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ScienceDirect
International Journal of Food Microbiology 117 (2007) 295–305
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

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

J. Sánchez, A. Basanta, B. Gómez-Sala, C. Herranz, L.M. Cintas, P.E. Hernández *

Departamento de Nutrición, Bromatología y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain

Received 13 April 2007; accepted 26 April 2007

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.
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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,

* Corresponding author. Tel.: +34 913943752; fax: +34 913943743.
E-mail address: ehernan@vet.ucm.es (P.E. Hernández).

0168-1605/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijfoodmicro.2007.04.012

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

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