Antimicrobial Potentials of Wild-type *Enterococcus* spp. from an Extreme Habitat and a Fermented Food – A Retrospective Mini-review

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The wild-type enterococcal strains are very well known for the production of bacteriocins active against both gram-positive and gram-negative bacteria. But reports on strains belonging to the genus enterococcus showing antifungal activity are relatively less abundant as compared to the bacteriocinogenic *Enterococcus* spp [45]. We showed in our study the broad-spectrum anti-*Candida* demonstration of an *Enterococcus faecium* strain isolated from the penguin rookery of the Antarctic region and anti-*Aspergillus* activity of *E. faecalis* recovered from cheddar cheese. Out of 240 isolates screened, one such isolate identified as *E. facium* (mentioned above)inhibited initially the *Candida albicans* NCIM 3471 and then six more *Candida* strains [47]. The partially purified anti-*Candida* peptide from the *E. faecium* strain revealed the twelve amino terminal amino acid residues. The analysis of the major twelve amino acid residues from the N-terminus, minor twelve N terminal amino acids residues, and the *de novo* sequences unravelled the presence and roles of LysinM motif, glycine-proline-glycine, and small stretch of bacteriocin-like motif suspected in the anti-*Candida* activity respectively [3,4]. Besides this, the indication about the correlation between secreted antigen (SagA/Bb) and the anti-*Candida* activity has been revealed [50,61].

In another earlier study conducted by us, the *E. faecalis* CHD 28.3 (previously known as *Lactococcus lactis* CHD 28.3) showed inhibition against different strains of mycelial mold including *Aspergillusflavus* var. *parasiticus*. The partial purification of the antimicrobial substance was achieved and was the partially purified substance was tested against the mold strain [45]. This chapter enables the retrospective review about the overall antimicrobial prowess and possible bio-control application of antimicrobial agents produced by *Enterococcus* spp. from several previously conducted study.

Keywords: Antimicrobial peptides; Anti-*Candida* factor; *E. faecalis*; *E. faecium*; bacteriocin

1. Introduction

Enterococci as ubiquitous microbes inhabit soil, food, water and gastrointestinal tract of humans and animals. A small number of enterococci are also found in oropharyngeal and vaginal secretions, and on the skin, especially in the perineal area and, consequently, they can be considered as normal inhabitants of the human organism [1]. Besides this, Enterococci play a major role in the development of the typical organoleptic characteristics of a variety of fermented foods such as cheeses, fermented sausages and vegetables where, in some cases, they are the predominant lactic acid bacteria [2,3]. The genus Enterococcus has acquired a special niche in a host of lactic acid bacteria (LAB) by virtue of its potential for producing various metabolites like lactic acid, hydrogen peroxide, bacteriocins and bacteriocin like inhibitory substance (BLIS). Many of these bacteriocins have been designated as enterocins, a new class of atypical bacteriocins from the genus Enterococcus; additionally a single strain of Enterococcus was reported to produce two different types of enterocins [4,5,6]. Many lactic acid bacteria (LAB) have proved their capability and versatility by producing highly diverse bacteriocins. Though these armamentariums are produced by LAB found in numerous fermented and non-fermented foods, nisin is currently the only bacteriocin widely used as a food preservative. During food fermentation enterocins were shown to control emergent pathogenic bacteria [7].

Going far beyond, the Enterococcus though not unarguably earned its pedestal as generally regarded as safe (GRAS) status LAB involved in food fermentation and preservation [8,9]. The genus *Enterococcus* currently comprises more than 26 species. *Enterococcus faecalis*, *E. faecium* and *E. durans* are predominant species most frequently found in dairy products [10]. Investigation on the microbiota of many traditional cheeses in the Mediterranean countries have indicated that enterococci play a pivotal role in the ripening of variety of cheeses, probably through proteolysis, lipolysis, and citrate utilization, hence enhancing their organoleptic attributes [11-12]. For example, a couple of Enterococcus strains (*Enterococcus mundtii* CRL35 and *E. faecium* ST88Ch), isolated from cheeses, were characterized and tested for their capability to control growth of *Listeria monocytogenes* 426 in experimentally contaminated fresh Minas cheese during refrigerated storage [13]. Enterococci are also present in other fermented foods, such as sausages and olives. However, their role in these products has not been fully elucidated. Besides this, of late, enterococci have demonstrated their probiotic potential to be used in functional foods [10-11]. Along with the genera like *Lactobacillus*, and *Bifidobacterium*, the genus Enterococcus has also curved their eminent niche to have bestowed with probiotic potentials [14-15]. Enterococcus species most commonly isolated from cheese are *E. faecalis* and *Enterococcus faecium,* followed by *E. durans* [10,16]. *E. durans* LAB18s isolated from "Minas Frescal" (a Brazilian soft cheese) showed several desirable traits for a probiotic strain [15]. A high prevalence of enterococci in

processed foods may be attributed to their resistance to heat, extreme salinity and harsh conditions during ripening of fermented foods [17-18]. Their paramount importance in cheese making and contribution to the promotion of the sensory characteristics is due to proteolytic and lipolytic ability, citrate utilization and aromatic volatile compounds production in various traditional cheeses in the Mediterranean and European countries [11,19, 20, 21-22]. They are also present in other fermented food products, such as sausages and olives [12]. Hassanzadazar et al. (2014) isolated *E. faecium* from Koopeh cheese and used them in the preparation of Iranian Ultrafiltered cheese and stated the probiotic potential of *E.faecium* in preparation of Koopeh cheese [23].

2. Molds as spoilers and Control of mold spoilage

Moulds are common spoilage organisms in different food and feed products. These spoiling moulds are responsible for causing great economic losses across the globe. Food and feed contamination by fungi and by their mycotoxins constitute potential health hazard to consumers [24]. The inhibition of growth of fungi in foods remains a big threat and challenge for food industries.

Antimicrobial activity of a cationic, heat-stable and hydrophobic bacteriocin S760 (enterocin) produced by *E. faecium* strain LWP760 was studied in detail [25]. It is also active against antibiotic resistant strains of *Staphylococcus aureus*, *Enterobacter cloacae, Acinetobacter baumannii* (with MICs of 0.05-3.0 mg/l), *Klebsiella pneumoniae* (with MICs of 6 mg/l), *Pseudomonas aeruginosa* (with MICs of 0.4-25.0 mg/1), as well against fungi belonging to species of *Candida albicans*, *Candida krusei* and *Aspergillus niger* (with MICs of 0.1-0.2 mg/l) [25]. Consumer demands for minimally processed foods and reduced use of chemical preservatives have stimulated research on antifungal lactic acid bacteria as biopreservatives [26]. Recently, a number of antifungal metabolites, *e.g.* cyclic dipeptides, phenyllactic acid, proteinaceous compounds, and 3-hydroxylated fatty acids have been isolated from lactic acid bacteria. From aforementioned examples, the applications of antifungal lactic acid bacteria including the *E. faecalis* in food and feed preservations are clearly understood. Many antifungal compounds or preservatives have been tried so far to observe the yeast inhibition in food storage, but the anti-yeast spectrum and effectiveness cannot meet the demand of disease treatment or food safety aspects [26, 27, 28]. In food industry, certain spoilage yeast could even survive in presence of preservatives at the maximum dose set by appropriate standards and legal abiding and strictures [29]. Furthermore, various antifungal agents and chemical additives have demonstrated health threat to the patients and consumers. A novel method of low side effects with inhibition against pathogenic and spoilage yeasts should therefore be developed.

3. Prophylactic effects of *E. faecalis*

The prophylactic effect of *E. faecalis* has been studied recently. The prophylactic effects of heat-killed cells of *E. faecalis* FK-23 (FK-23 preparation) on experimental candidiasis were investigated in normal and leukopenic mice [30]. The intraperitoneal administration of the FK-23 preparation into mice augmented the anti-*Candida* activity of immunocompromised peritoneal exudate cells obtained from the animals. These results suggested the potential usefulness of the FK-23 preparation as a prophylactic agent for the management of patients with opportunistic fungal infections [30].

4. Antifungal Potentials of *Enterococcus* spp.

Though an uncanny number of reports have been published so far on the bacteriocins like enterocins produced by *E faecalis* and *E. faecium* active only against gram-positive bacteria (Franz et al. 1996) [31], some exceptions with broad activity spectra have been described in recent years to show the ability to inhibit the gram-negative microorganisms; studies had reported earlier that the antimicrobial principles produced by LAB are inactive against Gram-negative bacteria and eukaryotic microorganisms such as yeasts or moulds [32]. Dalie et al. (2010) expressed the view that the main LAB endowed with their potentials to prevent or limit mycotoxinogenic mould growth belong to the genera *Lactococcus* and *Lactobacillus* and, to a lesser extent, to *Pediococcus* and *Leuconostoc* [26].

The reports on antifungal prowess of *E. faecium or E. faecalis* are relatively scanty [33]. Roy et al. [33] isolated numerous wild-type isolated many colonies of LAB and screened them using several types of moulds and an agar well diffusion assay on potato dextrose agar containing 0.1% Triton X-100.Though initially six colonies were identified for their antifungal activity against *Aspergillus flavus* IARI, only one out of them showed a broad-spectrum of antifungal activity against *A. flavus* IARI, *A. flavus* NCIM 555, *Aspergillus parasiticus* NCM *898* and *Fusarium* sp. This isolate was identified as *L. lactis* subsp. *lactis* CHD 28.3. *Aspergillus* IARI was the most sensitive indicator of the antifungal metabolite produced by this lactic strain.

It was demonstrated in one of the previous investigations [34] that the antifungal compounds such as phenyllactic acid and 4-hydrophenyllactic acid were produced by *Lactobacillus plantarum*. The antifungal activity of *L. plantarun* was due to many organic acids such as acetic and phenyllactic acids as suggested by Lavermicocca et al. [34-35]. In

addition, bacteriocin-like substances and other compounds were produced by *L. rhamnosus*, *L. plantarum* and *E. faecalis* [36-38].

Elsie et al. [38] reported the antifungal spectra of twelve LAB isolates against eight different moulds commonly associated with cheese spoilage and all isolates were displayed inhibition against *Penicillium solitum, Aspergillus versicolor* and *Cladosporium herbarum.* To determine their potential as biopreservatives in cheese, LAB isolates were inoculated into cottage cheese prior to the addition of *P. commune*. All *Lb. plantarum* isolates were found to prevent the visible growth of *P. commune* on cottage cheese by between 14 and >25 days longer than cottage cheese that contained either no added LAB or LAB that did not have antifungal activity [37]. As reported by Lavermicocca et al. (2000), *L. plantarum* 21B in sourdough bread showed antifungal activity in association with baker's yeast *S. cerevisiae*. According to them, phenyl lactic acid and p-hydroxy phenyl lactic acid responsible for phenylalanine metabolism were the two key factors in suppressing or controlling the mold growth.

Kivanc et al. (2014) reported the strong inhibitory activity of *E. durans* against three fungal strains (*Penicillium chrysogenum, P. griseofulvum* and *A. parasiticus*) and attributed the inhibition potential due to proteinaceous substances and organic acids [39]. Previously Belguesmia et al. [40] reported that the antifungal activity of duracin produced by *E. durans* A5-11. The same authors suggested that *E. durans* can be used as starter culture in foods. With their method, *E. durans* can be applied in biopreservation directly; however more studies were required to establish the roles of *E. durans* in this regard. With the recent augmented awareness and interest in food and feed safety throughout the world, LAB cultures with GRAS tag and antifungal, antimycotoxigenic and mycotoxin binding potential could be of immense value in combating mycotoxin menace. However, the introduction of large scale biopreservation of food demands careful safety assessment and risk analysis [26].

4.1 Antifungal proteins and peptides

A multitude of peptide bacteriocins from *Enterococci* have been purified and genetically characterized over the last several decades, but the antimycotic peptides/proteins are relatively less investigated from the *lactobacilli* and *enterococci* [40-41]. Veljovic et al. (2009) reported that 11 out of 26 Enterococcus isolates produced antimicrobial compounds when various lactococci and lactobacilli were used as indicator strains. Out of 11 Enterococcus isolates, 10 reportedly synthesized a bacteriocin-like substance of proteinaceous nature since antimicrobial activity was diminished by treatment with protease. Contrarily only one isolate, designated BG221, retained antimicrobial activity after treatment with protease indicating that it produces antimicrobial substance of non-proteinaceous nature [42]. The same workers demonstrated that all 26 isolates exhibited antimicrobial activity against *Candida pseudotropicalis*, and almost all against *Erwinia carotovora*. However, none of the enterococcal isolates showed any activity against *Pseudomonas aeruginosa PAO1, Candida albicans* and *Bacillus cereus* in the same study [42].

An antifungal protein active against *A. flavus* strain was isolated from a Cheddar cheese isolate *E. faecalis* CHD 28.3 from culture supernatant using three-step method ultrafiltration, anion exchange and gel filtration chromatography [45]. Employing a 10 kDa cut off membrane, ultrafiltration of the culture supernatant resulted in a recovery of 44.6% of the antifungal protein with 1.7 fold increase in the specific activity [45]. Anion-exchange chromatography using DEAE-Sepharose matrix followed by purification of the samples using high resolution gel filtration chromatography employing Superose-12 column/FPLC system led to a very low (~0.5%) recovery of antifungal activity with an increase in specific activity by 11.9 fold relative to initial value in crude supernatant. The molecular mass of the antifungal protein from the high resolution gel filtration was estimated to be around 11 KDa [45].

Stoyanova et al. (2010) reported two very promising and new bacteriocin-synthesizing strains 194 and K-205 that were isolated from raw cow milk and kurunga from Buryatia [46]. These strains demonstrated strong antibiotic-like activity up to 3600 and 2700 IU mL^{-1} as compared to nisin and up to 2500-1700 IU mL^{-1} as compared to fungicidal antibiotic nystatin. Treatment of the raw smoked sausages with cultural broth of *L. lactis* ssp. *lactis* 194 and K-205 inhibited growth of the mold *Eurotium repens* [46]. The promising results indicated that the treatment with lactococci strains may combat the fungal spoilage of food-stuff and prevent a contamination of raw smoked sausages by potential food-borne pathogens [46].

In 2011, Shekh et al. reported about isolation of a polar strain from the Antarctic region with anti-*Candida* activity [47]. The recovery of the antifungal isolate from the penguin rookery of the Antarctic area in the form *Enterococccus feacalis/faecium* became a fact after screening of 200-plus isolates. Later this isolate was characterized for identification by polyphasic approach and the molecular characterization of the antifungal compound(s) and its probable mode of action was partially accomplished. Very recently Zheng et al. (2015) reported a wide-spectrum antibacterial activity of the cell-free supernatant (CFS) produced by a strain *E. faecium* KQ 2.6 from peacock faeces against various gram-positive and gram-negative strains; however, the same CFS when applied against the fungal strains failed to inhibit the *C. albicans* and *Aspergillus niger* [48].

Using a *Caenorhabditis elegans* model of polymicrobial infection, Cruz et al (2013) discovered that *E. faecalis* and *C. albicans* negatively impact each other's virulence. Their study revealed that the inhibitor of *C. albicans* hyphal morphogenesis was a heat-stable secreted product from *E. faecalis* of molecular mass between 3 and 10 kDa, and generation of the signal was at least partially dependent on Fsr [49].

Continuing with previous study with cell free supernatant obtained from the Antarctic strain *E. faecium,* the anti-*Candida* protein (ACP) was purified by a three-step method by ultrafiltration, ammonium sulfate fractionation, dialysis, ion-exchange chromatography and gel filtration. The ion exchange fractions showing activity were pooled and resolved in Native-PAGE and analyzed by the zymogram before transferring it to the nylon membrane. The N-terminal sequencing was performed by the Edman degradation and the silver stained band was subjected to the de novo sequencing [50].

Comparing the partial *de novo* amino acid sequence of the antimycotic protein to other antimicrobial peptides and bacteriocins by using protein-protein BLAST in NCBI revealed no complete homology or identity with other known bacteriocins or AMPs. The *de novo* sequencing identified mainly three peptides WLPPAGLLGRCGR, WFRPWLLWLQSGAQYK and WLGNLFGLGPK with varying m/z values [50]. The last peptide sequence revealed a high percentage of glycine, proline, leucine and tryptophan. This kind of observation was not uncommon in many antimicrobial peptides including bacteriocins like enterocin and acidocin [50].

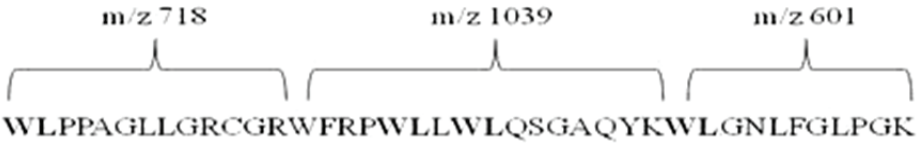

Fig. 1 *de novo* sequences identify three peptides with *m/z* ratios of 718, 1039, and 601 [50]. Based on the peaks and matching with MASCOT search, the respective peptides showed no significant match with any protein/peptide present in the data base. These peptides might be associated with the anti-*Candida* activity of *E. faecalis*.

4.1.1 N-terminal amino acid sequencing of the anti-*Candida* protein (ACP)

The first 12 N-terminal amino acid residues were identified by Edman degradation. The minor sequence obtained from the N-terminal sequencing was GPGGPGKSGDSL, and the same partial sequence was matched for homology. Complete homology was not found using the NCBI BLAST tool. Analysis of the major N-terminal sequence DEVYTVKS(S+S') GDSL revealed the presence of S', suggesting that serine is modified; this is a feature of class I antibiotics [51].

In a previous study, it was found that tenacin-3, an antifungal peptide was glycine-rich and therefore could enter the *C. albicans* cytoplasm [52], although tenacin-3 did not induce membrane permeabilisation. Linearpeptides with an extended structure were characterized by an unusual proportion of one ormore amino acids (most often proline, tryptophan, or glycine [53, 54]. Penaedinscharacterised from shrimps and prawns had a high content of Pro/ Arg/ Gly residues in theextended N-terminal domain [55]. Also ponericin G has glycineresidues flanking the central proline, resulting in a GPG motif with calculated grand averageof hydropathicity (GRAVY) of −0.683.20. The presence of Gly-Pro hinges in antimicrobialpeptides like oxypinins, ponericins, and cecropins support the antimicrobial potential ofACP, wherein a similar motif was observed (Shekh and Roy 2012). The regional flexibility provided by prolinewas sometimes enhanced by the presence of glycine residues (Cordes *et al*. 2002) [56]. In another recent report, apenaedin homologue, hyastatin from spider crab (Capinera, 2008) [57], was shown to possess a Pro/Glydomain similar to the N-terminal domain of penaedins that bind chitin tightly. Thisinformation buttresses the view that the N-terminal minor sequence GPGGPG of the anti-*Candida* protein in the present study could interact with the cell wall of *Candida* as a primerfor antimicrobial action [57]. However, the GPGG sequence was found to match with that of a known ABC transporter, ABC transporter peptide permease, and a hypothetical protein. The first 3 amino acid residues, GPG, matched the N-terminal sequence of enterocin 1071B (Balla *et al*. 2000 [58-59] and Franz *et al*. 2002). Likewise, the GPG sequence was also observed in EntC2 (Maldonado-Barragan *et al*. 2009). [60].

Based on the twelve amino acid residues from the N-terminal sequence determined in the our study [50], primers were designed and the PCR-amplified fragment of size 563 bp obtained using the purified genomic DNA (as template) of the producer strain was sequenced; the nucleotide sequence was analyzed by BLAST using the NCBI search [61]. The deduced amino acid sequence from nucleotide sequence generated from the PCR amplified fragment reveals the LysM motif that includes the DEVYTVKSGDSL. In an erstwhile mutational analysis study conducted by Onaga and Tiara (2008) [62], the LysM domain of PrChi-A was found to bind a chitin contributing significantly to the antifungal activity mediated by PrChi-A through their binding activity. The N-terminal sequence of PrChi-A was determined as DCTTYTVKSGDTCYAISQAN. This sequence was not homologous to the sequence of any plant chitinase, but very interestingly shares striking homology with the LysM of other proteins from several organisms [62]. LysM domains are also known to bind N-acetylglucosamine (GlcNAc)-containing glycan molecules including peptidoglycan from several bacteria and chitin from fungi [63-64]. The PrChi-A found in the plant kingdom was reported to bind to chitin in the fungal cell wall mainly through LysM domains and then it degraded the chitin by hydrolytic action. This led to disruption of the fungal cell wall and fungal growth inhibition [62].

The resulting protein sequence was blasted against the NCBI database with the best overall match showing 30 % identity [61] with the NIpC/P60 family [64]. In mass spectrometry analysis, the protein sequence matched upto 30%

with NlpC/P60 family protein present in the MASCOT database. NlpC/P60 is a large family of cell-wall related cysteine peptidases that are broadly distributed in bacteria, archaea and eukaryotes [64]. SagA of *E. faecium* belongs to NIpC/P60 and is a secreted antigen which binds to extracellular matrix proteins [65]. Teng et al. (2003) reported the N-terminal sequencing of the fibrinogen- binding protein revealing a 20-amino-acid sequence, DFDSQIQQQDQKIADLKNQQ, identical to the predicted N-terminal sequence of the mature SagA [66]. In our study, the significant peptides NQQADAQSQIDALESQVSEINTQAQDLLAK, DIADLQER, and VQAMTTMVK detected were matched with secreted antigen Sag A/SagBb proteins produced by the *E. faecium* strain [61]. NlpC/P60 proteins are often fused to auxiliary domains, many of which are known cell-wall binding modules (e.g. LysM domain) [68]. These auxiliary domains may be thought to function as targeting domains which localize their proteins to the cell wall [68]. The results obtained in this study reveal a so far not described function for enterococcal LysM domain protein and taken together our findings clearly indicate the presence of this auxiliary domain in the form of LysM domain and NIpC/P60.

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