Antibiotic resistance of enterococci and related bacteria in an urban wastewater treatment plant

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Abstract
The main objective of this work was to study the ecology of enterococci and related bacteria in raw and treated wastewater from a treatment plant receiving domestic and pretreated industrial effluents in order to assess the influence of treatment on the prevalence of antibiotic resistance phenotypes among this group of bacteria. The predominant species found in the raw wastewater were Entercoccus hirae, Entercococcus faecium and Entercoccus faecalis. Wastewater treatment led to a reduction in E. hirae (α < 0.1) and an increase in E. faecium (α < 0.1); the relative proportions of E. faecalis remained the same in the raw and in the treated wastewater. Among the isolates tested, no vancomycin resistance was observed among the enterococci. Entercoccus faecium and E. faecalis showed resistance prevalence values reaching 33%, 40% and 57% for the antibiotics ciprofloxacin, erythromycin and tetracycline, respectively. Antibiotic-resistant strains of enterococci were not eliminated by wastewater treatment. A positive selection of ciprofloxacin-resistant enterococci was indicated by a significant increase in resistance prevalence (α < 0.02) in treated wastewater compared with the raw wastewater.

Introduction
Enterococci are important commensal members of the intestinal microbiota of humans and animals. Despite their widespread distribution in food products, enterococci may be opportunistic pathogens and are frequently associated with nosocomial infections (Devriese et al., 1992; Klein, 2003). As a result of the high prevalence of acquired antibiotic resistance, enterococci are recognized as important active spreading agents of this type of resistance both at intra- and at interspecific levels (Klare et al., 2003). Recent studies, focusing on habitats related to human activity such as processing of food products, animal production or wastewater treatment, have provided new insights on the epidemiology and ecology of antibiotic-resistant bacteria (Kuhn et al., 2000; Blanch et al., 2003; Peters et al., 2003; Hayes et al., 2004). Despite the ubiquitous character of enterococci, their distribution in wastewaters and their fate during water treatment have been poorly characterized. This strongly limits our understanding of their role as indicators of faecal pollution, of the ecology of pathogenic strains or of their involvement in antibiotic resistance transmission.

Domestic wastewater treatment plants are important links in the water cycle in urban areas, and study of their microbial ecology may bring valuable insights for two main reasons. The microbial flora mirrors the commensal microorganisms of the human population of a particular area, and such studies provide evidence for the impact of human activity on water microbial ecology.

Here we examined the diversity of culturable enterococci and related bacteria present in raw and treated wastewater of a wastewater treatment plant receiving mainly domestic effluents, and evaluated the resistance prevalence of these microorganisms to six antibiotics.

Materials and methods
Wastewater treatment plant and sampling
Raw and treated wastewater samples were collected at four different times of the year (January, March, July and November 2004) from a wastewater treatment plant in northern Portugal. The treatment plant receives around
75% of the sewage drainage from a municipal area of more than 100,000 inhabitants and a population density above 1000 km$^{-2}$.

In the plant, raw wastewater consists of domestic sewage (around 70%) and pretreated industrial effluents (about 30%). Occasionally, storm water may enter the sewage network. Raw wastewater undergoes a preliminary treatment to remove voluminous solids, storage in a primary settling tank to remove settleable solids and a secondary biological treatment (activated sludge process). The treated wastewater from the secondary settling tank is discharged without any further treatment into a natural water stream. The total hydraulic retention time of the wastewater is approximately 12 h. One litre of each sample (effluents from the primary and secondary clarifiers) was collected in a sterile container, transported to the laboratory and analysed within a maximum period of 2 h.

**Wastewater analysis**

Water samples were analysed for determination of chemical oxygen demand (COD) and biochemical oxygen demand (BOD) according to standard methods (APHA, 1995). Microbiological analyses were performed using the membrane filtration method. Briefly, 1 mL serial dilutions of water samples were filtered and the membranes (cellulose nitrate, 0.45 μm pore size, 47 mm diameter, Albet, Barcelona, Spain) were placed onto four different media: plate count agar (PCA, Merck, New Jersey) for total heterotrophs, m-FC Agar (m-FC, Difco, Chicago, IL) for faecal coliforms, m-Endo-Agar-LES (m-Endo-LES, Difco) for total coliforms and m-Enterococcus Agar (m-Enterococcus, Difco) for total streptococci. After an incubation period of 24 h at 30, 35 and 44.5°C or 48 h at 35°C (for enterococci), the number of CFU in each culture condition was registered in the filtering membranes presenting between 20 and 80 typical colonies. Typical colonies on m-Endo LES were confirmed in Lauryl Tryptose Broth (LTB, Difco) and Brilliant Green Bile 2% (BGB, Difco), whereas isolates from m-FC and m-Enterococcus media were confirmed in EC medium (EC, Difco) and on Bile Esculin Agar (BEA, Merck), respectively.

Removal efficiencies of the wastewater treatment were calculated from: % removal = ($X_{raw} - X_{treated}$)/$X_{raw} \times 100$; log removal = log $X_{raw}$-log $X_{treated}$ where $X_{raw}$ and $X_{treated}$ were for total heterotrophs, total coliforms, faecal coliforms, total enterococci, COD or BOD in raw and treated wastewater, respectively (George et al., 2002).

**Isolation of bacteria and preliminary characterization**

Bacteria were isolated from the membrane filters containing countable CFU. Twenty-five to 100% of the colonies formed on m-Enterococcus agar were isolated and purified on PCA or, when necessary, on MRS agar (Man, Rogosa and Sharp, Amersham). Purified cultures were characterized by Gram staining, presence of catalase, growth and aesculin degradation on BEA medium, and growth at 45°C in Tryptone Soy Broth (TSB, Amersham) or in the same medium supplemented with 6.5% (weight in volume, w/v) NaCl. Isolates were maintained at -80°C in TSB supplemented with 15% (volume in volume, v/v) glycerol.

**Antibiotic resistance**

Antibiotic resistance phenotypes were tested based on the Kirby–Bauer method according to standard recommendations (National Committee for Clinical Laboratory Standards, 2003). Briefly, bacterial suspensions with an OD of 0.2, at 620 nm, were spread on Mueller Hinton agar (Oxoid) using a sterile cotton swab. Antibiotic discs of amoxicillin (AML, 25 μg), gentamicin (GEN, 10 μg), ciprofloxacin (CIP, 5 μg), sulphamethoxazole/trimethoprim (SXT, 23.75/1.25 μg), tetracycline (TET, 30 μg) and erythromycin (ERY, 15 μg) (all Oxoid) were placed on the surface of each inoculated plate. Additionally, resistance to vancomycin (VAN, 30 μg) and GEN (120 μg) was assayed for some of the strains. After 24 h of incubation at 35°C, the diameters of antibiotic inhibition of growth were measured and recorded as susceptible (S), intermediary (I) or resistant (R). The criteria used for these designations, based on inhibition zone diameters, were as follows (mm): AML 25: R < 14, I = 20–14, S ≥ 21; GEN 10: R < 14, I = 15–20, S ≥ 21; GEN 120: R < 7, S ≥ 10; CIP 5: R < 19, I = 19–21, S ≥ 22; ERY 15: R < 17, I = 21–17, S ≥ 22; TET 30: R < 17, S ≥ 19; SXT 25: R < 10, I = 9–15, S ≥ 16; VAN 30: S > 17. Escherichia coli ATCC 25922, Enterococcus faecalis DSM 2570, Pseudomonas aeruginosa DSM 1117 and Staphylococcus aureus DSM 1104 were used as controls in each experimental set. The inhibition zones observed for these organisms were compared with expected values. The average deviation of the diameters of inhibition zones measured for these organisms were compared with expected values. The average deviation of the diameters of inhibition zones measured for the control strains ranged between 0.1 and 0.2 mm.

Species distribution and the prevalence of antibiotic resistance or intermediary phenotype to each antibiotic among enterococci isolates from raw and treated wastewater were compared using the chi-squared test.

**RAPD typing**

Random amplified polymorphic DNA (RAPD) analysis was used to compare and cluster enterococci and related bacteria. The method used was based on that described by Tiago et al. (2004). Crude cell lysates were used as DNA templates for genotyping. Amplification reactions were performed with a total volume of 25 μL containing: 0.75 U Taq
polymerase, 1.5 mM MgCl$_2$ (Pharmacia Biotech), 0.2 mM of each dNTP, 1.0 µM primer M13 (5’GAGGGTGCGG TTCT3’) and 0.5 µL of crude cell lysates. After 5 min at 94 °C, samples were subjected to 45 cycles of amplification (Biometra), as follows: 1 min at 94 °C, 1 min at 34 °C and 2 min at 72 °C, and a final extension step of 10 min at 72 °C. Polymeric DNA fragments were analysed by electrophoresis in a 2% agarose gel in Tris-acetate-EDTA buffer. To allow alignment and comparison of profiles in different gels, the RAPD profile of *E. coli* ATCC 25922 generated under the same conditions was included in the extremities and in the centre of each gel.

The RAPD profiles were visually analysed and the presence or absence of each band was noted for each isolate. The resultant matrix was interpreted using a hierarchical clustering method, based on the Dice coefficient (SPSS 13.0 for Windows), in which isolates were grouped according to resemblance of their RAPD patterns. Clusters were defined for relative rescaled distances below 20%. As a quality control of the RAPD analysis and data interpretation, three isolates randomly selected among the test strains were successively processed together with the remaining strains. The rescaled distances obtained for each of these control strains was below 8%. Identification of those members comprising each cluster and validation of the typing method as discriminating different species were made through analysis of partial 16S rRNA gene sequences. Thus, 10–25% of the isolates of each cluster as well as others that could not be typed by RAPD analysis were selected for 16S rRNA gene sequence analysis.

**rRNA gene sequence analysis**

The extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were carried out as described previously (Rainey *et al.*, 1996). DNA sequences were determined using a model 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Primer 519R (5’TACCCGCGKGGCTG-3’) was used to generate partial 16S rRNA gene sequences comprising 420–450 nucleotides. The quality of the 16S rRNA gene sequences was checked manually using the Bioedit editor (Hall, 1999) and compared with sequences available in the EMBL/GenBank database using Blast network services and also with sequences in the Ribosomal Database Project II (RDP) (Cole *et al.*, 2005).

**Results and discussion**

**Wastewater characterization**

Microbiological analyses of the raw wastewater indicated that levels of total heterotrophs and total coliforms were about 10$^7$–10$^8$ CFU, and that levels of faecal coliforms and enterococci were about 10$^6$ CFU per 100 mL (Table 1). The number of organisms in each of these groups decreased following treatment, with reductions of 80–90% for total heterotrophs and total coliforms (i.e. in a range of 0.69–1.02 log$_{10}$) and of 84–96% for faecal coliforms and enterococci (i.e. in a range of 0.80–1.39 log$_{10}$). These microbial reduction values are in agreement with previous reports for similar wastewater treatment systems (Rose *et al.*, 1996; George *et al.*, 2002; Blanch *et al.*, 2003).

Relative decreases of COD and BOD observed in March and July ranged, respectively, from 76 to 88% and from 93 to 94%. Lower values were observed in November because of maintenance to the treatment plant: 42 and 47% of COD and BOD, respectively. This maintenance operation was responsible for the release of treated wastewater with COD and BOD values slightly higher than those legally established (125 and 25 mg O$_2$ L$^{-1}$, respectively) (Council Directive 91/271/EEC, 1991). Interestingly, the removal of microorganisms was not related to the wastewater COD or BOD reduction, given that in November the decrease in bacterial counts of the treated wastewater was similar to those recorded in January, March and July. These results suggest that the relative bacterial counts in treated and in raw

Table 1. Chemical and microbiological quality parameters of raw and treated water sampled at four different times of the year

<table>
<thead>
<tr>
<th></th>
<th>Raw water</th>
<th>Treated water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>January</td>
<td>March</td>
</tr>
<tr>
<td><strong>CFU x 10$^6$ 100 mL$^{-1}$</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total heterotrophs</td>
<td>52.0</td>
<td>200.0</td>
</tr>
<tr>
<td>Total coliforms</td>
<td>ND</td>
<td>139.0</td>
</tr>
<tr>
<td>Faecal coliforms</td>
<td>ND</td>
<td>2.5</td>
</tr>
<tr>
<td>Faecal streptococci</td>
<td>4.4</td>
<td>3.8</td>
</tr>
<tr>
<td>Oxygen demand (mg O$_2$ L$^{-1}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemical demand</td>
<td>ND</td>
<td>291.0</td>
</tr>
<tr>
<td>Biochemical demand</td>
<td>ND</td>
<td>233.0</td>
</tr>
</tbody>
</table>

ND, not determined.
wastewater may not be related to the metabolic activity of microbiota constituting the activated sludge.

**Isolation and characterization of enterococci and related bacteria**

During one year, a total of 167 Gram-positive, catalase-negative cocci were isolated from raw and treated wastewater. Among these isolates, 148 were confirmed as *Enterococcus* spp. based on their ability to grow and produce blackening on BEA, and to grow at 45 °C and in the presence of 6.5% NaCl, after 48 h of incubation. Nineteen isolates produced negative reactions to at least one of these tests and therefore were considered to represent nonenterococci.

Genotyping of the isolates led to the establishment of seven clusters (Figs 1 and 2), from which representative isolates were identified by analysis of their partial 16S rRNA gene sequences, comprising ~450 nucleotides. *Enterococcus hirae* isolates were included in a single cluster, comprising 65 members, defined at approximately 15% rescaled distance. *Enterococcus faecium* isolates were separated into two clusters formed, respectively, at about 12.5 and 15% rescaled distance. Within this latter cluster it was possible to distinguish a subcluster composed of three *E. avium* isolates. The isolates belonging to *E. faecalis* and *E. durans* formed two distinct clusters at 15% rescaled distance. Thirteen of the nonenterococcal isolates clustered together at about 20% rescaled distance and were identified as representing *Lactococcus lactis*. Six isolates could not be typed by RAPD, but 16S rRNA gene sequence analysis demonstrated that they were related to *Lactococcus raffinolactis* (~96%) (two isolates), *Streptococcus bovis* (~99%), *E. pseudoavium* (~100%) and *E. saccharolyticus* (~96%) (two isolates). Whereas the *Lactococcus* spp. strains were isolated from raw wastewater, the others were detected in treated wastewater.

**Enterococcal species distribution**

The most abundant species detected in raw wastewater was *E. hirae*, whereas *E. faecium* and *E. faecalis* were found in lower but similar proportions. The relative abundance of *E. faecalis* was similar in treated and in raw wastewater; it was the least abundant enterococcal species found in the treated wastewater (Fig. 3). Wastewater treatment led to a reduction in *E. hirae* (*z < 0.1*) but an increase in *E. faecium* (*z < 0.1*), with the two species reaching equivalent frequencies in treated wastewater. This result may reflect a higher intrinsic resistance to environmental stress of *E. faecium* in comparison with *E. hirae* (Renner & Peters, 1999).

The predominance of *E. hirae* and the increase in *E. faecium* during wastewater treatment were not observed by (Blanch et al., 2003) in a study involving a large number of enterococci isolated from urban wastewater treatment.
plants in Sweden, the UK and Spain. Blanch et al. also used m-Enterococcus agar as the isolation medium but pre-enrichment in Brain Heart Infusion was made in order to promote recovery of stressed cells. This latter procedure may explain the discrepancies observed between the results from the two studies.

In a comparison of enterococcal populations from human, animal and environmental origin in the UK, Spain, Denmark and Sweden, E. faecalis was found to be the most prevalent species, both in urban and in hospital sewage in Sweden, whereas in the same sample types, E. faecium was predominant in Spain and in the UK (Kuhn et al., 2003). The authors found a similar distribution of enterococci in surface water as in the treated sewage. According to Kuhn et al. (2003), E. hirae is predominant in environments associated with cattle and pig effluent. This may suggest that the high percentages of E. hirae found in the present study were due to contamination of agricultural land runoff and/or nonauthorized discharges from cattle and pig farms into the sewage system.

**Antibiotic resistance phenotyping**

A total of 133 enterococcal strains were characterized for their phenotype of antibiotic resistance/susceptibility (Table 2). Within the isolates tested, a low prevalence of resistance was observed for amoxicillin and sulfamethoxazole/trimethoprim. By contrast, in the isolates identified as E. faecalis and E. faecium, the prevalence of resistance observed to erythromycin, ciprofloxacin and tetracycline ranged between 23 and 57%. E. hirae clearly had the lowest prevalence of antibiotic resistance, a fact that may be due to its comparatively low resistance in water. Indeed, with such a short life span in water, these organisms have reduced ability to act as receptors in processes of horizontal gene transfer, and consequently will not have major relevance as disseminators of antibiotic resistance. The high levels of resistance to gentamicin (10 μg) were expected given that enterococci are intrinsically resistant to low concentrations of this antibiotic, although a few susceptible isolates were found. Twenty-seven enterococci isolates chosen at random were tested for resistance to a higher concentration of gentamicin (120 μg); only two isolates belonging to E. faecium were found to be resistant. Moreover, among the 65 isolates tested for resistance to vancomycin (30 μg), none was resistant.

Among the 12 isolates of Lactococcus lactis (one could not grow on Mueller Hinton agar), seven were resistant to ciprofloxacin and three presented with an intermediary phenotype. Considering that L. lactis is typically associated with food products and is used in food production, this result may indicate that regular and extensive antibiotic resistance screening may be desirable in strains of this and other genera used in similar procedures.

**Effect of wastewater treatment on antibiotic resistance phenotype**

Except for ciprofloxacin, the wastewater treatment did not select positively or negatively for antibiotic resistance phenotypes. Similarly, the treatment process was not related to any variation in the prevalence of isolates exhibiting resistance to more than one antibiotic. Indeed, for ciprofloxacin, wastewater treatment led to an increase in the prevalence of resistant enterococci. Moreover, the phenotype to ciprofloxacin resistance was three times higher in the treated than in the raw wastewater ($x < 0.02$). Despite the fact that horizontal gene transfer between enterococci has been described as a general mechanism of antibiotic resistance acquisition (Klare et al., 2003), this may not be the only explanation for the results observed in the present study. The results indicate that the significant increase in ciprofloxacin resistance may be due, at least in part, to the increase in the proportion of E. faecium, with high prevalence of resistance to ciprofloxacin, and the simultaneous decrease of E. hirae populations in the treated wastewater. Given the character of opportunistic pathogens together with their high survival rate during wastewater treatment and the high prevalence of the antibiotic resistance phenotype, monitoring levels of E. faecium and of E. faecalis is strongly recommended.

Our results clearly showed that in the plant studied, treatment is not efficient in eliminating commensal antibiotic-resistant enterococci from wastewater. According to these results, similar treatment plants may act as permanent suppliers of antibiotic-resistant bacteria to the environment, leading to a continuous dissemination and accumulation of resistant organisms in environmental water.
Antibiotic resistance of enterococci in wastewater

Table 2. Prevalence (%) of enterococci with resistance (or intermediary) phenotype for the antibiotics tested*

<table>
<thead>
<tr>
<th>Enterococcus faecalis</th>
<th>AML</th>
<th>GEN</th>
<th>ERY</th>
<th>CIP</th>
<th>SXT</th>
<th>TET</th>
<th>R &gt; 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of isolates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>133</td>
<td>2 (1)</td>
<td>50 (44)</td>
<td>27 (21)</td>
<td>18 (22)</td>
<td>1</td>
<td>32 (20)</td>
</tr>
<tr>
<td>Raw water</td>
<td>56</td>
<td>0</td>
<td>50 (41)</td>
<td>33 (7)</td>
<td>9 (20)</td>
<td>0</td>
<td>31 (18)</td>
</tr>
<tr>
<td>Treated wastewater</td>
<td>77</td>
<td>3 (1)</td>
<td>50 (46)</td>
<td>23 (30)</td>
<td>25 (23)</td>
<td>1</td>
<td>33 (21)</td>
</tr>
<tr>
<td>Other4</td>
<td>8</td>
<td>13</td>
<td>0 (100)</td>
<td>71</td>
<td>25</td>
<td>14</td>
<td>29 (25)</td>
</tr>
<tr>
<td>Enterococcus hirae</td>
<td>52</td>
<td>0</td>
<td>41 (46)</td>
<td>7 (2)</td>
<td>2</td>
<td>0</td>
<td>13 (4)</td>
</tr>
<tr>
<td>Enterococcus faecium</td>
<td>43</td>
<td>2 (2)</td>
<td>39 (61)</td>
<td>40 (40)</td>
<td>33 (26)</td>
<td>0</td>
<td>38 (30)</td>
</tr>
<tr>
<td>Other4</td>
<td>8</td>
<td>13</td>
<td>0 (100)</td>
<td>71</td>
<td>25</td>
<td>14</td>
<td>29 (25)</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>30</td>
<td>0</td>
<td>100</td>
<td>35 (35)</td>
<td>23 (60)</td>
<td>0</td>
<td>57 (30)</td>
</tr>
</tbody>
</table>

*None of the 65 isolates tested was resistant to vancomycin.

1Twelve isolates of Enterococcus hirae, one of Enterococcus faecalis and one of Enterococcus faecium were not able to grow on Mueller Hinton agar and were not tested for antibiotic resistance phenotype.

1R > 1. Resistant to more than one antibiotic; as all Enterococcus faecalis strains were resistant to GEN, this antibiotic was not considered for this analysis.

4Other refers to Enterococcus avium and Enterococcus durans isolates.

AML, amoxicillin; GEN, gentamicin; ERY, erythromycin; CIP, ciprofloxacin; SXT, sulfamethoxazole/trimethoprim; TET, tetracycline.

Ecology of antibiotic-resistant enterococci

The increased use of antimicrobials useful for treatment of infections is seen as an important factor contributing to the selection of bacteria with natural resistance or only weakly susceptible to target antibiotics. This explains the evident enhancement of infections caused by enterococci resistant to antibio-therapy (Klare et al., 2003). In a Portuguese medical study involving 12184 prescriptions of antibiotics corresponding to 11982 disease episodes, an annual antibiotic prescription frequency of 9.3% (prescriptions per 100 spondings) was reported (Observatório Nacional de Saúde. Médicos Sentinela (ONSA), 2002). In that study the most frequently prescribed antibiotics were penicillins (47%, of which 11% was amoxicillin), macrolides (16%, of which 2% was erythromycin), quinolones (15%, of which 7% was ciprofloxacin), cephalosporines (12%), sulfonamides (5%) and tetracyclines (2%). Comparing these data with the resistance prevalence values observed in the present study, it may be hypothesized that the clinical use of macrolides and quinolones has influenced the positive selection for resistant strains. By contrast, it seems that the clinical use of penicillins has not led to an increase in the prevalence of enterococci resistant to these antibiotics in wastewater.

Assessing the mechanisms of antibiotic resistance dissemination requires an integrated approach, including comparisons of the resistance prevalence among clinical, food and environmental isolates. In a study with E. faecium and E. faecalis isolated from animal and food products, Peters et al. (2003) observed that all of the 118 isolates were sensitive to ampicillin and to amoxicillin/clavulanic acid. In the present study, two strains of E. faecium and E. avium were found to be resistant to amoxicillin, confirming that environmental enterococci maintain high levels of sensitivity to beta-lactamic antibiotics. These observations are consistent with those obtained with clinical isolates (e.g. Fluit et al., 2000; Mutnick et al., 2003).

The observed levels of resistance to other antibiotics among nonclinical isolates, namely to tetracycline, erythromycin and ciprofloxacin, were higher both in the present study and in previous works. For tetracycline, resistance prevalence values of about 30% are reported for enterococci isolated from animal and food products (Peters et al., 2003; Hayes et al., 2004; Johnston & Jaykus, 2004), whereas among clinical and human isolates this prevalence is above 50% (Fluit et al., 2000; Mondino et al., 2003; Mutnick et al., 2003; Udo et al., 2003). The tetracycline resistance prevalence observed in the present study, mainly for E. faecalis, was closer to that observed for clinical isolates. The resistance of enterococci to macrolide antibiotics, namely erythromycin, is of concern because of the high prevalence of intermediary or resistance phenotypes observed in animal or food products or in wastewaters (Blanch et al., 2003; Peters et al., 2003; Hayes et al., 2004; Johnston & Jaykus, 2004; the present study). Intermediary levels of resistance to erythromycin (26–82%) in E. faecalis and E. faecium are reported by these authors. Moreover, Blanch et al. (2003) report prevalence values of erythromycin-resistant enterococci above 60% in raw, treated and hospital wastewaters, as well as in surface waters receiving the treated effluents, suggesting their environmental dissemination.

It was concluded in the present study that wastewater treatment may lead to the positive selection of enterococci showing a resistance phenotype to ciprofloxacin. Previous reports, focused both on animal or food products and on clinical isolates, have evidenced a widespread distribution of enterococci with intermediary or resistant phenotype to quinolone antibiotics, mainly in E. faecium, in which resistance prevalence may vary between 28% (Johnston & Jaykus, 2004) and 65% (Peters et al., 2003). In a study with enterococci isolated in medical centres from different European countries, Mutnick et al. (2003) reported a ciprofloxacin resistance prevalence of about 35%. Similar findings were reported by Fluit et al. (2000) in a European
surveillance study with isolates from urinary tract infections, one of the most frequent infections caused by enterococci; only 64% of the strains were found to be susceptible to ciprofloxacin. These reports, together with the present study, suggest that ciprofloxacin resistance prevalence in enterococci of food, animal and environmental origin is at similar levels to those observed in clinical isolates.

Enterococcus spp. resistant to vancomycin have been identified recently as a major issue of concern, as they have been found associated with nosocomial infections, in food products and even in sewage (Iversen et al., 2002; Blanch et al., 2003; Klare et al., 2003; Klein, 2003; Witte, 2004). The absence of vancomycin resistance among the enterococcal strains isolated during the present study may be due to the methodology used. According to Nayak et al. (2002), the disc diffusion assay may provide unreliable results for vancomycin resistance testing in enterococci, because the results do not correlate with those based on determination of resistance genes.

The underestimation of antibiotic resistance prevalence, using exclusively culturable organisms and phenotyping methods, has been highlighted recently (Schwartz et al., 2004; Volkmann et al., 2004). According to the evidence given in these studies, screening of total DNA for resistance genes provides additional insights into antibiotic resistance prevalence and dissemination. Such an approach represents an important complement to cultivation-dependent methods, allowing the detection of resistance genes in nonculturable bacteria and inferences to be made regarding the processes of horizontal gene transfer.

The present study provides evidence that antibiotic-resistant enterococci are not eliminated during wastewater treatment consisting of primary and secondary activated sludge processes. Moreover, a positive selection for antibiotic-resistant bacteria may occur during the overall treatment process. It was also observed that E. faecium, an opportunistic pathogen, was positively selected during wastewater treatment, with an increase in its relative proportion in the treated wastewater. Given that treated wastewater is usually released to rivers or coastal waters, a progressive change to the microbial ecosystem, namely in the antibiotic-resistant enterococci populations, may occur. This kind of change was evidenced recently by Novais et al. (2005). And events of this kind may explain the similar prevalence of resistance to some antibiotics found in food products, wastewater, or among human and clinical isolates.

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