *Streptococcus intermedius* | LA1443 | + |
| *Streptococcus parasanguinis* | L1541 | - |

[a] Abbreviations: C, colostrums; H, faeces; EV, vaginal exudates; FR, rectal frotis; M, meconium; L, breast milk; LA, amniotic liquid; P, skin; SC, umbilical cord blood.

[b] Sensitivity to cell-free supernatants of *E. faecalis* C901, assayed by the spot-on-lawn method. Sensitivity scale: + (<9mm), ++ (10-11 mm), +++ (11-12mm), and ++++ (12-14 mm), reflect the degree of sensitivity according to the diameter of the inhibition halo showed in brackets; -, resistant.

1 **Table 2.** Inhibitory spectrum of *Enterococcus faecalis* C901 against several bacteriocin producing and non-

2 producing strains.

3

| Bacterial species | Strain (bacteriocin)[a] | Source[b] | Sensitivity[c] | Reference |
|---|---|---|---|---|
| *Enterococcus faecium* | LP6T1a (L50A+L50B) | CIG | +++ | (11, 17) |
| | CTC492 (EntA+EntB) | CTC | + | (3, 9, 41) |
| | P13 (EntP) | FVM | +++ | (10) |
| *E. faecalis* | EFS2 (AS-48) | UG | +++ | (30, 37) |
| | OG1-X (AS-48) | UG | ++++ | (37) |
| | AE9, AE12, AE23 (Munt) | HRM | ++ | (7) |
| | PB1 (bac+) | HRM | +++ | |
| | EF1 | TNO | ++++ | |
| | CNRZ 135,136, 137 | INRA | +++ | |
| | CNRZ 34 (bac+) | INRA | - | |
| | BM4100WT | TNO | +++ | |
| | | | | |
| *Lactobacillus acidophilus* | NCDO 1748 | NCDO | - | |
| | ATCC 4356 | TNO | - | |
| *L. brevis* | LB9 | UV | - | |
| *L. casei* | NCDO 161 | NCDO | - | |
| *L. curvatus* | NCFB 2739 | NCDO | - | |
| *L. delbruecki* | ATCC 11842 | IPLA | - | |
| *L. fermentum* | ATCC1493 | ATCC | - | |
| | NCDO1750 | NCDO | - | |
| *L. hilgardii* | LB76 | UV | - | |
| *L. pentosus* | LPCO10 (plnS) | CIG | - | (28) |
| | 128/2 | CIG | - | |
| | CECT 4023 | CECT | - | |
| *L. plantarum* | NC8 (plNC8+ plnEF + plnJK) | MATFORSK | - | (13, 31, 32) |
| | LB6 | UV | - | |
| | CECT748 | CECT | - | |
| *L. reuteri* | DSM 20016 | TNO | - | |
| *L. salivarius* | NCFB 2747 | TNO | - | |
| *L. sakei* | NCFB 2714 | TNO | ++ | |
| *Lactococcus lactis* | | | | |
| *subsp. cremoris* | MG1363 | CIT | + | |
| | CNRZ117 | INRA | - | |
| *subsp. diacetylactis* | IPLA838 | IPLA | +++ | |
| *subsp. lactis* | IL1403 | INRA | ++++ | |
| | IPLA972 (lcn972) | IPLA | ++ | (36) |
| *Pediococcus parvulus* | P339 | UV | - | |
| *P. pentosaceous* | FBB63, P56 | TNO | - | |
| *Streptococcus thermophilus* | ST20 | TNO | - | |

45

46 [a]Bacteriocin produced is shown in brackets.

47 [b] Abbreviations: ATCC, American Type Culture Collection (Rockville, Md.); CECT, Colección Española de Cultivos Tipo (Universidad de Valencia,

48 Burjasot,Spain); CIG, Colección Instituto de la Grasa (Sevilla, Spain); CIT, Cranfield Institute of Technology (UK); CTC, Center of Technology of Meat

49 (IRTA; Girona, Spain); FVM, Facultad de Veterinaria, Universidad Complutense (Madrid, Spain); HRM, Hospital Ramón y Cajal (Madrid, Spain); INRA,

50 Institut National de la Recherche Agronomique (Jouy-en-Josas, France); IPLA, Intituto de Productos Lácteos de Asturias (Oviedo, Spain); Matforsk

51 (Ås, Norway); NCDO, National Collection of Dairy Organisms (Reading, UK); TNO, Nutrition and Food Research (Zeist, The Netherlands);UV,

52 Universidad de Valencia (Burjasot, Spain); UG, Universidad de Granada (Granada, Spain).

53 [c] Sensitivity to cell free supernatant of E. faecalis C901, assayed by the spot-on-lawn method. Sensitivity scale: + (<9mm), ++ (10-11 mm), +++

54 (11-12mm), and ++++ (12-14 mm), reflect the degree of sensitivity according to the diameter of the inhibition halo showed in brackets; -,

55 resistant.

56

**Table 3.** Minimal inhibition concentrations (MICs) of different antibiotics for *E. faecalis* C901.

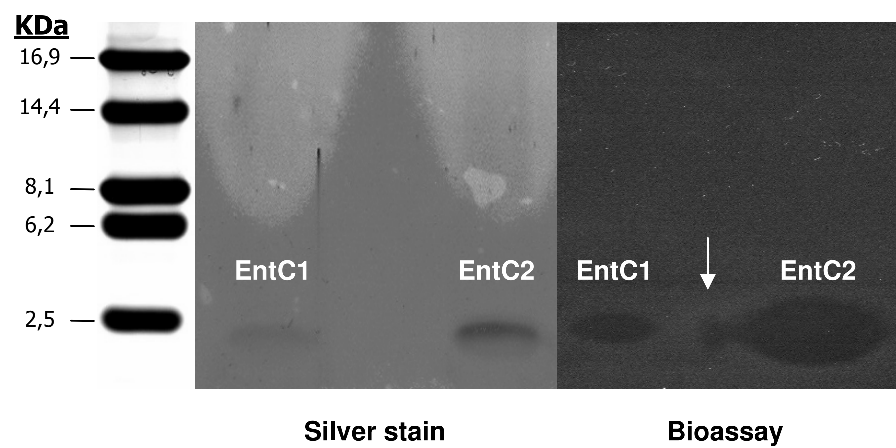
| **Antibiotic**[a] | **MICs (μg $ml^{-1}$)** | **S/R**[b] |
|---|---|---|
| AUG | 1/0.5 | 4/8 |
| AMP | 2 | ≤8/≥16 |
| CHL | 16 | ≤8/≥32 |
| CIP | 2 | ≤1/≥4 |
| CLI | >2 | |
| ERY | 2 | ≤0.5/≥8 |
| FOS | 32 | ≤64/≥256 |
| GEN | 128 | ≤500/>500 |
| IMI | 2 | |
| MUP | 256 | |
| NIT | 64 | |
| LNZ | 2 | ≤2/≥8 |
| OXA | >2 | |
| PEN | 4 | |
| Q/D | >4 | ≤1/≥4 |
| RIF | ≤1 | ≤1/≥4 |
| STR | ≤1000 | ≤1000/≥1000 |
| TEI | ≤0,5 | ≤8/≥32 |
| SXT | ≤1/38 | |
| TET | >8 | ≤4/≥16 |
| VAN | 4 | ≤4/≥32 |

[a] Antibiotic abbreviations are described in Materials and Methods

[b] NCCLS, 2002, (38) was used to determine sensitivity (S) or resistance (R)

Figure 1

A




B

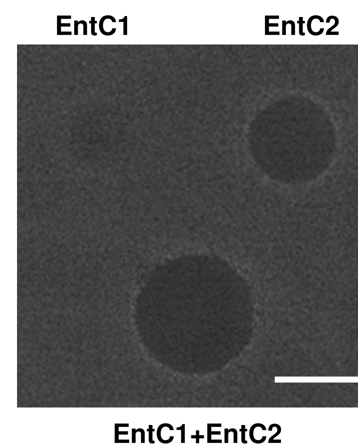
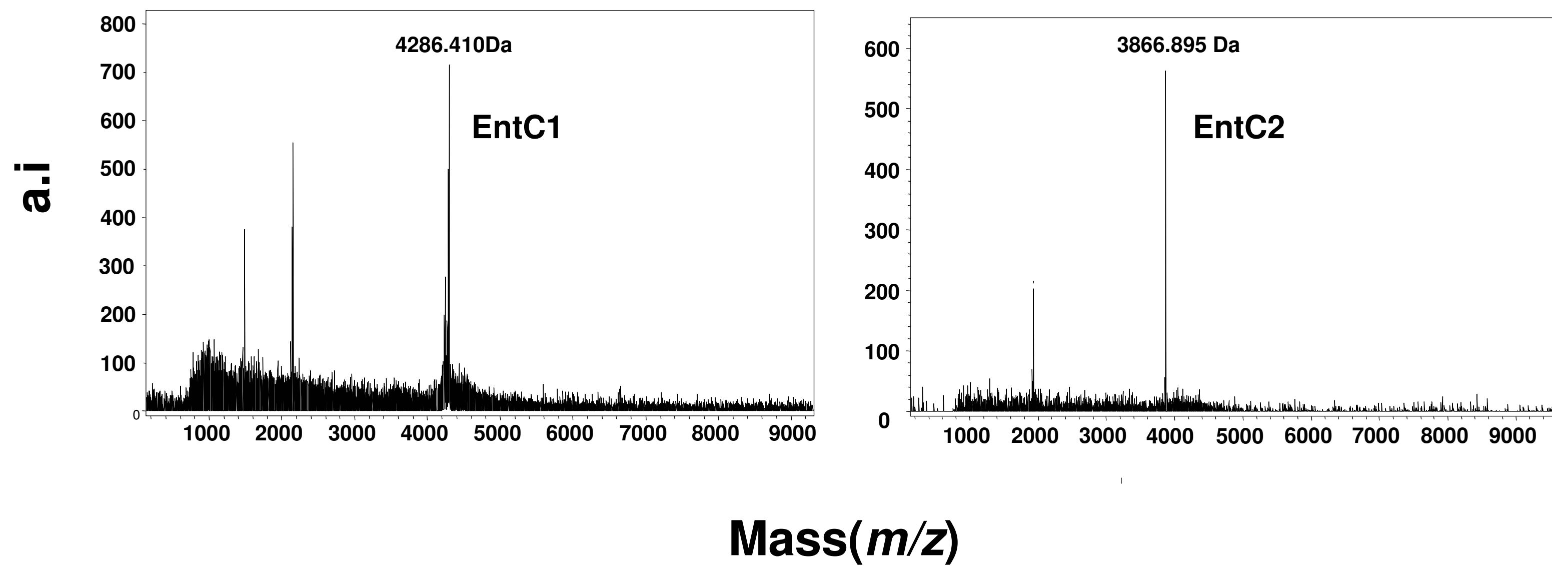


Figure 2



## Figure 3

| Peptide | Amino acid sequence and alignment | MW(t) | MW(e) | Reference |
|---|---|---|---|---|
| EntC1 (1071A) | ESVFSKIGNAVGPAAYWILKGLGNMSDVNQADRINRKKH | 4,286 | 4,284 | This study; (4, 5, 19) |
| LcnGα | GTWDDIGQGIGRVAYWVGKAMGNMSDVNQASRINRKKKH | 4,376 | 4,346 | (42) |
| LcnQα | *S*I G *V* K*A* | 4,260 | 4,260 | (55) |
| EntC2 | GPGKWLPWLQPAYDFVAGLAKGIGKEGNKNKWKNV | 3,869 | 3,867 | This study |
| 1071B | GPGKWLPWLQPAYDFVTGLAKGIGKEGNKNKWKNV | 3,899 | 3,898 | (4, 5, 19) |
| LcnGβ | KKWGWLAWVDPAYEFIKGFGKGAIKEGNKDKWKNI | 4,109 | 4,110 | (42) |
| LcnQβ | E G L | 4,018 | 4,018 | (55) |

Figure 4

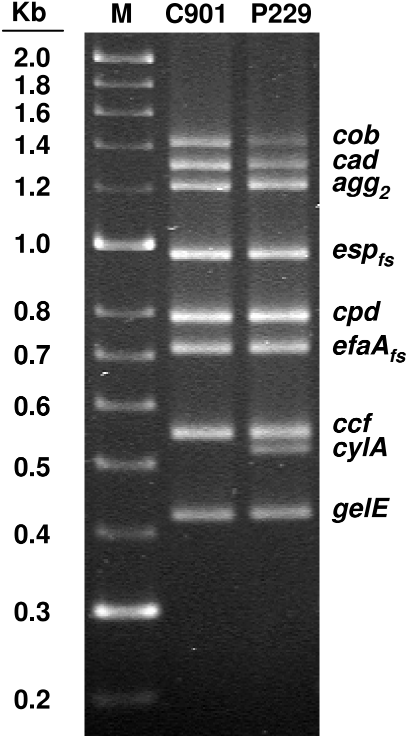


Figure 5