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Short communication

Incidence of virulence factors in enterococci from raw and fermented meat and biofilm forming capacity at 25 °C and 37 °C

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ABSTRACT

Twenty-nine *Enterococcus* strains from raw and fermented meat products were screened for the presence of virulence genes, including those for aggregation substances (*asa1* and *asa373*), cytolysin activator (*cylA*), collagen binding protein (*ace*), endocarditis antigen (*efaA*), enterococcal surface protein (*esp*) and gelatinase (*gelE*). Virulence gene occurrence, expression of gelatinase and pheromone aggregation was greater in *Enterococcus faecalis* than in *Enterococcus faecium* strains. All *E. faecalis* and 54% of *E. faecium* were positive for at least one or more virulence gene. The only strain of *Enterococcus gallinarum* tested also contained virulence genes. The effect of different growth temperatures (25 and 37 °C) on biofilm formation using polystyrene plates was also assessed. Strong biofilm formation occurred at lower than optimum temperature in all three species of enterococci. Neither *esp* nor *gelE* was necessary for biofilm formation and this relationship was species rather than strain specific. This study emphasizes the importance of enterococci as a reservoir of virulence genes and the potential for their genetic transfer to human strains following consumption of uncooked or undercooked contaminated meat.

1. Introduction

Enterococci inhabiting the gastrointestinal tract of humans and other animals have emerged as a major cause of nosocomial infections in recent decades (Franz et al., 1999). They can be found in other environments including soil, plants and water especially when contaminated with fecal materials (Aarestrup et al., 2002). Enterococci have been isolated from various foods of animal origin and they can be resistant to a wide variety of antibiotics commonly used in human medicine as well as those that are used for animal therapy, prophylaxis or for growth promotion (Jahan et al., 2013). They are also known for their capacity to exchange genetic information by conjugation (Clewell and Dunny, 2002) and may spread antibiotic resistance among non-pathogenic organisms (Cocconcelli et al., 2003; Fisher and Phillips, 2009).

Although enterococcal virulence factors are found more frequently among clinical strains, they are also detected in food isolates (Eaton and Gasson, 2001; Franz et al., 2001). Over the years several virulence factors have been identified in food enterococci which include: aggregation substances, cytolysin, pheromone, gelatinase, enterococcal surface protein and biofilm formation (Aslam et al., 2012; Barbosa et al., 2010; Foulquié Moreno et al., 2006; Franz et al., 2001; Martin et al., 2005; Ribeiro et al., 2011; Valenzuela et al., 2009). Occurrence of several virulence traits together with a high level of resistance to a wide variety of antibiotics suggests that enterococci may represent a reservoir of virulence and antibiotic resistant genes in the food chain which is a matter of concern (Ogier and Serror, 2008).

Biofilms can be defined as matrix-embedded bacterial populations adhered to a surface or to each other (Poulsen, 1999). Bacterial biofilms are more resistant to environmental stress than their free living counterparts and their attachment to food products or food contact surfaces accelerate spoilage, complicate cleaning and facilitate disease transmission. In the food industry biofilms produced by *Listeria monocytogenes*, *Salmonella* species, *Escherichia coli*, *Pseudomonas* species, *Brochothrix thermosphacta* and *Lactobacillus* species have been the subjects of greatest study (Di Bonaventura et al., 2008; Giaouris et al., in press; Rode et al., 2007). Although biofilms have been suggested to be important factors in the pathogenesis of enterococcal infection (Sandoe et al., 2003), few studies of biofilms have been done with food enterococci. Moreover, most of the studies on enterococcal biofilms have been conducted using optimum environmental conditions, allowing the cells to grow and divide normally. However, there are several studies on biofilms demonstrating that survival of enterococci involves stress–response mechanisms (George et al., 2005; Lleo et al., 2007). The present study examined the influence of suboptimal temperature exposure (25 °C), similar to conditions used for dry sausage fermentation, upon the ability of enterococci from meat to form biofilms. Very little information is currently available on the distribution of virulence genes in and biofilm formation by enterococci from fermented meat, especially in commercially fermented dry sausages and ham. Therefore, the current work also investigated the presence of virulence genes in antibiotic resistant enterococci isolated from raw sausage batter, commercially fermented sausage and dry cured ham.

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2. Materials and methods

2.1. Bacterial strains

Twenty nine enterococcal strains showing some antibiotic resistant traits isolated from raw and fermented meat, identified as *Enterococcus faecalis* (n = 14), *Enterococcus faecium* (n = 11) and *Enterococcus gallinarum* (n = 1), were examined. Reference strains of *E. faecalis* FAIR-E 324 (*asa1*$^+$, *cylA*$^+$, *efaA*$^+$, *gelE*$^+$ and *esp*$^+$), *E. faecalis* JH2-2 (*ace*$^+$; sex pheromone producer), *E. faecalis* OG1X:pCF10 (*asa373*$^+$) and *E. faecalis* ATCC 29212 (a strong biofilm producer) were used (Jahan et al., 2013). Enterococcal strains were grown from frozen stocks kept at −80 °C in Brain Heart Infusion Broth, BHIB (Difco, Fisher Scientific, Edmonton, AB, Canada) containing 50% (v/v) glycerol (Sigma, St. Louis, MO, USA) and were cultured on KF-Streptococcus Agar (Difco) at 37 °C.

2.2. Detection of virulence genes by PCR

DNA was extracted from overnight cultures grown at 37 °C in BHIB (Difco), using the ZR Fecal DNA kit according to the manufacturer's protocol (Zymo Research Corp., Orange, CA, USA). The sequences of oligonucleotide primers used to amplify virulence genes are listed in Table 1. Primers were synthesized by University Core DNA Services (University of Calgary, Calgary, AB, Canada) and PCR was performed as described before (Creti et al., 2004). Briefly, 2 μL of chromosomal DNA was used as the template in a final volume of 25 μL of PCR mixture, which contained 12.5 μL of PCR Master Mix 2× (Promega, Madison, WI, USA), 0.5 μL of each primer (25 pmol) and water to 25 μL. Thermal cycling conditions were: 1 cycle of denaturation (94 °C, 3 min); 36 cycles of denaturation (94 °C, 1 min), annealing (temperatures indicated in Table 1, 1 min), and extension (72 °C, 1 min), followed by a final extension (72 °C, 5 min).

2.3. Gelatinase assay

Production of gelatinase was determined using two methods. First Todd-Hewitt Agar (IBI Scientific, Peosta, IA, USA) containing 30 g/L gelatin (Difco) was used. After inoculation of pure test cultures previously grown at 37 °C for 24 h, the plates were incubated overnight at 37 °C and then cooled at 4 °C for 5 h. The appearance of a turbid halo around the colonies was considered positive for gelatinase activity. In the second method 0.8% Nutrient Broth (Oxoid Ltd., Basingstoke, England) with 12% gelatin (Difco) was used (Marra et al., 2007). Pure cultures were individually stabbed into tubes, incubated for 24 to 72 h at 37 °C and then held at 4 °C for 30 min. In tubes where an organism produced sufficient gelatinase, the gelatin remained liquefied upon cooling.

2.4. Pheromone clumping assay

The production of aggregation substances was determined in enterococcal isolates using the clumping assay described by Dunny et al. (1979) in the presence of the sex pheromone producing *E. faecalis* strain JH2-2. Briefly, *E. faecalis* JH2-2 was grown in Todd-Hewitt Broth, THB (IBI Scientific) for 18 h at 37 °C. The pheromone-containing supernatant was obtained by centrifuging at 10,823 ×*g* (Franz et al., 2001) and sterilized by autoclaving for 15 min. Each well of a 96 well polystyrene microtiter plate (Falcon no. 3072, Becton Dickinson and Co., Franklin Lakes, NJ, USA) containing 200 μL of serially diluted pheromone in THB was inoculated with 20 μL of each test culture (grown at 37 °C for 24 h) and incubated at 37 °C with shaking. Cell clumping (positive response) was examined at 2, 4, 6 and 18 h when samples were mounted on glass slides and observed by phase-contrast microscopy. Strains of *E. faecalis* OG1X:pAM373 and *E. faecalis* JH2-2 (which does not react with its pheromone) were used as positive and negative controls, respectively.

2.5. Biofilm assay

Detection of biofilm formation by enterococci in vitro was studied using the method described by Schlüter et al. (2009). Briefly, the strains were grown in Tryptic Soy Broth, TSB (Difco) with 1% glucose (Sigma, St. Louis, MO, USA) and incubated overnight at 37 °C and at 25 °C. The cells were pelleted, re-suspended in fresh medium and normalized to an absorbance of 1.00 at 595 nm using an Ultraspec 2000 spectrophotometer (Pharmacia Biotech, Cambridge, England). The cultures were diluted 1:40 and 200 μL of cells were dispensed into wells of a flat-bottom polystyrene microtiter plate (Becton Dickinson and Co.). The plates were incubated for 24 h at 37 °C and 25 °C without agitation to allow bacterial growth and formation of biofilm. After incubation, wells were washed with sterile phosphate buffered saline (PBS) and air-dried in a 60 °C incubator (Isotemp, Fisher Scientific) for 1 h. Biofilm formation was quantified using crystal violet staining and absorbance measured at 595 nm with a Synergy H4 Hybrid Micro-plate Reader (BioTek Instruments, Winooski, VT, USA). Biofilms were scored as: $OD_{595} < 0.120$, nonproducer; OD_{595} between 0.120 and 0.240, medium producer; $OD_{595} > 0.240$ to 0.70, strong producer (Schlüter et al., 2009); and $OD_{595} > 0.71$, very strong producer (Macovei et al., 2009).

2.6. Statistical analysis

Biofilm assays were performed for each strain in groups of 12 replicates and repeated twice. The statistical significance of mean differences ($p < 0.05$) was assessed by analysis of variance using JMP® 8.02 (SAS Institute Inc.) and differences in results between two temperatures were compared using Student's *t*-test.

Table 1
PCR primers and product sizes for detection of *Enterococcus* virulence determinants.

Gene	Primer sequence (5′ → 3′)	Product size (bp)	Annealing temperature (°C)	Reference
esp	TTGCTAATGCTAGTCCACGACC GCGTCAACACTTGCATTGCCGAA	932	61	Shankar et al. (1999)
gelE	ACCCCGTATCATTGGTTT ACGCATTGCTTTTCCATC	405	52	Eaton and Gasson (2001)
*asa*1	CCAGCCAACTATGGCGGAATC CCTGTCGCAAGATCGACTGTA	529	51	Creti et al. (2004)
*asa*373	GGACGCACGTACACAAAGCTAC CTGGGTGTGATTCCGCTGTTA	619	59	Creti et al. (2004)
cylA	GACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTTAC	688	54	Creti et al. (2004)
ace	GGAATGACCGAGAACGATGGC GCTTGATGTTGGCCTGCTTCCG	616	56	Creti et al. (2004)
efaA	GCCAATTGGGACAGACCCTC CGCCTTCTGTTCCTTCTTTGGC	688	56	Creti et al. (2004)