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Research Article

Different protein expression profiles in cheese and clinical isolates of *Enterococcus faecalis* revealed by proteomic analysis[†]

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Abstract

The use of *Enterococcus faecalis* in the food industry has come under dispute because of the pathogenic potential of some strains of this species. In this study, we have compared the secretome and whole-cell proteome of one food isolate (*E. faecalis* DISAV 1022) and one clinical isolate (*E. faecalis* H1) by 2-DE and iTRAQ analyses, respectively. Extracellular protein patterns differed significantly, with only seven proteins common to both strains. Notably, only the clinical isolate expressed various well-characterized virulence factors such as the gelatinase coccolysin (GelE) and the extracellular serine proteinase V8 (SprE). Moreover, various other putative virulence factors, e.g. superoxide dismutase, choline- and chitin-binding proteins and potential moonlighting proteins, have been detected exclusively in the secretome of the clinical isolate, but not in the food isolate. The iTRAQ analysis of whole-cell proteins of the two strains highlighted a stronger expression of

pathogenic traits such as an endocarditis-specific antigen and an adhesion lipoprotein in the pathogenic strain *E. faecalis* H1. Subsequently, six food isolates (including *E. faecalis* DISAV 1022) and six clinical isolates (including *E. faecalis* H1) were tested for the presence of gelatinase and protease activity in the culture supernatants. Both enzymatic activities were found in the clinical as well as the food isolates which clearly indicates that protease expression is strain specific and not representative for pathogenic isolates. Genetic analyses revealed that not only the gelatinase and serine protease genes but also the regulatory *fsr* genes must be present to allow protease expression.

Keywords:

2-DE;Endocarditis;fsr locus;Microbiology;Moonlighting proteins;Proteases;Superoxide dismutase

1 Introduction

Food fermentation processes are achieved by various microorganisms that together contribute to the final taste and organoleptic properties of a certain product. In cheese, the microbial population is generally composed of: (i) spontaneous bacteria originally present in the milk, (ii) starter strains selected for a specific performance and artificially introduced during industrial food production, (iii) contaminant bacteria accidentally present in a certain food sample (spoilage microorganisms). Generally, spontaneous food microbiota and starter strains contribute co-operatively to biotransformations, i.e. the conversion of macromolecules such as proteins, lipids, and fatty acids into smaller components, thereby modifying the cheese texture and resulting in particular flavors 1. In contrast, spoilage bacteria can negatively influence the organoleptic properties of cheese 2, are able to produce harmful molecules such as bioactive biogenic amines 3 and are potentially pathogenic for humans 4. It is becoming more and more evident that a clear separation between “safe” and “harmful” bacteria in the context of foodstuffs is artificial, as in some cases starter strains have been proven to be potentially pathogenic 5.

A good example for a group of organisms expressing beneficial as well as pathogenic traits are members of the genus *Enterococcus* 6. Species belonging to this genus are ubiquitous and inhabit very heterogeneous niches such as soils, vegetables, food and the gastro-intestinal tract of humans and animals. Enterococci tolerate high NaCl concentrations (up to 6.5%), are viable under a wide pH (4.6–9.6) and temperature range (10–45°C) and can survive thermal treatments such as pasteurization (30 min at 62.8°C) 7.

Indigenous enterococci are considered essential for cheese flavor in most Southern European countries due to their proteolytic and esterolytic activity and to their diacetyl production accompanying citrate metabolism 8. They may get into milk either directly from animal feces or indirectly from contaminated water, milking equipment or the bulk storage tank 9. Enterococci, moreover, can be employed as starter strains for cheese production 10 and *Enterococcus faecalis* strains are even sold as probiotics in the product Symbioflor. However, unlike other lactic acid bacteria (LAB), enterococci are not considered as “Generally Recognized As Safe” (GRAS).

On the other hand, it is very well established that enterococci are among the most common nosocomial pathogens 11: due to their natural antibiotic resistance 12, they are often found in patients treated with antimicrobial agents for long periods, e.g. after trauma, during anticancer chemotherapy and after organ transplantation 13. The pathogenic potential of *Enterococcus* strains is especially linked to the production of β-hemolysins 14, hyaluronidases, proteinases and other extracellular or surface-bound adhesion or aggregation substances 9, 15 that enable colonization/invasion of host tissues. Genetic studies on *E. faecalis* have revealed the presence of a

so-called “pathogenicity island”, probably acquired by horizontal gene transfer as suggested by a different G+C content from the remainder of the genome 16. This genomic region encodes for aggregation substance, cytolysins and other virulence-associated proteins 17.

Some of the pathogenic traits found in clinical strains, i.e. coccolysin (gelatinase E) and the extracellular serine proteinase V8, are only expressed at high cell-densities and thus under control of a two-component regulatory *fsr* quorum sensing (QS) system 18–20. The *fsr* QS system consists of the oligopeptide pheromone FsrB, the histidine kinase sensor FsrC and the response regulator FsrA 21, 22 and has so far only been detected in clinical isolates of *E. faecalis*.

Genotypic and phenotypic analyses have demonstrated a statistically significant higher frequency of virulence factors, especially related to adhesion, hydrolytic enzymes and cytolysins, in clinical enterococci isolates compared with food-isolates or starter strains 23. However, enterococci with proven pathogenic potential, both at the phenotypic and genotypic level, were also isolated from food sources (e.g. different cheeses) 24. A recent secretome analysis of *E. faecalis* JH2-2, a culture collection strain, revealed the presence of various pathogenicity-associated traits, including coccolysin and proteins such as elongation factor Tu, chaperone DnaK and glycolytic enzymes, which might exhibit a second function, e.g. adhesion to host tissue 25.

The present study aims to evaluate the potential risks of using *E. faecalis* as starter cultures or even probiotics by comparative proteome analyses of one representative food- and one clinical isolated strain of *E. faecalis.* Moreover, the genomes of several strains derived from both the hospital and cheese were tested for the presence of genes coding for the virulence-related proteins gelatinase E (GelE) and serine protease V8 (SprE) and for their regulatory *fsr* system.

2 Materials and methods

2.1 Bacterial strains and culture conditions

The 12 *E. faecalis* strains used in this study are listed in Table 1: six of these were isolated from different Piedmont cheeses and the other six were isolated from hospitalized patients. *E. faecalis* DISAV1022 and *E. faecalis* H1, the strains used for the proteome analysis, were isolated from Robiola of Roccaverano, a Piedmont goat cheese, and from urine, respectively. All strains were maintained in an M17 medium at −24°C in 0.5 mL aliquots with 0.5 mL 40% v/v glycerol. Bacterial cultures were grown in the M17 medium in closed screw-cup bottles at 37°C without shaking. Bacterial growth was monitored by optical density at 600 nm (OD_{600}). For all proteomic, phenotypical and genetic experiments, culture supernatants or cells were recovered in the middle of the exponential growth phase. For both proteome analyses and phenotypical assays three biological replicates were performed.

Table 1. Origin of *E. faecalis* strains analyzed in previous studies 28 and in this study

Cheese-isolates		Clinical isolates	
Name	**Origin**	**Name**	**Origin**
E. faecalis DISAV1022	Robiola of Roccaverano	*E. faecalis* H1	Uroculture
E. faecalis B	Robiola of Roccaverano	*E. faecalis* H2	Open surgical wound
E. faecalis C	Robioletta	*E. faecalis* H3	Hemoculture
E. faecalis D	Robiola of Roccaverano	*E. faecalis* H4	Uroculture
E. faecalis E	Toma	*E. faecalis* H5	Hemoculture
E. faecalis F	Toma	*E. faecalis* H6	Hemoculture

2.2 Proteome analyses: Comparative proteome analyses of extracellular proteins by 2-DE

2.2.1 Extraction of extracellular proteins

Cells of three replicate 1.5 L cultures were harvested by centrifugation (3500×*g*, 4°C, 20 min) and culture supernatants were sterile-filtered through stericup 0.22 µm filters (Millipore). The extracellular proteins were precipitated with 16% w/v trichloroacetic acid while stirred overnight at 4°C. The resulting suspensions were then ultracentrifuged (35 000×*g*, 4°C, 90 min) and the pellets washed with 100% ethanol (35 000×*g*, 4°C, 20 min). The protein pellets were then dried, pulverized and resuspended in the smallest possible volume of 50 mM Tris-HCl, pH 7.3. The obtained samples were extracted with phenol as previously described 26. Briefly, 1 mL of phenol was added to 1 mL sample aliquots and the mixtures were incubated for 10 min at 70°C and for 5 min at 0°C and then centrifuged (7000×*g*, room temperature, 5 min). The upper phase and the solid interphase were discarded and 1 mL of MilliQ water was added to the lower phase, which was then incubated for 10 min at 70°C and for 5 min at 0°C and then centrifuged (7000×*g*, room temperature, 5 min) again. The upper phase was discarded and 1 mL of ice-cold acetone was added to the lower phase before incubating overnight at −20°C. Precipitated proteins were recovered by centrifugation (15 000×*g*, 20 min, 4°C) and washed with ice-cold acetone (15 000×*g*, 20 min, 4°C). Pellets were pulverized and resuspended in Tris-HCl 50 mM, pH 7.3. Proteins were quantified by the Bradford assay (BioRad) 27.

2.2.2 2-DE: Isoelectric focusing

The isoelectric focusing was performed using two different immobilized pH gradient (IPG) strips (GE Healthcare) as described elsewhere 28. Briefly, 13 cm IpG strips (GE Healthcare) with linear (L) pH gradients ranging from pH 4 to 7 and pH 6 to 11 were used. For the pH 4–7 strips, 350 µg of proteins were solubilized in rehydration solution A (6.5 M urea, 2.2 M thiourea, 4% w/v CHAPS, 5 mM Tris-HCl pH 8.8, 0.5% v/v IpG buffer (GE Healthcare), 100 mM DTT) and loaded by in-gel rehydration. IEF was performed using IPGphor (GE Healthcare) at 20°C with 78 000 Vh after 12 h of rehydration. For the pH 6–11 strips, 1100 µg of proteins were solubilized in 100 µL of rehydration solution B (6.5 M urea, 2.2 M thiourea, 4% w/v CHAPS, 5 mM Tris-HCl pH 8.8, 0.5% v/v IpG buffer (GE Healthcare), 1.2% w/v DeStreak (GE Healthcare)) and loaded by anodic cup loading 29 on strips previously rehydrated with 250 µL of rehydration solution B for 6 h. IEF was performed using IPGphor (GE healthcare) at 20°C with 28 000 Vh.

2.2.3 SDS-PAGE

After IEF strips were incubated at room temperature in 6 M urea, 30% v/v glycerol, 2% w/v SDS, 50 mM Tris-HCl pH 8.6, supplemented with 2% w/v DTT for 15 min and subsequently with 4.5% w/v iodoacetamide for 15 min. They were then sealed on top of 1.0 mm vertical second-dimensional gels as described previously 30. SDS-PAGE was carried out on 11.5%T and 3.3%C acrylamide homogenous gels. The running buffer consisted of 25 mM Tris, 192 mM glycine, 0.1% SDS. Running conditions were 11°C, 600 V constant voltage, 20 mA/gel, 60 W for 15 min, 11°C, 600 V constant voltage, 40 mA/gel, 80 W for about 2.5 h as previously described 30. Molecular markers were from the low Mr Electrophoresis Calibration kit (GE Healthcare). Gels were automatically stained using Processor Plus (Amersham Biosciences) with freshly prepared Neuhoff stain (Colloidal Coomassie Blue) 31.

2.2.4 Image and statistical analyses

2-DE gels were digitized with Personal Densitometer SI (Amersham Biosciences). Image analysis and spot detection were performed with Progenesis PG200 software (Non Linear Dynamics). Spot detection was automatically performed by using the algorithm named "2005 detection". After the establishment of some user seeds, matching was automatically performed and manually verified. For both strains two technical replicates and three biological replicates were performed. All the spots were identified but only spots present in all the technical and biological replicates were further considered as a significative result.

2.2.5 Protein identification by MS: Sample preparation

Samples for MS analyses were prepared according with Riedel et al. 26. Briefly, all visible spots were excised from the stained 2-DE gels, transferred to 96-well plates, and subjected to in-gel tryptic digestion. The excised gel plugs were destained with 50% v/v methanol in 100 mM $(NH_4)HCO_3$, pH 8.0 for 1 h at 37°C and washed twice with water and once with 100 mM $(NH_4)HCO_3$. The gel pieces were then dehydrated for 10 min in 80% v/v ACN and dried 30 min at 40°C after removing the solvent. Seven µL of trypsin solution (10 ng/µL in 5 mM Tris-HCl, pH 8.4, sequencing grade, Promega, Madison, WI, USA) were added to the dried gel pieces in each well and then allowed to re-swell for 10 min. The gel slices were overlayed with a further 20 µL Tris-HCl (5 mM, pH 8.4) and incubated for 3 h at 37°C. Samples were stored at −20°C. Prior to mass spectrometry analysis, 0.7 µL aliquots of the peptide solutions were mixed with 2.1 µL of matrix solution (0.5% w/v CHCA in 70% ACN/0.1% TFA), and immediately spotted in volumes of 0.7 µL onto a MALDI plate and allowed to air-dry.

2.2.6 MS

The samples were analyzed, as previously described 26, on a 4700 Proteomics Analyser MALDI TOF/TOF system (Applied Biosystems). The instrument was equipped with an Nd:YAG laser operating at 200 Hz. All mass spectra were recorded in positive reflector mode, and they were generated by accumulating data from 5000 laser pulses. First, MS spectra were recorded from the standard peptides on each of the six calibration spots, and the default calibration parameters of the instrument were updated. Subsequently, MS spectra were recorded for all sample spots on the plate and internally calibrated using signals from autoproteolytic fragments of trypsin. Up to five spectral peaks per spot that met the threshold criteria were included in the acquisition list for the MS/MS spectra. Peptide fragmentation was performed at collision energy of 1 kV and a collision gas pressure of approximately 2.5×10^{-7} Torr. During MS/MS data acquisition, a method with a stop condition was used. In this method, a minimum of 3000 laser pulses and a maximum of 6000 laser pulses were allowed for each spectrum.

2.2.7 Protein identification

MS and MS/MS data were searched using Mascot version 1.9.05 (Matrix Science) as the search engine 32. All searches were initially performed against the nonredundant NCBI library (http://ncbi.nlm.nih.gov). GPS (Global Proteomics Server) Explorer Software (Applied Biosystems) was used for submitting data acquired with the MALDI-TOF/TOF mass spectrometer for database searching. The following search settings were used: maximum number of missed cleavages: 1; peptide tolerance: 25 ppm; MS/MS tolerance: 0.2 kDa. Carboxyamidomethylation of cysteine was set as fixed modification, and oxidation of methionine was selected as variable modification.

2.3 Protein location prediction

Protein location was predicted by using PsortB software (http://www.psort.org/psortb/) 33.

2.4 Comparative proteome analyses of whole-cell proteins by iTRAQ

2.4.1 Protein extraction and labeling for iTRAQ analysis

Cells of three replicate 1.5 L cultures were harvested by centrifugation (3500×*g*, 4°C, 20 min). To extract the whole-cell proteins, bacterial cell pellets were resuspended in 4 mL of 0.5 M triethylammonium bicarbonate (TEAB) buffer containing 1% Triton X-100 and sonicated four times for 1 min on ice. After pelleting the cell debris (20 000×*g*, 30 min), proteins were precipitated from the supernatant with 6 volumes of acetone at −20°C overnight. Protein pellets were washed with acetone, dried at room temperature and solved in 50 mM Tris-HCl, pH 7.5. Proteins were further purified by phenol extraction as described above. The protein pellets were finally resolved in 0.5 M TEAB buffer and stored at −80°C. The protein concentration was quantified according to Bradford 27 (BioRad). Subsequently, 100 µg of each protein sample were dried and resolved in 20 µL 0.05% SDS as stated in the iTRAQ Reagents Protocol (Applied Biosystems). After reduction and alkylation of cysteine residues, peptides were digested with trypsin and labeled with iTRAQ reagents. Two independent protein analyses were performed, in which the peptides were labeled with different iTRAQ reagents. In analysis I (1. biological replicate) we used tag 114 for *E. faecalis* DISAV1022 peptides and tag 115 for *E. faecalis* H1 peptides. In analysis II (2. biological replicate) we used tag 116 for *E. faecalis* DISAV1022 peptides and tag 117 for *E. faecalis* H1 peptides. Prior to LC-MS/MS the labeled peptides from each analysis were pooled.

2.4.2 Strong cation exchange chromatography (SCX)

The iTRAQ samples were purified and pre-fractionated by cation-exchange HPLC (2.1 mm×200 mm SCX-column, PolySULPHOETHYL A, 5 mm, 300-Å, PolyLC, Columbia, USA) using a gradient between the mobile phase A (10 mM KH_2PO_4, 25% ACN/water, pH 3.0) and mobile phase B (10 mM KH_2PO_4, 25% ACN/water, and 350 mM KCl, pH 3.0). Peptides were eluted at a flow rate of 0.3 ml/min over the following gradient: 10 min 100% mobile phase A, 40 min 0–50% mobile phase B, 10 min 100% mobile phase B. A total of 27 fractions were collected and pooled according to the relative intensity of their SCX spectra into four master-fractions of similar peptide concentrations. The four pools were desalted via C-18 columns (Sep-Pak cartridge, Waters).

2.4.3 Nano-LC separation and MALDI target spotting of tryptic peptides

The iTRAQ samples were analyzed by nano-LC-MALDI-TOF/TOF MS as described by Carranza et al. 34. For this, peptides were separated on an Ultimate chromatography system (Dionex – LC Packings) equipped with a Probot MALDI spotting device. A 5 µL of the desalted SCX pools were injected by a Famos autosampler (Dionex – LC Packings) directly onto a 75 µm×150 mm reversed-phase column (PepMap 100, 3 mm; Dionex – LC Packings). Peptides were eluted at a flow rate of 300 nL/min by using the following gradient: 0–10 min, 0% solvent B; 10–105 min, 0–50% solvent B; and 105–115 min, 50–100% solvent B. Solvent A contained 0.1% TFA in 5% ACN/water, and solvent B contained 0.1% TFA in 80% ACN/water. For MALDI analysis, the column effluent was directly mixed with MALDI matrix (3 mg/mL CHCA in 70% ACN/water, 0.1% TFA) at a flow rate of 1.1 µl/min via a µ-Tee fitting. Fractions were automatically deposited every 10 s onto a MALDI target plate (Applied Biosystems) using a Probot microfraction collector. A total of 416 spots were thus collected from each SCX run.

2.4.4 MALDI-TOF/TOF-MS

MALDI plates were analyzed on a 4800 MALDI TOF/TOF system (Applied Biosystems) equipped with an Nd:YAG laser operating at 200 Hz according with Carranza et al. 34. All mass spectra were recorded in positive reflector mode and generated by accumulating data from 800 laser shots. First, MS spectra were recorded from peptide standards on each of the six calibration spots, and the default calibration parameters were updated. Second, MS spectra were recorded for all sample spots on the MALDI target plate (416 spots per sample, 4 samples per plate). The MS spectra were recalibrated internally based on the ion signal of neurotensin peptide (Sigma). Spectral peaks that met the threshold criteria and were not on the exclusion list were included in the acquisition list for the MS/MS spectra. The threshold criteria and settings described by Widmer et al. 35 were used: Mass range: 800–4000 Da; minimum signal-to-noise (*S/N*) for MS/MS acquisition: 100; maximum number of peaks/spot: 8. Peptide CID was performed at a collision energy of 1 kV and a collision gas pressure of approximately 2.5×10^{-6} Torr. During MS/MS data acquisition, a method with a stop condition was used. In this method, a minimum of 1000 shots (20 sub-spectra accumulated from 50 laser shots each) and a maximum of 2000 shots (40 sub-spectra) were allowed for each spectrum. The accumulation of additional laser shots was halted whenever at least 6 ion signals with an *S/N* of at least 60 were present in the accumulated MS/MS spectrum, in the region above *m/z* 200.

2.4.5 Protein identification and relative quantification

GPS (Global Proteomics Server) Explorer Software (Applied Biosystems) was used for submitting data acquired with the MALDI-TOF/TOF mass spectrometer for database searching. The MS and MS/MS data were searched using Mascot version 2.1.0 (Matrix Science) as the search engine 32. The following search settings were used: maximum missed cleavages: 1; maximum number of signals per spectrum: 55; peptide mass tolerance: 35 ppm MS/MS tolerance: 0.2 or 0.25 Da. ITRAQ labeling of lysine and of the N-terminal amino group of peptides and methyl methanthiosulfonate (MMTS) derivatization of cysteine were specified as fixed modifications. All searches were performed against a database containing all known sequences of the genus *Enterobacter,* as well as against a current Uniref100 database. Moreover, common contaminants such as trypsin and keratin were added to both databases to avoid false-positive identification of proteins.

The ratio of peak areas between iTRAQ reporter ions 114 and 115 (analyses I) and reporter ions 116 and 117 (analyses II) was used to determine the relative abundance of proteins in each protein sample. For normalization the ratio of each protein sample from iTRAQ analyses I and II were multiplied. The root of the product was then extracted and further referred to as “normalized”. The mean, standard deviation and *p*-values to estimate statistical significance of protein quantification were calculated by the MASCOT software. Proteins were assigned as differentially regulated when the regulation factors were higher than 1.5 in both independent analyses.

2.5 Phenotypical assays

Cells of all the 12 tested *E. faecalis* strains were grown in 150 mL of M17 medium to the middle of the exponential growth phase. Supernatants were separated by centrifugation and filtration (see extraction of extracellular proteins) and concentrated to a final volume of 3 mL by using Amicon Ultra-15 Centrifugal Filter Units with a cut-off of 10 kDa (Millipore). These concentrated supernatants were used for all phenotypical assays.

2.5.1 Gelatinase assay

The gelatinase substrate was prepared as a 3% w/v gelatin solution in 50 mM Tris-HCl, pH 7.3 buffer. At room temperature this gelatin solution is liquid, while at 4°C it becomes solid. To 4 mL aliquots of this gelatin solution, 400 µL of the concentrated supernatants were added and the

mixtures incubated for 2 h at 37°C. Thereafter, they were incubated overnight at 4°C to allow the gelatin to solidify where culture supernatants did not exhibit any gelatinase activity.

2.5.2 Protease assay

The protease substrate was prepared as a 1% w/v azoalbumin solution in 50 mM Tris-HCl, pH 7.5 containing 5 mM EDTA to inhibit metalloprotease activity. To 250 µL of this substrate solution, 150 µL of the concentrated supernatants were added and the mixtures incubated for 6 h at 37°C. The enzymatic reactions were stopped by adding 1.2 mL of 10% w/v trichloroacetic acid. The mixtures were incubated 15 min at room temperature and then centrifuged at 15 000×*g* for 15 min. Six hundred microliter of supernatant were mixed with 750 µL 1 M NaOH. Proteolytic activity was determined by measuring the optical density at 440 nm (OD_{440}). The blank was a mixture of 150 µL concentrated supernatant and 250 µL of substrate solution with 1.2 mL of trichloroacetic acid without incubation 36, 37. These experiments were performed in five analytical replicates for each of three biological replicates.

2.6 Genetic experiments

2.6.1 DNA extraction

DNA extraction was performed as described by Dolci et al. 38. Briefly, cells from 2 mL of bacterial culture were centrifuged (16 100×*g*, 20 min, room temperature) in tubes containing 0.3 g of glass beads (Sigma). Fifty microliter of lysozyme (50 mg/mL) were added to the pellet which was then incubated 1 h at 37°C. Three hundred microliter of lysis buffer (2% v/v Triton-X100, 1% w/v SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and 300 µL of a mixture of phenol:chloroform:isoamylic alcohol (25:24:1) were added and the mixtures were homogenized in a bead beater instrument (Fast Prep24, MP Biomedicals). Thereafter, 300 µL of TE buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0) were added to the tubes and a centrifugation step (16 100×*g*, 10 min, 4°C) was performed. The DNA in the aqueous phase was precipitated with 1 mL of ice-cold ethanol, collected by centrifugation (16 100×*g*, 10 min, 4°C), and washed briefly in 150 µL of 70% ethanol. The pellets were dried, resuspended in 50 µL of MilliQ water and incubated 30 min at 37°C with light shaking. The extracted DNA was quantified using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies) and stored at −20°C. DNA was standardized to a final concentration of 50 ng/µL before PCR amplification.

2.6.2 PCR amplification

To verify the presence of the gelatinase gene *gelE* and of the extracellular serine protease gene *sprE*, the following primers were used: *gelE* fwd (5′-CGG AAG GCG TTA CTG TTG ATT CAG-3′) and *gelE* rev (5′-CAT TCA ACG CAC CTG ATT GTC-3′) for *gelE* and *sprE* fwd (5′-GAA GTG GCA GAT ACA ACC GAA GCG CC-3′) and *spr*E rev (5′-ATT GGT GAA CCA GAT TGA CCG CCG-3′) for *sprE*. The presence of the three components of the regulatory *fsr* locus (*fsrA*, *fsrB* and *fsrC*) were verified by using the primers described by Nakayama et al. 39.

PCR amplifications were performed in a final volume of 25 µL using the MyCycler System (Biorad) and the reaction mix consisted of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 3.0 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 1.25 U *Taq* polymerase (Eppendorf), 0.2 µM of each primer and 1 µl of the extracted DNA standardized to 50 ng/µL.

The reaction conditions were: denaturation at 95°C for 60 s, annealing for 60 s and extension at 72°C for 2 minutes, for a total of 35 cycles. An initial 5 min denaturation step at 95°C and a final

7 min extension at 72°C were used. The annealing temperatures were 50°C for coccolysin, 68°C for extracellular serine protease and 48°C for *fsr* locus components.

PCR products were separated on 1% agarose gels containing 0.5 μg/mL ethidium bromide, visualized under UV light and photographed. A 1 kB ladder (MBI Fermentas) was used as size markers. PCR products were sequenced by MWG Biotech.

2.6.3 Rep PCR

Rep-PCR fingerprinting was performed with the single oligonucleotide primer (GTG)5 (5′-GTGGTGGTGGTGGTG-3′) 40–42. The PCR reaction was performed in a final volume of 25 μL containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 2 mM primer, 0.75 U *Taq* polymerase (Applied Biosystem) and 50 ng of DNA obtained as described above. PCR amplification was carried out by using the following protocol: 95°C for 5 min as initial step, 95°C for 30 s, annealing at 40°C for 30 s and 65°C for 8 min for the next 30 cycles, 65°C for 16 min concluded the amplification. Rep-PCR products were resolved by electrophoresis on 2% agarose gels containing 0.5 μg/mL ethidium bromide for 2 h at a constant voltage of 120 V in 1× TBE buffer (10 mmol/L Tris borate, 1 mM EDTA, pH 8.0).

Gels containing the Rep-PCR profiles of strains were normalized by using the 1 kb molecular ladder loaded in each gel and subjected to cluster analysis with BioNumerics software (Applied Maths). The Pearson product moment correlation coefficient was used to calculate the similarities in profile patterns and dendrograms were obtained by means of the unweighted pair group method using the arithmetic average (UPGMA) clustering algorithm Dolci et al. 38.

3 Results

Secreted enzymes and released surface-bound proteins play an important role not only in adaptive responses to environmental changes, but also in host–pathogen interactions, and often serve as virulence determinants. In the present proteome analysis of two representative *E. faecalis* isolates from raw milk cheese (DISAV1022) and the human urogenital tract (H1), both acidic and high p*I* exoproteomes as well as whole-cell proteins were therefore compared by 2-DE and iTRAQ, respectively.

3.1 Extracellular proteomic profiles

To minimize contamination with cytosolic proteins released by lysed cells, extracellular proteins were recovered from the culture broth in the middle of the exponential phase as shown in Fig. 1. A total of 33 proteins were identified for *E. faecalis* H1, of which 24 were present on pH 4–7 gels, eight on pH 6–11 gels and one protein, a choline-binding protein, was present on both gels (Fig. 2A and B and Table 2). A total of 16 proteins were identified for *E. faecalis* DISAV1022, of which eight were present on pH 4–7 gels and eight on pH 6–11 gels (Fig. 2C and D and Table 3). Analysis with the program PsortB version 3.0, which predicts the sub-cellular localization of proteins, revealed a presumed extracellular location for 21 out of the 33 identified proteins in *E. faecalis* H1 (Table 2) and for eight out of the 16 identified proteins in *E. faecalis* DISAV1022 (Table 3). Protein expression profiles of the clinical and the cheese-derived strains appeared quite different: only seven proteins were found in common for both *E. faecalis* DISAV 1022 and *E. faecalis* H1, namely, the elongation factor Tu, SalA, SalB, an autolysin precursor, a sulfatase domain protein and the 50S ribosomal protein L6. No potentially pathogenic extracellular factors were detected for the cheese-isolated strain. By contrast, two well-characterized virulence factors, coccolysin and the extracellular serine proteinase V8, were identified for the clinical isolate, along with other factors

favoring infection, like superoxide dismutase, a choline-binding protein, a chitin-binding protein and potential moonlighting enzymes such as glyceraldyhyde 3-phosphate dehydrogenase, 2,3-bisphosphoglycerate mutase and enolase. Furthermore, two antimicrobial enzymes enterolysin A and endolysin were only detected in the secretome of the clinical isolate.

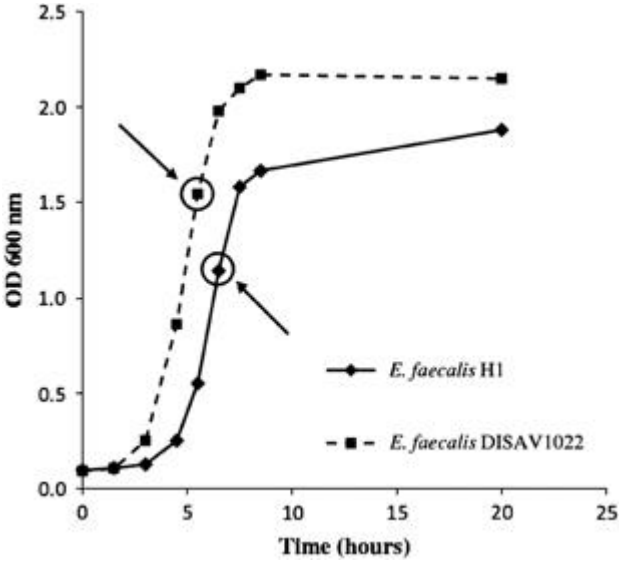

Figure 1.

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Growth curve of *Enterococcus faecalis* H1 and *Enterococcus faecalis* DISAV 1022 in M17 medium. Arrows indicate the harvesting time point.

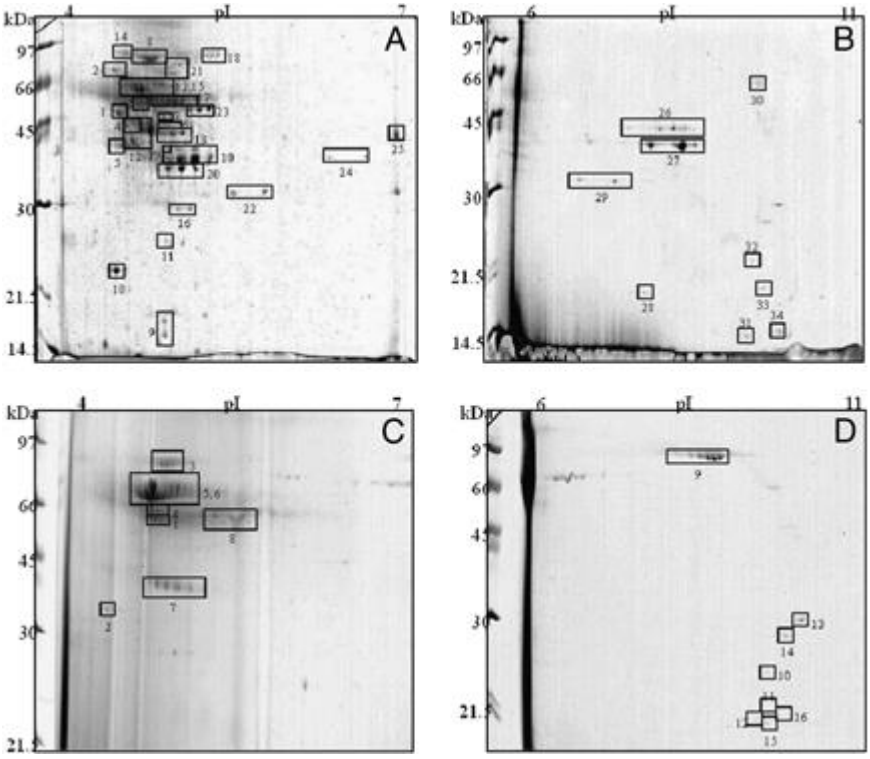

Figure 2.

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Two-dimensional gel electrophoresis (2-DE) of extracellular proteins of *Enterococcus faecalis* H1 (A and B) and *Enterococcus faecalis* DISAV1022 (D and C). The proteins were separated on Immobiline Dry strips with linear pH gradients from 4 to 7 (A and C) and 6 to 11 (B and D), followed by an SDS PAGE on 11.5% polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue G-250.

Table 2. Identified proteins from the 2-DE extracellular maps of *E. faecalis* H1

Protein name	Spot no.	Protein designation (Uniprot ID/gene designation)	p*I* calc	MW obs	MW calc	Peptides no.	Sequence coverage (%)	Protein score	PsortB predicted location
Autolysin putative	30	E6FVX3/*HMPREF9518_01333*	9.31	77 03 775	77 06 77	18	41	466	Extracellular
Bacterial extracellular solute-binding protein	4	E6FJV7/*HMPREF9519_00258*	4.79	47 58 479	47 47 6	12	40	281	Extracellular/cell wall
Basic membrane protein family	5	E6FQV7/*HMPREF9519_02401*	4.88	37 78 372	37 77 9	15	56	700	Extracellular/cell wall
Chaperone protein dnaK	2	Q835R7/*dnaK*	4.59	65 54 653	65 58 4	17	44	500	Cytosol
Chitin-binding protein	28	Q838S1/*EF_0362*	8.78	21 12 214	21 13 7	10	60	304	Extracellular
Choline-binding protein	25	Q830V7/*EF_2662*	8.76	42 42 428	42 39 8	10	32	347	Extracellular
Choline-binding protein	26	Q830V7/*EF_2662*	8.76	42 42 428	42 39 8	10	35	341	Extracellular
Coccolysin	10	Q833V7/*EF_1818*	4.99	55 52 556	55 50 3	11	36	858	Extracellular
Elongation factor Tu	3	Q839G8/*Tuf*	4.73	43 41 437	43 38 7	5	23	111	Cytosol
Endo-β-*N*-acetylglucosaminidase	22	Q830C5/*EF_2863*	5.96	34 54 348	34 56 9	7	36	620	Extracellular
Endolysin	24	Q834Z1/*ply-2*	6.53	40 32 402	40 29 0	9	35	618	Extracellular
Enolase	1	Q9K596/*eno*	4.56	46 48 462	46 51 1	19	66	848	Cytosol
Enterolysin A	29	Q9F8B0/*enlA*	9.30	37 30 370	37 32 2	2	9	138	Extracellular
Formate acetyltransferase	18	Q834N1/*pflB*	5.31	84 70 846	84 58 8	21	29	659	Cytosol

Protein name	Spot no.	Protein designation (Uniprot ID/gene designation)	p*I* calc	MW obs	MW calc	Peptides no.	Sequence coverage (%)	Protein score	PsortB predicted location
Fumarate reductase flavoprotein subunit, putative	17	Q831F4/*EF_2556*	5.26	53883	53883	8	27	653	Membrane
Glucose 6-phosphate isomerase	7	Q835G1/*pgi*	4.96	49370	49473	8	35	510	Cytosol
Glyceraldehyde 3-phosphate dehydrogenase	13	Q833I8/*gap-2*	5.03	35092	35177	10	56	407	Cytosol
Glycosyl hydrolase, family 20	8	Q839P8/*EF_0114*	4.96	94412	94906	22	40	654	Membrane
Penicillin-binding protein	21	Q830D1/*EF_2857*	5.54	77081	77885	18	37	404	Membrane
Phosphoglycerate kinase	6	Q833I9/*Pgk*	4.90	42842	42739	13	50	694	Cytosol
Phosphate-binding protein	27	P85173/*HPBP*	7.77	38873	38353	16	56	703	Extracellular
Putative uncharacterized protein	9	Q835B5/*EF_1462*	4.99	16458	16849	10	70	517	Extracellular/cell wall
SalA antigen	15	Q93LK4/*EF_3060*	5.07	51301	50093	7	19	452	Extracellular
SalB antigen	12	Q93LK3/*EF_0394*	5.01	47335	47532	10	39	357	Extracellular
Serine proteinase, V8 family	20	Q833V8/*sprE*	5.54	31040	31059	9	42	344	Extracellular
Sulfatase domain protein	23	Q835V8/*EF_1264*	6.20	79796	80701	18	39	1010	Extracellular/membrane
Superoxidedismutase [Fe]	11	Q838I4/*sodA*	4.99	22368	22769	6	53	276	Extracellular
Thiamin biosynthesis lipoprotein ApbE, putative	19	Q82Z24/*EF_3255*	5.36	39427	39124	14	57	544	Membrane/extracellular
2,3-bisphosphoglycerate dependent phosphoglycerate mutase	16	Q839H4/*gpmA*	5.09	26804	26700	6	65	490	Cytosol

Protein name	Spot no.	Protein designation (Uniprot ID/gene designation)	p*I* calc	MW obs	MW calc	Peptides no.	Sequence coverage (%)	Protein score	PsortB predicted location
5'nucleotidase family protein	14	Q839U0/*EF_0062*	5.04	140433	143392	24	28	890	Cell wall
50S ribosomal protein L6	31	Q839E9/*RplF*	9.63	19739	19940	8	44	440	Cytosol
50S ribosomal protein L13	33	Q02VM0/*rplM*	9.82	16418	16419	3	24	101	Cytosol
50S ribosomal protein L17	34	Q839D8/*rplQ*	10.13	14946	14846	12	75	421	Cytosol
50S ribosomal protein L21	32	Q836X6/*rplU*	9.66	11913	11514	9	94	264	Cytosol

Table 3. Identified proteins from the 2-DE extracellular maps of *E. faecalis* DISAV1022

Protein name	Protein designation (Uniprot ID/gene designation)	Spot no.	p*I* calc	MW obs	MW calc	Peptides no.	Sequence coverage (%)	Protein score	PsortB predicted location
Autolysin putative	E6HZJ9/*HMPREF9499_00188*	9	9.31	77035	77122	19	37	497	Extracellular
Putative extracellular protein	D4EVX9/*HMPREF9377_01699*	4	4.91	43512	43889	10	32	255	Extracellular
Hypothetical protein EF3184	Q82Z86/*EF_3184*	2	4.86	26427	26443	7	29	346	Extracellular
N-acetylmuramoyl-L-alanine amidase	F2MN31/*OG1RF_10197*	3	4.89	54606	54643	22	51	512	Extracellular
Pheromone cAD1 lipoprotein	Q82Z23/*EF_3256*	7	5.27	33179	33142	13	40	321	Extracellular/cell wall
Sal A antigen	Q93LK4/*EF_3060*	6	5.07	51014	50930	16	41	564	Extracellular
Sal B antigen	Q93LK3/*EF_0394*	5	5.01	47354	48064	13	39	329	Extracellular
Sulfatase domain protein	C7VC25/*EFNG_00204*	8	6.2	79968	79987	20	44	384	Extracellular/membrane
S1-RNA-binding domain	C7VST3/*EFKG_02605*	11	9.65	17375	17157	11	71	280	Cytosol

Protein name	Protein designation (Uniprot ID/gene designation)	Spot no.	p*I* calc	MW obs	MW calc	Peptides no.	Sequence coverage (%)	Protein score	PsortB predicted location
Translation elongation factor Tu	Q839G8/*tuf*	1	4.74	43 418	43 388	14	52	245	Cytosol
30S ribosomal protein S4	Q82ZI6/*rpsD*	13	9.84	23 219	23 234	16	79	229	Cytosol
30S ribosomal protein S12	C2JS98/*rpsL*	16	11.1	15 324	15 305	8	61	173	Cytosol
50S ribosomal protein L3	Q839G4/*rplC*	14	10.08	22 703	22 717	12	71	274	Cytosol
50S ribosomal protein L6	C7W8B2/*rplF*	10	9.63	19 397	19 455	8	44	171	Cytosol
50S ribosomal protein L24	Q839F3/*rplX*	12	9.66	11 108	11 115	4	39	215	Cytosol
50S ribosomal protein L27	Q836X4/*rpmA*	15	10.13	10 152	10 159	2	35	238	Cytosol

3.2 iTRAQ analysis of whole-cell proteins

iTRAQ analysis of *E. faecalis* H1 and *E. faecalis* DISAV1022 whole-cell proteins led to the identification of 599 proteins commonly expressed by both strains (Supporting Information Table S1). Among these, 21 were significantly upregulated and 18 were significantly down-regulated in *E. faecalis* H1 as compared with *E. faecalis* DISAV1022 (Tables 4 and 5). Most interestingly, *E. faecalis* H1 proteins possibly involved in pathogenicity, i.e. the endocarditis-specific antigen, a chitin-binding protein, an adhesion lipoprotein and a two-component regulatory system for alkaline phosphatase activation, appeared to be up-regulated compared to the cheese isolate. Among the proteins comparatively up-regulated in *E. faecalis* DISAV1022 were three different proteins involved in sugar up-take from the external medium and otherwise proteins not involved in pathogenicity.

Table 4. Proteins identified by iTRAQ analysis on the whole-cell fraction, as more expressed in *E. faecalis* H1 than in *E. faecalis* DISAV1022

Protein identification	Rank	ID (Uniprot)/gene designation	MW (calc)	Peptides no.	Total ion score	Fold change expression
Adhesion lipoprotein (EF0095)	514	Q8KU66/*ef0095*	41 860.44	1	102.59	3.53
Alkaline phosphatase synthesis transcriptional regulatory protein PhoP	781	Q834F2/*phoP*	30 220.59	1	45.07	1.57

Protein identification	Rank	ID (Uniprot)/gene designation	MW (calc)	Peptides no.	Total ion score	Fold change expression
Chaperone protein clpB	48	Q831Y7/*clpB*	10 7376.7	10	840.40	1.51
Chitin-binding protein, putative	419	Q838S1/*EF_0362*	22 998.16	1	145.12	2.42
CoA-binding domain protein	528	Q834M9/*EF_1616*	17 418.36	2	97.98	2.53
Cobalt transport family protein	764	Q839D3/*EF_0239*	31 508.26	1	47.38	2.00
Decarboxylase, putative	374	Q838D6/*EF_0634*	78 298.33	3	173.23	1.60
Dps family protein	314	Q82Z45/*EF_3233*	19 507.99	4	210.49	2.26
Endocarditis-specific antigen	551	Q832Z2/*EF_2076*	40 152.48	2	93.44	2.04
Glutamyl-aminopeptidase	125	Q82ZL6/*pepA*	42 245.81	7	498.91	1.89
Glycosyl hydrolase, family 1	391	Q839A6/*EF_0271*	59 204.47	4	161.97	2.14
Glycosyl hydrolase, family 1	742	Q831B5/*EF_2597*	59 064.36	1	50.34	4.89
Lipoprotein, putative	384	Q839T2/*EF_0071*	100 101.1	2	165.66	1.95
Oxidoreductase, pyridine nucleotide-disulfide family	585	Q82Z22/*EF_3257*	78 643.34	2	84.38	2.71
Peptidase, M20/M25/M40 family	178	Q82Z92/*EF_3178*	45 373.69	4	387.11	2.06
PTS system, β-glucoside-specific IIABC component	636	Q831B4/*EF_2598*	71 239.41	1	73.70	3.02
Purine nucleoside phosphorylase	577	Q839I0/*deoD-2*	27 837.46	2	86.09	1.62
Putative uncharacterized protein	108	Q837R1/*EF_0770*	21 606.27	6	543.40	1.87
Putative uncharacterized protein	474	Q833L4/*EF_1933*	14 993.58	1	119.65	27.24
Putative uncharacterized protein	691	Q82ZD4/*EF_3130*	12 550.37	1	61.32	2.72
Sensor protein	767	Q836Q7/*EF_1051*	62 883.3	1	46.90	1.87

Table 5. proteins identified by iTRAQ analysis on the whole-cell fraction, as more expressed in *E. faecalis* DISAV1022 than in *E. faecalis* H1

Protein identification	Rank	ID (Uniprot)/gene designation	MW (calc)	Peptide no.	Total ion score	Fold change expression
ABC transporter, ATP-binding protein	622	Q833B4/*EF_2050*	30 252.31	1	76.77	2.00
Acyl carrier protein, putative	257	Q830B0/*acpP1*	93 30.78	2	273.81	1.81

Protein identification	Rank	ID (Uniprot)/gene designation	MW (calc)	Peptide no.	Total ion score	Fold change expression
Alkyl hydrogen peroxide reductase	696	O30738/*ahpC*	22 702.04	1	60.21	2.29
α1,2-mannosidase, putative	452	Q832K9/*EF_2217*	86 234.94	2	129.55	1.84
D-Alanine-poly(phosphoribitol) ligase subunit 2	809	Q830N2/*dltC*	90 17.69	1	42.32	1.59
Glycosyl hydrolase, family 65	76	Q82ZA9/*EF_3157*	93 216.47	9	668.51	1.68
Lipoprotein, putative	177	Q833X8/*EF_1796*	25 156.3	4	338.57	1.81
NAD-dependent epimerase/dehydratase family protein	487	Q832Q5/*EF_2165*	39 275.57	2	115.22	1.66
NADPH-dependent FMN reductase domain protein	382	Q834F7/*EF_1698*	18 391.44	2	166.51	3.17
Nucleoside diphosphate kinase	241	Q836S2/*EF_1036*	16 963.47	4	287.14	1.60
Probable tautomerase	401	Q830C9/*EF_2859*	7869.3	2	155.42	2.91
PTS system, IIA component	756	Q836T9/*EF_1018*	12 576.35	1	48.19	1.74
PTS system, IIABC components	158	Q834W2/*EF_1516*	75 848.23	5	422.49	1.98
PTS system, IIBC components	246	Q832L3/*EF_2213*	55 719.89	4	283.64	1.85
Putative uncharacterized protein	49	Q834B6/*EF_1753*	67 339.99	10	832.44	1.55
Putative uncharacterized protein	469	Q838D2/*EF_0638*	20 172.07	1	121.01	25.32
TPR domain protein	192	Q834T0/*EF_1553*	48 042.44	5	357.07	1.51

3.3 Phenotypical assays

As a next step we wanted to elucidate whether the origin of the strain is linked to the presence or absence of two selected virulence factors, namely the gelatinase coccolysin and the serine protease V8, which were found exclusively in the hospital strain H1. To this end, enzymatic assays were performed with 12 isolates from cheese and hospitalized patients (Table 1), including the two strains whose proteome profiles were analyzed before.

The results of the gelatinase activity assays are shown in Fig. 3: only one of the cheese-isolated strains (*E. faecalis* E) displayed gelatinase activity, while three out of six hospital-isolated strains (*E. faecalis* H1, *E. faecalis* H3 and *E. faecalis* H5) were able to degrade gelatin. Results of the serine protease activity assay (Fig. 4) are in good agreement with the gelatinase assay: As expected, strains displaying gelatinase activity also possessed serine protease activity.

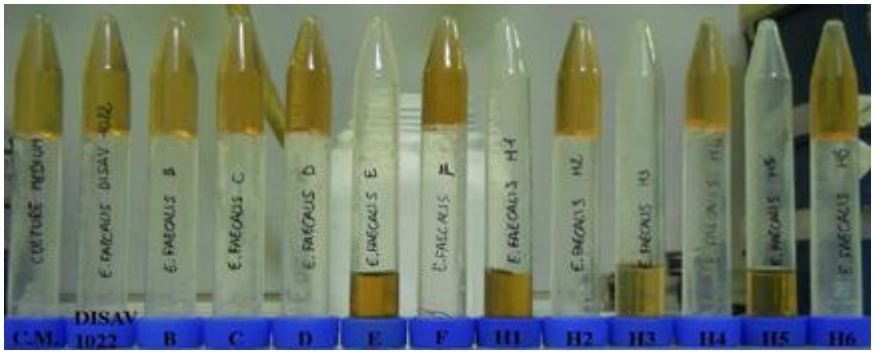

Figure 3.

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Gelatinase activities of sterile culture supernatants from food and clinical isolates of *Enterococcus faecalis*. Cultures were grown until the mid-exponential phase in M17 medium and sterile filtered. 50-Fold concentrated supernatants were incubated with 4 mL of 3% p/v gelatin. The strains producing coccolysin appear in the liquid state because of the ability of the enzyme to digest gelatin. C.M. is the culture medium, the negative control. The strains from *E. faecalis* DISAV 1022 to *E. faecalis* F are the cheese isolates; the strains from *E. faecalis* H1 to *E. faecalis* H6 are the clinical isolates.

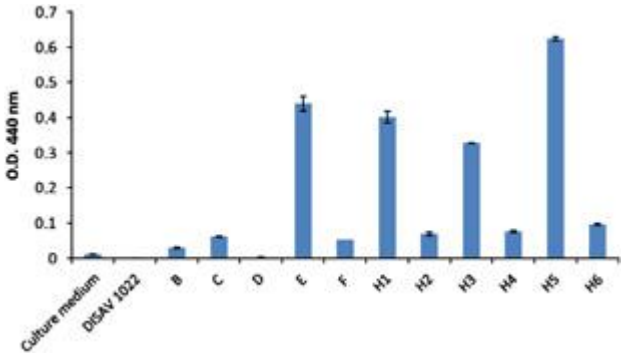

Figure 4.

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Serine protease activities of sterile culture supernatants from food and clinical isolates of *Enterococcus faecalis*. Cultures were grown until the mid-exponential phase in M17 medium and sterile-filtered 50-fold concentrated supernatants were incubated with 1% azoalbumin and 65 mM EDTA (inhibition of metallo proteases) for 6 h at 37°C. Proteolytic activity is proportional to the optical density (OD) at 440 nm. Each bar represents the average from three independent experiments. Error bars represent the standard errors of the means. C.M. is the culture medium, the negative control. The strains from *E. faecalis* DISAV 1022 to *E. faecalis* F are the cheese isolates; the strains from *E. faecalis* H1 to *E. faecalis* H6 are the clinical isolates.

3.4 Genetic analyses of genes coding for protease and the regulatory QS system

To investigate whether the lack of gelatinolytic and proteolytic activities was due to the absence of the genes encoding coccolysin (*gelE*) and extracellular serine proteinase V8 (*sprE*), a PCR analysis was performed. As shown in Table 6, *gelE* and *sprE* genes were present in four out of six cheese isolates (*E. faecalis* DISAV1022, *E. faecalis* B, *E. faecalis* D, *E. faecalis* E) and in three out of six hospital isolates, namely *E. faecalis* H1, *E. faecalis* H3 and *E. faecalis* H5. Both the amplicon size and sequence confirmed the functionality of the protease genes (data not shown).

Since both *gelE* and *sprE* are under positive control of the *fsr* locus, both the cheese and the clinical isolates were tested for the presence of genes coding for this regulatory system (*fsrA*, *fsrB* and *fsrC*) by PCR. Four out of six cheese isolates (*E. faecalis* DISAV1022, *E. faecalis* B, *E. faecalis* E and *E. faecalis* F) and three out of six clinical isolates (*E. faecalis* H1, *E. faecalis* H3 and *E. faecalis* H5), possessed the whole *fsr* locus. The clinical isolate *E. faecalis* H6 appeared to have *fsrB* and *fsrC* but not *fsrA*. Two cheese isolates (*E. faecalis* C and *E. faecalis* D) and one clinical isolate (*E. faecalis* H4) appeared to have *fsrB*, but not *fsrA* and *fsrC*, while *E. faecalis* H2 did not possess any component of the *fsr* locus. Interestingly, the size of the *fsrA* PCR product of *E. faecalis* DISAV1022 and *E. faecalis* B was twice as large as expected: the sequencing of the amplicons highlighted the insertion of *sep1, a* gene of plasmid origin, inside the *fsrA* locus (Supporting Information figure).

3.5 Rep PCR

All strains were analyzed by the fingerprinting method Rep-PCR in order to determine strain biodiversity. As shown in Fig. 5, the strains can be grouped in three clusters and are clearly distinguishable based on their origin. Cluster analysis revealed a clear distinction between human and food isolates. In particular, the first cluster contains the hospital strains H4, H5 and H6, the second cluster contains all the strains isolated from cheese and the third cluster contains the other three strains isolated from hospitalized patients (H1, H2 and H3). It is interesting to highlight that the food isolates are more homogeneous from a genetic point of view with a similarity coefficient of 85%. Conversely, there is a significant dissimilarity among the group of hospital isolates, which are also clearly different from the food isolates (Fig. 5).

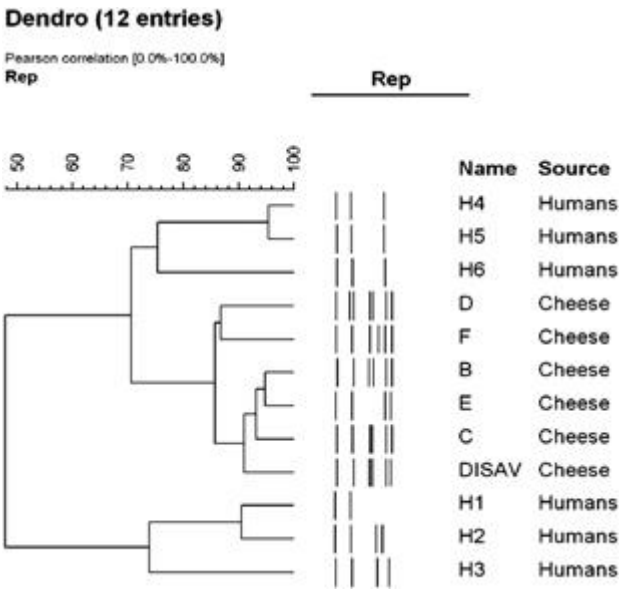

Figure 5.

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Genetic biodiversity analyses of six cheese- isolated and six clinical isolated *E. faecalis* strains. The origin of the strains analyzed in previous studies (*E. faecalis* DISAV 1022) 28 and in this study is also shown.

Table 6. PCR-based evaluation of the presence of genes coding for selected potential virulence factors gelatinase GelE and serine protease V8 SprE. The whole *fsr* locus that regulates *gelE* and *sprE* expression was analyzed too.

Pathogenicity grade	Strain	Structural genes *gelE*/*sprE*	Regulatory genes *fsr* locus				Phenotypical assays
			FsrA	*fsrB*		*fsrC*	
High pathogenicity potentia	*E. faecalis* H1	+	+	+	+	+	
	E. faecalis H3	+	+	+	+	+	
	E. faecalis H5	+	+	+	+	+	
	E. faecalis E	+	+	+	+	+	
Low pathogenicity potential	*E. faecalis* H2	−	−	−	−	−	
	E. faecalis H4	−	−	+	−	−	
	E. faecalis H6	−	−	+	+	−	
	E. faecalis C	−	−	+	−	−	
	E. faecalis F	−	+	+	+	−	
Not clear pathogenicity potential	*E. faecalis* D	+	−	+	−	−	
	E. faecalis DISAV1022	+	+ (higher amplification product)	+	+	−	
	E. faecalis B	+	+ (higher amplification product)	+	+	−	

4 Discussion

The pathogenic potential of a microbial species can be linked to exotoxin production, immune system evasion and/or suppression strategies and host invasiveness. All these features are genetically determined by either chromosomal or plasmid DNA, and single strains can differ from common species features.

Pathogenic traits are often found on the cell-surface or secreted. In the present paper we therefore compared the extracellular and whole-cell proteomes of two representative strains belonging to the same species, *E. faecalis*, but isolated from different ecological niches (cheese and human urogenital tract), to investigate whether the habitat selects for strains with site-specific protein profiles.

Proteins detected in the secretome of both analyzed *E. faecalis* strains (DISAV1022 and H1), SalA, SalB and the sulfatase domain family protein, have also been described as secreted proteins of the laboratory model strain *E. faecalis* JH2-2 by Benachour and co-workers 25. SalA and SalB are the major secreted antigens in *E. faecalis* 43. SalB expression is triggered by stress 44 and Breton and co-workers demonstrated that it is involved in resistance to various stress conditions including bile salts, NaCl, SDS, ethanol, heat shock and alkaline and acid pH 45. Despite its amino acid sequence similarity to SalB, SalA has been predicted to be a secreted lipase 43.

The elongation factor Tu is a well-known cytosolic protein involved in protein synthesis 46. Interestingly, it is frequently found extracellularly 47 even though it lacks any export signal sequence. Also in *E. faecalis* JH2-2 25, EF Tu has been found in the surface-associated protein fraction. In *Lactobacillus johnsonii*, surface-located EF Tu has been proven to act as a mucin-binding protein 47. Since this protein also lacks any cell-wall anchoring motif, its release from the cell might be induced under specific conditions, such as variation of pH 48 or cell-wall turnover 49.

Other proteins common to both strains and also found in *E. faecalis* JH2-2 by Benachour 25 were autolysins, cell-wall degrading enzymes 50 involved in peptidoglycan maturation and degradation. Moreover, two proteins involved in peptidoglycan renewal 51, *N*-acetylmuramoyl-L-alanine amidase and endo-β-*N*-acetylglucosaminidase, were identified in the cheese and the clinical isolate, respectively.

Interestingly, two other lysins, endolysin and enterolysin A, were only found in the clinical isolate *E. faecalis* H1. Endolysins are phage-encoded lytic enzymes, whose target is similar to that of autolysins. They digest bacterial peptidoglycan at the end of the phage lytic cycle, thus allowing the release of the viral progeny into the external environment 51. Endolysins display lytic activity against various Gram-positive bacteria and thus may be useful in infection control of pathogenic multidrug-resistant enterococci and food-contaminating Gram-positive bacteria 52, 53. Enterolysin A is a class II heat-stable bacteriocin produced by *E. faecalis* and is active against different strains of lactobacilli, lactococci and enterococci. It has a domain with significant sequence homologies to two bacteriophage lysins 54. The presence of these lysins in the hospital isolates might be due to specific conditions in the urogenital tract which might select for the expression of traits to suppress the growth of other uropathogenic species.

A choline-binding and a chitin-binding protein were exclusively detected for *E. faecalis* H1. The pathogen *Streptococcus pneumoniae* requires choline-binding proteins for efficient epithelium colonization and invasion 55. More recently, a specific streptococci choline-binding protein, LytB, was reported to have *N*-acetylglucosaminidase activity and is involved in cell separation during bacterial replication while also facilitating host-cell invasion 56. Hammerschmidt and co-workers 57 reported that, in pneumococci, choline-binding proteins are involved in adhesion, either to host cells or to target host extracellular matrix proteins.

Sanchez and co-workers characterized the function of chitin-binding proteins in *Lactobacillus plantarum* 58, which revealed a non-hydrolytic accessory protein essential for the degradation of chitin by chitinases. Chitin-binding proteins bind to *N*-acetylglucosamine present in the chitin exoskeleton of arthropods and fungi (including yeasts) and in a wide variety of polymers such as mucins 58. They may therefore also play a role in adhesion to human epithelial cells. Generally, chitin-binding proteins are surface-bound, yet significant amounts have also been found extracellularly for *L. plantarum* 58. The finding of a chitin-binding protein in the clinical isolate but not in the cheese-derived *E. faecalis* strain seems to indicate a good adaptation to an ecosystem in which yeasts and fungi can also occur.

An interesting aspect of this study is the expression of several potential moonlighting proteins 59, 60 present in the hospital isolate but absent from the cheese-isolate. In particular, five glycolytic enzymes (glucose 6-phosphate isomerase, glyceraldheyde 3-phosphate dehydrogenase, phosphoglycerate kinase, 2,3-bisphosphoglycerate mutase and enolase) and the chaperon protein dnaK, known to be involved in stress response when present in the cytosol, were exclusively found in the H1 secretome. Several papers have shown that glycolytic enzymes, beside their innate cytosolic function, may exhibit a second completely unrelated function in both prokaryotes 61 and eukaryotes 62 when expressed in different cellular compartments (moonlighting function), controlling multiple biological and physiopathological processes 63. All mentioned proteins have been shown to be able to bind human plasminogen (abundant in plasma and extracellular fluids) and, in the presence of self-produced or host-available factors 61, 64, to convert plasminogen into plasmin. The latter can further activate other proteases resulting in the degradation of the extracellular matrix, thereby enabling microbial migration through host tissue and crossing of the mucosal barrier, and thus facilitates host invasion 65.

The exoproteome of the hospital isolate *E. faecalis* H1 contained, in contrast to the cheese isolate, an extracellular manganese superoxide dismutase (SOD). This enzyme may be considered a potential virulence factor for two reasons: (i) a recent study has demonstrated that SOD is linked to the antibiotic resistance of this species. In particular, it was shown that Δ*sodA* mutants were significantly more susceptible to bactericidal drugs than the wild-type strain 66. (ii) SOD has been shown to eliminate or attenuate the toxic effect of H_2O_2 and other oxygen-reactive species released by granulocytes and macrophages during phagocytosis and thus impairing infection 67.

Most interestingly, the coccolysin (GelE) and the extracellular serine proteinase V8 (SprE) were exclusively detected on extracellular 2-DE maps of *E. faecalis* H1). GelE and SprE have been described as major virulence factors of the genus *Enterococcus* 18. Coccolysin is an extracellular zinc-containing metalloproteinase related to aureolysin from *Staphylococcus aureus* and elastase from *Pseudomonas aeruginosa* 68; it was purified and characterized from *E. faecalis* 69, highlighting its ability to hydrolyze gelatin, collagen, fibrinogen, casein, fibrin, endothelin-1, bradykinin LL-37 and the complement components C3 and C3a 70. The extracellular serine proteinase V8 is a secreted 26-kDa protease that shares homology with *S. aureus* V8 protease 18. The respective coding genes *gelE* and *sprE* are located in the same operon and are co-transcribed 19.

The evaluation of the distribution of these two virulence factors in a larger number of isolates (six from cheese and six from hospitalized patients) by enzymatic activity assays revealed the presence of gelatinase and protease activities in one cheese isolate (*E. faecalis* E) and in three hospital isolates (*E. faecalis* H1, *E. faecalis* H3, *E. faecalis* H5). Since these activities are probably due to GelE and SprE, these finding confirmed the literature reported co-transcription of the corresponding *gelE-sprE* genes. Notably, PCR analyses proved that three cheese isolates (*E. faecalis* DISAV1022, *E. faecalis* B, *E. faecalis* D), which do not exhibit protease activity, bear the *gelE/sprE* structural genes.

A very interesting study correlated the expression of virulence associated genes to particular environments: Hew *et al*. employed quantitative RT-PCR to show that *clpP*, *clpX*, *gls24*, *agg*, *efaA*, *gelE*, $cylBL_L$ genes were differentially expressed when *E. faecalis* was exposed to different culture media 71.

On the other hand, *gelE/sprE* genes are under the positive control of the *fsr* regulatory system consisting of three genes *fsrA*, *fsrB*, *fsrC* which are needed to induce gelatinase and serine protease expression 72.

The presented experimental evidence suggests that the absence of GelE/SprE activity in *E. faecalis* DISAV1022, *E. faecalis* B and *E. faecalis* D, despite the presence of their respective coding genes, might be due to the absence or modification of these regulatory genes. Indeed, *E. faecalis* D only possesses *fsrB*, but lacks both *fsrA* and *fsrC*. Sequencing of the *fsrA* amplicon of *E. faecalis* DISAV1022 and *E. faecalis* B revealed an insertion inside the gene of *sep1*, a gene that is normally located on plasmids, which leads to an impaired function of the resulting polypeptide and is responsible for the lack of proteolytic activity. *Sep1* is a sex factor that allows recombination between microbial strains and is normally coded by transmissible plasmids that have evolved by repeated recombination and insertion of different transposable elements 73. It has already been demonstrated by Reinscheid et al. that phenotypical alterations of *Streptococcus agalactiae* can be due to s*ep1* insertions into the *pcsB* gene 74. Notably, some of the analyzed strains (both of cheese and human origin), although not possessing the structural genes for *gelE/sprE*, reveal in their genome the presence of the entire *fsr* locus or at least part of it. One might speculate that the *fsr* regulatory system of these strains is (i) simply the remainder of a complete and functional QS system or (ii) is involved in the regulation of other genes. Bourgogne and co-workers, analyzing transcripts of *fsrB* deletion mutants, demonstrated that this locus modulates, both positively and negatively, the expression of numerous genes, among them *bopD* which is important for biofilm formation, and genes encoding for the surface proteins EF0750 to -0757 and EF1097 75. These authors, furthermore, hypothesize that the *fsr* system can affect mRNA stability.

An interesting protein which was exclusively identified in the secretome of the cheese isolate *E. faecalis* DISAV1022 is a 33 kDa pheromone cAD1 lipoprotein. The sex pheromone cAD1 is a small hydrophobic peptide secreted by plasmid-free *E. faecalis* strains, which stimulates conjugative transfer of the plasmid pAD1 from plasmid-bearing *E. faecalis* donors. When pAD1 is acquired by the recipient, the synthesis of cAD1 is repressed. Acquiring pAD1 is useful since this plasmid, besides bearing the genetic determinants for the surface-associated pheromone sensor, also encodes genes involved in the synthesis of bacteriocins and hemolysin 76. The genetic determinant of cAD1, designated *cad*, encodes a 309 amino acid lipoprotein precursor with the last eight residues representing the cAD1 octapeptide. A second gene, named *eep*, is required for cAD1 pheromone biosynthesis and encodes for a zinc-metalloprotease, probably involved in the processing of the cAD1 pheromone precursor to give rise to the final octapeptide at the cytoplasmic membrane level 77. The 33 kDa protein, found in the non-pathogenic *E. faecalis* DISAV1022, is consistent with the 309 amino acid lipoprotein precursor of the mature hydrophobic octapeptide pheromone. It has been established previously that pheromone production is more frequent in *gelE*-negative *E. faecalis* strains 78, 79.

iTRAQ analyses of the whole-cell proteins of *E. faecalis* H1 and *E. faecalis* DISAV1022 revealed the overexpression of an endocarditis-specific antigen, probably involved in biofilm formation and host-tissue colonization, in *E. faecalis* H1. *E. faecalis* is known as common cause of infective endocarditis 80. Another protein that was found to be over-expressed in *E. faecalis* H1 compared to the cheese isolate is the adhesion lipoprotein EF0095, confirming that the strain is able to colonize host tissues. This higher adhesion ability is also underlined by the up-regulation of the chitin-binding protein, already found also in the extracellular 2-DE maps and able to bind *N*-acetylglucosamine units of the mucin present in tissues during inflammations 58. In the clinical isolate the sensor kinase and response regulator of a two-component regulatory system inducing the expression of the alkaline phosphatase PhoP were found to be up-regulated 81: alkaline phosphatase is a well-known virulence factor because it may play an important role in host colonization due to its ability to promote biofilm formation 82. Moreover, higher levels of both glutamyl aminopeptidase and peptidase belonging to the M20/M25/M40 family suggest that *E. faecalis* H1 also exhibits a higher proteolytic potential than *E. faecalis* DISAV1022.

Among the most interesting proteins over-expressed in *E. faecalis* DISAV 1022 were three components of the phosphotransferase system (PTS system IIA component, IIABC components and IIBC components), a complex used by bacteria for sugar uptake from the external medium. The up-regulation of this sugar transport system as well as of an α-mannosidase is surprising, since most sugars are consumed during cheese production.

The iTRAQ results seem to highlight a tendency of enolase to be over-expressed in the *E. faecalis* DISAV1022 whole-cell proteome, although its fold-factor was under the threshold of 1.5; interestingly this protein was also found in the extracellular proteome of *E. faecalis* H1. It can be speculated that the two strains biosynthesize similar amounts of the protein, but that only *E. faecalis* H1 exports part of it, probably to exploit some of its well recognized moonlighting functions.

In conclusion, a comparison of the extracellular and whole-cell proteomes of a cheese and clinical isolate of *E. faecalis* has unveiled significant differences in their protein profiles. Among the proteins detected exclusively or found over-expressed in the clinical strain were several important pathogenic traits and potential virulence factors, i.e. gelatinase E, extracellular serine proteinase V8, endocarditis-specific antigen, choline-binding protein, chitin-binding protein, adhesion protein, superoxide dismutase and various potential moonlighting proteins, which might be involved in the activation of proteolytic cascades. In general, besides the discussed pathogenicity factors, a habitat-imprinting effect has been observed: for instance, chitin-binding protein, *penicillin*-binding protein (PBP), endolysin and enterolysin A were found only in the clinical *E. faecalis* strain. The competition with fungi in the human host, the frequent administration of antibiotics (PBP) and co-existence of antagonist bacteria may account for these, expected, differences. Furthermore, hospital environmental pressure seems to lead to higher strain genetic diversity (Rep-PCR) than food habitats.

One of the most studied pathogenicity factors of enterococci are the two proteases GelE and SprE. Our finding that these proteases were expressed by both cheese and clinical isolates suggests that expression of these virulence factors is determined by the strain and not by the habitat. To our knowledge this is also the first report describing the presence of the *gelE/sprE* regulatory *fsr* system in an *E. faecalis* food isolate. Bacterial genome plasticity, the possibility of horizontal gene transfer between food strains and gut microflora and the rapid adaptation of bacteria to changing environments might transform originally harmless food-strains into potentially pathogenic strains. Our results certainly underline the need for an accurate characterization of strains at the genome, proteome and phenotypic level prior to their use as starter cultures or probiotics.

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