



Detailed Soluble Proteome Analyses of a Dairy-Isolated *Enterococcus faecalis*: A Possible Approach to Assess Food Safety and Potential Probiotic Value

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Enterococci are common inhabitants of the gastrointestinal tracts of humans and animals and thanks to their capability to tolerate different environmental conditions and their high rates of gene transfer, they are able to colonize various ecological niches, as food matrices. *Enterococcus faecalis* bacteria are defined as controversial microorganisms. From one side they are used as food starters, bio-control agents and probiotics to improve human or animal health. From the other side, in the last two decades enterococci have emerged as important nosocomial pathogens, because bearing high-level of resistance to antibiotics and several putative virulence factors. In this study, the soluble proteome quantitation data (LC-MS/MS) of the food-isolated strain *E. faecalis* D27 (dairy-isolate) was compared with the soluble proteome quantitation data of the pathogenic *E. faecalis* UW3114 (urinary tract infection isolate) and with the one of the health promoting strain *E. faecalis* Symbioflor1, respectively. The comparison of cytosolic protein expression profiles highlighted statistically significant changes in the abundance of proteins mainly involved in specific metabolic pathways, nutrient transport, stress response, and cell wall modulation. Moreover, especially in the dairy isolate and the clinical isolate, several proteins with potential pathogenic implications were found, such as serine proteases, von Willebrand factor, serine hydrolase with beta lactamase activity, efflux transporter, and proteins involved in horizontal gene transfer. The analysis of the extracellular proteome provided interesting results concerning proteins involved in bacterial communication, such as pheromones and conjugative elements and also proteins able to interact with human components. The phenotypic characterization evaluating (i) biofilm formation (ii) hemolytic activity on blood agar plates (iii) protease activity (iv) gelatinase (v) antibiotic resistance pattern, enabled us to elucidate the risks associated with the poor characterized foodborne *E. faecalis* D27.

Keywords: foodborne bacteria, gel-free proteomics, virulence, antibiotic resistance, host interaction

INTRODUCTION

Enterococci are ubiquitous gram-positive bacteria that can be found in various ecosystems, ranging from soil, surface waters, plants, the gastrointestinal tract (GIT) of animals and humans as well as in foods. Enterococci also emerged as important pathogens, since they are one of the major cause of both nosocomial and outpatient-associated infections (1), including clinical manifestations such as urinary tract infections, endocarditis, primary bacteremia and meningitis (2).

In the last decades, enterococci were widely studied because they have intrinsic (absence of target, impermeability, absence of uptake mechanism) and, often, acquired (antibiotic degrading or modifying enzymes) resistance to antibiotics (3, 4). Generally, intrinsic/natural resistance is chromosomally-encoded and therefore non-transmissible, whereas acquired resistance can be plasmid-mediated and hence transmissible by genetic recombination (5). An increasing number of multidrug resistant enterococci has been detected as the predominant microbiota under antibiotic pressure, predisposing hospitalized patients to severe infections and mortality (6). Some virulence factors and antibiotic resistances determinants are carried on mobile genetic elements (MGE) (7), supporting the theory of enterococci as reservoir for antibiotic resistance determinants found in pathogens (8, 9). A large number of reports has focused on the presence or absence of virulence determinants in enterococcal isolates from different origins (food and living being) (10, 11). In particular, the higher incidence in clinical isolated enterococci of genetic elements encoding for virulence factors indicate that these genes enhance their ability to colonize humans, increasing the infection level, as suggested by virulence studies on bacterial mutants in animal models (12).

Within enterococci species, whereas *E. faecium* harbors more antibiotic resistance traits, *E. faecalis* shows a higher potential for virulence, because it synthesizes many proteins that facilitate interaction with environmental (both biotic and abiotic) surfaces, biofilm formation and host colonization (13, 14). Some of these proteins, such as aggregation substance, cytolysin, enterococcal surface protein, gelatinase and protease, are considered to be potential virulence factors (15, 16).

Enterococcus faecalis appears to be typically associated with the human GIT (17) and it is one of the first lactic acid bacteria (LAB) to colonize the intestine of the newborn (18). In healthy human GIT, this bacterium is harmless and present in low abundance, however it can cause life-threatening infections during antibiotic-induced dysbiosis (19). *E. faecalis* is usually carried by food, due to its widespread distribution in the environment. In particular it is often isolated from food of animal origin such as meat (20), milk (21), and cheese (22). Its presence may indicate a natural contamination during manufacturing processes, in some cases as a consequence of fecal contamination caused by poor hygiene (23). Otherwise, *E. faecalis* is used as starter culture (24) to carry out fermentative processes, indeed it plays an important role in cheese ripening and aroma development. However, some authors have reported the presence of antimicrobial resistance and virulence determinants in enterococci found in foods, including cheeses (25, 26).

Therefore, a food contaminating *E. faecalis* could represent a possible intermediate vehicle for the transmission of pathogenic traits. In this case, bacteria could act as vectors for the dissemination of antibiotic resistance determinants and virulence factors via the food chain to the consumer, a risk that has so far been poorly addressed (27–29). Actually, it has been observed that human gut enterococci are probably acquired from food since they possess the same antibiotypes and toxinogenic profiles than cattle-, pig-, and sheep- isolated enterococci (30).

Despite the risks associated to *E. faecalis* have led to define these bacteria as controversial (31) they are currently present not only in fermented food but even used as health supplements and probiotics by the pharmaceutical industry. Enterococcal probiotics are usually utilized as “food supplements” in the form of encapsulated or lyophilized pharmaceutical preparations (32). This choice is partly motivated by the lower acid resistance of enterococci compared to lactococci and lactobacilli, that render them less suitable to survive the gastric pH transit. These bacteria are thus ingested in high number to achieve functional or probiotic effects especially for treatment of diseases such as irritable bowel syndrome, diarrhea, antibiotic associated diarrhea, or for health improvement such as cholesterol levels lowering or immune regulation (33, 34).

For all these reasons (possible pathogenicity/virulence determinants or transferable antibiotic resistance characters), a careful selection of *E. faecalis* strains for food supplementation should be done and a constant monitoring on new food-isolated enterococcal strains is required to limit the propagation of virtually harmful microorganism. However, evaluating these risks only by genome analysis of strains is limiting since gene function annotation is based just on sequence homologies with genes present in databanks. Therefore, some evidences should be validated by functional investigations as well as by studying bacterial protein profiles and separately analyze sub-proteomes.

In this view, the present study intended to characterize *Enterococcus faecalis* D27, isolated from cheese, with the specific aim to elucidate if this strain harbors pathogenic traits or rather reveals probiotic features. For this reason, we also examine both a pathogenic (clinically isolated) and a probiotic strain belonging to the same species, to understand the similarities and differences among all the three strains by comparing phenotypic traits, proteomic, and biochemical profiles.

MATERIALS AND METHODS

Bacterial Strains and Cultured Condition

In this study three strains of *Enterococcus faecalis* (present in our collection), with different origin were investigated: D27 (isolated from an artisanal cheese), Symbioflor 1 (commercial probiotic strain) and UW3114 (clinical isolated strain, bearing the pathogenicity island of *E. faecalis* MMH594) (35). All strains were maintained in BHI medium (Oxoid, Munich, Germany) at -20°C in 0.5 mL aliquots with 0.5 mL 40% v/v glycerol. For both proteome analyses and phenotypical assays three biological replicates were performed. Bacterial cultures were grown in the BHI medium at 37°C with slight agitation (180 rpm). Culture supernatants (extracellular proteome) and cells

(cytosolic proteome) were recovered by centrifugation (10,000xg, 10 min, 4°C) from the same bacterial culture in the early stationary growth phase.

Sample Preparation for Proteomic Analyses

Cytosolic Proteins

The intracellular protein extract was obtained as previously described by Zühlke et al. (36). Briefly, cell pellet was washed with TE buffer and lysed by glass beads (diameter: 0.1–0.11 mm) using a Precellys 24 Homogenizator (Peq Lab, Erlangen, Germany). In-solution digestion of protein extracts with trypsin was done according to the method described previously (37). Proteins were dissolved in 50 mM TEAB/0.1% RapiGest™ SF (Waters, Milford, MA, USA), reduced with TCEP (tris-(2-carboxyethyl)phosphine hydrochloride, Invitrogen, Carlsbad, CA, USA) for 45 min at 60°C and alkylated with iodoacetamide (Sigma, Steinheim, Germany) for 15 min at room temperature. Proteins were digested with 0.5 mg trypsin in a 1:200 ratio (Promega, Madison, MA, USA) for 6 h under gentle agitation at 37°C. Desalting of peptides was achieved using a standard protocol (38). For absolute quantification, a tryptic digest of yeast alcohol dehydrogenase (ADH1, Waters, USA) was added into the samples to final concentration of 50 fmol/μL.

Extracellular Proteins

The extracellular protein extract was obtained as previously describe by Lassek et al. (39). Briefly, 10% TCA were added to the cell free supernatant and incubated o/n at 4°C. Samples were centrifuged (10,000 x g, 1 h, 4°C). Protein pellet was washed with 70% ethanol (in the last step 96% ethanol was used). After drying using a vacuum centrifuge (Concentrator plus, Eppendorf) proteins were resuspended in 8M urea/2M thiourea buffer and centrifuged (16,000 x g, 10 min, RT) to recovery the supernatant. Proteins were separated using a Criterion™ TGX™ precast gel (4–20%, 12+2 Well Comb, 45 μL, 1 mm) (Biorad, Hercules, CA, USA), fitting with Mini Protean II-Apparatus (140 V for 75 min, in glycine running buffer). The digestion of the extracellular proteins was performed as described by Lassek et al. (39). Briefly, the excised gel pieces, from SDS-PAGE, were destained using 50% (v/v) methanol in 100 mM NH₄HCO₃. Gel pieces were dehydrated and modified trypsin (sequencing grade, Promega, Fitchburg, WI) was added to a final ratio of 1:10 (trypsin/sample) in 50 mM Tris/HCl, pH 7.5, and the sample incubated at 37°C overnight. Peptides were iteratively extracted from the gel using an ultrasonic bath for 15 min.

The protein concentration of both intracellular and extracellular extracts was determined using Roti Nanoquant (Roth, Germany) according to the manufacturer's instructions.

MS Analysis

Cytosolic Proteins

The separation of the peptide mixture from intracellular proteins was performed using a nanoACQUITY™ UPLC™ system (Waters, USA) following the protocol described by Zühlke et al. (36). The obtained data were searched against a randomized

E. faecalis OG1RF database (NCBI, version 2015-09-01) with added laboratory contaminants and yeast ADH1 sequence and sequences from the proteins encoded by the pathogenicity island of E. faecalis MMH594 (5,490 entries). For positive protein identification the following criteria had to be met: 1 fragment ion matched per peptide, 5 fragment ions matched per protein, 1 peptide matched per protein; 2 missed cleavages allowed, primary digest reagent: trypsin, fixed modification: carbamidom ethylation C (+57.0215), variable modifications: deamidation N, Q (+0.9840), oxidation M (+15.9949), pyrrolidonecarboxylic acid N-TERM (−27.9949). The protein false discovery rate (FDR) was set to 5%. For the final analyses, only identifications based on at least two peptides were considered. A protein had to be identified in at least two out of three technical replicates per biological replicate. In addition, for the final analysis protein had to be present in two out of three biological replicates per time point, which reduced FDR on protein level to <0.7%.

Extracellular Proteins

Peptide mixtures resulting from in-gel tryptic cleavage were separated by RP chromatography using an EASY-nLC (ThermoFisher Scientific, Waltham, USA). Fractionated peptides were loaded onto the analytical column at a flow rate of 700 nL min^{−1} in 100% buffer A (0.1% acetic acid) and separated using a binary 87-min gradient from 5 to 35% of buffer B (0.1% acetic acid in acetonitrile) at a constant flow rate of 300 nL min^{−1}. The EASY-nLC was coupled to an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). After a full survey scan in the Orbitrap (m/z range from 300 to 2,000, resolution 30,000, lock mass option-enabled [lock mass 445.120025]) MS/MS experiments in the LTQ XL were performed for the six most abundant precursor ions (CID). Unassigned charge states and singly charged ions were excluded from fragmentation; dynamic exclusion was enabled after 30 s. For protein identification, spectra were searched against a database of E. faecalis OG1RF (NCBI, version 2015-09-01) containing sequences of all predicted proteins from its genome, including reverse sequences and sequences of common laboratory contaminants and sequences from the proteins encoded by the pathogenicity island of E. faecalis MMH594 (5488 entries). Database searches using Sorcerer SEQUEST (version v. 27 rev. 11, Thermo Scientific) and Scaffold 4.4.1 (Proteome Software, Portland, OR, USA) as well as statistical analysis was done as described earlier (40).

Quantification Methods for Proteome Analysis of E. faecalis Strains

Cytosolic Proteins

The protocol that was used for the extraction of cytosolic proteins does not involve any steps that might lead to loss of proteins and is compatible with downstream in-solution digestion of proteins. For this reason, the label-free LC-IMSE approach was chosen for identification and quantification of proteins to reliably determine the abundances of cytosolic proteins. In particular,

the absolute quantitative data were obtained by applying a LC-IMSE approach in combination with the Hi3 quantification method (41, 42).

Extracellular Proteins

Extraction of extracellular proteins is based on precipitation and subsequent solubilization of proteins in a buffer that is not compatible with in-solution digestion. Therefore, separation by SDS-PAGE and in-gel digestion of proteins was applied, allowing to obtain reliable quantitative results based on relative spectral abundances. Relative quantitative data were obtained using a label-free LC-MS/MS approach and using normalized spectral counts (NSAF) for quantification of identified proteins (43). In this approach, the spectral counts of a protein are divided by its length and normalized to the sum of spectral counts/length in a given analysis.

Database Deposition and Graphic Representation of Data

The mass spectrometry proteomics data have been sent to the Proteome Xchange Consortium via the PRIDE partner repository (44) with the dataset identifier PXD011701 for the intracellular proteins and identifier PXD011660 for the extracellular proteins. For functional prediction and classification of both cytosolic and extracellular proteins, the analysis pipeline ProPhane was used (40, 45) and Voronoi treemaps were generated using Paver (Decodon, Greifswald, Germany; <http://www.decodon.com/paver/>). Thanks to the Voronoi treemaps, it is possible to visualize the bacterial proteome, grouping the proteins according to their function, sub-function or specific protein name (the functional prediction is based on TIGRFAMS and Cluster of orthologous groups (COG)).

Phenotypic Analyses

The *E. faecalis* isolates were analyzed for activity of different known virulence factors namely, biofilm formation, gelatinase, protease, and hemolytic activity.

Protease Assay

The assay was performed on the cell-free supernatant as described by Pessione et al. (16), using Azocasein solution (1% Azocasein, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA) instead of Azoalbumin.

Gelatinase Assay

The assay was performed on the cell-free supernatant as described by Pessione et al. (16). Briefly, 4 ml aliquots of a solution of 3% (p/v) gelatine in 50 mM pH 7.3 Tris-HCl were distributed in 15 ml tubes. At room temperature gelatin solution appears to be liquid, while at 4°C it becomes solid. The tubes were incubated at 4°C for 1 h to evaluate the ability of solution to solidify and then incubated at 37°C to bring it at the liquid state again. Four hundred microliters of the concentrated bacterial extracellular extracts were added to the liquid solutions and the mixtures were incubated 2 h at 37°C, the optimum for gelatinase activity. After that, they were incubated overnight at 4°C to allow

to gelatin to solidify again if gelatinase was not present in the extracellular extracts.

Hemolytic Assay

Cells from a liquid culture were plated on Columbia Agar base (Oxoid, Munich, Germany) plates enriched with 5% defibrinated horse blood (TCS Biosciences, Botolph Claydon, UK). The hemolytic activity was checked after 48 h examining the cultures for signs of β -hemolysis (clear zones around colonies), α -hemolysis (green zones around colonies), γ -hemolysis (no sign of hemolysis).

Biofilm Formation

In vitro biofilm formation was investigated as described by Laverde Gomez et al. (35) with slight modification. Bacterial cells from exponential growth phase were incubated for 48 h at 37°C in 96-well microliter plates. The planktonic culture was removed and the biofilm (placed on the bottom of the well) was washed with PBS and stained with crystal violet solution (Roth, Karlsruhe, Germany). The absorbance at OD_{595nm} of the 99.8% ethanol used to destain the biofilm was measured. The biofilm formation was determined as OD_{600nm}/OD_{595nm} (ratio between culture and destaining solution absorbance).

Determination of Antimicrobial Susceptibility

The three strains were tested for susceptibility to 18 antibiotics, including different antibiotic classes such as aminoglycosides and glycopeptides, using the broth microdilution method according to DIN58940 (46). The following antibiotics were used: penicillin, ampicillin, gentamycin, streptomycin, vancomycin, teicoplanin, daptomycin, clindamycin, erythromycin, ciprofloxacin, moxifloxacin, tetracycline, tigecycline, rifampicin, linezolid, mupirocin, chloramphenicol, and trimethoprim/sulfamethoxazole. The test was performed according to EUCAST breakpoints definitions (v 8.1) or to ECOFF (epidemiological cut-off) values when no breakpoints were defined (www.eucast.org). As a quality control, also the *E. faecalis* ATCC29212 was tested in this experiment, as recommended by EUCAST guidelines.

RESULTS

The extracellular and intracellular proteomic profiles of the food-isolate *Enterococcus faecalis* D27 (from now referred to as D27) were compared to the profiles of the probiotic *E. faecalis* Symbioflor 1 (from now referred to as Symb1) and the pathogenic *E. faecalis* UW3114 (from now referred to as UW3114), in order to assess the potential risks and benefits associated to the not well-characterized *E. faecalis* D27. Phenotypic aspects connected to virulence/pathogenesis were also investigated.

Gel-free Proteomic Analyses

In order to highlight the proteins directly or partially related to pathogenicity possible present in each of the three investigated strains, the (NCBI) database of *E. faecalis* OG1RF was used for protein identification. Moreover, to identify proteins encoded by the pathogenicity island (PAI) of UW3114 the

database also contained the sequences of the PAI of strain MH594, since many of the proteins encoded by this PAI were also found on the PAI of UW3114 (35). Only proteins present in at least two of the three biological replicates, were considered for comparative analysis. The proteomic pattern obtained for the food strain D27, was compared to those of the probiotic strain Symb1 and the clinical isolate UW3114 separately. This approach allowed to establish: (i) which proteins are expressed by both matched bacteria but in different amounts and (ii) which proteins belong to the so called on/off proteins, that are synthesized by one strain but not by the other.

In the intracellular compartment of D27, Symb1, and UW3114 we identified 889, 883, and 860 proteins, respectively (Tables S1, S2). The classification of intracellular proteins in functional groups reveals similar profiles among the three strains (Figure 1), including proteins involved in: (I) protein synthesis, (II) energy metabolism (III) metabolism (metabolite transport and energy conversion), (IV) protein fate and (IV) poorly characterized proteins. The most striking difference is the presence of the high-abundant protein Gls24-like protein Ef0055 in strain UW3114, located on the pathogenicity island. In the secretome we identified 440, 370 and 253 proteins for D27, Symb1, and UW3114, respectively (Tables S3, S4). Only proteins with extracellular, cell wall, cell membrane or unknown sub-localization in the localization prediction by pSortb (version 3.0.2) were considered for the discussion.

Comparison Food/Probiotic

Concerning the cytosolic compartment, 794 shared proteins (88 % of the identified proteins), between D27 and Symb1, were identified by mass spectrometry analysis (Table S1). Figure 2A shows a general overview on the different proteins amount in D27 in comparison to Symb1 (color variation in a scale from red, abundant in D27 to blue, abundant in Symb1). Quantitative differences were detected, especially for proteins involved in metabolism, cell envelope, information storage and processing. Interestingly, the “poorly characterized” protein area comprises a high number of significant differentially expressed proteins (Figure 2A).

The proteins that are more abundant in D27 as compared to Symb1 are 37 and, among them the conserved protein UCP028846 (18.27 fold change - OG1RF_11419), tagatose-biphosphate aldolase (11.95 fold change - OG1RF_10434), 1-phosphofructokinase (7.70 fold change - OG1RF_10431), exonuclease SbcC (6.88 fold change - OG1RF_12058) and glycosyl hydrolase (6.05 fold change - OG1RF_12425) show the highest fold change values. For what concern the 95 proteins present in D27 and absent in Symb1, are worth mentioning the hemolysin (OG1RF_10438) and the penicillin-binding protein 1B (OG1RF_11450) (Table S1).

D27 and Symb1 share 344 extracellular proteins that represent about the 90% of the identified proteins. The Voronoi treemap (Figure 2B) provides an overview of the differentially expressed proteins between the two strains according to the quantitative data [based on the normalized spectrum abundance factors (NSAF)]. As suggested by the graphic

visualization, most of the common proteins show a comparable level in two strains considered. Forty proteins are more abundant in D27 as compared Symb1, showing fold changes ranging between 5.63 and 2.05, most belonging to membrane transporters and cell-wall biosynthetic enzymes. The proteins present in D27 and absent in Symb1 are 95, where the gelatinase (OG1RF_11526) is the most interesting identified protein (Table S3).

Comparison Food/Pathogen

The cytosolic identified proteins shared by the dairy-isolate D27 and the clinical isolate UW3114 are 769, corresponding to the 87% of the total identified proteins (Table S2). As noticed previously, comparing D27 and Symb1, most of the proteins were assigned to the functional groups of metabolism-involved, cell envelope, information storage/processing and poorly characterized proteins (Figure 3A). The proteins more abundant in D27, as compared to UW3114, are 43 (Table S2) and among them the aldehyde-alcohol dehydrogenase (9.47 fold change - OG1RF_10627), phosphoglycerate mutase (5.50 fold change - OG1RF_12264), formate acetyltransferase (5.30 fold change - OG1RF_11329), 2-dehydropantoate 2-reductase (5.20 fold change - OG1RF_11367), uracil phosphoribosyltransferase (5.14 fold change - OG1RF_11432) show the highest fold change values. For what concern the 120 proteins present in D27 and absent in UW3114, are worth mentioning the hemolysin A (OG1RF_10716) and the β -lactamase (OG1RF_11969).

In the extracellular compartment of the D27 and UW3114, 226 proteins expressed by both strains were found (Table S4). The Voronoi treemap (Figure 3B) outlines how most of the proteins (red cells) are more abundant in the food isolate compared to the clinical strain. In particular, 116 proteins were more abundant in D27 compared to UW3114, showing fold change ranging between 21.06 and 2.01 (Table S4). Besides membrane transporters and cell-wall biosynthetic enzymes are worth mentioning a penicillin-binding protein C (13.75 fold-change - OG1RF_10724) and a β -lactamase (6.98 fold-change - OG1RF_11219). The different protein profiles between strains, indicated by the high fold change variation of the common proteins, is also supported by the presence of 212 proteins only identified in D27 and absent in UW3114 (Table S4). Among these, the most interesting are: choloylglycine hydrolase (AAM75246.1_39), efflux transporter (RND family - OG1RF_10301), PFL4705 family integrating conjugative element protein (OG1RF_12168) and chitin-binding protein (OG1RF_12499) (Table S4).

Phenotypic Tests

Evaluation of Enzymatic Activities Related to Pathogenicity

Protease and hemolysis were evaluated in the three strains in study, being the most common enzymatic activities associated to virulence, and the results are reported in Figure 4. As shown in Figure 4A, the strain UW3114 displays a 2-fold more intense protease activity than the food-isolate D27. As respect to the probiotic, no comparison can be done since

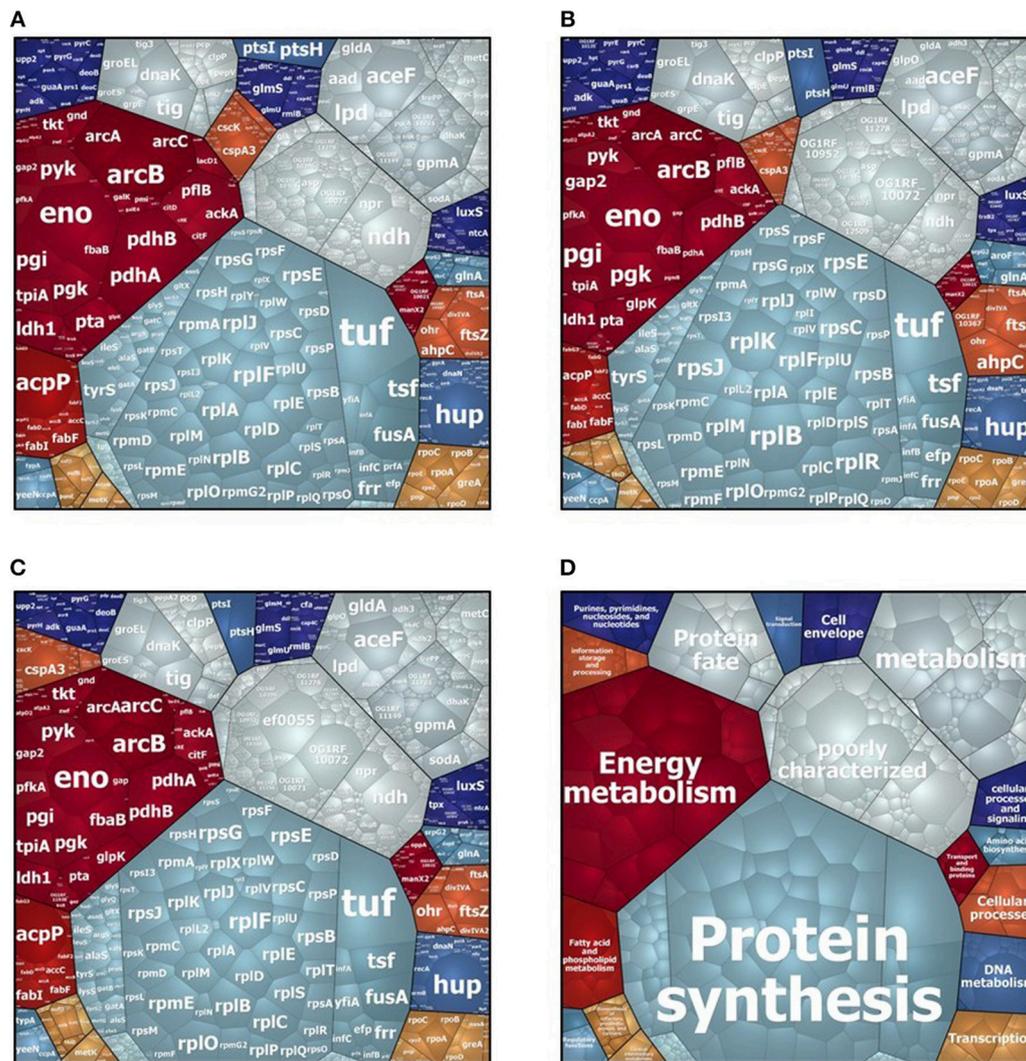


FIGURE 1 | Voronoi treemap visualization of cytosolic proteins of *E. faecalis* D27 (A), *E. faecalis* Symbioflor 1 (B) and *E. faecalis* UW3114 (C). Each protein is represented by a small cell, proteins are clustered according to their functional classification, cell-size correlates with protein abundance. (D) Represents functional organization of proteins.

for the latter measured absorbance values were negative. Concerning hemolysis, only the clinical isolate UW3114 shows hemolytic activity, against horse blood cells, where a β -hemolysis pattern (formation of clear halos) was observed (Figure 4B). Concerning gelatinase all the three strains were negative (data not shown).

Biofilm Formation

The ability to produce extracellular matrix was evaluated by growing bacteria on an abiotic solid surface, to allow biofilm formation. Although the three strains were able to secrete extracellular polymeric substances, biofilm quantification (using crystal violet staining) demonstrated that for the clinical strain biofilm is 8-fold more abundant than for Symb1 and 4-fold more than for D27 (Figure 5).

Antibiotic-Resistance Profiles

To get general insights in the antibiotics susceptibilities and resistances of the analyzed *E. faecalis* strains, 18 different antibiotics were tested and the results reported in Table 1. All the three isolates showed species-specific resistance to mupirocin (MUP) and also to clindamycin (CLI). These results were expected in enterococci, and confirmed by the test on the reference strain *E. faecalis* ATCC29212. None of the isolates showed resistance to glycopeptide compounds (vancomycin and teicoplanin) or to penicillin antibiotics (penicillin and ampicillin). Both the probiotic and the dairy-isolated strain D27 showed to be susceptible to 16 analyzed antibiotics. Conversely, the clinical isolate UW3114 was found to be resistant to 8 antibiotics, among which erythromycin. Erythromycin-resistance is ascribable

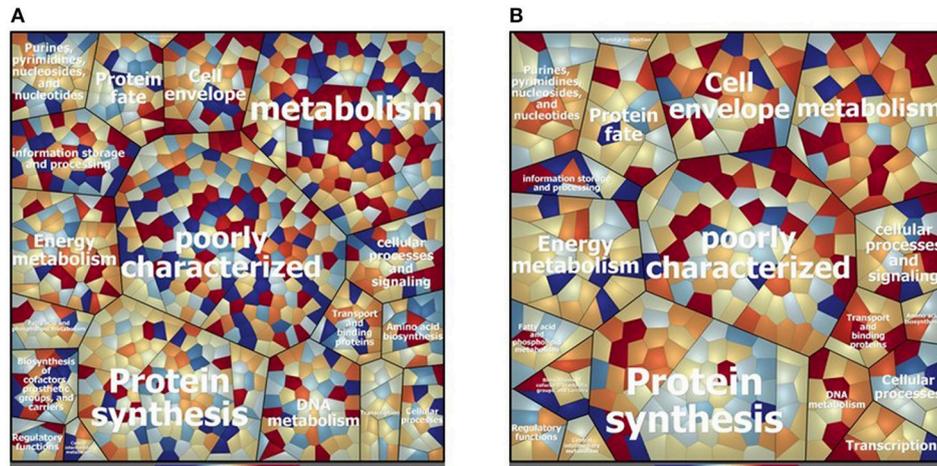


FIGURE 2 | Voronoi treemap visualization depicting the different protein pattern of *E. faecalis* D27 and *E. faecalis* Symbiofor 1 in the cytosolic (A) and extracellular (B) compartment. The proteins are grouped according to their predicted functional classification. Variation of relative protein amounts are indicated by color: red indicates higher amount in D27, blue higher abundance in Symbiofor 1.

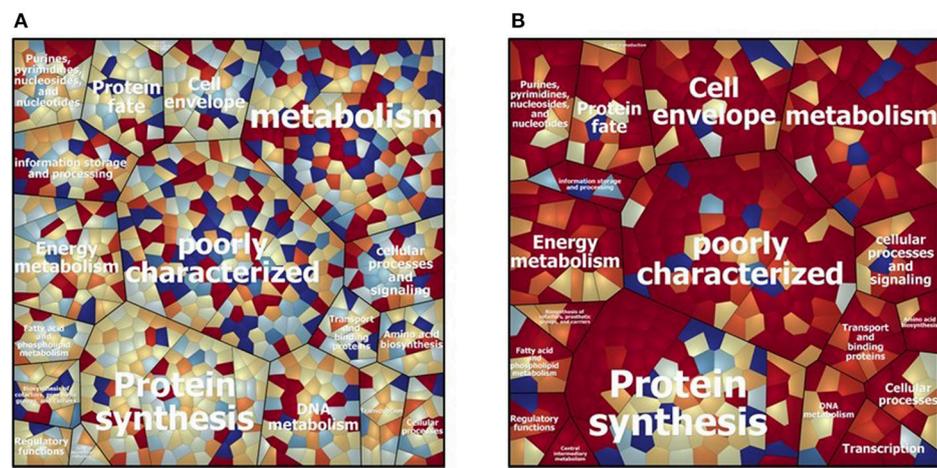


FIGURE 3 | Voronoi treemap visualization depicting the different protein pattern of *E. faecalis* D27 and *E. faecalis* UW3114 in the cytosolic (A) and extracellular (B) compartment. The proteins are grouped according to their predicted functional classification. Variation of relative protein amounts are indicated by color: red indicates higher amount in D27, blue higher abundance in UW3114.

to the presence of the PAI, as described before by Laverde Gomez (35).

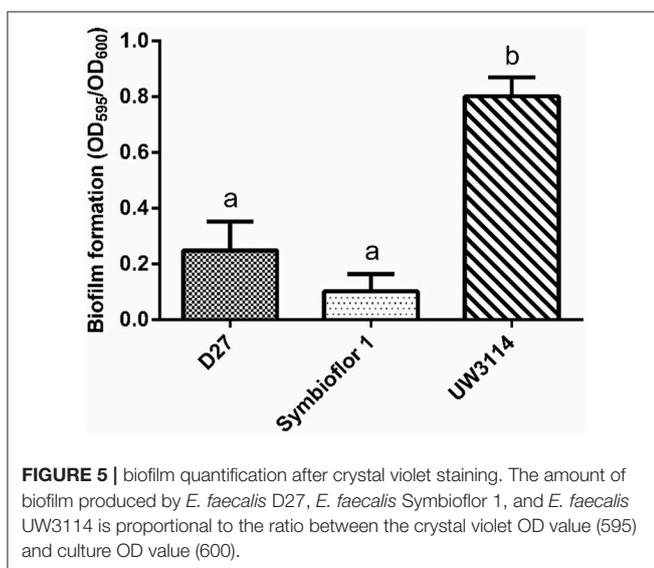
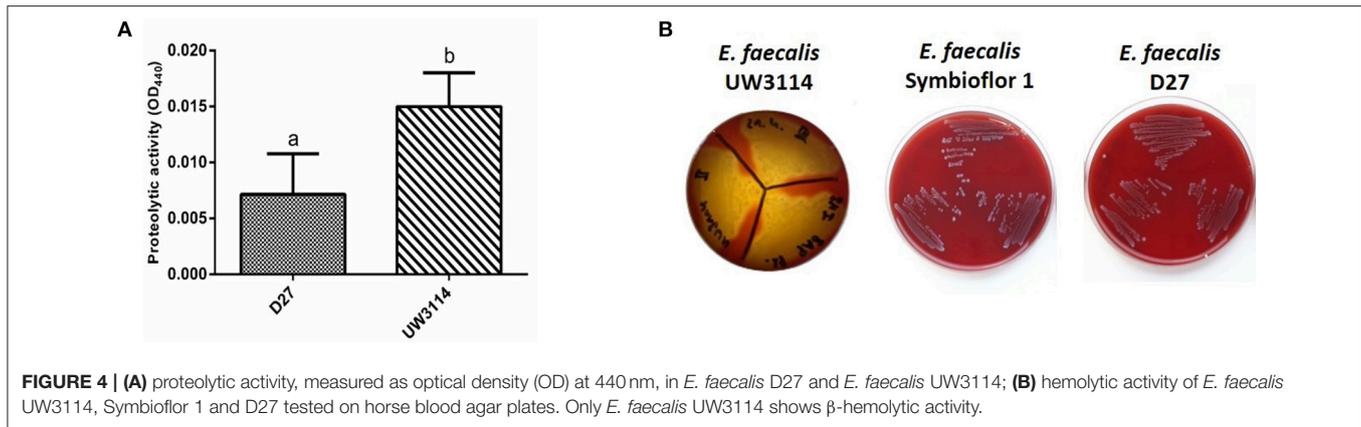
DISCUSSION

Establishing the safety of a food-isolated strain is of primary importance especially when the controversial species *E. faecalis* is concerned. Actually, enterococci are not generally regarded as safe (GRAS) by e.g., the FDA. Proteomics is a valuable strategy to detect virulence characters. Furthermore, comparison with clearly recognized pathogenic and probiotic bacteria is an added value to highlight differences useful to classify the food-isolated strains as safe.

The *E. faecalis* in study D27 was compared to a clinical isolate UW3114 and to a commercialized probiotic Symb1. Several proteins display a significant fold-change as compared to the other two enterococci or are exclusively present in the food isolate. Of these, some are worth discussion because of their implication in pathogenicity, antibiotic resistance, gene transfer and possible human-host interaction.

Proteins Involved (or Possibly Involved) in Pathogenicity

Several proteins connected with pathogenic traits are expressed by the dairy-isolate D27. The protein named “secreted antigen” was found to be a serine protease by database search (BLAST



comparison with *E. faecalis* strains). The serine protease HtrA (OG1RF_12305) was expressed by both D27 and UW3114, however it was 2-fold more abundant in the clinical isolate. In the secretome, a different serine protease SprE (OG1RF_11525) was detected only in the hospital-isolated strain UW3114. Although D27 does not express SprE, another serine protease with pathogenic implications was found, the S41A family carboxy-terminal peptidase (OG1RF_11392). It has been reported that, in a murine sepsis model, disease caused by a S41-deficient mutant of *S. aureus* was less severe than that caused by the WT strain; this result demonstrates the role of these proteins in Gram-positive pathogenicity (47). A further proteolytic enzyme found in both D27 and UW3114 secretome is gelatinase (OG1RF_11526), one among the most important virulence factors in *E. faecalis*. The presence of genes for gelatinase E (GelE) and serine protease V8 (SprE) is usually considered a marker to identify potential pathogenic strains (48). However, it was already observed that a cheese-isolated *E. faecalis* strain does not show gelatinase or protease activity when submitted to phenotypic tests, even if possessing the abovementioned genes (16). This phenomenon

was also observed in strains D27 and UW3114, where the presence of gelatinase enzyme does not support gelatinase activity. Two different types of hemolysins were present: hemolysin (OG1RF_10438) and hemolysin A (OG1RF_10716). D27 expresses both enzymes, whereas both the probiotic and the clinical strains express only one of them. However, the clinical isolate is the only strain showing β -hemolysis *in vitro*, as expected (Figure 4B). Actually, it is long time established that the presence of the *cyl* operon is indispensable but not sufficient for the hemolytic activity, since different enzymes are involved and a proteolytic cleavage is required for protein activation (49). The influence of environmental factors on gene expression is a well-known phenomenon. For Enterococcus, the effect of multiple external factors on pathogenic character expression has been described since 1998 (50).

Finally, a LemA family protein (OG1RF_10353) was found in D27 and UW3114, with less than 2-fold change in abundance. This protein family was well characterized in the *Pseudomonas* genus, where it was identified as a transmembrane histidine protein kinase (HPK) sensor-regulators, involved in lesion formation in the host (51).

Proteins Involved in Antibiotic Resistance

As a general rule, it has to be highlighted that in Enterococci, antibiotic resistance is based upon three main mechanisms: (a) intrinsic or natural resistance (b) acquired resistance and (c) tolerance. The intrinsic resistance is a genus-related feature and it is generally linked to insensitivity of the bacterial cell to the antibiotic molecules either through absence of the uptake mechanism (5), or to a modification of the antibiotic targets such as the PBPs (penicillin binding proteins) (52) or to efflux systems (53). Discovered in the 1980 (54) and referred to also as “intrinsic insensitivity” it is the main cause of the difficulty of treating enterococcal infections (55). The acquired resistance, on the contrary, is often based upon production of enzymes modifying or degrading the antibacterial molecule and it is transmissible by genetic recombination. Hence, this last mechanism is more dangerous since it can allow spread of the resistance character among strains (5).

Two classes of proteins were found that could confer antibiotic resistance to the *E. faecalis* strains under investigation: the

TABLE 1 | Antibiotic susceptibility (green) and resistance (red) of the three tested strains of *E. faecalis*, D27, UW3114, and Symbioflor 1, and the quality control strain *E. faecalis* ATCC29212, as recommended by EUCAST guidelines.

	D27	UW3114	Symbioflor1	ATCC29212
PEN	2	8	4	2
AMP	2	4	4	2
GEN	≤64	512	≤64	≤64
STR	≤128	2,048	≤128	≤128
VAN	2	2	4	4
TPL	≤1	≤1	≤1	≤1
DAP	≤2	≤2	≤2	≤2
CLI	>8	>8	8	8
ERY	≤1	>16	≤1	≤1
CIP	≤2	32	≤2	≤2
MFL	≤0.125	>2	≤0.125	0.25
LNZ	≤2	≤2	≤2	≤2
TET	≤0.5	>16	≤0.5	>16
TGC	≤0.063	≤0.063	≤0.063	≤0.063
RAM	2	≤1	4	≤1
SXT	≤0.032	≤0.032	≤0.032	≤0.032
CMP	8	8	8	8
MUP	>32	>32	>32	>32

PEN, penicillin; AMP, ampicillin; GEN, gentamycin; STR, streptomycin; VAN, vancomycin; TPL, teicoplanin; DAP, daptomycin; CLI, clindamycin; ERY, erythromycin; CIP, ciprofloxacin; MFL, moxifloxacin; LNZ, linezolid; TET, tetracycline; TGC, tigecycline; RAM, rifampicin; SXT, trimethoprim/sulfamethoxazole; CMP, chloramphenicol; MUP, mupirocin.

true resistance marker β -lactamase and the penicillin-binding proteins (PBPs) that can be involved in resistance if a mutation in their sequence occurs. The three strains show to possess a heterogeneous set of these proteins.

As far as the membrane-located PBPs are concerned, their relative abundance evaluation has the limitation of a low reliability, because the extraction procedure could not have been homogeneous in the three samples analyzed. PBPs (e.g., PBP2B, PBP3, PBP4, PBP5) are involved in the peptidoglycan formation. However, it was observed that the presence of a high molecular weight variant of PBP5 decreased susceptibility of *E. faecium* to ampicillin and other β -lactams (56, 57). In *E. faecalis*, resistance to both imipenem and ampicillin due to amino acid mutation (520 and 605 residues) in the PBP4 was observed (58). In the cytosolic proteome of the studied strains, the PBP1A (OG1RF_10925) seems 2.5-fold more abundant in D27 compared to Symb1; the PBP1B (OG1RF_11450) is exclusively expressed by D27; the PBPC (OG1RF_10724) was found in large amount in D27, compared to the other two strains. Furthermore, extracellular proteins identification in the food strain confirmed the presence of PBPs, with some differences respect the cytosolic compartment. PBP1A that in the cytosol was found more abundant in D27 compared to Symb1, in the extracellular compartment appears to be more abundant in the probiotic strain. Therefore, it seems that this abundance is rather due to compartmentalization than to higher gene expression.

Three different β -lactamases were found, both in the intra- and extra-cellular proteomes. Two of them are metallo-hydrolases, one exclusively expressed in Symb1 (OG1RF_10969) and the other (OG1RF_11863) 2.5-fold more abundant in the clinical isolate compared to the food-isolate; the last is a serine-hydrolase (OG1RF_11969) exclusively expressed in D27. The antibiotic inactivation based on hydrolytic activity is mostly effective when applied to β -lactam antibiotics. In particular, there are two main molecular strategies employed by β -lactamases to hydrolytically cleave the β -lactam ring of penicillins and cephalosporins: through the action of an active site Ser-nucleophile, or through activation of water via a Zn^{2+} center (59). As reported in the literature, the proteins described above are responsible for insensitivity to β -lactam antibiotics in enterococci (60). Furthermore, this type of resistance seems to be transmissible (5).

The identification of a high variety of proteins, possibly involved in resistance, found in the proteomes of the three strains in study, suggests that resistance events could occur also in the probiotic and the dairy-isolate that share a similar antibiotic type. Actually, probiotics were already described as harboring resistance traits (61). However, in spite of these protein profiles, the antibiotic susceptibility testing revealed a general sensitivity pattern toward beta-lactams in all the three strains considered (Table 1). As far as the PBPs are concerned, it is possible that they are active and not mutated forms as those described in *E. faecalis* by Ono (58). Regarding beta lactamases, it is possible to hypothesize that the expression of these proteins is a condition necessary but not sufficient to engender resistance, suggesting that other factors can play a role in the occurrence of resistant phenotypes.

As far as the food-borne *Enterococcus* D27 is concerned, an efflux transporter (RND family - OG1RF_10301) has also been found. Efflux pumps, responsible either of multidrug resistance mechanisms or of single antibiotic extrusion, have been described in LAB as well (62). In the strain D27 this efflux system could be involved in the clindamycin resistance pattern observed (Table 1), whereas the resistance to mupirocin observed seems to be due to other mechanisms as it has long been established (63). On the other hand, both these resistances are present in all the three strain considered, suggesting that they can be based upon an intrinsic resistance mechanism as frequently described for Enterococci (55).

Proteins Involved in Horizontal Gene Transfer

The PFL4705 family integrating conjugative element protein (OG1RF_12168) was found both in the food-isolate D27 and in the probiotic strain Symb1, with comparable expression levels. Often, virulence determinants are acquired as mobile elements during gene transfer processes also mediated by this protein and these genetic exchanges are frequent between *E. faecalis* strains (35, 64). The second interesting protein more expressed by D27, is the pheromone cAM373 (OG1RF_11130). It was characterized also in *S. aureus* and corresponds to a

heptapeptide (AIFILAS) located within the C-termini of the signal sequences of putative pre-lipoproteins. This hydrophobic, linear peptide molecule acts as signals that facilitate the conjugative transfer of a specific category of plasmids referred to as pheromone-responsive plasmids (65). This specific type of inducible plasmid represents an important mechanism for dissemination of antibiotic resistance and virulence among *Enterococcus* strains (66). These findings also suggest that the food ecosystem is a suitable environment for favoring cell-to-cell communication.

In the extracellular fraction, a second pheromone, cAD1 (OG1RF_12509), more expressed by the dairy-isolate, was found. In *E. faecalis*, this octapeptide sex pheromone was identified as responsible of an induced mating response by donors carrying the hemolysin plasmid pAD1 or related elements (67). This result, although expected for the clinical isolate is surprising for the food strain that generally do not need to acquire hemolysins in its ecological niche.

Proteins Involved in Human Host/Environment Interactions

The von Willebrand factor (vWF) type A domain protein (OG1RF_10869) is an example of potential pathogenicity-associated protein more expressed in the secretome by the food-isolate strain compared to the probiotic and clinical isolates (fold-change 2.21 and 2.16, respectively). vWF is a huge multimeric protein, well-characterized in humans, where it triggers platelet adhesion in areas of vascular damage. In particular, the domain A1 mediates platelet adhesion under flow in areas of vessel injury through the platelet glycoprotein Ib α (GPIb α) (68). Most prokaryotic vWF domains have not been investigated in detail, however, Konto-Ghiorgi et al. (69) showed that the GBS PilA tip pilin vWF domain was important for pilus-mediated bacterial adhesion to human alveolar and intestinal epithelial cells *in vitro*. The last newsworthy evidence is the variety of lipoproteins produced by the dairy-isolate. Even if the function and the localization is still unknown for most of them it is well-recognized that lipoproteins in Gram-positive bacteria represent about the 25% of the surface associated proteins, which could play a major role in bacterial virulence processes (70).

Among up-regulated proteins in D27, choloylglycine hydrolase (AAM75246.1_39) represents an interesting enzyme since it catalyzes the initial “gateway” reaction in the bacterial metabolism of CBAs (conjugated bile acids). The reaction consists in a deconjugation of CBAs to liberate free primary bile acids (BAs; cholic acid or chenodeoxycholic acid) and amino acids (71). CBAs have been suggested to repress bacterial growth in the small intestine by means of direct antimicrobial effects, up-regulation of host mucosal defenses, or synergistic action of both mechanisms (72). The expression of this enzyme by the food-isolate can increase the ability of the strain to survive to CBAs action in the gut, thus enhancing the probiotic potential of D27. This is partly expected, because *Enterococci* generally display a high degree of resistance to bile salts (73). Considering that, all the three strains share the presence of several proteins involved in mucosa and mucus adhesion (e.g., enolase, EFTu,

EFTs, DnaK, Clp, GroEL, chitin-binding protein) (74, 75), it is possible to hypothesize that the foodborne D27 can also share the same attitude to persist in the human host by adhering to the gut mucosa. However, D27 ability to form biofilm, although higher than Symb1, is four-fold lower than the one observed in the pathogenic UW3114.

CONCLUSION

The results obtained in the present investigation comparing a food-isolated *E. faecalis* D27 with a patented probiotic and a pathogenic isolate, underline that there is the need of a detailed typing of strains, either employed in food fermentation or foodborne contaminants, to avoid risks of virulence dissemination.

The probiotic strain *E. faecalis* Symbioflor 1, although bearing some resistance traits, possess several proteins that support its role as a probiotic, namely involved in stress response, that are of primary importance both for the shelf-life of the probiotic preparation and for bacterial survival in the human gastrointestinal tract. The clinical isolate *E. faecalis* UW3114 was the only strain synthesizing some of the ascertained virulence determinants such as the serine protease SprE and displaying the highest levels of antibiotic resistance included aminoglycosides. All these results were expected.

As far as the foodborne isolate is concerned, *E. faecalis* D27 besides showing a high metabolic activity (confirmed by the up-regulation of several proteins involved in hydrolysis, energy metabolism, and nutrient transport) also displays some proteins whose function could be hazardous. Among these, antibiotic resistance factors (although not all expressed in the tested conditions) and horizontal gene transfer involved proteins (PFL4705 family integrating conjugative element), are a clear evidence that recombination events (both in food and in the GIT) can cause antibiotic resistance spread. Other possible virulence factors are the vWF type A domain protein, and some proteases involved in pathogenicity, like the serine protease HtrA, the S41A family carboxy-terminal peptidase and LemA. On the other hand, the presence in D27 of a high number of proteolytic enzymes is probably linked to the advantage of possessing proteases in a food matrix like cheese that is very rich in proteins. It is worth to highlight that single approaches for typing bacteria to find pathogenicity factors all have the limitation of considering characters (genes or proteins) that not always are disclosed.

Considering the heterogeneous habitat in which food microorganisms live, modifications of their proteomic patterns can also occur both by interaction with other bacteria (genetic exchanges) and during contact with the host. The results obtained in the present investigation are far to be exhaustive since new genes and proteins involved in pathogenesis in different *E. faecalis* strains are repeatedly reported in the literature. However, taken together, these data demonstrate the importance of carefully and periodically characterize food-isolated enterococcal strains to ascertain their safety before employing them for human consumption. In this context, in parallel to genetic analysis, complementing gel-free proteomics (especially the

analyses concerning secreted “marker-factors”) and phenotypic tests proved to be a valuable tool to assess these features.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fnut.2019.00071/full#supplementary-material>

Table S1 | The table includes the cytosolic proteins identified in at least two of the three biological replicates of *E. faecalis* D27 and *E. faecalis* Symbioflor 1 and the proteins found only in one *E. faecalis* strain and absent (off) in the other strain. The fold-change is given by the ratio between the average of the protein abundance (fmol/ng) in *E. faecalis* D27 and the average of the protein abundance (fmol/ng) in *E. faecalis* Symbioflor 1. Accession, protein name, description, localization (obtained by pSortb), main role and subrole are reported to give information on the

protein function. For each protein, the abundance (fmol/ng) for all the biological replicates, as well as the average of the abundance are reported for both *E.*

faecalis D27 and *E. faecalis* Symbioflor 1. The Repcount refers to the number of technical replicates in which the protein was identified.

Table S2 | The table includes the cytosolic proteins identified in at least two of the three biological replicates of *E. faecalis* D27 and *E. faecalis* UW3114 and the proteins found only in one *E. faecalis* strain and absent (off) in the other strain. The fold-change is given by the ratio between the average of the relative abundance (fmol/ng) in *E. faecalis* D27 and the average of the relative abundance (fmol/ng) in *E. faecalis* UW3114. Accession, protein name, description, localization (obtained by pSortb), main role and subrole are reported to give information on the protein function. For each protein, the abundance (fmol/ng) for all the biological replicates, as well as the average of the abundance are reported for both *E. faecalis* D27 and *E. faecalis* UW3114. The Repcount refers to the number of technical replicates in which the protein was identified.

Table S3 | The table includes the secreted proteins identified in at least two of the three biological replicates of *E. faecalis* D27 and *E. faecalis* Symbioflor 1 and the proteins found only in one *E. faecalis* strain and absent (off) in the other strain. The fold-change is given by the ratio between the average of the abundance [normalized spectral abundance factor (NSAF)] in *E. faecalis* D27 and the average of the abundance (NSAF) in *E. faecalis* Symbioflor 1. Accession, protein name, description, localization (obtained by pSortb), main role and subrole are reported to give information on the protein function. For each protein, the abundance (NSAF) for all the biological replicates are reported for both *E. faecalis* D27 and *E. faecalis* Symbioflor 1.

Table S4 | The table includes the secreted proteins identified in at least two of the three biological replicates of *E. faecalis* D27 and *E. faecalis* UW3114 and the proteins found only in one of *E. faecalis* strain and absent (off) in the other strain. The fold-change is given by the ratio between the average of the abundance [normalized spectral abundance factor (NSAF)] in *E. faecalis* D27 and the average of the abundance (NSAF) in *E. faecalis* UW3114. Accession, protein name, description, localization (obtained by pSortb), main role and subrole are reported to give information on the protein function. For each protein, the abundance (NSAF) for all the biological replicates are reported for both *E. faecalis* D27 and *E. faecalis* UW3114.

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