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# Antimicrobial and safety aspects, and biotechnological potential of bacteriocinogenic enterococci isolated from mallard ducks (*Anas platyrhynchos*)

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**Abstract**

Samples from the intestinal content and carcasses of mallard ducks (*Anas platyrhynchos*) were evaluated for enterococci with antimicrobial activity, presence of genes coding bacteriocins and their expression, and potential virulence factors. *Enterococcus faecalis* comprised the largest enterococcal species with antagonistic activity followed by *E. faecium*, *E. hirae*, *Enterococcus* spp., and the non-enterococci. Although all *E. faecalis* isolates manifested a potent direct antimicrobial activity, no activity was detected in supernatants of most producer cultures. However, all *E. faecium* isolates showed antimicrobial activity in their supernatants and encoded bacteriocins, although the occurrence in the isolates of several enterocin genes did not always correlate with a higher antagonistic activity in supernatants. The *efaAfm* determinant was the only virulence gene detected in *E. faecium*, while *E. faecalis* showed a larger number of virulence determinants, and *E. hirae* did not carry any of the virulence genes examined. The rapid identification of genes coding described bacteriocins permits recognition of isolates that are potentially producers of novel bacteriocins. Purification of the antimicrobial activity of *E. hirae* DCH5 and *Lactococcus garvieae* DCC43 revealed unique chromatographic fragments after MALDI-TOF mass spectrometry analysis, suggesting the antagonistic peptides were purified to homogeneity. Bacteriocinogenic *E. faecium* and *E. hirae* isolates may be considered hygienic for production of bacteriocins, and potentially safe due to their low incidence of potential virulence genes and susceptibility to most clinically relevant antibiotics. However, the presence among the enterococci of *E. faecalis* strains with a potent antagonistic activity and multiple virulence factors, raises concerns regarding their potential pathogenicity to consumers.


*Keywords:* *Enterococcus*; Enterococci; Enterocins; Virulence factors; Mallard ducks (*Anas platyrhynchos*)

## 1. Introduction

The enterococci are lactic acid bacteria (LAB) that are important in environmental, food, and clinical microbiology. These bacteria play a beneficial role in the development of the sensory characteristics of fermented foods and have been successfully used as starter and adjunct cultures, and as probiotics. Detrimental aspects may, among others, include their consideration as indicators of faecal contamination, the spoilage of foods and the production of toxic amines (Franz et al., 2003; Foulquié-Moreno et al., 2005). Many enterococci also produce a diverse and heterogeneous group of ribosomally synthesized antimicrobial peptides or bacteriocins, generically referred to as enterocins (Cintas et al., 2001; Foulquié-Moreno et al., 2005). Most bacteriocins from LAB are synthesized as inactive prepeptides containing an N-terminal extension. The mature peptides are often cationic, amphiphilic, membrane-permeabilizing molecules, divided into classes (Cotter et al., 2005; Fimland et al., 2005). Bacteriocins may inhibit pathogenic bacteria with a beneficial impact as protective cultures (Cotter et al., 2005).

However, the enterococci are now becoming recognized as important causes of nosocomial and to a lesser extent community-acquired infections. Typical enterococcal infections occur in hospitalized patients with underlying conditions representing a wide spectrum of severity of ilness and immune modulation. Moreover, due to the higher incidence of infections by enterococci in young, older and immunocompromised patients, and to their extended resistance to antibiotics, they are being considered as emerging pathogens (Franz et al., 2001; Pillar and Gilmore,

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2004). The majority of infections are caused by either *E. faecalis* or *E. faecium* (Kayser, 2003; Nallapareddy et al., 2005). However, strains of *E. gallinarum* (Dargere et al., 2002), *E. hirae* (Poyart et al., 2002), and *E. mundtii* (Higashide et al., 2005) have been also implicated as responsible of endophtalmitis and native valve endocarditis in humans. Motile enterococci, such as *E. casseliflavus* are rare causes of enterococcal bacteremia (Pappas et al., 2004). An outbreak of *E. faecium*-related sepsis has been also documented to spread from pigs to humans (Lu et al., 2002).

Clinical isolates of enterococci involved in human infections and antibiotic resistance are also producers of bacteriocins or bacteriocin-like molecules (Shankar et al., 2002; Nallapareddy et al., 2005) and, in addition, many enterococcal isolates of different origin carry potential virulence factors (Eaton and Gasson, 2001; Franz et al., 2001; Semedo et al., 2003a; Martín et al., 2006). Mobile genetic elements coding virulence determinants can be transferred to food associated enterococci (Cocconcelli et al., 2003), and participate in molecular communication between bacteria of the animal and human microflora (Saavedra et al., 2003), giving the enterococci a new dimension regarding their potential pathogenicity for immunocompromised persons.

Therefore, the presence of enterococci in foods may have a beneficial effect for their role as antimicrobial agents but also a possible concern as microorganisms involved in nosocomial and opportunistic infections (Foulquié-Moreno et al., 2005; Martín et al., 2005; Yousif et al., 2005; Martín et al., 2006). The safety of foods containing enterococci is an issue that the food industry must carefully address (Franz et al., 2003; Foulquié-Moreno et al., 2005). Thus, of great interest would be to evaluate the hygienic (antimicrobial activity) and safety aspects (virulence factors) related to the presence of enterococci in the intestine and carcasses of wild animal species, profitable for human consumption and available from hunting (Martín et al., 2006). The mallard duck (*A. platyrhynchos*) is a common and widespread dabbing duck which breeds throughout the temperate and sub-tropical areas of North America, Europe and Asia. In Spain more than five million small game birds, including mallards, are shot down per year. In this study, enterococci isolated from mallard ducks have been evaluated for their antimicrobial activity, presence of genes encoding bacteriocins and their expression, and potential virulence factors.

## 2. Materials and methods

### 2.1. Microbiological analysis, indicator strains and bacteriocinogenic assays

Samples from the intestinal content and carcasses of mallard ducks (*A. platyrhynchos*) were evaluated for the microbiological selection of enterococci after cultivation on (i) Slanetz and Bartley medium (Oxoid Ltd., Basingstoke, UK) at 44 °C for 48 h, (ii) Kanamycin Aesculin Azide agar (KAA) medium with the kanamycin selective supplement (Oxoid) at 40 °C during 48 h, and (iii) Columbia agar base medium with the Staph/Strep selective supplement (Oxoid) in air supplemented with 5% $CO_2$ at 37 °C for 48 h. Indicator strains used for determination of the antimicrobial activity of selected isolates are shown in Table 1. The antimicrobial activity of isolates was screened by the stab-on-agar test, while the antimicrobial activity of cell-free culture supernatants was screened by an agar diffusion test (ADT) and, when stated, by a microtiter plate assay (MPA) (Cintas et al., 2000; Martín et al., 2006). Supernatants were subjected to proteolytic treatment with α-chymotrypsin, trypsin and proteinase K (Sigma Chemical Co., St. Louis, MO, USA), at 1 mg/ml for 37 °C during 2 h, to ascertain the protein nature of their antagonistic activity. When stated *Pediococcus damnosus* CECT 4797 was used as the indicator microorganism.

### 2.2. Enterococcal genus and species identification

The assignment of antagonistic isolates to the genus *Enterococcus* and identification of the *E. faecium*, *E. faecalis*, *E. gallinarum*, *E. flavescens,* and *E. casseliflavus* species was performed using genus-and species-specific PCR primers (Martín et al., 2006). *E. faecalis* V853, *E. faecium* BM4147, *E. gallinarum* BM4174, *E. casseliflavus* CECT 969$^T$, and *E. flavescens* CECT 970$^T$ were used as the positive control strains. Oligonucleotide primers were obtained from Sigma-Genosys Ltd. (Cambridge, UK). The identity of the *Enterococcus* sp. DCH5 isolate and the non-enterococcal DCC43 strain was evaluated by comparing their whole-cell protein profile, determined by analyzing their cell extracts by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), with protein profiles of previously described bacteria by Marc Vancanneyt, University of Ghent (BCCM/LMG Culture Collection, Ghent, Belgium).

### 2.3. PCR-detection of enterocin structural genes and potential virulence factors

PCR amplification of known structural genes of enterocin A (*entA*), enterocin B (*entB*), enterocin P (*entP*), enterocin L50 (*entL50A-entL50B*), enterocin Q (*entqA*), mundticin KS (*munKS*), enterocin AS-48 (*as-48*), bacteriocin 31 (*bac31*), enterocin 1071A and 1071B (*ent1071A-ent1071B*), enterolysin A (*enlA*), and the cytolysin (hemolysin-bacteriocin) precursor ($cylL_L$-$cylL_S$), was performed with specific bacteriocin PCR primers (Martín et al., 2006). Some of the indicator strains listed in Table 1 have been also used as positive controls for amplification of *entA*, *entB*, *entP*, *entL50A-entL50B*, *entqA*, and *as-48*. *E. faecalis* F2 was used as a positive control for detection of $cylL_L$-$cylL_S$. No positive controls were available for detection of genes *bac31*, *munKS, ent1071A-ent1071B,* and *enlA*. Primers EntA-F and EntA-R have been also used for amplification of a 380-bp fragment of genes *entA-entiA* of *E. faecium* T136, while primers EntP-F and EntP-R have been used for amplification of a 423-bp fragment of genes *entP-orf2* of *E. faecium* P13 (Martín et al., 2006). Primers GL50B-F (5′-AAAACATATAGTCAGTC TCAATCACTG-3′) and EntL50-R2 (Martín et al., 2006) have been used for amplification of a 1860-bp fragment of genes *orfG-entL50B* of *E. faecium* L50, while primers EL50B-F (5′-ATAACGCCCCCTTATGCTTT-3′) and EntL50-R2 (Martín et al., 2006) amplify a 1053-bp fragment of genes *orfE-entL50B*. Primers EntL50-R1 (Martín et al., 2006) and L50AD-R (5′-GAAAGGCCTACGGCTCAAG-3′) amplify a 1502-bp fragment of genes *entL50A-orfC*, and primers EntL50-R1 and

L50AC-R (5′-CTAACGGCTTCAGCATTTGA-3′) amplify a 1832-bp fragment of genes *entL50A-orfD*. The primer pairs L50J40-F (5′-AAAAATGATTGGAGGAGTTATATTATG-3′) and L50J40-R (5′-CGAATGTTTACACAACAAAAAAC-3′), and the primer pairs L50J45-F and L50J45-R have been used, respectively, for amplification of a 400-bp and 450-bp fragments covering partly the upstream and downstream nucleotide sequence of genes *entL50A-entL50B* of *E. faecium* L50 (Cintas et al., 1998). Primers used for amplification of genes *agg* (aggregation substance), *gelE* (coding for gelatinase), *cylL$_L$-cylL$_S$* (cytolysin precursor), *cylM* (posttranslational modification of cytolysin), *cylB* (transport of cytolysin), *cylA* (activation of cytolysin), *esp* (enterococcal surface protein), *efaAfm* and *efaAfs* (cell wall adhesins of *E. faecium* and *E. faecalis*, respectively), and *cpd*, *cob*, and *ccf* (sex pheromones) were those described by Eaton and Gasson (2001) and Martín et al., (2006). The primer pairs RHCT1 and RHCT2 (Hickey et al., 2003) have been also used for amplification of the *cylL$_L$L$_S$M* cytolysin fragment. *E. faecalis* P4 has been used as a positive control for amplification of gene *agg*, *E. faecalis* OG1s for gene *gelE*, *E. faecalis* P36 for gene *esp*, *E. faecalis* EBH1 for gene *efaAfs*, *E. faecium* F10 for gene *efaAfm*, and *E. faecalis* F2 for genes *cpd*, *cob* and *ccf*.

### 2.4. PCR analysis, DNA sequencing and other DNA manipulations

PCR-amplifications were performed from total bacterial DNA obtained using the Wizard® DNA Purification Kit (Promega, Madrid, Spain) in 50 µl reaction mixtures with 1–3 µl of purified DNA, 70 pmol of each primer and 1 U of Platinum® *Taq* DNA polymerase (Invitrogen, Madrid, Spain). Samples were subjected to an initial cycle of denaturation (97 °C for 2 min), followed by 35 cycles of denaturation (94 °C for 45 s), annealing (41 to 67 °C for 30 s) and elongation (72 °C for 30 s to 2 min), ending with a final extension step at 72 °C for 7 min in a DNA thermal cycler Techgene (Techne, Cambridge, UK). PCR resulting fragments were analyzed by electrophoresis in 2% agarose (Pronadisa, Madrid, Spain) gels, with the Gel Doc 1000 documentation system (Bio-Rad, Madrid, Spain). When required, PCR-generated fragments were purified by a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) or by agarose gel electrophoresis followed by extraction with a QIAquick Gel Extraction Kit (QIAGEN). Determination of the nucleotide sequence of the PCR-purified fragments was performed using the ABI PRISM® BigDye™ Terminator cycle sequencing kit and the automatic DNA sequencer ABI PRISM, model 377 (Applied Biosystems, Foster City, CA, USA) at the DNA sequencing service Sistemas Genómicos (Valencia, Spain).

### 2.5. Production of gelatinase and hemolysin, and antibiotic susceptibility testing

Production of gelatinase and investigation of hemolysis were performed as described (Martín et al., 2006). Antimicrobial susceptibility patterns were determined by soft agar overlay

Table 1
Antimicrobial activity of representative isolates and classification into different inhibitory groups[a]

| Isolate | Group of inhibition | Indicator microorganisms[b] | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *L. monocytogenes* | | | | *Lb. sakei* 2714 | *E. faecium* | | | *L. lactis* BB24 | *E. faecalis* | | | *P. acidilactici* 347 | *P. pentosaceus* FBB61 |
| | | 935 | 5105 | 4032 | ScottA | | L50 | P13 | T136 | | INIA4 | P4 | F2 | | |
| DCH2 | I | 9.3 | 12.8 | 6.0 | 9.0 | 9.8 | 7.7 | 8.2 | – | – | – | 7.5 | 5.3 | – | 5.5 |
| DEC7 | II | 14.6 | 13.9 | 14.1 | 12.4 | 12.9 | 11.1 | 11.7 | 6.8 | 11.0 | 9.2 | 9.9 | 7.7 | 4.6 | 7.8 |
| DCH3 | III | 5.2 | 12.7 | 5.8 | 3.3 | 8.5 | – | 10.3 | 9.7 | – | 10.4 | 6.9 | 5.1 | 9.9 | 9.7 |
| DAC9 | IV | – | 6.1 | 7.8 | 6.5 | 12.5 | 5.4 | 7.8 | 7.6 | 6.0 | 6.5 | – | 4.3 | 7.6 | 8.0 |
| DAC2 | V | 4.6 | 10.7 | 13.9 | 9.3 | 9.6 | 9.7 | 9.9 | 8.2 | – | 6.5 | 5.6 | 6.6 | – | 7.8 |
| DAC7 | VI | – | 7.8 | 10.2 | – | 10.1 | 6.8 | 7.8 | 9.9 | – | 7.0 | 6.3 | 5.1 | – | 3.4 |
| DAC10 | VII | – | – | – | – | 7.2 | – | 5.5 | – | – | – | 6.5 | 5.6 | 5.7 | – |
| DAC37 | VIII | – | – | 6.5 | – | – | – | – | – | – | – | – | – | – | – |
| DCC17 | IX | – | 5.7 | – | – | 7.1 | 7.4 | 6.9 | 6.7 | 5.1 | 9.8 | – | – | 8.0 | 11.6 |
| DBH9 | X | – | – | – | – | 11.0 | 6.3 | 7.5 | 7.4 | 6.4 | 6.8 | – | – | 6.8 | 9.1 |
| DBC4 | XI | – | 7.6 | – | 7.9 | 9.6 | 5.9 | 6.2 | 7.7 | 4.4 | – | – | – | 5.9 | 9.5 |
| DBC9 | XII | – | 7.1 | – | 6.2 | 9.9 | 6.1 | 6.8 | 6.6 | 5.7 | 4.9 | – | – | 6.4 | 8.2 |
| DBC16 | XIII | – | – | – | – | 8.7 | – | 5.2 | – | – | – | – | – | 5.4 | – |
| DCH5 | XIV | – | 10.0 | – | – | 5.1 | 5.8 | 7.6 | 9.1 | – | 6.3 | 5.8 | 6.4 | – | – |
| DCH37 | XV | 4.2 | 6.7 | 5.9 | 6.4 | – | – | 6.2 | 10.2 | – | 10.1 | 5.2 | 4.8 | 8.7 | 8.6 |
| DCC14 | XVI | – | 7.1 | – | – | 8.9 | 8.5 | 8.4 | 8.7 | – | 6.0 | – | – | – | – |
| DCC32 | XVII | – | – | – | – | 7.9 | 6.0 | 7.9 | 6.7 | 5.0 | – | – | – | 5.6 | 10.6 |
| DCC43 | XVIII | – | 5.7 | – | 5.4 | 4.0 | – | 5.6 | – | 6.9 | – | – | – | 4.6 | – |
| DDC28 | XIX | – | 12.8 | 8.8 | 12.4 | 11.4 | 11.1 | 12.0 | – | 8.7 | 9.4 | 10.6 | 8.6 | – | 7.9 |
| DEC23 | XX | 6.1 | 5.4 | 6.0 | 6.6 | 6.7 | 5.0 | – | 5.4 | – | 5.2 | – | – | 6.4 | 6.6 |

[a] Antimicrobial activity as determined by the stab-on-agar test. Results as the diameter of the halos of inhibition in millimeters (mm). (−) antimicrobial activity not detectable.

[b] *L. monocytogenes* 935, 5105 and 4032 were obtained from the CECT (Colección Española de Cultivos Tipo, Valencia, Spain), and *L. monocytogenes* ScottA was obtained from the DNBTA (Facultad de Veterinaria, Universidad Complutense, Madrid, Spain). *Lb. sakei* 2714 was obtained from the NCFB (National Collection of Food Bacteria, Reading, UK). *E. faecalis* INIA4 was obtained from the INIA (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain). *E. faecalis* P4 and F2 were obtained from the IFR (Institute of Food Research, Norwich, UK) and *P. pentosaceus* from the TNO (Nutrition and Food Research, Zeist, The Netherlands). *Lactococcus lactis* BB24 (producer of nisin A), *E. faecium* T136 (producer of EntA and EntB), *E. faecium* P13 (producer of EntP), *E. faecium* L50 (producer of EntP, EntL50A, EntL50B, and EntQ), and *P. acidilactici* 347 (producer of PedA-1), were from our own LAB collection.

disc diffusion on Diagnostic Sensitivity Test (Oxoid) agar plates. Antibiotic-containing disks (Oxoid) were those of ampicillin (10 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), clindamycin (2 μg), erythromycin (15 μg), fusidic acid (10 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), norfloxacin (10 μg), penicillin G (10 IU), rifampicin (5 μg), streptomycin (10 μg), tetracycline (30 μg), and vancomycin (30 μg). Inhibition zone diameters were measured after overnight incubation of the plates at 37 °C. Resistance phenotypes were recorded as recommended (NCCLS, 2002).

### 2.6. NCI-ELISAs, purification of enterocins and mass spectrometry analysis

Polyclonal antibodies with predetermined specifity for enterocin P (Gutiérrez et al., 2004), enterocin L50 (EntL50, EntL50A and EntL50B) and enterocin Q (Criado et al., 2006), and enterocin A (Martínez et al., 2000) were used. The presence of bacteriocins in the supernatants of isolates of interest was detected and quantified using a non-competitive indirect enzyme-linked immunosorbent assay (NCI-ELISA), as described (Martín et al., 2006). The antimicrobial activity of selected isolates was also purified as described (Cintas et al., 2000; Gutiérrez et al., 2004; Martín et al., 2006). Briefly, supernatants from early stationary phase 1-liter cultures grown in MRS broth at 32 °C were subjected to precipitation with ammonium sulfate, applied to gel filtration PD-10 columns and further subjected to cation-exchange (SP Sepharose Fast Flow) and hydrophobic-interaction (Octyl Sepharose CL-4B) chromatographies, followed by reverse-phase chromatography in a $C_2$ to $C_{18}$ column (PepRPC HR 5/5) integrated in a FPLC system (RP-FPLC). Fractions with high bacteriocin activity were mixed and rechromatographed on the reverse-phase column to obtain chromatographically pure bacteriocin. During purification, bacteriocin activity was calculated by an MPA. Purified fractions from the last RP-FPLC step were subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Gutiérrez et al., 2006; Martín et al., 2006). All chromatographic columns and supporting gels were from Amersham Biosciences Europe GmbH (Cerdanyola, Spain).

## 3. Results

### 3.1. Identification of isolates with antimicrobial activity

Samples from the intestinal content and carcasses of mallard ducks were evaluated for antagonistic isolates with direct antimicrobial activity against selected indicator bacteria, and their antimicrobial spectra permitted their classification in 20 inhibition spectrum groups (I to XX) (Table 1). From the antagonistic isolates, 31 (2 isolates of group I, 6 isolates of group

Table 2
PCR-amplification of bacteriocin structural genes from isolates of the intestinal content and carcasses of mallard ducks *(Anas platyrhynchos)*

| Isolate | Group of inhibition | Supernatant activity | Species [a] | *entP* | *entL50A-entL50B* | *entqA* | *entA* | *entB* | *enlA* |
|---|---|---|---|---|---|---|---|---|---|
| DCH2 | I | + | *Fcium* | + | + | − | + | + | − |
| DCH39 | I | + | *Fcium* | − | − | − | + | + | − |
| DEC7 | II | + | *Fcium* | − | − | − | + | + | − |
| DEC8 | II | + | *Fcium* | − | − | − | + | + | − |
| DBH18 | II | + | *Fcalis* | − | + | − | − | − | − |
| DBH20 | II | + | *Fcalis* | − | + | − | − | − | − |
| DBC5 | II | − | *Fcalis* | − | − | − | − | − | − |
| DCH9 | II | − | *Fcalis* | − | − | − | − | − | − |
| DCH3 | III | + | *Fcium* | + | + | + | − | − | − |
| DCH1 | III | + | *Fcium* | + | + | + | − | − | − |
| DAC9 | IV | − | *Fcalis* | − | − | − | − | − | + |
| DAC8 | IV | − | *Fcalis* | − | − | − | − | − | + |
| DAC2 | V | + | *Fcium* | + | − | − | + | + | − |
| DAC7 | VI | + | *Fcium* | + | − | − | − | − | − |
| DAC10 | VII | − | *Fcalis* | − | − | − | − | − | − |
| DAC37 | VIII | + | *Enter* | − | − | − | − | − | − |
| DAC41 | IX | − | *Fcalis* | − | − | − | − | − | + |
| DCC17 | IX | − | *Fcalis* | − | − | − | − | − | − |
| DBH9 | X | − | *Fcalis* | − | − | − | − | − | + |
| DBC4 | XI | − | *Fcalis* | − | − | − | − | − | + |
| DBC9 | XII | − | *Fcalis* | − | − | − | − | − | + |
| DBC16 | XIII | − | *Fcalis* | − | − | − | − | − | − |
| DCH6 | XIV | + | *Hirae* | − | − | − | − | − | − |
| DCH5 | XIV | + | *Hirae* | − | − | − | − | − | − |
| DCH37 | XV | + | *Fcium* | + | + | + | − | − | − |
| DCH38 | XV | + | *Fcium* | + | + | − | − | − | − |
| DCC14 | XVI | + | *Fcium* | + | + | − | + | − | − |
| DCC32 | XVII | − | *Fcalis* | − | − | − | − | − | − |
| DCC43 | XVIII | + | *Garvieae* | − | − | − | − | − | − |
| DDC28 | XIX | + | *Fcium* | − | − | − | + | + | − |
| DEC23 | XX | − | *Fcalis* | − | − | − | − | − | + |

[a] *Fcium* is *E. faecium*; *Fcalis* is *E. faecalis*; *Enter* is *Enterococcus* sp., *Hirae* is *E. hirae*, and *Garvieae* is *L. garvieae.*

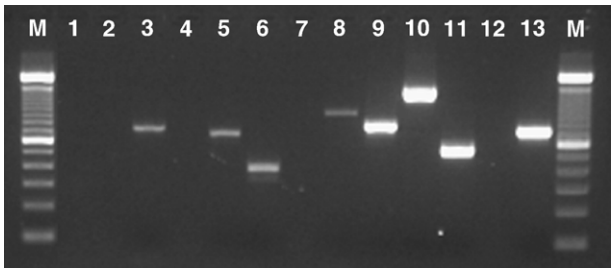


Fig. 1. PCR products from purified DNA from *E. faecalis* DBC4 amplified with primers for known virulence factors: lane 1, $cylL_L$-$cylL_S$ (324-bp); lane 2, $cylL_LL_SM$ (2659-bp); lane 3, *cylM* (742-bp); lane 4, *cylB* (843-bp); lane 5, *cylA* (701-bp); lane 6, *gelE* (419-bp); lane 7, *agg* (1553-bp); lane 8, *esp* (933-bp); lane 9, *cpd* (782-bp); lane 10, *cob* (1405-bp); lane 11, *ccf* (543-bp); lane 12, *EfaAfm* (735-bp); and lane 13, *EfaAfs* (705-bp).

II, 2 isolates of groups III, IV, IX, XIV, XV, and 1 isolate each from the rest of the antagonistic groups), were further identified. It was determined (Table 2) that all but one isolate (DCC43, group XVIII) amplified a 112-bp fragment for assignment to the genus *Enterococcus*. Those isolates that permitted further amplification of a 550-bp fragment were assigned to the species *E. faecium*, while those who amplified a 941-bp fragment were assigned to the species *E. faecalis*. However, enterococci from groups VIII and XIV could not be assigned to any of the enterococcal species evaluated. By SDS-PAGE comparaison of whole-cell protein profiles, the unrecognized *Enterococcus* sp. DCH5 and *Enterococcus* sp. DCH6 isolates were identified as *E. hirae*, while the unidentified DCC43 isolate was identified as *Lactococcus garvieae* (results not shown).

### 3.2. Detection of enterocin structural genes and potential virulence factors

Purified DNA of selected enterococci was subjected to PCR-amplification to determine the existance of structural genes coding described enterocins. All *E. faecium* isolates amplified either a 216-bp fragment of gene *entP*, a 172-bp fragment of *entA*, a 126-bp fragment of *entB*, a 286-bp fragment of enterocin L50 (*entL50A-entL50B*) or a 105-bp fragment of *entqA* (Table 2). However, only the *E. faecalis* isolates DBH18 and DBH20 showed detectable antimicrobial activity in their supernatants with amplification of the 286-bp fragment fragment coding enterocin L50. The rest of the *E. faecalis* isolates did not manifest antimicrobial activity in their supernatants, although 54% of them amplified a 1770-bp fragment of gene *enlA*. None of the *E. faecium* or *E. faecalis* isolates, nor the *Enterococcus* sp. DAC37, the *E. hirae* isolates DCH5 and DCH6, and the *L. garvieae* DCC43 showed amplification fragments with the primers used. None of the evaluated strains showed PCR-amplification fragments for genes $cylL_L$-$cylL_S$, *as-48*, *munKS*, and *ent1071A-ent1071B*.

The presence in the selected isolates of genes coding potential virulence factors was also evaluated (results not shown). All *E. faecium* isolates amplified a 735-bp fragment coding *efaAfm*, while all *E. faecalis* isolates amplified the 705-bp fragment of *efaAfs*, the 419-bp fragment of the *gelE* determinant, and the 782-bp and 543-bp fragments of the sex pheromone genes *cpd* and *ccf*. All but three *E. faecalis* isolates also amplified the 1405-bp fragment of the sex pheromone gene *cob*. Only *E. faecalis* DCH9 and *E. faecalis* DAC10 amplified the 1553 fragment of the *agg* gene, while *E. faecalis* DBC4 was the only isolate to amplify the 933-bp fragment of the gene *esp*, the 742-bp fragment of the gene *cylM* and the 701-bp fragment of the gene *cylA* (Fig. 1). All *E. faecalis* isolates were phenotypically hemolysin (Hly) and gelatinase (GelE) positive. However, the *E. hirae* isolates DCH5 and DCH6 and *L. garvieae* DCC43 did not show any amplification fragment for potential virulence factors with the primers used. Phenotypes recorded from antimicrobial susceptibility patterns indicated that all *E. faecium* and *E. faecalis* isolates were resistant to nalidixic acid and

Table 3
Production of bacteriocins and antimicrobial activity of *Enterococcus faecium* strains isolated from the intestinal content and carcasses of mallard ducks (*Anas platyrhynchos*)

| Isolate | Group of inhibition | Production of EntP (ng/ml) [a] | Production of EntL50A (ng/ml) [a] | Production of EntL50B (ng/ml) [a] | Production of EntQ (ng/ml) [a] | Production of EntA (ng/ml) [a] | Antimicrobial activity (BU/ml) [b] |
|---|---|---|---|---|---|---|---|
| DCH2 | I | 960 | 0 | 0 | – | 2020 | 12,918 |
| DCH39 | I | – | – | – | – | 2070 | 12,768 |
| DEC7 | I | – | – | – | – | 2297 | 22,183 |
| DEC8 | I | – | – | – | – | 1772 | 16,526 |
| DCH1 | III | 5366 | 23 | 28 | 705 | – | 2724 |
| DCH3 | III | 5793 | 37 | 47 | 903 | – | 3709 |
| DAC2 | V | 1642 | – | – | – | 2705 | 14,141 |
| DAC7 | VI | 1071 | – | – | – | – | 446 |
| DCH37 | XV | 4729 | 33 | 39 | 2120 | – | 3274 |
| DCH38 | XV | 3235 | 65 | 72 | – | – | 10,743 |
| DCC14 | XVI | 498 | 0 | 0 | – | 0 | 262 |
| DDC28 | XIX | – | – | – | – | 2233 | 20,783 |
| L50 | NA | 5488 | 55 | 58 | 1102 | – | 16,230 |
| P13 | NA | 7800 | – | – | – | – | 7770 |
| T136 | NA | – | – | – | – | 2450 | 12,428 |

(−) no presence of the structural gene.
NA: control strains, not ascribed to inhibition groups.
[a] Detection and quantification by a NCI−ELISA.
[b] Antimicrobial activity as determined by a microtiter plate assay (MPA), with *P. damnosus* CECT 4797 as indicator microorganism.

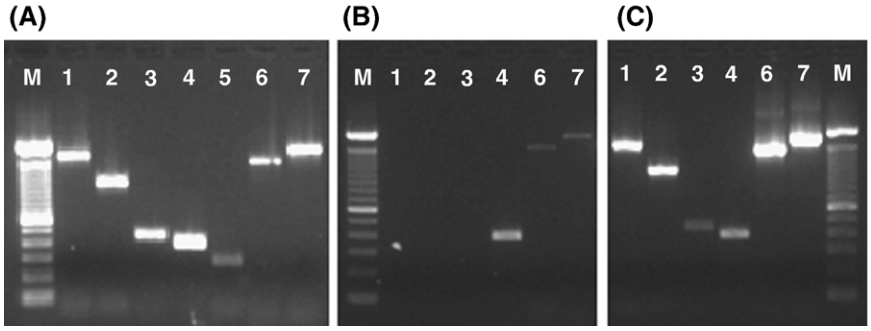


Fig. 2. PCR-amplification fragments of purified DNA from *E. faecium* L50 (A), *E. faecium* DCH2 (B), and *E. faecium* DCC14 (C), using the following primer pairs specific for the EntL50 operon and adjacent genes: lane 1, GL50B-F/EntL50-R2; lane 2, EL50B-F/EntL50-R2; lane 3, L50J45-F/L5045-F; lane 4, L50J40-F/L50J40-R; lane 5, EntL50-R1/EntL50-R2; lane 6, EntL50-R1/L50AD-R; and lane 7, EntL50-R1/L50AC-R. M, nucleic acid molecular size marker 100 bp DNA ladder.

streptomycin, while most of them were also resistant to kanamycin and clindamycin. Some *E. faecium* isolates were resistant to ciprofloxacin while many *E. faecalis* isolates were resistant to rifampicin and gentamicin. *E. faecium* DCH39 and *E. faecium* DEC28 showed resistance to erythromycin and tetracycline, and *E. faecium* DDC28 to tetracycline and chloramphenicol. All enterococci were sensitive to penicillin G, ampicilin, vancomycin, teicoplanin, chloramphenicol, fucsidic acid, norfloxacin, and nitrofurantoin.

### 3.3. Evaluation of *E. faecium* isolates for production of bacteriocins

Representative *E. faecium* isolates carrying structural genes for production of known bacteriocins were evaluated for production and functional expression of their bacteriocins in supernatants of producer cells. The NCI-ELISA data (Table 3), determined that *E. faecium* DCH2 of the group of inhibition I was a low producer of EntP, with no production of either EntL50A and/or EntL50B, suggesting that the structural *entL50A-entL50B* genes are silent for production of enterocin L50 (EntL50). Similarly, *E. faecium* DCC14 of the group of inhibition XVI and carrying the same structural genes as *E. faecium* DCH2 was a much lower producer of EntP than *E. faecium* DCH2 with no production of either EntL50A, EntL50B, and EntA, and with an antimicrobial activity that is only 1.7 to 2.2% of that of the control strains *E. faecium* L50 and *E. faecium* T136. Production of EntA by *E. faecium* DCH39 (group of inhibition I), *E. faecium* DEC7 and *E. faecium* DEC8 (group of inhibition II), and *E. faecium* DDC28 (group of inhibition XIX) was 72 to 94% of that of the control *E. faecium* T136, although the antimicrobial activity of the isolates DEC7, DEC8, and DDC28 was 25 to 45% higher than that of *E. faecium* T136.

The NCI-ELISA data also confirmed the production of EntP and EntA by *E. faecium* DAC2 (group of inhibition V), while *E. faecium* DAC7 (group of inhibition VI) produced 14% of the EntP and 6% of the antimicrobial activity of the control strain *E. faecium* P13. When production of EntP, EntL50, and EntQ was quantified in *E. faecium* DCH1 and *E. faecium* DCH3 (group of inhibition III), and *E. faecium* DCH37 (group of inhibition XV), the production of EntP by all isolates was similar to that of *E. faecium* L50 while the production of EntL50 was 44 to 67% for production of EntL50A and 81% for production of EntL50B with larger fluctuations for production of EntQ as compared to that of *E. faecium* L50, whereas the antimicrobial activity of the supernatants was of 17 to 22% of that of *E. faecium* L50. However, *E. faecium* DCH38 (group of inhibition XV) showed a lower production of EntP and a higher production of EntL50 than *E. faecium* L50. Similarly, *E. faecium* DCH38 was a non-EntQ producer confirming previous data on the absence of the structural gene *entqA* in this strain. Nevertheless, *E. faecium* DCH38 shows an antimicrobial activity that is 66% of that of *E. faecium* L50.

### 3.4. Nucleotide sequencing of genes coding bacteriocins

To determine if the different production of bacteriocins and/or differences in the antimicrobial activity of the distinct

Table 4
Purification of the antimicrobial activity of *Enterococcus hirae* DCH5

| Purification stage | Volume (ml) | Total $A_{254}$ [a] | Total activity ($10^3$ BU) [b] | Specific activity [c] | Increase in specific activity (fold) [d] | Yield (%) |
|---|---|---|---|---|---|---|
| Culture supernant | 1000 | 6200 | 5569 | 898 | 1 | 100 |
| Ammonium sulphate precipitation | 100 | 1420 | 9058 | 6379 | 7 | 162 |
| Gel filtration chromatography | 200 | 558 | 7550 | 13,530 | 15 | 135 |
| Cation-exchange chromatography | 50 | 7.05 | 2913 | 413,191 | 460 | 52 |
| Hydrophobic interaction chromatography | 10 | 0.73 | 2597 | 3,557,534 | 3961 | 46 |
| Reverse phase chromatography | 0.1 | 0.006 | 718 | 119,666,666 | 133,259 | 13 |

[a] Absorbance at 254 nm multiplied by the volume in mililiters.
[b] Antimicrobial activity in bacteriocin units per mililiter (BU/ml) multiplied by the total volume.
[c] Specific activity is the number of bacteriocin units divided by the total $A_{254}$.
[d] The specific activity of a fraction divided by the specific activity of the culture supernatant.

enterococcal isolates as compared to that of the reference strains, would be attributable to subtle small polymorphic genetic differences in or near the structural gene coding mature bacteriocins, an extended PCR-generated fragment and determination of their corresponding nucleotide sequence was performed from outside structural genes coding for EntP, EntA, and EntL50 (EntL50A and EntL50B). PCR amplification of purified DNA from *E. faecium* strains, producers of EntP, with primers EntP-F/EntP-R (results not shown), generated in all but one case, that of *E. faecium* DCH2, a 423-bp fragment, whose nucleotide sequence was identical to that of the same nucleotide fragment of the reference strain *E. faecium* P13. Similarly, PCR-amplification of purified DNA from *E. faecium* DCH2 and *E. faecium* DCC14 with primers L50J40-F/L50J40-R showed amplification of a 400-bp fragment corresponding to the *entL50A-entL50B* genes of *E. faecium* L50. However, when a PCR-nested was performed to determine the location of *entL50A-entL50B* respect to that of *E. faecium* L50, the results of

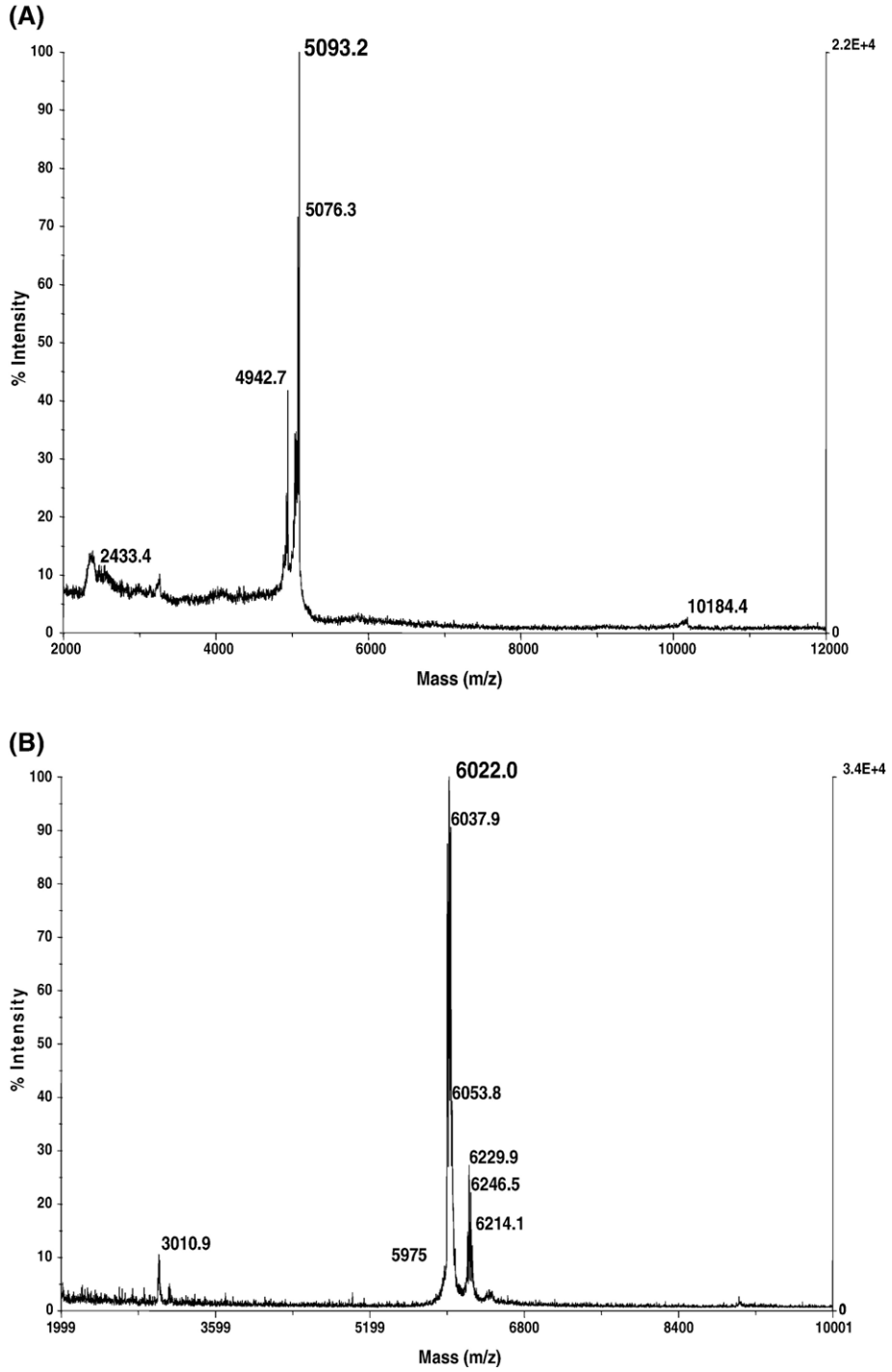


Fig. 3. Mass spectrometry analysis of the purified antimicrobial peptide of *E. hirae* DCH5 (A), and *L. garviae* DCC43 (B).

Fig. 2 suggest that *E. faecium* DCH2 do not show the nucleotide fragment corresponding to the three ORFs (*orfE, orfF, orfG*) located upstream the start codon of *entL50A*, while a weak signal suggest the possible existance downstream of *entL50B* of the two ORFs (*orfC, orfD*). For *E. faecium* DCC14, the *entL50A-entL50B* fragment seems to be allocated within the same genetic fragment as in *E. faecium* L50. The PCR-amplification of purified DNA from *E. faecium* strains, producers of *EntA*, with primers EntA-F/EntA-R (results not shown), generated in all cases a 380-bp fragment, whose nucleotide sequence was identical to that of the same nucleotide fragment of the reference strain *E. faecium* T136. PCR-amplification of purified DNA from *E. faecium* strains producers of EntP, EntL50 and EntQ, with primers EntP-F/EntP-R and L50J45-R/L5045J-R (results not shown), showed amplification of a 423-bp fragment of genes *entP-orf2* of the reference strain *E. faecium* P13, and a 450-bp fragment covering the *entL50A-entL50B* genes of *E. faecium* L50. In all cases, the nucleotide sequence of the PCR-generated 423-bp fragments and the 450-bp fragments was identical to that obtained from the *E. faecium* L50 reference strain.

### 3.5. Purification of the antimicrobial activity of E. hirae DCH5 and L. garvieae DCC43

The rapid genetic detection of enterocin structural genes has facilitated the recognition of enterococci as producers of bacteriocins previously described, or their consideration as producers of potentially novel bacteriocins. The narrow host range antimicrobial activity of the *Enterococcus* sp. DAC37 isolate (group VIII) precluded efforts towards purification of its antagonistic activity, while most of the *E. faecalis* isolates did not manifest a measurable antimicrobial activity in supernatants of cultures grown in MRS (de Man, Rogosa and Sharpe) broth. Purification of the antimicrobial activity *E. hirae* DCH5 (group XIV), permitted an 133,259-fold increase of its specific activity and a 13% recovery of the initial antimicrobial activity (Table 4). MALDI-TOF mass spectrometry analysis of the purified antagonistic activity of *E. hirae* DCH5 showed a major peak of 5,093.2 Da, suggesting the eluted peptide was purified to homogeneity (Fig. 3A). Purification of the antagonistic activity of *L. garvieae* DCC43 also permitted the recovery of a major peak of antimicrobial activity, with an 4,099,378-fold increase of its specific activity and a final recovery of a 209% of the initial antimicrobial activity (Table 5). MALDI-TOF mass spectrometry analysis of the purified activity showed a major peak of 6,022 Da (Fig. 3B), suggesting the eluted peptide was purified to homogeneity.

## 4. Discussion

A 14% (70 out of 500) of the isolates recovered from mallard ducks and selected after growth on selective media for enterococci, displayed antagonistic activity. However, other LAB such as *L. garvieae* DCC43 are isolated on media selective for enterococci, confirming their lack of sufficient selectivity (Domig et al., 2003). The *E. faecalis* isolates comprised the largest enterococcal species with antimicrobial activity (48%) followed by *E. faecium* (39%), *E. hirae* (6%), and other yet unidentified enterococcal isolates. *E. cecorum* is also a reported component of the mallards flora (Murphy et al., 2005), whereas *L. garvieae* clearly contributes to the microbial ecology of Italian fermented sausages (Rantsiou et al., 2005). A higher percentage of enterococci with antagonistic activity, and the predominance of *E. faecium* as the most active enterococcal species has been observed for enterococci isolated from wood pigeons (Martín et al., 2006).

Representative enterococci with antimicrobial activity were evaluated for the presence of genes coding bacteriocins. Well characterized enterocins produced by enterococci are the cytolysin (hemolysin-bacteriocin) precursor (Gilmore et al., 1994), enterocin AS-48 (Martínez-Bueno et al., 1994), bacteriocin 31 (Tomita et al., 1996), enterocin A (Aymerich et al., 1996), enterocin B (Casaus et al., 1997), enterocin P (Cintas et al., 1997), enterocin L50 (EntL50A and EntL50B) (Cintas et al., 1998) and enterocin Q (Cintas et al., 2000), enterocin 1071A and 1071B (Balla et al., 2000), mundticin KS (Kawamoto et al., 2002), and enterolysin A (Hickey et al., 2003; Nilsen et al., 2003). Three more enterocins characterized are enterocin SE-K4 (Eguchi et al., 2001), enterocin RJ-11 (Yamamoto et al., 2003) and enterocin EJ97 (Sánchez-Hidalgo et al., 2003). In this work, PCR-amplification of genes coding described bacteriocins has shown that amongst the antagonistic *E. faecium* isolated from mallards, 1 (8%, group VI) hold the gene *entP*, 4 (33%, groups I, II, and XIX) carry the genes *entA* and *entB*, 1 (8%, group V) hold the genes *entP*, *entA*, and *entB*, 3 (25%, groups III and XIV) carry the genes *entP*, *entL50A-entL50B*, and *entqA*, 1 (8%, group XVI) hold the genes *entP*, *entL50A-entL50B*, and *entA*, and 1 (8%, group I) hold the genes *entP*, *entL50A-entL50B*, *entA*, and *entB*. Similarly, amongst the antagonistic *E. faecalis* isolates, 2 (13%, group II) hold the gene *entL50A-entL50B*, 7 (46%, groups IV, IX, X, XI, XII, and

Table 5
Purification of the antimicrobial activity of *Lactococcus garvieae* DCC43 [a]

| Purification stage | Volume (ml) | Total $A_{254}$ | Total activity ($10^3$ BU) | Specific activity | Increase in specific activity (fold) | Yield (%) |
|---|---|---|---|---|---|---|
| Culture supernant | 1000 | 26,600 | 63 | 2.3 | 1 | 100 |
| Ammonium sulphate precipitate | 100 | 2550 | 797 | 312 | 135 | 1265 |
| Gel filtration chromatography | 200 | 1760 | 496 | 281 | 122 | 787 |
| Cation-exchange chromatography | 50 | 1 | 723 | 723,000 | 314,347 | 1147 |
| Hydrophobic interaction chromatography | 10 | 0.1 | 489 | 4,893,000 | 2,127,391 | 776 |
| Reverse phase chromatography | 0.9 | 0.014 | 132 | 9,428,570 | 4,099,378 | 209 |

[a] Abbreviations as in Table 4.

XX) carry the gene *enlA*, and 6 (40% groups II, VII, IX, XIII, and XVII) did not produce PCR-amplification fragments for any of the bacteriocin primers evaluated (Table 2). No isolates carrying genes *cylL*$_L$-*cylL*$_S$, *entAS-48*, *ent1071A-ent1071B*, *munKS*, and *bac31*, were detected. Thus, there was no correlation between the presence of known enterocin genes and the antagonistic spectrum of the isolates, although such a correlation has been found in other enterococci (Martín et al., 2006). Nevertheless, the presence of *entA*, *entB*, *entP*, *entL50A-entL50B*, and *entqA* genes, either alone or in combination with others, seems to be common in enterococci from human, animal, clinical, food, agricultural, and environmental sources (Herranz et al., 1999; Cintas et al., 2000; De Vuyst et al., 2003; Foulquié-Moreno et al., 2003; Ben Omar et al., 2004; Martín et al., 2006).

The antagonistic enterococci were further evaluated for potential virulent genes. In this study, none of the *E. faecium* strains harboured potential virulence factors, except the cell wall adhesin *efaAfm*, which agrees with others results (Eaton and Gasson, 2001; Martín et al., 2006). However, it should be noted that the lack of sex pheromones in *E. faecium* may reflect sequence divergence rather than absence of gene transfer pheromones, since sex pheromone crosstalk between *E. faecium* and *E. faecalis* has been reported (Eaton and Gasson, 2001). However, all *E. faecalis* isolates evaluated in this work carried the *efaAfs*, *cpd*, and *ccf* genes, and most of them also the *cob* gene. Sex pheromone production in enterococci is not a desirable trait, since it may promote adquisition of antibiotic resistance and other linked traits from other enterococci and lead to increased virulence (Eaton and Gasson, 2001). The absence of a complete *cylMBA* fragment in the *E. faecalis* isolates evaluated may also reflect the absence of a true β-hemolysis or that the observed hemolysis is due to an still unrevealed molecule or mechanism. Others have reported the incidence of *cylMBA* in non-hemolytic *E. faecalis* strains (Eaton and Gasson, 2001), and a higher incidence of hemolytic enterococcal strains with the *cyl*L$_L$-*cylL*$_S$ gene (De Vuyst et al., 2003; Semedo et al., 2003a). Insertion/deletion events leading to truncated or absent *cyl* genes, and possible sequence divergences in genes coding for the structural subunits of the cytolysin, may also explain the difficulties to tackle genes involved in production of active cytolysins (Semedo et al., 2003b; Pillar and Gilmore, 2004). Two *E. faecalis* isolates amplified a genomic fragment of the size of the *agg* gene, while only *E. faecalis* DBC4 carried *esp*. This result contrasts with others in which 33 to 36% of the *E. faecalis* isolates of food origin produced the cell-wall associated protein Esp (Eaton and Gasson, 2001; Franz et al., 2001). The absence of *esp* is, nevertheless, a positive attribute for *E. faecalis*. All *E. faecalis* strains harbour more virulence determinants than do the *E. faecium* strains, and the existence of multiple virulence factors in *E. faecalis* may contribute to the competitiveness of such potentially pathogenic enterococci in mallards. However, neither *Enterococcus* sp. DAC37, nor *E. hirae* DCH5 and *E. hirae* DCH6 and *L. garvieae* DCC43 carried any of the potential virulence factors examined. A specific cause for concern and contributing factor to pathogenesis of enterococci is their resistance to a wide variety of antibiotics. However, most of the enterococci evaluated in this study are susceptible to most clinically relevant antibiotics.

Evaluation of the production and functional expression of bacteriocins by *E. faecium* (Table 3), revealed that *E. faecium* DCH2 and *E. faecium* DCC14 were silent for production of EntL50 (EntL50A and EntL50B) and EntA. Although nucleotide sequencing did not reveal polymorphic differences in or near *entL50A-entL50B* and *entA* genes, PCR-nested experiences (Fig. 2) suggest that *E. faecium* DCH2 does not hold the three tightly clustered ORFs (*orfE*, *orfF*, and *orfG*), presumably involved in processing and secretion of EntL50 in *E. faecium* L50 (Cintas et al., 1998). The low production of EntP by *E. faecium* DCH2, as compared to that of the control strains *E. faecium* P13 and *E. faecium* L50, may be assigned to the absence of a 423-bp *entP-orf2*-derived fragment in *E. faecium* DCH2, which suggest that genetic modifications of the promoter region of *entP* may affect transcription, while those in *orf2* may affect immunity/transport of EntP. Indeed different expression levels of bacteriocins may be a result of promoter activity. These modifications do not constitute unusual situations in LAB (Martín et al., 2006). Meanwhile, the higher antimicrobial activity of *E. faecium* DEC7, *E. faecium* DEC8, and *E. faecium* DDC28, lower producers of EntA than the control *E. faecium* T136, may suggest that they are producers of a more stable EntA or producers of still unknown bacteriocins. The co-production of EntA and EntP by *E. faecium* DAC2 generates supernatants with a higher antagonistic activity than that of the control *E. faecium* P13 and *E. faecium* T136, while the lower production of EntP by *E. faecium* DAC7 may reflect phenotypic and genetic diversity among enterococcal strains affecting production of bacteriocins. All evaluated isolates carrying the genes *entP*, *entL50A-entL50B*, and *entqA*, were producers of enterocins at concentrations similar to that of the reference strain *E. faecium* L50. Differences in bacteriocin production and antimicrobial activity among such isolates may be resonably ascribed to their multiple bacteriocin production, phenotypic and genetic diversity of the bacteriocinogenic strains, and to still unknown factors (Gutiérrez et al., 2004; Martín et al., 2006). The occurrence of several enterocin structural genes in the *E. faecium* isolates does not always correlate with a higher bacteriocin activity in their supernatants.

The rapid determination of the absence of genes coding known enterocins permits detection of isolates potentially producing novel bacteriocins. Relevant to this study has been the observation that most *E. faecalis*, except *E. faecalis* DBH18 and *E. faecalis* DBH20, showed a potent direct antimicrobial activity (Table 3), but no activity was detected in supernatants of producer cells. Although the antagonistic activity of *E. faecalis* isolates producing EnlA is detected only in solid media (Nilsen et al., 2003), and *E. faecalis* cells express cytolysin by a quorum-sensing mechanism in response to target cells (Coburn et al., 2004), further studies should be directed towards a deeper elucidation of the antimicrobial and hemolytic activity of the antagonistic *E. faecalis* strains isolated from mallards. The linkage of production of antimicrobial substances to other virulence factors may contribute to the competitiveness of virulent *E. faecalis* in food and the gastrointestinal tract.

In this study, purification to homogeneity of the antimicrobial activity of *E. hirae* DCH5 revealed the presence of a major antagonistic peptide of 5093 Da (Fig. 3A), whereas purification of the antimicrobial activity of *L. garvieae* DCC43 revealed an antagonistic peptide with a molecular mass of 6022 Da (Fig. 3B). This purification procedure has permitted purification to homogeneity of many bacteriocins, including most known enterocins (Aymerich et al., 1996; Cintas et al., 2000; Gutiérrez et al., 2004), although it was not appropriate for purification of the antimicrobial compound(s) of *E. columbae* PLCH2 (Martín et al., 2006). A bacteriocin, designated hiraecin S, not purified to homogenety, has been reported to be produced by *E. hirae* C311 isolated from bovine faeces (Siragusa, 1992). Garviecin L1-5 with a molecular weight of about 2.5 kDa is produced by *L. garvieae* L1-5, isolated from bovine milk (Villani et al., 2001). Further work is in progress to determine the primary structure of the bacteriocins produced by *E. hirae* DCH5 and *L. garvieae* DCC43 by combined amino acid and DNA sequencing.

Mallard ducks hold in their intestinal content and carcasses a diverse enterococcal population with antimicrobial activity. Bacteriocinogenic *E. faecium* and *E. hirae* isolates may be considered hygienic due to production of enterocins, and potentially safe for their low incidence of potential virulence genes and susceptibility to most clinically relevant antibiotics. However, the presence among the enterococci of *E. faecalis* strains with a potent antagonistic activity and multiple virulence factors, gives the enterococci a possible concern regarding their potential pathogenicity for immunocompromised persons. In the sake of a good microbiological practice, it should be proposed not to feed the consumer with high levels of virulence factor-loaded enterococci (Martín et al., 2006). Accordingly, it should be recommended that mallards and other small game be eviscerated and defeathered longer before consumption. Nevertheless, some of the antagonistic enterococci and their enterocins evaluated in this study may find potential applications in the food and feed industry as producers of natural antimicrobial compounds, for production of food ingredients with antimicrobial activity, and for their evaluation as protective cultures and/or probiotics, provided their hygienic and safety status is further evaluated.

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