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Food-Borne Enterococci and Their Resistance to Oxidative Stress

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Enterococci are important food-borne pathogens that cause serious infections. Several virulence factors have been described including aggregation substance, gelatinase, cytolysin, and enterococcal surface protein. The ability to cause infections is mainly dependent on the response to oxidative stress due to the production of reactive oxygen species by immune cells. The aim of our study was to analyze the resistance of enterococcal strains from food to clinically relevant antiseptic agents with regard to the presence of selected virulence factors, and to uncover potential mechanisms of the antioxidative resistance. Eighty-two enterococcal isolates from Bryndza cheese were tested using in vitro growth assays to study the ability of these isolates to survive exposure to antiseptic agents – hydrogen peroxide, hypochlorite, and chlorhexidine. Virulence genotypes of the isolates were determined by PCR, and RT real time PCR was used for gene expression under oxidative stress. Resistance against antiseptic agents depends on the concentration of applied chemicals, on the time of exposure, but also on virulence factors of the enterococcal strains. Oxidative stress induces the expression of antioxidative enzymes and down-regulates the expression of prooxidative enzymes. These effects are dependent on the virulence genotype of the enterococcal strains. These findings are important for future research, especially concerning the role of enterococci in oral diseases.

Keywords: *Enterococcus faecalis*, reactive oxygen species, oxidative stress, free radicals, infection, virulence

The genus Enterococcus contains various species of gram-positive bacteria commonly present in mammalian organisms, including humans, in the food microflora and in the environment. The physiology of these generally nonpathogenic organisms enables their ubiquitous occurrence and ability to survive and grow under harsh conditions. They have a unique tolerance against oxidative stress, grow in salty and acidic environment, at a wide range of temperature (10-45°C), at pH 9.6, in the presence of 6.5% NaCl, and are able to survive at 60°C (Sherman, 1937). Enterococci naturally colonize the human intestinal tract as part of the commensal flora. Due to probiotic characteristics, some enterococcal strains are intentionally used as part of food supplements. On the other hand, some strains can cause severe and clinically important nosocomial infections, frequently with a high level of antibiotic resistance (Giraffa *et al.*, 1997).

The source of pathogenic enterococci can be the exogenous environment, or endogenous microflora naturally present in the human gut after their translocation (Huycke *et al.*, 1998). With a frequency of 12%, enterococci are the second to third most important and clinically relevant bacterial genus causing hospital acquired infections. Only two of all known species of this genus are considered to be responsible for the vast majority of human enterococcal infection. *E. faecalis* was reported as the predominant enterococcal species, accounting for almost 90% of all clinical enterococci isolates, while *E. faecium* was isolated in up to 15% of clinical cases (Hidron *et al.*, 2008). The most frequent diseases caused by enteroccocci strains reported were urinary tract infections and wound infections especially in immunocompromised and postsurgical patients. In these cases, similar to other infections, an interaction among various bacteria was documented, additionally enterococci synergistically increase infectivity of bacterial mixtures (Low *et al.*, 2001). Intensive care unit patients are at high risk of acquiring bacteremia infections caused by enterococci. Up to 63% of enterococcal isolates causing clinically relevant bacteremia are multi-drug resistant (Shaked *et al.*, 2006). Cases of infection with high-level of gentamycin resistance isolates are more frequently associated with serious disease and higher mortality (Jang *et al.*, 2010).

Transfer of DNA between bacteria can happen via different mechanisms. In enterococci, cell-cell contact is dependent on the presence of the aggregation substance. This adhesion factor is encoded by genes localized on sex pheromone plasmids (Galli and Wirth, 1991) and facilitate the exchange of genetic material between bacterial cells. *E. faecalis* strains with the *asa1* gene encoding the best described aggregation substance protein bind to several extracellular proteins like fibronectin, thrombospondin or collagen type I (Rozdzinski *et al.*, 2001). Enterococci can directly kill neighboring cells, either prokaryotic or eukaryotic. The mechanism lies in the disruption of cell membrane by the cytolysin complex (Haas and Gilmore, 1999). It consists of two different components that are post-transcriptionally modified and act together in the process of membrane disruption. The presence of enterococcal surface protein, Esp, is tightly correlated with biofilm production in

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clinical isolates of *E. faecalis*. Direct disruption mutagenesis of the coding gene results in a loss of phenotype, the loss of ability to form biofilms on abiotic surfaces (Toledo-Arana *et al.*, 2001). A crucial virulence factor of *E. faecium* is the microbial metalloendopeptidase called gelatinase. The nucleotide sequence the *gelE* gene encodes an open reading frame with homology to Bacillus proteinase and Pseudomonas elastase. Although the mature active enzyme consists of 318 amino acid residues, it is produced as a larger preproenzyme (Su *et al.*, 1991). Activation requires not only the cleavage of a N-terminal signal sequence but also processing of the C-terminus, which is unique among the known metalloproteases of Gram-positive bacteria (Del Papa *et al.*, 2007).

The potential of enterococci to survive the attack of immune cells and to cause infections is mainly dependent on their ability to resist oxidative stress, as free radicals and other reactive oxygen species are used by neutrophils and macrophages as part of the primary non-specific immune reaction. The clinical importance of antioxidative enzymes was shown for superoxide dismutase (Bizzini *et al.*, 2009), NADPH peroxidase and other enzymes (La Carbona *et al.*, 2007). The response to oxidative stress and its regulation in enterococci are described in detail in a published review (Riboulet *et al.*, 2007). Whether known virulence factors of enterococci are linked with survival under oxidative stress is currently unknown.

The aim of our study was to analyze the resistance of enterococcal strains from food to oxidative stress and to clinically relevant antiseptic agents with regard to the presence of selected virulence factors. In addition, expression of genes related to oxidative stress was analyzed to uncover potential mechanisms of antioxidative resistance.

Materials and Methods

Bacterial strains and their identification

Enterococcal strains isolated from Bryndza cheese samples gathered in Liptov and Tisovec regions (Slovakia) in 2000 and 2001 were analyzed *in vitro*. Eighty-two strains previously phenotyped as *E. faecium* and *E. faecium* were grown at 37°C in LB medium from glycerol stocks. Multiplex PCR was used for the determination of strain specificity. Target genes suitable for discrimination are D-alanine: D-alanine ligases as published previously (Dutkamalen *et al.*, 1995). Hot start master mix plus (QIAGEN, Germany) was used for the PCR. Templates for PCR were prepared from overnight cultures of enterococci in LB medium. Five microliter of the culture were suspended in 195 μl of deionized water in PCR plates. After thorough mixing, bacterial suspensions were heated for 5 min at 95°C in a thermal cycler and centrifuged. The supernatant was used as the PCR template.

Detection of virulence factors

Virulence factors were genotyped by using a previously published multiplex PCR targeted at *asa1*, *gelE*, *cylA*, and *esp* genes (Vankerckhoven *et al.*, 2004). The same template prepared for species identification was also used for genotyping virulence factors. Hot start master mix plus (QIAGEN) was used for the PCR. Agarose electrophoresis was used for PCR product identification and size determination.

In vitro exposure assays

All selected enterococcal strains were subjected to *in vitro* exposure assays. Overnight cultures of the strains in LB medium in deep-well plates were inoculated (40 μl) into fresh LB medium (150 μl) and the particular exposure agent (10 μl). Exposure agents included hydrogen peroxide, chlorhexidine and sodium hypochlorite (all chemicals, Merck, Germany). Deionized water served as the control. All growth assays were done in polystyrene microplates (Sarstedt, Germany) to enable direct OD 600 measurements (Tecan, Austria). OD measurements were performed every hour for 5 h and after an overnight growth at 37°C in duplicates.

Gene expression analysis

In the hydrogen peroxide assay, RNA was isolated with the RNeasy 96 kit (QIAGEN) after 5 h of cultivation in 0.025% hydrogen peroxide. Concentration and purity of the RNA was checked using the Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). Isolated RNA was used as a template for RT real time PCR. One-step QuantiFast SYBR Green RT-PCR kit (QIAGEN) was used for the analysis of gene expression on iCycler iQ5 (Bio-Rad, USA) and ABI PRISM 7900HT sequence detection system (Applied Biosystems). RecA was chosen as a housekeeping gene. Ct values were collected with the proprietary software at a constant fluorescence threshold. Delta delta Ct method was used for the relative quantification of expression of the particular genes. Melting curve analysis was performed to check the specificity of the PCR products.

Statistical analysis

All data were analyzed using the Graphpad Prism 5, SPSS 16, and XLStatistics 5 software. ANOVA with post hoc LSD test was used for the analyses. P-values less than 0.05 were considered significant. Data are presented as Mean±SD. In graphs, significance is shown using asterisks and plus signs: *denotes $p<0.05$; **$p<0.01$; ***$p<0.001$ in comparison to the respective virulence genes negative group; $^{+}$denotes $p<0.05$; $^{++}p<0.01$; $^{+++}p<0.001$ in comparison to *E. faecium*.

Results

Analyzed strains of enterococci showed similar abilities to grow at 37°C under control conditions. There were no significant differences between groups divided according to virulence genotyping. In addition, the growth of *E. faecium* and *E. faecalis* strains did not differ. At the concentration of 0.2%, all of the analyzed chemicals were able to prevent the growth of enterococci (data not shown). At a lower concentration of 0.025%, all of the analyzed virulence genes seem to improve the survival of strains under oxidative stress induced by hydrogen peroxide. This is especially clear and significant for ASA and marginally insignificant for GEL (Fig. 1). Interestingly, chlorhexidine did not prevent growth at this concentration. No significant differences between the analyzed groups were seen in cultivation with chlorhexidine either.

Short-time exposure to antiseptic chemicals is more relevant for clinical use. Although exposure to 0.2% hydrogen peroxide clearly slowed down the growth of enterococci, ASA+ and GEL+ strains were considerably better able to resist oxidative stress. In addition, if exposed to hypochlorite or chlorhexidine, GEL+ and CYT+ strains had higher absorbance values in comparison to strains without these virulence factors. *E. faecium* performed worse than *E. faecalis* when exposed to chlorhexidine (Fig. 2).

Hydrogen peroxide is not only a widely clinically used anti-

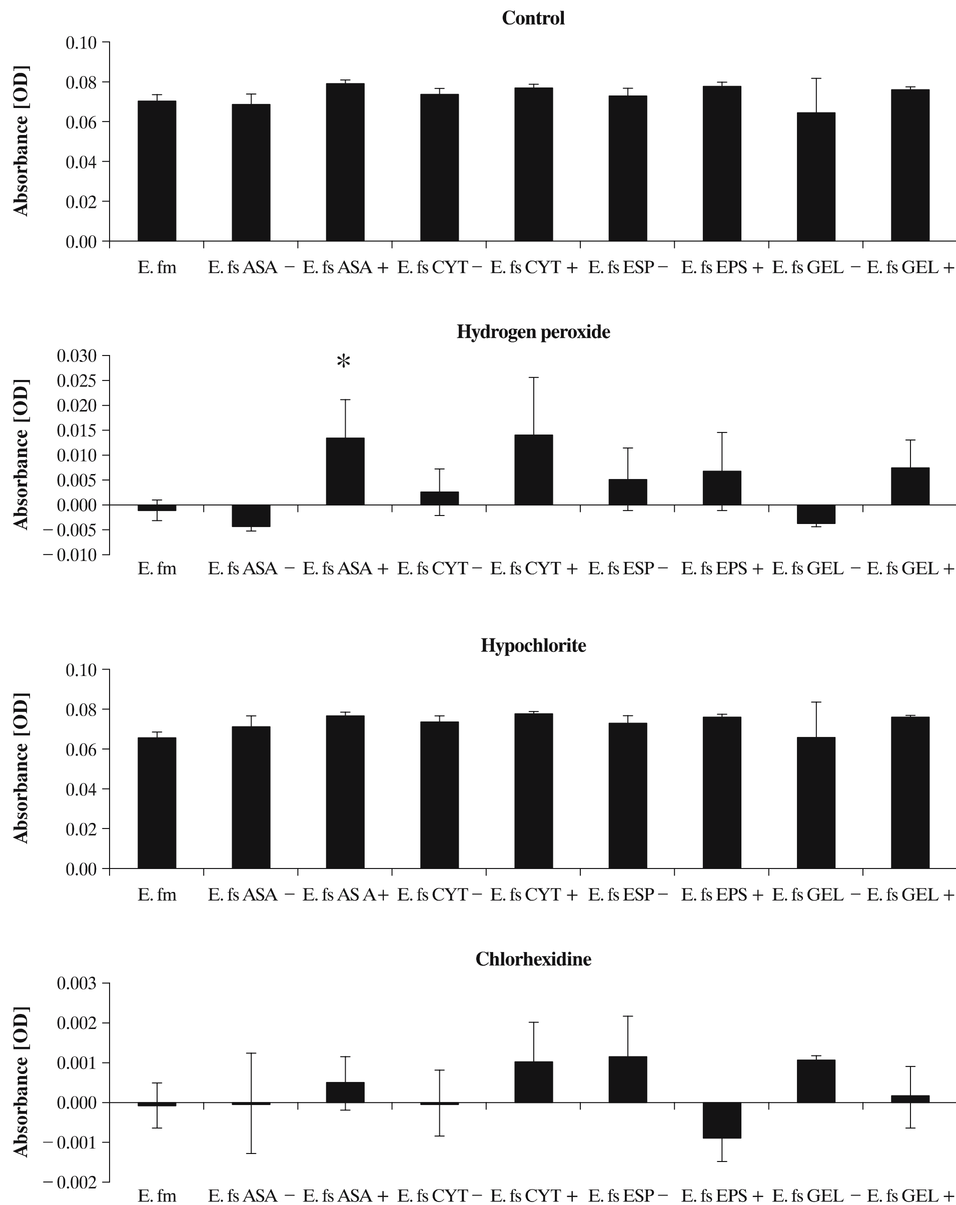

Fig. 1. Growth slope (5 h) of enterococcal strains grouped according to species and virulence genotype in LB medium enriched with 0.025% of the respective chemicals.

septic agent, but it also belongs to reactive oxygen species and induces oxidative stress. Thus, the expression of genes related to oxidative stress was analyzed in enterococci cultivated with 0.025% hydrogen peroxide using RT real time PCR. Oxidative stress decreased the expression of NADH oxidase (NOX), an enzyme catalyzing the production of superoxide anion radical. NADH peroxidase (NPR) expression was not affected. Other analyzed antioxidative enzymes were up-regulated by cultivation with hydrogen peroxide, modeling oxidative stress conditions. Superoxide dismutase (SOD) expression was increased similarly in all groups of strains (Fig. 3). On the contrary, there were significant effects of the presence of virulence genes regarding the expression of catalase and alkylhydroperoxide reductase (AHPC). Especially, ESP+ and GEL+ strains could better respond to hydrogen peroxide by increased expression of antioxidative enzymes (Fig. 4).

Discussion

Enterococci are important food-borne pathogens, but they also belong to normal physiological gastrointestinal flora. Clinically important enterococcal infections include bacteremia and sepsis, endocarditis and urinary tract infections. However, the possibilities of detecting bacteria by sensitive cultivation and especially by molecular methods, helped to uncover the role of enterococci in oral diseases (Rams *et al.*, 1992).

In vitro studies revealed that the main virulence function of *E. faecalis* in endodontic infections is probably the ability

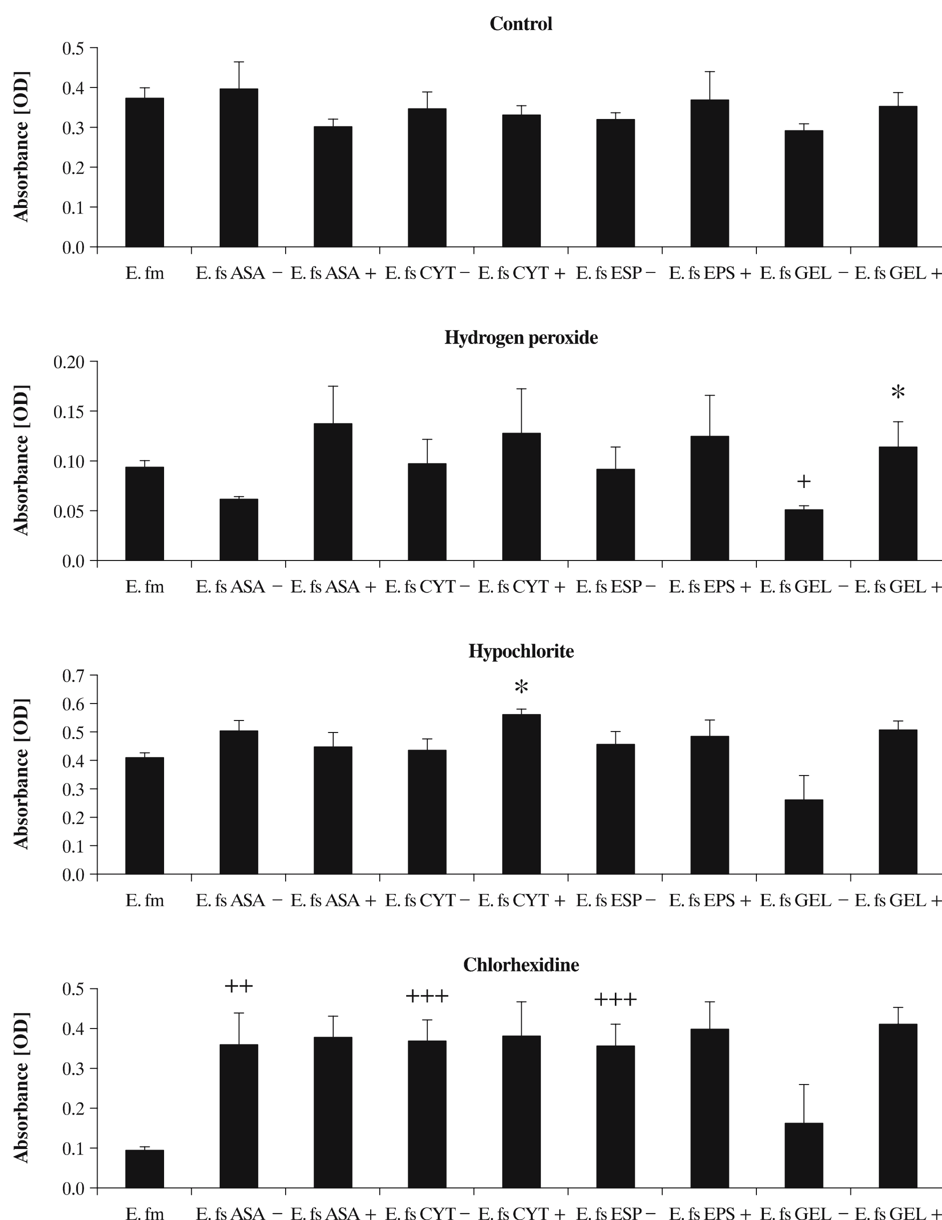

Fig. 2. Absorbance of enterococcal strains grouped according to species and virulence genotype after 4 min exposure to 0.2% of the respective chemicals.

to bind to collagen, which might be related to several described virulence genes (Love, 2001). Another important virulence determinant is pH resistance. In a direct comparison, *E. faecalis* is similarly resistant to acidic environment and even more resistant to alkali conditions than *S. mutans* (Nakajo *et al.*, 2006). Virulence factors of enterococci and their role in endodontic infections were reviewed previously (Kayaoglu and Orstavik, 2004).

Antimicrobial agents like hydrogen peroxide, sodium hypochlorite and especially chlorhexidine are used routinely in the clinics for disinfection of the root canal. The ability of enterococci to survive under these conditions is of special importance for recurrent endodontic infections. *In vitro* studies try to model the situation and analyze the antimicrobial effects of these chemicals against *E. faecalis* (Dametto *et al.*, 2005). Although the results are not consistent between studies, in most experiments depending on the concentration used, these antimicrobials are effective in killing clinical isolates of *E. faecalis* (Dunavant *et al.*, 2006; Davis *et al.*, 2007). The antimicrobial activity seems to not be affected by time since preparation of the teeth accompanied the infection (Oliveira *et al.*, 2007).

The results of our *in vitro* assays show that clinically used antiseptic agents are effective. However, their effect on enterococcal strains depends on time and concentration. Diluted solutions acting for a short time might not be effective, especially, if the target enterococci possess some of the virulence factors that were revealed as protective against hydrogen per-

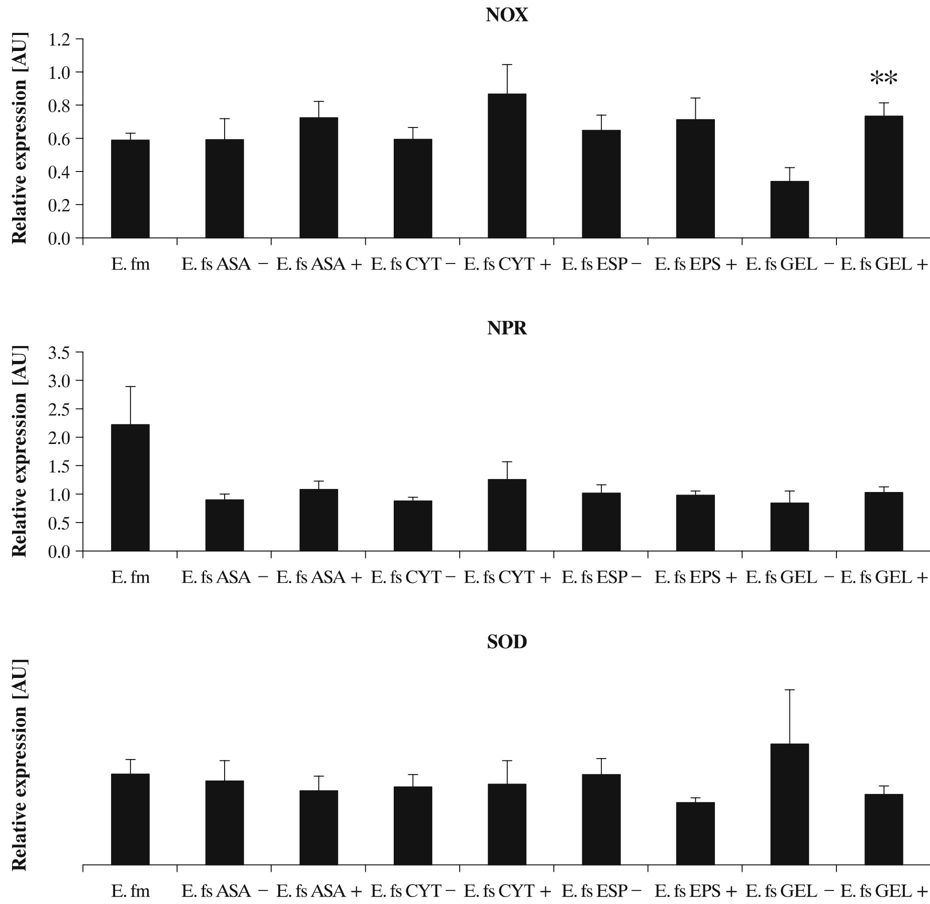

Fig. 3. Changes in expression of genes related to oxidative stress in enterococcal strains grouped according to species and virulence genotype cultivated with 0.025% hydrogen peroxide in comparison to control conditions. NOX, NADH oxidase; NPR, NADH peroxidase; SOD, superoxide dismutase.

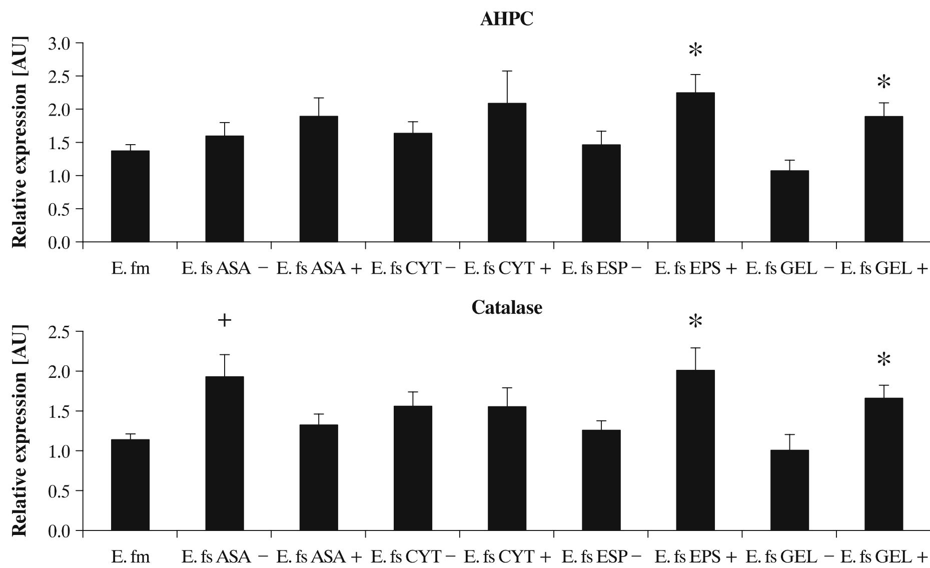

Fig. 4. Changes in expression of genes related to oxidative stress in enterococcal strains grouped according to species and virulence genotype cultivated with 0.025% hydrogen peroxide in comparison to control conditions. AHPC, alkylhydroperoxide reductase.

oxide, hypochlorite or chlorhexidine treatment. These include gelatinase and the aggregation substance. The mechanism of the protection can currently only be speculated, but the interaction between the surface of the bacterial cell and the environment, including free radical producing leukocytes may play a major role.

An additional mechanistic insight can be drawn from the analysis of gene expression in enterococci under oxidative stress. Gelatinase-positive strains and strains with the enterococcal surface protein especially had a more robust response to hydrogen peroxide, including a stronger induction of antioxidative enzymes like alkylhydroperoxide reductase and catalase that can metabolize and detoxify hydrogen peroxide from acting as a reactive oxygen species. Potentially of clinical importance is the finding that gelatinase-positive strains were able to maintain the expression of the superoxide-producing NADH oxidase even under oxidative stress. This might point towards active use of free radicals as virulence factors.

In conclusion, the ability of these strains to resist oxidative stress and clinically used antiseptic agents depends not only on time of exposure and concentration of the used chemicals, but also on the virulence genotype of the enterococcal strains.

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