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Assessment of tetracycline and erythromycin resistance transfer during sausage fermentation by culture-dependent and -independent methods

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ABSTRACT

The food chain is considered one of the main routes of antibiotic resistance diffusion between animal and human population. The resistance to antimicrobial agents among enterococci could be related to the efficient exchange of transferable genetic elements. In this study a sausage model was used to evaluate the persistence of antibiotic resistant enterococci during meat fermentation and to assess horizontal gene transfer among bacteria involved in meat fermentation. *Enterococcus faecalis* OG1rf harbouring either pCF10 or pAMβ1 plasmid was used as donor strain. The analysis of population dynamics during fermentation confirmed that the human isolate *E. faecalis* OG1rf was able to colonize the meat ecosystem with similar growth kinetics to that of food origin enterococci and to transfer the mobile genetic elements coding for tetracycline and erythromycin resistances. Transconjugant strains were detected after only two days of fermentation and increased their numbers during ripening even in the absence of selective antibiotic pressure. By means of culture-dependent and -independent molecular techniques, transconjugant strains carrying both tetracycline and erythromycin resistance genes were identified in enterococci, pediococci, lactobacilli and staphylococci groups. Our results suggest that the sausage model provides a suitable environment for horizontal transfer of conjugative plasmids and antibiotic resistance genes among food microbiota.

1. Introduction

The Genus *Enterococcus* is part of the microbiota involved in flavour and texture development of some type of cheeses and cured meats (Foulquie Moreno et al., 2006). In particular, *Enterococcus faecalis* and *Enterococcus faecium* are present in raw meat and increase their number during the fermentation processes (Hugas et al., 2003; Fontana et al., 2009). Despite their commensal nature, enterococci have become an increasingly important cause of infections in hospitalized patients and are among the most frequent nosocomial pathogens worldwide (Klare et al., 2005; Hidron et al., 2008). A contributing factor in their pathogenesis is their emerging resistance to a wide variety of antibiotics (Araoka et al., 2011), moreover, enterococcal strains harbouring antibiotic resistance genes have been detected throughout the food chain, from animals to ready to eat food products (Ribeiro et al., 2011; Vignaroli et al., 2011).

Analyses of enterococcal genomics support the hypothesis that the frequent acquisition of antibiotic resistance genes is related with the common presence of mobile genetic elements such as plasmids, bacteriophages, transposons and pathogenicity islands (van Schaik and Willems, 2010). Among these mobile genetic elements, the pheromone responsive plasmid pCF10, mainly described in *E. faecalis* (Hirt et al., 2005) shows high frequency of conjugal transfer due to the presence of the bacterial adhesin, coded by the *prg*B gene, that mediates the binding between enterococcal cells (Dunny, 2007). This plasmid harbours also *tetM*, the tetracycline resistance determinant gene, present on the Tn916-like conjugative transposon Tn925, which is stably integrated into the plasmid DNA (Clewell et al., 1995). Conjugative gene transfer using pCF10 were reported in feed and feed manufacturing environment (Channaiah et al., 2010); the gastrointestinal tract of human (Egervarn et al., 2010) and house fly (Akhtar et al., 2009).

A second well-described conjugative plasmid pAMβ1 occurring in enterococci has a broad host range and carries resistance to erythromycin encoded on *ermB* (Clewell et al., 1974; Kruse and Sorum, 1994). The conjugative transfer of this plasmid has been shown to occur among various bacterial pathogens as well as among antibiotic susceptible strains isolated from different ecological niches (Morelli et al., 1988; Kruse and Sorum, 1994).

Although the role of the food chain as a possible source of antibiotic resistant microorganisms has been proposed (Cocconcelli

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et al., 2003; Rizzotti et al., 2005, 2009; Nawaz et al., 2011), few studies have been conducted to evaluate the transfer of antibiotic resistance genes between bacterial strains normally found during food fermentation. (Toomey et al., 2009a,b; 2010) reported on the ability of wild-type antibiotic resistance determinants *erm*B and *tet*M, present in lactic acid bacteria (LAB) strains isolated from food sources, to be transferred to recipient strains *in vitro* and *in vivo*.

The aim of this study was to evaluate the persistence of antibiotic resistant enterococci during the sausage fermentation process as well as to assess the transfer of mobile genetic elements carrying antibiotic resistance determinants in complex food microbial communities.

2. Material and methods

2.1. Bacterial strains and growth conditions

All donor and recipient strains used in this study are listed in Table 1. The two donor strains *E. faecalis* OG1rf:pCF10 (Dunny et al., 1978) carrying pCF10, and. *E. faecalis* OG1rf:pAMβ1, carrying pAMβ1 (Clewell et al., 1974) were grown overnight at 37 °C on BHI broth (Oxoid, Basingstoke, England) containing when appropriate tetracycline or erythromycin plus rifampicin. All *Lactobacillus, Pediococcus,* and *Enterococcus* and *Staphylococcus* strains were grown overnight on MRS broth (Merck), M17 broth (Oxoid), and BHI broth (Oxoid), respectively, at 37 °C. The strains used as recipients have been previously isolated from fermented foods; all of them were susceptible to tetracycline, erythromycin and rifampicin (Table 1), as assessed by the microdilution method according to Clinical and Laboratory Standards Institute (CLSI, 2005). Antibiotics were purchased from Sigma Chemical Co (Schnelldorf, Germany) and used at the following concentrations; vancomycin, 32 and 64 μg/ml; rifampicin, 50 μg/ml; tetracycline, 10 μg/ml; and erythromycin, 50 μg/ml.

2.2. Mating experiments in a food model

A fermented sausage model was used to assess horizontal gene transfer among bacteria. First, pork meat batter (pork meat, 85.5% w/w, and pork fat, 10% w/w) was sterilized by gamma ray irradiation treatment (6 KGy) to eliminate the adventitious microbiota present in the raw meat (Bioster, Bergamo, Italy). The absence of viable bacterial cells was checked by plating on PCA (Oxoid). Then, all the following procedures were performed using sterile tools under a sterile cabinet. To perform mating experiments, the batter was mixed with a sterile solution of glucose (1%, w/v), NaCl (3%, w/v), flavouring additives (0.5%, w/v) and nitrite (0.017%, w/v). From the whole mince a total of 15 sausages have been prepared to perform the different experiments in triplicate. Practically, as control of growth dynamics during sausages fermentation, three sausages were inoculated with 10^5 CFU/g of the donor strain *E. faecalis* OG1rf:pCF10, three sausages with 10^5 CFU/g of the donor strain *E. faecalis* OG1rf:pAMβ1 and three sausages with the recipients strains (Table 1). To asses the gene transfer, three sausages were inoculated with 10^5 CFU/g of the donor *E. faecalis* OG1rf:pCF10 together with all the recipient strains (pCF10 experiment) and three sausages with 10^5 CFU/g of the donor *E. faecalis* OG1rf:pAMβ1 together with all the recipient strains (pAMβ1 experiment). After different inoculation procedures, the mixed batter was stuffed into artificial casings and fermented for 1 day at 23 °C, 95% relative humidity (RH), followed by 27 day incubation at 15 °C at 89% RH. Sampling was performed at days 0, 2, 7, 14, 21 and 28 during the fermenting process for each type of sausages and then, samples were used for microbiological and molecular analysis.

2.3. Microbiological analysis

Sausage samples (10 g) were homogenized in 90 ml of peptone water for 3 min using 1.25 μm filter bags (Biochek, Foster City, Calif.) in a stomacher (Stomacher Lab-Blender 400, A.J. Seward Lab. London, England). Tenfold dilution were made and then plated on the selective agar.

The donor strains *E. faecalis* OG1rf:pCF10 and *E. faecalis* OG1rf:pAMβ1 were enumerated on Slanetz and Bartley medium (SB, Oxoid) with rifampicin plus tetracycline and rifampicin plus erythromycin, respectively, and incubated at 37 °C during 24 h. *Lactobacillus* recipient strains (Table 1) were enumerated on MRS agar pH 5.5 (Merck) with 32 μg/ml vancomycin at 37 °C for 24 h under restriction oxygen conditions, using anaerobic jars containing Anaerocult A gas packs (Merck, Darmstadt, Germany). *Pediococcus* recipient strains were enumerated on M17 agar with vancomycin (64 μg/ml) at 37 °C for 24 h and microscopic observation was used to differentiate them from lactobacilli.

Table 1
Bacterial strains used in this study.

Strains	Source	MICs (μg/ml)			Determinants of antibiotic resistance	
		ery	tet	rif	*tet M*	*ermB*
Donor strains						
Enterococcus faecalis OG1rf (pCF10)	Clinical	<4	>10	>4	+	–
Enterocuccus faecalis OG1rf (pAMβ1)	Clinical	>50	<4	>4	–	+
Recipient strains						
E. faecalis UC8840	Cheese	<4	<4	<4	–	–
E. faecium UC8842	Cheese	<4	<4	<4	–	–
E. durans UC8841	Cheese	<4	<4	<4	–	–
Lactobacillus rhamnosus UC10060	Sausage	<4	<4	<4	–	–
Lactobacillus sakei UC8704	Sausage	<4	< 4	<4	–	–
Lactobacillus curvatus UC8990	Sausage	<4	<4	<4	–	–
Pediococcus acidilactici UC10129	Sausage	<4	<4	<4	–	–
Staphylococcus carnosus UC8838	Sausage	<4	<4	<4	–	–
Staphylococcus equorum UC8839	Sausage	<4	<4	<4	–	–
Staphylococcus vitulinus UC8837	Sausage	<4	<4	<4	–	–
Staphylococcus xylosus UC8836	Sausage	<4	<4	<4	–	–

ery: erythromycin; tet: tetracycline; rif: rifampicin.
UC: Culture Collection of Universita Cattolica Sacro Cuore, Piacenza-Cremona.
MIC: Minimum Inhibitory Concentration.