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■ Prevalence and antimicrobial susceptibility of *Enterococcus* spp. in ready-to-eat salads (dips), the environment and the personnel of a salad processing plant in Northern Greece

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■ Παρουσία και αντιμικροβιακή ευαισθησία των *Enterococcus* spp. στις έτοιμες για κατανάλωση σαλάτες, το περιβάλλον και το προσωπικό ενός εργοστασίου παραγωγής σαλατών στη Βόρεια Ελλάδα

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ABSTRACT. A total of 225 samples from the dips (cheese, roe, egg plant and tzatziki dip), the environment and the handlers of a salad manufacturing plant in Northern Greece were examined for the presence and antibiotic susceptibility of *Enterococcus* spp. *Enterococcus faecium* was the isolated species from 12% of the samples. 38.1% and 15% of egg plant and cheese dip samples were positive, respectively. Among the dip ingredients, 30% of mayonnaise, 20% of feta cheese and 10% of myzithra were positive. Positive were, also, 8.6 % of the environmental samples, 20% from handlers' skirts, 20% from handlers' gloves and 16.7% from their nasal cavity. All isolates were sensitive to glycopeptides (vancomycin and teicoplanin). Isolates from handlers' nasal cavities were resistant to penicillin and ampicillin, while one of them was, also, resistant to chloramphenicol. All isolates from the environment, the dips and their ingredients were susceptible to these three antibiotics, but they presented resistance to more than 3 antibiotic categories. Ready-to-eat foods may be potential source of contamination to humans of multidrug resistant enterococci. Further research is needed to elucidate their epidemiology in these foods.

Keywords: *Enterococcus* spp., antimicrobial susceptibility, ready-to-eat dips, environment.

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ΠΕΡΙΛΗΨΗ. Διακόσια εικοσιπέντε δεύγματα από τις σαλάτες (τυροσαλάτα, ταραμισαλάτα, μελιτζανοσαλάτα και τζατζίκι), το περιβάλλον και το προσωπικό ενός εργοστασίου παραγωγής σαλατών στην Βόρεια Ελλάδα εξετάστηκαν για την παρουσία των *Enterococcus* spp. και την αντιμικροβιακή αντοχή τους. Απομονώθηκε *Enterococcus faecium* σε ποσοστό 12% των δειγμάτων. Ποσοστά 38,1% και 15% των δειγμάτων μελιτζανοσαλάτας και τυροσαλάτας, αντίστοιχα, ήταν θετικά. Τα ποσοστά απομόνωσης από τα συστατικά των σαλατών ήταν 30% για τη μαγιονέζα, 20% για τη φέτα και 10% για τη μυζήθρα. Θετικά ήταν, επίσης, 8,6% των δειγμάτων από το περιβάλλον, 20% από τις ποδιές, 20% από τα γάντια και 16,7% από τις οινικές κοιλότητες των εργαζομένων. Όλα τα απομονωθέντα στελέχη ήταν ευαίσθητα στα γλυκοπεπτίδια (βανκομυκίνη και τεικοπλανίνη). Τα δύο απομονωθέντα στελέχη από τις οινικές κοιλότητες των εργαζομένων ήταν ανθεκτικά στην πενικιλλίνη και την αμπικιλλίνη, ενώ ένα από αυτά ήταν επιπλέον ανθεκτικό στη χλωραμφενικόλη. Όλα τα στελέχη που απομονώθηκαν από το περιβάλλον, τις σαλάτες και τα συστατικά τους ήταν ευαίσθητα στα τοία αυτά αντιβιοτικά, αλλά παρουσίασαν αντοχή σε περισσότερες των τριών κατηγορίες αντιβιοτικών. Τα έτοιμα προς κατανάλωση τρόφιμα μπορεί να είναι δυνητικές πηγές μόλυνσης του ανθρώπου με πολυναθεκτικούς στα αντιβιοτικά εντεροκοκους.

Λεξεις ενρετηρίασης: *Enterococcus* spp., αντιμικροβιακή ευαίσθηση, έτοιμες για κατανάλωση σαλάτες, περιβάλλον

INTRODUCTION

Enterococci naturally inhabit the human and animal intestinal tracts. Although they were considered as microorganisms with low pathogenicity to humans, they have been recently incriminated as causative agents of a variety of severe infections, especially in immunocompromised patients and nosocomial settings (Chatterjee et al. 2007). *Enterococci* are frequently isolated from food products of animal origin (Chingwaru et al. 2003, Citak et al. 2004). Their counts in fermented meat and dairy products could be more than 10^5 CFU/g (Tzanetakis et al. 1992, Franz et al. 1999, Giraffa et al. 2000).

The presence of enterococci in food is highly controversial, as they are considered indicators of faecal contamination and responsible for the spoilage of meat products (Franz et al. 1999, Godfree et al. 1997). In addition, they are important in flavour development of various types of cheese, bioprotection in dairy and meat products and beneficial as probiotics (Coppola et al. 1988, Litopoulou-Tzanetaki et al. 1993, Centeno et al. 1996).

There are many reports of enterococci resistant to penicillin, tetracycline, chloramphenicol, erythromycin, gentamicin, lincomycin, rifampicin, teicoplanin, vancomycin and other antimicrobial agents (Giraffa et al. 2000, Pavia et al. 2000, Wilson and McAfee 2002, Chingwaru et al. 2003, Citak et al. 2004). During the recent years, an increasing prevalence of vancomycin-resistant enterococci (VRE) has been observed, causing a serious problem in hospitals, especially in intensive care units. The problem is, also, present in Greek hospitals, where unusual phenotypes of *Enterococcus faecalis* (penicillin- and imipenem-resistant, but

ampicillin-susceptible) have been observed (Maniatis et al. 2001, Pournaras et al. 2004, Metzidie et al. 2006).

The aim of the present study was to investigate the prevalence and antimicrobial susceptibility of *Enterococcus* spp. in the environment, the personnel and the products of a ready-to-eat (RTE) salads manufacturing plant in Greece. This is the first attempt to study the presence and antibiotic susceptibility of *Enterococcus* spp. in a salad manufacturing plant in Greece.

MATERIALS AND METHODS

Sampling procedures

A salad manufacturing plant in Northern Greece was investigated. A total of 82 samples from four kinds of dips (20 cheese, 20 roe, 21 egg plant and 21 tzatziki dips) and 40 samples from 4 of their basic ingredients (10 from feta cheese, 10 from myzithra, 10 from mayonnaise and 10 from roasted egg plant pulp) were tested. The ingredients of the cheese dip (tyrosalata) were: feta cheese, myzithra (whey cheese), yoghurt, soybean oil and hot chili pepper; of tzatziki dip: yoghurt, cucumber and garlic; of the egg plant dip (melitzanosalata): roasted egg plant, mayonnaise, garlic, spices and vinegar; and of the roe dip (taramosalata): paste of salted fish roe, bread and soybean oil.

In addition, 71 environmental swab samples (36 during production time and 35 immediately after the application of sanitation program), 12 samples from handlers' nasal cavities, 10 samples from their gloves and 10 from their skirts were, also, examined bacteriologically.

Samples from dips and basic ingredients were taken from batches of four different days of production in a six month period. Environmental and handlers'

samples were collected in two different occasions.

Portions of 250g of the dips and their basic ingredients were aseptically collected in the factory and were placed into stomacher bags. The examined surfaces of the plant were walls, floors, drainage lids, refrigerator knobs, mixer tanks and accessories, pulping machines, working tables, filling spoons, reusable containers, plastic frames and tanks.

One hundred cm² of surfaces and whole handlers' hands were swabbed twice by the wet-dry double swab technique using sterile cotton swabs moistened with 0.1% peptone water containing 0.85% NaCl, followed by a second swabbing using a dry swab. The two swabs were pooled in one sample. Swabbing was carried out in the middle of the working day and after the application of the sanitation program.

Samples from handlers' nasal cavities were taken by swabbing each person's nostrils using one sterile cotton swab per nostril that was previously moistened in the same medium as the swabs for surface sampling. The two cotton swabs were pooled in one sample into tubes containing 10 ml of buffered peptone water (BPW) (LAB M, Lancashire, UK).

All samples were transferred to the laboratory inside cold portable insulated boxes and processed within 3 h of collection.

Isolation, enumeration and identification of *Enterococcus* spp.

Enumeration of enterococci was performed only in dips and their basic ingredients. Portions of 25g were taken aseptically from the samples and placed into stomacher bags containing 225 ml BPW, homogenized for 1 min in a stomacher (Lab Blender 400, A.J. Seward and Co. Ltd., London) and then prepared 10-fold dilutions. One ml from each dilution was plated using pour plating technique onto Kanamycin Aesculin Azide agar (KAA, LAB M, Lancashire, UK) and incubated for 24-48 hours at 37±0.5 °C. For the detection of less than 10 CFU/g, the first dilution was incubated for enrichment at 37°C for 24 h. One loopful of the enriched culture was spread plated onto the same agar and incubated at 37°C for 24-48 h.

Swabs were overnight incubated in BPW broth at 37±0.5 °C for enrichment and then 0.1 ml of each tube was plated onto KAA agar and incubated aerobically at 37±0.5 °C overnight.

If growth was observed, two typical black colonies were isolated on TSYE agar (LAB M, Lancashire, UK) for further investigation. Presumptive identification of the isolates, was based on tests for Gram staining, catalase and oxidase production, growth at 10°C and 45°C, growth in the presence of 6.5% NaCl and at pH 9.6 in Tryptone Soy Broth (LAB M, Lancashire, UK).

The lower detection limit level was less than 1 log CFU/g. Isolates were identified at species level on the basis of biochemical characterization by the semi-automated system WIDER (Francisco Soria Melguizo, Madrid, Spain), using the Gram positive minimal inhibitory concentration/identification (MIC/ID) panels. The following biochemical tests were included in these panels: acidification of arabinose, cellobiose, lactose, mannitol, ribose and saccharose; the use of esculin, arginine and urea; the production of phosphate, α-glucosidase and β-glucuronidase; the transformation of pyruvate in acetoin; growth in the presence of optochin, bacitracin and novobiocin; growth in the presence of 6.5% sodium chloride and haemolysis.

E. faecium ATCC 19434, *E. faecalis* ATCC 19433, *E. durans* ATCC 19432 and *E. hirae* LMG 6399 were used as quality control strains.

Antibiotic susceptibility tests

All isolates were tested for antibacterial susceptibility to 20 antibiotics used regularly in Greek hospitals. MIC was evaluated according the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2006) in the semi-automated system WIDER (Francisco Soria Melguizo, Madrid, Spain), using the Gram Positive MIC/ID Panels.

The antibiotics were: beta-lactams (penicillin, ampicillin, oxacillin and amoxicillin/clavulanic acid), cephalosporins (cefazolin and cefotaxime), aminoglycosides (streptomycin 1000, gentamicin, gentamicin 500 and amikacin), glycopeptides (vancomycin and teicoplanin), fluoroquinolones (levofloxacin), macrolides (erythromycin), lincosamides (clindamycin), streptogramins (quinupristin/dalfopristin), oxazolidinones (linezolid), rifamycins (rifampin), chloramphenicol, fosfomycin and trimethoprim/sulfamethoxazole.

RESULTS

In total, *Enterococcus* spp. was isolated from 27/225 (12%) samples. All of them were identified as

Enterococcus faecium. Briefly, *E. faecium* was isolated from 38.1% of the egg plant dip, 15% of the cheese dip, 30% of mayonnaise, 20% from feta cheese and 10% of myzithra cheese (Table 1). The mean counts of enterococci were 2 ± 0.9 log CFU/g in cheese dips, 1 ± 0.59 log cfu/g in egg plant dips, 1 ± 0.9 log CFU/g in mayonnaise, 5 ± 0.8 log CFU/g in feta cheese and 3 ± 0.7 log cfu/g in myzithra cheese (Table 1). *Enterococcus* spp. was not isolated from tzatziki, roe dip and roasted egg plant pulp.

Three of the 35 (8.6%) environmental swab samples, which were taken from mixers and filling spoons after their sanitation, were *E. faecium* positive (Table 1). These areas were not sampled during production because they were covered with dips.

E. faecium was isolated from 16.7% from handlers' nasal cavities, 20% from their gloves and 30% from their skirts (Table 1).

All 25 isolates of *E. faecium* from the dips, their ingredients and the plant environment were multidrug resistant (MDR), showing resistance at least to five antibiotics up to nine. All of them (100%) were resistant to oxacillin (MIC > 4 mg/L), cefazolin (MIC > 4 mg/L), cefotaxime (MIC > 4 mg/L), gentamicin (MIC > 8 mg/L) and amikacin (MIC > 16 mg/L) (Tables 2 and 3). Furthermore, 96% of them were also resistant to rifampin (MIC > 2 mg/L), 80% to erythromycin (MIC > 2 mg/L), 64% to clindamycin (MIC > 2 mg/L) and 40% to fosfomycin (MIC > 64 mg/L). Eight isolates presented intermediate resistance to quinupristin/dalfopristin (MIC = 2 mg/L), six to erythromycin (MIC = 2 mg/L), three to levofloxacin (MIC = 4 mg/L) and one to rifampin (MIC = 2 mg/L) (Table 3). A different resistance pattern was observed in the two human isolates, as they were resistant to penicillin (MIC > 8 mg/L) and ampicillin (MIC > 16 mg/L) and one of them was, also, resistant to chloramphenicol (MIC > 8 mg/L), while the isolates from the environment and food were susceptible to these antibiotics (Table 2). None of the isolates was resistant to glycopeptides (vancomycin and teicoplanin) and to gentamicin 500 µg/ml.

DISCUSSION

In the present study, *E. faecium* was detected in RTE dips, as well as in the food processing environment and the handlers. All isolates were multidrug resistant (MDR), as they were resistant to more than three antibiotic categories. However, differences in

Table 1. Isolation and enumeration of *Enterococcus faecium* in RTE dips, the environment and the handlers of the salad manufacturing plant.

Sample origin	Number of samples	Percentage of positive samples (%)	Counts as log cfu/g
Dips			
Cheese dips	20	15	2 ± 0.9
Roe dips	20	-	-
Tzatziki	21	-	-
Egg plant dips	21	38.1	1 ± 0.59
Basic ingredients			
Roasted egg plant	10	-	-
Mayonnaise	10	30	1 ± 0.9
Feta cheese	10	20	5 ± 0.8
Myzithra cheese	10	10	3 ± 0.7
Environment			
During production	36	-	ND*
After sanitation	35	8.6	ND
Handlers			
Gloves	10	20	ND
Skirt	10	30	ND
Nasal cavities	12	16.7	ND
Total	225	12	

*ND= Not done

antibiotic resistance patterns were observed. Based upon these phenotypic resistance patterns that discriminate the isolates, an epidemiological link might be present between isolates from mayonnaise and handlers' gloves and skirts, as they presented identical antibiotic pattern (Table 2). Similarly, identical patterns were observed among isolates from myzithra and feta cheese (which were bought from the same dairy plant) and cheese dip, suggesting a common source. Studies in Greece have shown that dairy products are normal habitat for enterococci with population ranging from 10^5 to 10^7 CFU/g (Tzanetakis and Litopoulou-Tzanetaki 1992, Litopoulou-Tzanetaki et al. 1993), although they are not used as starter cultures, in spite of the beneficial role of *E. faecium* in ripening of feta (Litopoulou et al. 1993, Sarantinopoulos et al. 2002).

The isolated enterococci from humans showed distinguished antibiotic pattern, totally different from the patterns of the other isolates (Table 2). This difference is an indication that no cross-contamination between humans and dips occurred.

None of the enterococci was resistant to glycopeptides, not even the isolates from two handlers that

Table 2. Antimicrobial resistance of *Enterococcus faecium* isolates recovered from RTE dips, the environment and the handlers of the salad manufacturing plant.

Isolate origin	No of isolates	Antimicrobial resistance pattern
Cheese dips	3	AMC, CFT, CFZ, E (I)**, FOS, G, OX*, RIF
Egg plant dips	4	AMC, CFT, CFZ, CL, E, G, OX, Q/D (I), RIF
	3	AMC, CFT, CFZ, E, FOS, G, OX, Q/D (I), RIF
	1	AMC, CFT, CFZ, CL, E, FOS, G, OX, Q/D (I), RIF (I)
Mayonnaise	3	AMC, CFT, CFZ, CL, E, G, OX, RIF
Feta Cheese	2	AMC, CFT, CFZ, E (I), FOS, G, OX, RIF
Myzithra cheese	1	AMC, CFT, CFZ, E (I), FOS, G, OX, RIF
Filling spoons	2	AMC, CFT, CFZ, CL, E, G, LEV (I), OX, RIF
Mixer accessories	1	AMC, CFT, CFZ, CL, E, G, LEV (I), OX, RIF
Handlers' skirts	3	AMC, CFT, CFZ, CL, E, G, OX, RIF
Handlers' gloves	2	AMC, CFT, CFZ, CL, E, G, OX, RIF
Handlers' nasal cavities	1	AM, P
	1	AM, CH, P

(*) Amikacin (AMC), amoxicillin/clavulanic acid (A/C), ampicillin (AM), cefazolin (CFZ), cefotaxime (CFT), chloramphenicol (CH), clindamycin (CL), erythromycin (E), fosfomycin (FOS), gentamicin (G), gentamicin (G500), levofloxacin (LEV), linezolid (L), oxacillin (OX), penicillin (P), quinupristin/dalfopristin (Q/D), rifampin (RIF), streptomycin (S1000), vancomycin (V) and teicoplanin (TEC).

(**) Intermediate resistance

had been hospitalized a few weeks ago and two others that used to visit their relatives in intensive care units. In spite of these findings, the presence of strains resistant to glycopeptides cannot be excluded. Other authors reported similar results for food isolates (Mannu et al. 2003, Peters et al. 2003, Majhenic et al. 2003, Messi et al. 2006, Barbosa et al. 2009). Some other authors obtained isolates from foods of animal origin resistant to vancomycin (Klein et al. 1998, Van den Braak et al. 1998, Gomes et al. 2008, Sabia et al. 2008, Kolumn et al. 2009).

The high resistance frequency to erythromycin that was found in food isolates in our study is in agreement with the findings of other authors (Teuber et al. 1999, Mannu et al. 2003, Peters et al. 2003, Ben Omar et al. 2004, Barbosa et al. 2009). This may be explained by the fact erythromycin resistance plasmids and transposons are commonly found among enterococci (Murray 1990).

It has been reported that enterococci isolated from Turkish white cheese (Citak et al. 2004) were resistant to streptomycin, oxacillin and erythromycin more than to other antibiotics with corresponding values of 89.1%, 81.1% and 93%, respectively. Concerning resi-

stance to vancomycin and teicoplanin, they reported a high value of 86.1% and 66.3%, respectively.

It is of medical interest that 32% of the isolates showed intermediate resistance to the streptogramin quinupristin/dalfopristin (Table 3), which is a new antimicrobial agent used in human medicine for the treatment of infections with vancomycin-resistant *E. faecium*.

The findings of our study, concerning the susceptibility of enterococci from foods to clinically important antibiotics, such as penicillin, ampicillin, vancomycin and gentamicin, are similar to those reported from other authors (Franz et al. 2001, Lopes et al. 2003, Mannu et al. 2003, Peters et al. 2003, Cosentino et al. 2004, Canzek Majhenic et al. 2005).

Enterococci isolated from fermented sausage were resistant to streptomycin and isolates from Emmentaler and Appenzeller cheeses showed high frequency of resistance to erythromycin, gentamicin, vancomycin and tetracycline (Teuber et al. 1996).

Enterococci isolated from Spanish RTE foods (dairy and meat products, olives etc.) were susceptible to ampicillin, penicillin, streptomycin and gentamicin

Table 3. Percentage of resistance to antibiotics of *Enterococcus faecium* isolated from RTE dips and the environment.

Antibiotics	No of isolates	Percentage
Amikacin	25	100%
Cefazolin	25	100%
Cefotaxime	25	100%
Clindamycin	16	64%
Erythromycin	20	80%
	6 (I)*	24%
Fosfomycin	10	40%
Gentamicin	25	100%
Levofloxacin	3 (I)	12%
Oxacillin	25	100%
Quinupristin/Dalfopristin	8 (I)	32%
Rifampin	24	96%
	1 (I)	4%

* I= Intermediate resistance

500µg/ml (Ben Omar et al. 2004).

Although virulent strains resistant to antibiotics have been isolated from food products and animals, it is difficult to assess the role of the food chain as a possible source of resistant enterococci and, except from very few cases, there is not clear indication of foodborne infections caused by enterococci (Franz et al. 1999).

Lu et al. (2002) reported an outbreak from ampicillin-resistant, vancomycin susceptible *E. faecium* strain in China, where thousands of domestic pigs died from haemorrhagic shock and 40 farmers were hospitalized with severe illness after contact with sick pigs, while 12 of them died due to respiratory failure and shock.

Contaminated food may enable the transfer of bacteria from animals to humans, contributing to the spread and persistence of antibiotic resistance. According to Ogier and Serror (2008), dairy products represent a potential reservoir of antimicrobial resistance and virulence traits which could be transferred from food strains to human strains in the human intestinal tract. Fermented food, such as cheese and cured sausages, may contribute to the distribution of antibiotic-resistant bacteria (staphylococci and enterococci) to the consumer (Teuber et al. 1999).

E. faecium has the potential for gene transfer, which may occur inter- or intra- enterococcal species (Boyle et al. 1993, Quednau et al. 1998), as well as among other

bacterial genera, such as *Staphylococcus* spp. (Noble et al. 1992). Recent reports have documented horizontal transfer of vancomycin resistance determinant (vanA gene) from *E. faecalis* to methicillin-resistant *Staphylococcus aureus* (MRSA), resulting in MRSA strains with increased resistance to vancomycin (Weigel et al. 2003).

The identification of the source of contamination and the transmission routes of enterococci to humans may contribute to the development and application of measures to minimize this risk. Food chain seems to be among these sources. In that respect, control of MDR bacteria in foods is an important issue for the safety of consumers, who are at highest risk, and constitutes a special challenge because of their robust nature, their wide distribution and their stability in the external environment (Franz et al. 1999). The use of strains that have beneficial impact on food safety and human health and, at the same time, the exclusion of virulent strains from food products consist a scientific challenge.

Processing of RTE dips does not include any decontamination step for vegetative pathogens, such as heat treatment, either during production or before consumption. The only existing hurdles to control microbial growth are NaCl concentration, pH, a_w and cold storage. Apart from refrigeration, the range of the values of the other hurdles in the dips seems not to be capable to restrict the growth of *Enterococcus* spp. In that respect, control of pathogens relays upon strict application of good hygiene and manufacturing practices in order to eliminate the risk of spreading of virulent and MDR *Enterococcus* spp.

Although enterococci are not considered important as foodborne pathogens, their spread through the food chain represents a so-called indirect zoonotic problem (Bager et al. 1997, Bates 1997). The antibiotic sensitivity of enterococci isolated from foods, especially RTE, has not been investigated in detail, except from some dairy and meat products. The present study, although limited in one plant, provides information concerning distribution of enterococci and probable transmission routes in a Greek ready-to-eat salad processing plant. Further investigation is needed to elucidate the epidemiology of enterococci in RTE foods, such as dips and salads, as they may be potential source of human infections. This knowledge is highly recommended for a proper risk assessment to clarify the possible health hazard for consumers related to the presence of MDR enterococci in foods.

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