



Dear Colleagues,

The 1st International Symposium on Enterococci in Foods, is organized by the participants of the FAIR-CT97-3078 project, under the auspices of the Food and Agro-Industrial Research (FAIR) Programme of the European Commission, and hosted by the Federal Institute for Health Protection of Consumers and Veterinary Medicine (BgVV), Berlin. The Symposium will focus on the discussion of Enterococci present in fermented foods and their functional and safety properties and has been designed to bring together scientists and R&D and production managers of food and pharmaceutical industries from all over the world.

Enterococci occur and grow in a variety of fermented foods. The presence of enterococci in food products has long been considered as an indication of poor sanitary conditions during production and processing. On the other hand, fermented foods containing enterococci, have a long history of safe use. It is also claimed that enterococci play an important role in the development of the organoleptic properties of the fermented foods. Furthermore, many enterococcal strains have interesting biotechnological traits, such as bacteriocin production, and functional properties such as probiotic features.

The last few years however, this view is changing because of the increasing incidence of enterococci in nosocomial infections, and the widespread occurrence of antibiotic-resistant enterococci, especially in hospital environments. Many studies have consistently found vancomycin resistant enterococci in clinical human samples, in animal samples as well as in foods.

Over the past four years, the European Commission has funded the project "Enterococci in Food Fermentations. Functional and Safety Aspects" (FAIR-CT97-3078). The project generated new data on enterococci in respect to physiology, typing, taxonomy, as well as aspects of functionality, safety and technological performance. For the first time, this Symposium, will provide a unique point of entry for new and established scientists interesting in learning the state-of-art from current leaders in the field.

We would like to dedicate the symposium to the memory of our reknowned colleague

**Prof. Dr. Jos Huis in 't Veld**

who made major contributions in the field of probiotics.

George Kalantzopoulos  
Co-ordinator of the  
FAIR-CT97-3078 Project

Dieter Arnold  
Acting Director  
of the BgVV

Günter Klein  
Local Organizing  
Committee

# Enterococci in Foods Functional and Safety Aspects Scientific Programme

**Thursday, 30 May 2002**

- 08:00 - 09:30**            **Registration and poster set up**
- 09:30 - 09:45**            **Opening ceremony**  
Welcome by **D. Arnold**, Acting Director of the BgVV  
Welcome by **G. Kalantzopoulos**, Coordinator of the FAIR-CT97-3078 project  
Welcome by **D. Bennink**, European Commission
- 09:45 - 10:30**            **W.H. Holzapfel and C.M.A.P. Franz** (Federal Research Centre for Nutrition,  
Germany)  
General overview lecture on enterococci
- 10:30 - 12:45**            **Session 1 Ecology, Taxonomy and Physiology of Enterococci**  
Chair: W.H. Holzapfel, E. Tsakalidou
- 10:30 - 11:15**            **K.H. Schleifer** (TU Muenchen, Germany) and **G. Klein** (BgVV, Germany)  
Ecology, taxonomy and physiology of enterococci
- 11:15 - 12:00**            Coffee break and poster viewing
- 12:00 - 12:15**            **M. Vancanneyt, E. Tsakalidou, G. Kalantzopoulos, W. Holzapfel, F.  
Dellaglio, T. Cogan, L. De Vuyst, A. Lombardi, K. Kersters and J. Swings**  
Genotypic characterization of *E. faecalis* and *E. faecium* strains and  
correlation with their origin and functional and safety properties
- 12:15 - 12:30**            **F. Leroy and L. De Vuyst**  
Modelling the effects of temperature, pH and environmental stress conditions  
on bacteriocin production by *E. faecium* RZS C5
- 12:30 - 12:45**            **M.C. Rea and T.M. Cogan**  
Citrate, pyruvate and sugar metabolism in *Enterococcus faecalis* FAIR E-239
- 12:45 - 14:00**            **Lunch break**

- 14:00 - 17:15**                    **Session 2    Functionality of Enterococci in Foods**  
 Chair: T. Cogan, G. Kalantzopoulos
- 14:00 - 14:45                    **G. Giraffa** (Istituto Sperimentale Lattiero Caseario, Italy)  
 Functionality of enterococci in dairy products
- 14:45 - 15:30                    **M. Hugas** (Meat Technology Center-CeRTA, Spain)  
 Functionality of enterococci in meat products
- 15:30 - 16:30                    Coffee break and poster viewing
- 16:30 - 16:45                    **M.R. Foulquié Moreno, M.C. Rea, T.M. Cogan and L. De Vuyst**  
 Applicability of a bacteriocin-producing *E. faecium* as a co-culture in cheddar cheese manufacture
- 16:45 - 17:00                    **M. El Soda**  
 Selection of *E. faecium* strains for Egyptian cheesemaking
- 17:00 - 17:15                    **L. De Vuyst**  
 ENTIP: ENTero cocci Industrial Platform
- 17:15 - 18:15**                    Poster viewing
- 19:00**                                **Conference dinner**

**Friday, 31 May 2002**

- 09:00 - 12:15**                    **Session 3    Functionality of Enterococci in the GI Tract**  
 Chair: G. Reuter, L. De Vuyst
- 09:00 - 09:45                    **S.P. Borriello** (Central Public Health Laboratory, United Kingdom)  
 Gut function and the normal flora
- 09:45 - 10:30                    **C. Vael** and **H. Goossens** (University of Antwerp, Belgium)  
 Enterococci as probiotics: Chances and challenges
- 10:30 - 11:00                    Coffee break and poster viewing
- 11:00 - 11:15                    **K.J. Domig, H.K. Mayer and W. Kneifel**  
 EU-Project SMT-CT98-2235: Methods for the official control of probiotics (microorganisms) used as feed additives: Enterococci
- 11:15 - 11:30                    **P. Becquet**  
 EU Assessment of enterococci as feed additives
- 11:30 - 11:45                    **J. Benyacoub, G. Czarnecki-Maulden, C. Cavadini, T. Sauthier, R. Anderson, E.J. Schiffrin and T. von der Weid**  
*E. faecium* SF68 stimulates immune functions in young dogs
- 11:45 - 12:00                    **J. Huebner**  
 Protective immune response against enterococcal infections
- 12:00 - 12:15                    **A. Rincé, Y. Le Breton, J.-C. Giard, S. Flahaut, A. Hartke and Y. Auffray**  
 Physiological and molecular aspects of the bile salts response of *E. faecalis*
- 12:15 - 13:30**                    **Lunch break**

<b>13:30 - 17:15</b>	<b>Session 4 Safety Aspects of Enterococci</b> <u>Chair:</u> G. Klein, F.H. Kayser
13:30 - 14:15	<b><u>M. Stiles</u></b> (University of Alberta, Canada) Safety aspects of enterococci from the food point of view
14:15 - 14:45	<b><u>F.H. Kayser</u></b> (University of Zurich, Switzerland) Safety aspects of enterococci from the medical point of view
14:45 - 15:30	Coffee break and poster viewing
15:30 - 15:45	<b><u>I. Klare</u>, <u>G. Werner</u> and <u>W. Witte</u></b> Occurrence and spread of antibiotic resistances in <i>E. faecium</i>
15:45 - 16:00	<b><u>F. Schwarz</u>, <u>V. Perreten</u> and <u>M. Teuber</u></b> Molecular structure and evolution of the conjugative multiresistance plasmid pRE25 of <i>Enterococcus faecalis</i> isolated from a raw fermented sausage
16:00 - 16:15	<b><u>P.S. Cocconcelli</u></b> Gene transfer of antibiotic resistance and virulence determinants among enterococci in food
16:15 - 16:30	<b><u>J. Peters</u>, <u>K. Mac</u>, <u>H. Wichmann-Schauer</u>, <u>L. Ellerbroek</u> and <u>G. Klein</u></b> Species distribution and antibiotic resistance patterns of enterococci isolated from food of animal origin in Germany
16:30 - 16:45	<b><u>K. Mac</u>, <u>H. Wichmann-Schauer</u>, <u>C. Dittmar</u>, <u>J. Peters</u> and <u>L. Ellerbroek</u></b> Detection of species and resistance genes in enterococci of animal origin by multiplex PCR
16:45 - 17:00	<b><u>I. Kühn</u>, <u>A. Iversen</u>, <u>L.G. Burman</u>, <u>B. Olsson-Liljequist</u>, <u>A. Franklin</u>, <u>M. Finn</u>, <u>F. Aarestrup</u>, <u>A-M. Seyfarth</u>, <u>A.R. Blanch</u>, <u>H. Taylor</u>, <u>J. Caplin</u>, <u>M.A. Moreno</u>, <u>L. Dominguez</u>, <u>I. Herrero</u> and <u>R. Möllby</u></b> Epidemiology and ecology of enterococci, with special reference to antibiotic resistant strains, in animals, humans and the environment
17:00 - 17:15	<b><u>R. Gelsomino</u>, <u>M. Vancanneyt</u>, <u>T. M. Cogan</u> and <u>J. Swings</u></b> The effect of raw-milk cheese consumption on the enterococcal faecal flora
<b>17:15 - 18:00</b>	<b>Conclusions</b> W.H. Holzapfel, G. Kalantzopoulos, F.H. Kayser, G. Klein
<b>18:00 - 18:15</b>	<b>Closing ceremony</b>
<b>18:30</b>	<b>Farewell reception</b>

# Oral Presentations

## General overview of the enterococci

Wilhelm H. Holzapfel, Claudia Guigas and Charles M.A.P. Franz

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As a group, the enterococci were first described by Thiercelin in 1899. Although these “diplococci from intestinal origin” were suggested as separate genus in those early days, it took more than 80 years before their genus status was finally recognised. The enterococci constitute a major genus of the lactic acid bacteria (LAB). Their “robust” nature and adaptability are indicated by their association with a wide variety of habitats, e.g. surface waters, soil, plants, and in the gastrointestinal tract of humans and animals. They are also associated with foods, most likely as a result of contamination from plant or animal sources, but they also seem to play some role in numerous fermented foods. Their wide distribution in nature, when compared to other LAB, is probably explained by their persistence and their resistance to growth inhibitory factors such as acidity, salt, drying, heat and chemical sanitising agents.

The association of enterococci with some foods may be detrimental, e.g. their role in spoilage of heat-treated, packaged processed meats. In cheese production they may also be considered as indicators of poor hygiene. On the other hand, they have also been suggested to play an important part in the ripening and aroma development of some traditional cheeses and fermented sausages. These beneficial activities have been attributed to proteolytic, lipolytic and esterolytic activities. Bacteriocin production among enterococcal strains isolated from foods, has also been viewed as a possible beneficial aspect. This has been proposed to find application in the biopreservation of fermented foods such as cheeses and sausages, thereby safeguarding these foods from adulteration by foodborne pathogens such as *Listeria monocytogenes*. Enterococci indeed produce a vast array of different bacteriocins and some strains shown to be capable even of multiple bacteriocin production. Enterococcal bacteriocins, or enterocins, may show similarity to bacteriocins produced by other LAB genera, for example to ‘pediocin-like’ bacteriocins (Ent A, EntP, mundticin) or to two-peptide bacteriocins (Ent1071 A&B). Yet, they also produce some very distinct and unusual bacteriocins such as the bacteriocin/haemolysin called cytolysin, which contains lanthionine and  $\beta$ -methylanthionine residues similar to the lantibiotic nisin, but which in contrast to nisin consists of two peptides and is active against eukaryotic cells.

Some enterococcal strains have been used successfully as human probiotics. Their success as probiotics has been attributed to factors such as acid and bile resistance, bile salt hydrolase activity, production of antimicrobials and their ability to survive and compete in the gastrointestinal tract. On the other hand, some strains of enterococci have also become recognised as important nosocomial pathogens causing bacteraemia, endocarditis and other infections. Some strains may therefore be considered as typical opportunistic pathogens, and may cause infections of immunocompromised patients, e.g. as a result of an underlying disease. This problem is also related to the fact that particular strains exhibit intrinsic or extrinsic antibiotic resistances, and that some strains show multiple antibiotic resistance. Especially vancomycin resistant strains have caused serious and difficult to treat infections within the nosocomial setting. Recent studies show that some food isolates may also harbour some virulence traits; thereby *E. faecalis* appears to harbour more virulence factors and with a higher frequency than *E. faecium* strains. Transfer of virulence factors encoded on pheromone-response plasmids may occur *in vitro* or in the gastrointestinal tract in animal model studies. However, many virulence traits described so far (Esp, Ace, Efa<sub>fs</sub>, Efa<sub>fm</sub>) appear to be chromosomal. The question in the safety and acceptability of enterococci in the food and probiotic situation is still heavily debated. It should however be emphasised that some well defined strains with a long history of safe use are known. Moreover, the particular role and benefits of enterococci in certain food fermentations deserve increased attention in research.

# **Ecology, Taxonomy and Physiology of Enterococci**

## Ecology, taxonomy and physiology of enterococci

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During the last 20 years 24 enterococcal species have been described. Moreover, three new species, '*E. azikeevi*', '*E. phoeniculicola*' and '*E. rottae*' have been proposed but not yet validly published. *Enterococcus seriolicida* has been excluded from the genus *Enterococcus* and reclassified as *Lactococcus garvieae*. Many of the more recently described new enterococcal species differ significantly in their physiological and biochemical properties from those of typical enterococci. Therefore, genotypic methods are necessary to identify them reliably as members of the genus *Enterococcus*. Based on extensive rRNA sequence analyses seven species groups can be distinguished within the genus *Enterococcus*.

A comprehensive set of rRNA targeted oligonucleotide probes has been designed for the identification of enterococci at different taxonomic levels: genus, species group and species, respectively. To avoid misidentifications due to the presence of identical probe target sequences in phylogenetically diverse organisms a nested set of probes, targeting independent sites or genes (16S/23S rRNA), was applied. These probes were immobilized on microwell plates or glass slides (DNA chips) and used for reverse hybridization with labeled target DNA. The target comprising 16S and 23S rDNA was in vitro amplified and simultaneously labeled using polymerase chain reaction and general primers targeting a wide spectrum of bacteria. An even more sensitive, but also more time-consuming alternative was the specific amplification of two adjacent enterococcal rDNA fragments. The probe set was evaluated and optimized against all type strains of enterococcal species and a selection of strains belonging to Gram-positive bacteria with low DNA G+C content. The probe set was used to detect and identify enterococci in drinking water and ice cream. Moreover, microcolony hybridization was applied as a fast and reliable method to identify viable, reproductive enterococci.

In addition to genotypic identification methods there is a need for reliable conventional phenotypic identification schemes for simple and rapid determination of enterococcal species in food or in the gastrointestinal tract (GIT). Only a limited number of enterococcal species is of importance for the ecology of the GIT or the food microflora, including *E. faecalis*, *E. faecium*, *E. durans/hirae*, *E. gallinarum* and *E. casseliflavus*. After genus identification the differentiation within these species can include e.g. mannitol and arabinose fermentation and growth at 50°C. Methyl-alpha-D-glucopyranoside can be used as an additional discriminatory feature. Widely used commercial identification systems may fail to precisely identify rare species. Ecological aspects should also be taken into account. In the human GIT *E. faecium* is the most common species whereas in most animal species *E. faecalis* is the predominant species. Especially in foods of animal origin (cheese, pork meat, beef, poultry meat) also *E. faecalis* is most frequent and *E. faecium* is rarely isolated. This is of special interest as glycopeptide resistance is most often found in human clinical *E. faecium* strains as well as in *E. faecium* from the environment or animal samples and less frequent in *E. faecalis* strains. EU experts propose as safety criteria for probiotics in feed additives the exclusion of resistances or the lack of transferability. This proposal can also be applied to enterococci in foods. Specific resistances must be excluded, but transferability or acquisition of resistance (e.g. vancomycin) cannot be excluded per se. However, technologically used strains should differ from clinical strains concerning their resistance patterns and transfer rates.

## Genotypic characterization of *Enterococcus faecalis* and *Enterococcus faecium* strains and correlation with their origin and functional and safety properties

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In the framework of the research project “Enterococci in Food Fermentations. Functional and Safety Aspects” (FAIR-CT97-3078) about 400 enterococcal strains have been collected, biochemically characterized, identified at the species level, and deposited in the FAIR-E collection (centrally maintained at the BCCM/LMG Bacteria Collection). The species *Enterococcus faecalis* and *Enterococcus faecium* largely dominated the isolates whether they originated from food, animals or humans. The occurrence of both species in nosocomial infections has questioned their safety in (fermented) foods, where they contribute to the ripening and product flavour. This has led to numerous epidemiological studies dealing with the prevalence of vancomycin-resistant *E. faecium* in hospitals or their assumed transmission from animals to humans. The present work aimed to reveal the genomic relationships between vancomycin-resistant and susceptible *E. faecium* and *E. faecalis* strains from diverse sources and to correlate these data with available functional and safety properties in foods and probiotics.

*E. faecium* and *E. faecalis* strains were genotypically typed using pulsed-field gel electrophoresis (PFGE) of *Sma*I restriction patterns, random amplified polymorphic DNA (RAPD)-PCR and amplified fragment length polymorphism (AFLP). In both species, PFGE demonstrated that the majority of the strains originated from different clonal lineages. In *E. faecium*, two main genomic groups (I and II) were obtained in both RAPD-PCR and AFLP analyses. DNA-DNA hybridization values between representative strains of both groups demonstrated a mean DNA-DNA reassociation level of only 71%. The two groups could be further subdivided into four and three subclusters in RAPD-PCR and AFLP analyses, respectively, and a high correlation was seen between the subclusters generated by these two methods. Subclusters of group I were to some extent correlated with origin and pathogenicity of the strains. Host specificity was, however, not confirmed. In *E. faecalis*, a much higher homogeneity was observed among strains of the species in both RAPD-PCR and AFLP analyses. However, the congruency between groupings obtained by both approaches was very low. When correlating the typing results with origin and safety properties, no clusters with unique biochemical features could be delineated.

The main conclusion is that for both, *E. faecalis* and *E. faecium*, the presence of safety properties (e.g. antibiotic resistance and potential virulence factors) and functional properties (e.g. antimicrobial activity) is strain-specific. For *E. faecium*, however, the incidence of some of these features correlated well with the intraspecific grouping and may be useful information for the selection of strains for cultures in food or probiotic preparations.

## **Modelling the effects of temperature, pH, and environmental stress conditions on bacteriocin production by *Enterococcus faecium* RZS C5**

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Several enterocins, bacteriocins produced by enterococci, are characterised by a strong antilisterial activity. *Enterococcus faecium* RZS C5, a natural isolate from cheese, produces such a bacteriocin.

Bacteriocin production by *E. faecium* RZS C5 occurs in a growth-associated way, as is generally observed for lactic acid bacteria. However, whereas for most lactic acid bacteria production continues until the late active growth phase or the stationary phase, enterocin production by *E. faecium* RZS C5 is limited to the very early growth phase and is switched off at a well defined cell density of 0.4 g CDM l<sup>-1</sup>. On the other hand, at constant pH values higher than 6.5, enterocin production is maintained until the end of the active growth phase. In the latter case, however, antilisterial activity is reduced. The relation between bacteriocin production switch-off and cell concentration suggests that enterocin production is severely regulated. A kinetic model was set up for the description of the early enterocin activity peak. Furthermore, the influence of pH and temperature on the kinetics of growth and bacteriocin production of *E. faecium* RZS C5 was investigated. At constant pH 6.5, high enterocin activity was obtained in the temperature range 20-35°C. At 35°C, enterocin activity could only be detected between pH 5.5 and 8.0.

Because a stimulating effect of harsh environmental conditions on the bacteriocin production by lactic acid bacteria has been suggested, the influence of environmental stress on the enterocin production by *E. faecium* RZS C5 at a controlled temperature of 35°C and constant pH 6.5 was analysed in a kinetic way. It turned out that no straightforward statement about the effect of environmental stress on bacteriocin production could be made; the effect appeared to be dependent on the type of stress applied. Oxidative stress did not interfere with cell growth or bacteriocin activity. In contrast, salt stress decreased both the cell growth and the specific bacteriocin production. Nevertheless, moderate levels of sodium chloride improved bacteriocin activity because they increased the biomass concentration at which bacteriocin production was shut off. Environmental stress due to limitations in sugar or complex nutrients did not affect the early shut-off mechanism or the specific bacteriocin production. However, bacteriocin stability decreased or increased at low levels of sugar or complex nutrients, respectively.

## Citrate and pyruvate metabolism in enterococci

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Citrate metabolism is an important technological characteristic of lactic acid bacteria (LAB). However, unlike other LAB, there is little information available on citrate metabolism in enterococci. *E. faecalis* FAIR E-239 grew in a basal medium (BM) (MRS without citrate, glucose, acetate or T80) but it grew better in BM containing citrate (BMC) indicating that citrate was used as an energy source. However, citrate utilisation per unit of biomass was curvilinear. Citrate utilisation per unit of biomass was linear when the strain was grown in filter-sterilised spent medium containing citrate (SMC). In filter-sterilised spent medium containing citrate and glucose (SMCG), citrate was not utilised until all the glucose was used. Similar results were obtained with *E. faecalis* FAIR E-237 and E-255 and *E. faecium* FAIR E-238 and E-371. Other metabolisable sugars, e.g. fructose, also inhibited citrate utilisation by FAIR E-239 while non-metabolisable sugars, such as lactose and galactose, did not. Sucrose, which was metabolised slowly, also resulted in slow utilisation of citrate. The ability to metabolise pyruvate was not inhibited by glucose. In a mixture of citrate and pyruvate in SM, citrate utilisation was not affected by the addition of pyruvate, but pyruvate utilisation did not begin until the citrate was depleted. When glucose was added to cells actively growing on citrate, glucose metabolism began immediately, but citrate utilisation was not switched off for a considerable time. The addition of pyruvate to cells growing on citrate did not affect citrate utilisation but the addition of citrate to cells growing on pyruvate slowed down pyruvate metabolism while citrate was metabolised. These results show that enterococci differ from other LAB in that they can utilise citrate as an energy source and that the presence of a fermentable carbohydrate in the medium prevents them from metabolising citrate.

# **Functionality of Enterococci in Foods**

## Functionality of enterococci in dairy products

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Enterococci have important implications in the dairy industry. They occur as non-starter microflora in a variety of cheeses, especially artisan cheeses produced in the southern Europe from raw or pasteurised milk, and in natural milk or whey starter cultures. Enterococci play an acknowledged role in the development of organoleptic characteristics during ripening of many cheeses and they have been also used as components of cheese starter cultures. The positive influence enterococci may have on cheese seems due to specific biochemical traits such as lipolytic activity, citrate utilisation, and production of aromatic volatile compounds. Some enterococci of dairy origin have also been reported to produce bacteriocins (enterocins) inhibitory against food spoilage or pathogenic bacteria, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Vibrio cholerae*, *Clostridium* spp., and *Bacillus* spp. The technological application of enterocins, which were shown to be produced during cheese manufacture, led to propose enterococci as adjunct starter or protective cultures in cheeses. There is then evidence that enterococci, either added as adjunct starters or present as non-starter microflora, could find potential application in the processing of some fermented dairy products. Literature data seem, however, to suggest that the complex biochemical and ecological phenomena explaining the technological functionality of the enterococci in dairy products are still to be fully determined. Clearly, the clinical research on enterococci underlines also that the safety of dairy products containing enterococci is an issue that the industry must carefully address before proceeding to their application.

**Keywords:** enterococci, non-starter microflora, starter adjuncts, protective cultures, artisan cheeses, dairy products.

## Functionality of enterococci in meat products

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Enterococci are normal constituents of the natural microbiota of many dry fermented sausages manufactured without starter cultures, specially in slightly fermented sausages of high pH (>5.3). These are traditional products of southern mediterranean countries with a long history of safe consumption.

Many enterococci isolated from these kind of sausages have the ability to produce bacteriocins. *Enterococcus faecium* CTC492, isolated from slightly fermented sausages, is a competitive strain in the meat environment producing two bacteriocins Enterocin A and Enterocin B with a spectrum of activity comprising *Listeria monocytogenes*, *Staphylococcus aureus* and spoilage slime-producing lactic acid bacteria (LAB).

In the last decade, the knowledge on the LAB bacteriocins has been dramatically improved. But the applied aspects have not been deserved the same attention. There are several reasons that might explain this fact. Bacteriocins do not have a broad host range, thus their effect on the improvement of the overall acceptability and shelf life extension is not so evident in many cases compared to other chemical preservatives. In every food system there are many factors affecting the effectivity of bacteriocins like the food composition, additives, physico-chemical conditions etc.. and last but not least legislation on food additives is very restrictive and has not been modified. However, most of the above limitations can be solved, mainly through the combination of bacteriocins and other antimicrobials with complementary host ranges like sodium lactate or physical preservation techniques as high hydrostatic pressure and by the application of spray-dried or freeze-dried fermented liquors specified as cultured milk proteins in the ingredients list instead of purified bacteriocins of the bacteriocinogenic culture.

In fact, several successful applications from enterococci bacteriocins have been reported, mainly concerning their effectiveness in eliminating *Listeria monocytogenes* from fermented and slightly fermented sausages, in fresh meat packed under vacuum or modified atmospheres, in vacuum-packed and sliced cooked ham, in paté and in vacuum-packed frankfurter sausages either through casing application, in the meat emulsion or in plastic films. But also in the shelf life extension of fresh meat, the slime prevention in sliced vacuum-packed cooked ham and in inhibiting *Salmonella* and *E.coli* in pressurized modelized cooked pork.

As conclusion, bacteriocinogenic enterococci could be used to solve some hygienic and spoilage problems still occurring in meat products despite the implementation of HACCP but current regulation is hampering their application.

## Applicability of a bacteriocin-producing *Enterococcus faecium* as a co-culture in Cheddar cheese manufacture

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Bacteriocins are ribosomally synthesised, extracellularly released, antibacterial peptides. The strains *Enterococcus faecium* RZS C5 and *E. faecium* DPC 1146 produce the listericidal bacteriocins enterocin B and enterocin A, respectively. These two enterocins belong to the class II bacteriocins, which are small, cationic, hydrophobic, heat-stable peptides that are not post-translationally modified, except for cleavage of a leader peptide from the prebacteriocin peptide. *E. faecium* RZS C5 was studied during batch fermentation in both a complex medium and in milk to understand the influence of environmental factors, characteristic for milk and cheese, on both growth and bacteriocin production. Fermentation conditions were chosen in view of the applicability of *in situ* enterocin production during Cheddar cheese production. Enterocin production by *E. faecium* RZS C5 in a complex medium (MRS) started in the early logarithmic growth phase, and enterocin activity decreased during the stationary phase. The effect of pH on enterocin production and decrease of activity was as intense as the effect on bacterial growth. Enterocin production took place optimally at pH 5.5. The use of lactose instead of glucose increased the production of enterocin, and at higher lactose concentrations production increased more and loss of activity decreased. When 2.0 % NaCl was added, the production was not affected, however a higher decrease in the bacteriocin activity was observed. The production in skimmed milk was lower and was detected mainly in the stationary phase. When casein hydrolysate was added to the milk, enterocin production was higher and started earlier, indicating the importance of an additional nitrogen source for *E. faecium*. For co-cultures of *E. faecium* RZS C5 with the starters used during Cheddar cheese manufacture, no antimicrobial activity was detected during the milk fermentation. Furthermore, the applicability of *E. faecium* RZS C5 and *E. faecium* DPC 1146 strains was tested in Cheddar cheese manufacture on pilot scale. Enterocin production took place from the beginning of the cheese manufacturing and was stable during the whole ripening phase of the cheese. This indicates that both an early and late contamination of the milk or cheese can be combated with a stable, *in situ* enterocin production. The use of such a co-culture is an additional safety provision beyond good manufacturing practices.

## **Selection of *Enterococcus faecium* strains for Egyptian cheesemaking**

### Morsi El Soda

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Five hundred *Enterococcus* cultures were isolated from the Egyptian cheeses Ras and Domiatti and from the traditional Egyptian fermented milks Laban Rayeb, Zabadi and Kishk.

Identification was accomplished using API 20 STREP and SDS-PAGE electrophoresis.

Cultures identified as *Enterococcus faecium* which represented 70% of the isolated enterococci were then selected to meet production requirements i.e. high cell yield, a good separation of the biomass and the stability of the biomass after lyophilization as well as the technological requirements i. e. acidifying activity, autolytic properties, proteolytic activity and esterolytic activity.

As far the production requirements were concerned it was found that the average cell yield was 1.2 mg/lit. Ninety five of the strains showed a good separation of the biomass and 70% revealed a good resistance to lyophilization.

Concerning the technological requirements of the cultures as a general rule, *E. faecium* are not high acid producers, on the other hand, all the strains exhibited an aminopeptidase activity hydrolysing leucyl 4-nitroanilide faster than arginyl, alanyl, prolyl and glycylic derivatives.

*E. faecium* showed active esterolytic activity towards 4-nitrophenyl derivatives and 2-nitrophenyl derivatives of fatty acids. Strains differed in their rate of autolysis and as a general rule the autolysis rate was lower than 25% after 120 h of incubation at 10°C.

Egyptian Ras and Domiatti cheese were made in the presence and absence of selected *E. faecium* strains. The cheese containing *E. faecium* exhibited higher levels of free fatty acids and amino acids. The organoleptic evaluation of the different cheeses revealed a preference to the *E. faecium* containing cheese, which suggest a desirable role of this microorganism during the ripening of Egyptian cheeses.

## **ENTIP: ENTerococci Industrial Platform**

Luc De Vuyst

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The ENTerococcus Industrial Platform (ENTIP) was an Industrial Platform associated with the EU-FAIR Project FAIR-CT97-3078, a European Commission Shared Cost Research Project funded within the EU-FAIR Program. This project dealt with the functional and safety aspects of enterococci in food fermentations. The platform has been established as a deliverable of this project and due to the interest of the subject by the industry.

All partners of the FAIR project were involved in this platform. Four industrial partners were ENTIP members: a French dairy association, a Swiss pharmaceutical company, a Danish starter culture company, and a European producer of probiotics. The platform was coordinated from the Vrije Universiteit Brussel by Prof. Dr. ir. Luc DE VUYST. Meetings were organised at six monthly intervals. The companies had first access to up-to-date knowledge and project results, first access to protocols established within the consortium, and direct involvement in the research tasks.

The objectives of the ENTIP Industrial Platform were:

- the financing and promotion of research and development on enterococci within the European Commission;
- the provision of a forum whereby companies meet with research groups focusing on enterococci;
- to help developing an opinion on the application of enterococci in food and probiotic preparations;
- to disseminate information about enterococci to opinion leaders, scientists, consumers and cheese makers;
- to ensure the potential exploitation of research results on enterococci.

Thanks to the collaborative efforts of all FAIR and ENTIP partners, three brochures have been produced for dissemination:

Brochure I: a catalogue of Enterococci of the FAIR-E collection, listing approximately 400 enterococcal strains with some of their physiological and biochemical properties.

Brochure II: a brochure for non-specialists on the safety and health promotion aspects of Enterococci. It deals with the antibiotic resistance, potential virulence factors and probiotic properties of Enterococci.

Brochure III: a brochure on the use of Enterococci in cheese manufacture. It deals with the technological characteristics of selected enterococcal strains and their use as starter or adjunct cultures, including the project results of the cheese trials.

For more information on the ENTerococcus Industrial Platform, see the ENTIP webpage on url <http://imol.vub.ac.be/IMDO/IMDO.html/>

# **Functionality of Enterococci in the GI Tract**

## Gut function and the normal flora

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The microbial flora of the gastrointestinal tract is the most complex microbial ecosystem of the mammalian body. There are up to  $10^{12}$  viable bacteria per gram of gut content, and up to 400-500 different species. This means that we each of us harbour more bacteria in our gut than there have ever been people on the planet. This usually symbiotic relationship can be disturbed by a number of factors, including of course, antimicrobials. This massive collection of bacteria has immense biochemical potential, which could be viewed as another organ of the body, and can have both advantageous and disadvantageous effects on the host. They can for example, protect us from infection with *Clostridium difficile* (colonisation resistance), or from cancer. Conversely, they may contribute to cancer, and exacerbate acidureas. It would be suprising if host gut physiology was not also influenced by the gut flora. This presentation will also therefore try to show how what we accept as normal gut structure and function is in fact the end-product of a complex set of interactions between ourselves and the gut flora.

Further reading:

Borriello S P (1990): The influence of the normal flora on *Clostridium difficile* colonisation of the gut. *Ann Med* **22**: 61-7.

Borriello S P (1984): Bacteria and gastrointestinal secretion and motility. *Scand J Gastroenterol* **19** (Suppl 93): 114-121

## Enterococci as probiotics: Chances and challenges

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Enterococci are used as probiotics in many European countries; most of them are *E. faecium*, but some preparations also include *E. faecalis*. There has been serious concern of the use of such organisms as biotherapeutic agents, because *E. faecium* harbour genes coding for antibiotic resistance whereas *E. faecalis* may have genes which have been associated with virulence.

In our presentation we will address the epidemiology of antibiotic resistance of enterococci and the association between virulence properties and human infection. We will also discuss the colonisation of enterococci, including probiotic agents, in healthy volunteers. Based on these data we will conclude on the chances and challenges of using enterococci as probiotics.

We have conducted several studies on the epidemiology of vancomycin-resistant enterococci (VRE) in Europe. In general, these and other studies, have shown that colonisation with VRE seems to be endemic in healthy people and farm animals; outbreaks still occur occasionally. European VRE of sporadic human origin as well as animal isolates are usually susceptible to many antibiotics and are highly polyclonal. Outbreak isolates appear to be more antibiotic resistant (although outbreaks due to "susceptible" VRE have been reported) and are mostly *vanA*-positive *E. faecium*. Very recently, we participated in a European surveillance study, including 13 clinical microbiology laboratories in 8 EU countries. Between March and Jun 2001, investigators prospectively collected 1314 isolates of enterococci and processed 3499 stool samples, to assess the prevalence of high level resistant VRE among clinical and faecal isolates, respectively. This study showed that *E. faecium* remains the predominant species and most VRE are of the VanA phenotype. The rates of VRE among the countries varied significantly: while in some countries the prevalence is still low, in others surprisingly high rates with multi-resistant VRE were noticed. Finally, we were able to compare the prevalence of gastrointestinal colonisation with VRE among patients admitted at the University Hospital Antwerp with the prevalence of VRE colonisation in May 1996, using the same stool culture method and identification of VRE. The prevalence of VanA-positive VRE (all *E. faecium*) decreased significantly from 19/335 (5.7%) in 1996 to 3/353 (0.6%) in 2001 ( $P=0.0007$ ). These studies suggest that the epidemiology of VRE is rapidly changing in Europe

We also investigated the presence of cytolysin (*cytA*), gelatinase(*gelE*), aggregation substance (*asa*) and enterococcal surface protein (*esp*) in 181 *E. faecalis* and 89 *E. faecium* isolates collected from patients with clinical disease and from the stools of healthy students. In *E. faecalis* *cytA* was demonstrated more frequently in urinary tract isolates, *gelE* and *asa* were found more frequently in blood isolates and *esp* was demonstrated more frequently in urinary tract and wound isolates. No association between antimicrobial resistance and the presence of a virulence factor was observed in *E. faecalis*. In *E. faecium* the only virulence factor that could be demonstrated was *esp*, present in 7/48 clinical isolates (15%). Ampicillin resistance was present in 26% of *E. faecium* isolates. A significant correlation occurred with *esp* and ampicillin resistance ( $P < 0.0001$ ) but not with resistance to vancomycin. These data suggest that *esp* is a virulence factor in *E. faecium* that can be acquired prior to vancomycin resistance.

Subsequently, we investigated 11 probiotic *E. faecium* strains for the presence of these virulence and resistance factors. All probiotic strains were negative for *esp,asa, cytA* and *gelE* and no resistance to clinical important antimicrobials was detected.

Finally, we studied the colonisation of enterococci in 13 healthy volunteers challenged with a probiotic, *E. faecium* (7 volunteers), or placebo (6 volunteers). The purpose was to examine the prevalence of enterococci as normal inhabitants of the gastrointestinal tract, to characterise enterococci isolated from the stools and to investigate the kinetics of *E. faecium* SF68 following a 5-day consecutive oral dose. The enterococcal faecal flora was investigated in these volunteers until 12 months after the ingestion of this probiotic or placebo. Enterococcal isolates were identified by PCR and typed by pulsed-field gel electrophoresis. We noticed that the enterococcal faecal flora changed rapidly in time (many different enterococcal species and pulsed-field types) and that the probiotic strain could not be recovered from stool specimens in most volunteers after 1 week (2 volunteers remained colonised for 7 and 9 weeks respectively, but the proportion of *E. faecium* SF68 had decreased significantly).

These data suggest that the intrinsic enterococcal faecal flora is not very stable in the gut. Knowledge of the resistance and virulence properties and of the colonisation in the human gut of enterococci provides opportunities to assess the safety of commercialised probiotics and to develop new probiotic strains.

## **EU-Project SMT-CT98-2235: Methods for the official control of probiotics (microorganisms) used as feed additives: Enterococci**

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This project was initiated by a Dedicated Call and is based on the regulations of the Council Directive 70/524/EEC concerning additives in feeds and the application of probiotics in feeds. According to the Official Journal of the European Communities (11.9.96 No C263/3), eleven genera of bacteria, 3 genera of yeast and one mould have been approved for temporary use within specified member states and are in line with the provisions of Council Directive 93/113/EC on the use of marketing of enzymes, micro-organisms and their preparations in animal nutrition.

The project was undertaken to develop and validate methods for six probiotic micro-organism Species (enterococci, lactobacilli, bifidobacteria, pediococci, bacilli and yeast). The protocols should include selective enumeration methods and procedures of microbiological identification.

The project partnership consisted of the following organisations:

- Central Science Laboratory (CSL, York, UK), coordination, Yeasts
- University of Agricultural Sciences, Vienna (IMB, Vienna, Austria), Enterococci
- University of Caen (LMA, Caen, France) Lactobacilli
- Dairy Products Research Centre (Teagasc, Fermoy, Ireland) Bifidobacteria, Pediococci
- Gaiker Laboratories (Gaiker, Bilbao, Spain) Bacilli.

Aiming at the selective enumeration of the genera either present as a single component or in combination with other micro-organisms, a variety of culture methods applying selective and non-selective agar media were screened and the most suitable selected. Strategies for microbial identification were based on molecular biological methods which had to be adopted according to recent developments. All methodological protocols developed were brought into ISO format and validated.

Starting with a screening of published methods for culturing and enumerating enterococci, the first step was the establishment of a collection of enterococcal strains used as probiotics in animal feeds and of isolates and reference strains from culture collections. Based on a multitude of publications, 12 commercially available media were selected for further testing and then compared with regard to their usefulness and selectivity. Moreover, selected strains of lactobacilli, pediococci and streptococci were included for reference purposes. Bile Esculin Azide Agar was shown to be the most suitable medium. It was demonstrated that the API 20 Strep test combination (bioMérieux) had a good discriminatory power for the most important probiotic species *E. faecium* and therefore can be used as a confirmation method of presumptive enterococcal colonies harvested from the plates. The enumeration method was validated in a collaborative study involving 20 laboratories from 12 European countries.

For identification of probiotic strains, the performance of molecular biological methods based on Polymerase Chain Reaction (PCR) and Pulsed Field Gel Electrophoresis (PFGE) were investigated using 74 enterococcal test strains. Randomly Amplified Polymorphic DNA (RAPD) also showed good identification results and can be used for fast identification, but does not serve as a reference method because of its pronounced variability due to local laboratory conditions. A commonly used PFGE protocol had to be slightly adopted and was used with nine restriction endonucleases to type the whole enterococci strain collection. This method had a very good discriminatory power. The PFGE protocol was brought into ISO format and recommended as a standard method for checking the identity of enterococcal strains, which are used as probiotic feed additives.

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## EU Assessment of enterococci as feed additives

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### 1. Regulatory Position of Micro-organisms in the feed area

Micro-organisms such as enterococcal strains are used as probiotics in feed, as it has been shown that micro-organisms had a positive effect on the gut flora, especially for young animals or during feed transition phases. This favourable effect on the gut flora is perceived by the farmers as an improvement of the health status of the animals (e.g. less diarrhea) and results in significant improvement on animal performances (e.g. growth rate, feed conversion ratio).

Micro-organisms have been used since the end of the 80s in the animal feeds and were strictly regulated in 1993, when they were introduced under the scope of Council Directive 70/524/EEC on feed additives. After a transition period, which ended in year 2000, any micro-organisms should be assessed by the EU bodies and authorised by a Commission Regulation, before it can be placed on the market for use in feedingstuffs (Pre-market authorisation).

### 2. Regulatory framework for enterococci

Council Directive 70/524/EEC on feed additives is based on three main principles:

- pre-market authorisation
- positive list principle: only products which are listed in the annex of the directive can be used in animal feed under the conditions stated in this annex (animal categories, minimum and maximum dosages in feedingstuffs, incompatibilities with other feed additives)
- the authorisation is given after a thorough risk assessment of the particular strain on human and animal health as well as the environment. Feed additives (including enterococcal strains) should also be completely identified and analytical methods for their control, as such or in feed are described. Finally, they should provide evidence of their efficacy in animal production.

Therefore, an applicant (a person who wants to place on the market a new enterococcal strain preparation or use an approved product for another application (new animal category)) shall produce a dossier following strict guidelines and submit it to the authorities.

The current process involves a first evaluation of the dossier by a Rapporteur Member State, promoting the dossier at EU level). This first evaluation time frame varies from country to country and depends on the quality of the submitted dossier. However, it cannot last more than one year.

After this first assessment, The Rapporteur Member State submits the dossier for a second evaluation, by the other member states and the Scientific bodies of the Commission (Scientific Committee for Animal Nutrition). This second assessment lasts between 15 to 30 months.

When, after these two evaluations, a product is authorised, the authorisation is firstly provisional (4 years) and becomes indefinite, when sufficient proof of efficacy are shown.

### 3. Guidelines for authorisation

In order to build a dossier for the evaluation, the applicant shall follow the guidelines, as published in Commission Directive 94/40/EEC (currently under review). The guidelines are composed of four parts (summary, identity and methods for control, efficacy and safety). For the three last parts, detailed evaluation methods are proposed to ensure that the product complies with Council Directive 70/524/EEC.

Concerning safety, the requirements are very detailed and concern:

- the safety for the target animal categories, based on a tolerance test (10 times at least the recommended maximum incorporation rate in feed during at least 4 weeks)
- the safety for the consumer and the environment: presence of toxins and virulence factors as well as antibiotic resistance and transferability are assessed
- The safety for the workers (at the workplace) based on requirements of Council Directive 89/391/EEC and especially assessment of the presence of toxins and virulence factors as well as antibiotic resistance

## ***Enterococcus faecium* SF68 stimulates immune functions in young dogs**

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The gut microflora plays a crucial role in several physiological functions of the host. Lactic acid bacteria (LAB) have been shown to influence the immune system of humans and some laboratory animals. Among LAB, *Enterococcus faecium* (SF68) has been shown to exert inhibitory effects against some enteropathogens, including enterotoxigenic *E. coli*, salmonellae, shigellae, and clostridia. In addition, Enterococci are normal inhabitants of the colonic flora of humans and animals, thus providing a rationale for their use as a component of functional foods. Weaning, stress, illness, and dietary changes are all conditions that affect the intestinal microflora of pets and for which probiotics might be beneficial. However, no studies have to date addressed the immune stimulating and modulating properties of probiotics in pets. Therefore, our objective was to address the capacity of SF68, when incorporated in dry dog food, to stimulate immune functions in young dogs. Puppies were allotted to different groups receiving a control diet or a diet containing SF68. Blood samples were obtained from the dogs following their usual vaccination against canine distemper virus (CDV) at weeks 10, 18, 31, and 44 of trial. Both cellular and humoral markers of the canine immune system were analyzed. We have observed that peripheral blood mononuclear cells (PBMC) from puppies fed SF68 were able to proliferate more than those from control upon activation with different mitogens. While the proportions of T cell subsets were not affected by the probiotic, we observed a significant increase in mature B cells in puppies receiving SF68. No changes in the amount of total IgG in the plasma were observed. However, in line with the increased B cell population, the amounts of specific anti-CDV IgGs were significantly higher in the group of puppies fed SF68 and were maintained at elevated levels throughout the study. These results suggest that SF68 administration increased the priming of naïve B cells in response to CDV vaccination. This study shown for the first time that a dry dog food containing viable SF68 significantly enhanced cell-mediated and humoral immune functions in dogs.

## Protective immune response against enterococcal infections

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Enterococci are important pathogens, especially in immunocompromised patients. Because of multiple antibiotic resistances, some infections caused by these bacteria can be impossible to treat with currently available antibiotics. A better understanding of the interaction between enterococci and the human host defense system will help in the development of new options for prevention and treatment of these otherwise untreatable infections.

We previously showed that enterococci possess a capsule that is the target of opsonic antibodies. Purified capsular polysaccharide material induced antibodies that protected animals from enterococcal infections. These antigens could be used for the development of immunotherapies to prevent and/or treat infections in hospitalized patients.

Preparations of enterococci have been used for decades as immune stimulants, although the precise mechanism of action has not been elucidated. We hypothesize that polysaccharide antigens might be responsible for the effects ascribed to the oral administration of enterococcal preparations since most protein antigens will be denatured in the gastrointestinal tract.

We studied a probiotic *Enterococcus faecalis* strain by immune electron microscopy and by an opsonophagocytic assay and ELISA using sera raised against four different enterococcal capsular polysaccharides. Our results indicate that this strain is highly susceptible to opsonization, and capsular polysaccharide prepared from this strain reacted with all sera tested. Immune electron microscopy outlined a capsule-like structure. Purified polysaccharide material was extracted with trichloroacetic acid, and we will present preliminary compositional and structural data for this antigen. These results suggest that the probiotic enterococcal strain studied possesses a capsular polysaccharide antigen that is the target of opsonic antibodies. The probiotic strains seems to be more susceptible to opsonophagocytic killing than are most clinical isolated studied so far. Further studies are under way to evaluate if the formation of these antibodies is induced by oral application of the respective probiotic preparation.

## Physiological and molecular aspects of the bile salts response of *Enterococcus faecalis*

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Microbial inhabitants in the gastrointestinal tract are presumably challenged by toxic detergent-like compounds such as bile salts in the duodenal loop. Analysis of the susceptibility of *Enterococcus faecalis* towards bile salts (sodium cholate, sodium deoxycholate [1:1]) revealed that killing with 0.3% bile salts was nearly instantaneous. Bile salts tolerance was observed after extremely short pretreatment of *E. faecalis* cells with a sublethal concentration of 0.08% bile salts (flash adaptation). Cross tolerance were also observed after incubation with 0.08% bile salts. In order to analyze this physiological response of *E. faecalis*, a library of insertional mutants was constructed and screened for mutants sensitive to bile salts. This allowed the isolation and the characterization of 10 mutants affected in resistance to bile salts. Insertion loci of two mutants corresponded to genes of unknown function, while the amino acids sequences deduced from the other loci were homologous to proteins related to DNA repair, oxidative response, transcriptional regulation, dGTP hydrolysis, membrane composition or cell wall synthesis. Further characterization of one mutant revealed that the insertion within *the E. faecalis sagA* gene led to a decrease of the resistance towards numerous independent physicochemical stresses, to modifications of the cell wall integrity and to perturbations of cell division with septation anomalies. In an other hand, bidimensional electrophoreses allowed to observe during a pretreatment of *E. faecalis* cells with a sublethal concentration of 0.08% bile salts the induction of the synthesis of 45 proteins. Six of them, amplified in response to at least 6 different physicochemical treatments, were called General Stress Proteins (Gsp 62 to Gsp67). Immunological detection experiments led us to identify the proteins Gsp66 and Gsp67 as DnaK and GroEL respectively. Sequencing of the N-terminal extremities of Gsp62, 63, 64 and 65 was carried out on proteins separated by preparative 2D gel electrophoresis and permitted to determine their 25 to 39 first amino acids and to found the corresponding genes in the sequence of the *E. faecalis* V583 genome (<http://www.tigr.org>). Transcriptional analyses of the 3 genes *gsp62*, *gsp64* and *gsp65* showed induction when cells were subjected to hyperosmotic, oxidative, heat, acid or detersive treatments (bile salts and SDS) with a proportionality between mRNA amounts observed for each gene which suggested that the 3 genes may belong to the same regulon. Another gene (*gls24*), previously identified as a glucose starvation induced gene, was shown to be also strongly induced under a bile salts treatment. The transcriptional initiation site of each gene was determined by 5'RACE PCR or primer extension experiments. The 133 amino acid protein deduced from *gsp65* shares homologies with organic hydroperoxide resistance (Ohr) and OsmC proteins. Knock out of *gsp65* revealed an increased sensitivity of the mutant to the tert-butyl hydroperoxide (tBOOH) and to ethanol and demonstrated that Gsp65 is an Ohr protein. A *gsp64* mutant showed a sensibility toward alkaline, ethanolic or oxidative stresses while disruption of *gsp62* had no significant effet on *E. faecalis* stress resistance. Further investigations are in progress to elucidated the *gsp62* and *gsp64* function. Mutation of *gls24* led to a greater sensitivity to a bile salts challenge as well as changes in cell morphology. These results argue once more for the capacity of bile salts to induce cross resistance.

# **Safety Aspects of Enterococci**

## Safety aspects of enterococci from the food point of view

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The enterococci are natural inhabitants of the mammalian gastrointestinal tract and, as such, they were among the earliest bacteria to be described. They were included in Lancefield's group D streptococci. In 1984 the genus *Enterococcus* was established with the inclusion of *E. faecalis* and *E. faecium* from the group D streptococci. Since then an additional 17 species have been described, but *E. faecalis* and *E. faecium* remain the enterococci of greatest importance. The enterococci were generally considered to be harmless but undesirable in foods because of the attitude of Public Health agencies to them as faecal contaminants. Exceptions were made for the so-called "*Streptococcus durans*." The enterococci are resistant to drying, heat and sanitizing agents. They grow from 10 to 45°C, survive heating at 62.8°C for 30 min, tolerate 6.5% NaCl and 40% bile, and grow between pH 4.0 and 9.6. They persist in the extraenteral environment and they are ubiquitous in many food processing establishments and can become an important part of the food microflora. The status of enterococci as harmless bacteria in foods is being challenged by their growing importance in nosocomial infections and the emergence of vancomycin resistant strains (VRE). The enterococci are highly susceptible to genetic change because of the apparent ease of plasmid transfer afforded by the aggregation characteristics of many strains. The control of enterococci in foods has assumed a new level of importance in food processing and food microbiology.

## Safety aspects of enterococci from the medical point of view

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Enterococci occur in a remarkable array of environments, because of their ability to grow and survive under harsh conditions. Thus, they can be found in soil, food, water, and a wide variety of animals. The major habitat of these organisms is the gastrointestinal tract of humans and animals. In humans, they make up a significant portion of the normal gut flora ( $10^5 - 10^7/g$  of stool). Smaller numbers are found in oropharyngeal secretions, vaginal secretions, and on the skin. Although enterococci belong to the normal flora of humans, these organisms can cause infectious disease.

Most clinical isolates of the genus *Enterococcus* are *E. faecalis*, which account for approximately 80-90% of enterococci isolated in the clinical microbiology laboratory. *E. faecium* accounts for 5-10% of isolates. Recent evidence suggests that the prevalence of *E. faecium*, especially of multiresistant strains, is increasing in large tertiary care hospitals. Occasionally, isolates of *E. durans*, *E. avium*, *E. casseliflavus*, *E. gallinarum*, *E. raffinosus*, and *E. hirae* are encountered clinically.

Surprisingly little is known about the factors that contribute to the ability of enterococci to cause infections. Many strains of *E. faecalis* isolated from clinical cases produce a cytolysin (hemolysin) exhibiting tissue damaging capacity. Further extracellular products often observed in clinical isolates is a proteinase (gelatinase) and is extracellular superoxide. Furthermore, many of the clinical isolates possess the aggregation substance on the surface and an extracellular surface protein, both contributing to the adherence of enterococci to eucaryotic cells. Some strains of *E. faecalis* are resistant to multiple antimicrobials. Most clinical strains of *E. faecium* do not exhibit specific virulence factors. However, resistance to multiple antibiotics is often observed in clinical strains of this species. The ultimate role of all these factors in enterococcal pathogenicity remains to be determined. What is clear, however, is that enterococci are not as intrinsically virulent as organisms such as *Staphylococcus aureus* or *Streptococcus pyogenes*.

Despite the low pathogenicity, enterococci are often isolated from patients suffering from urinary tract infections, bacteremias, intraabdominal and pelvic infections, wound and tissue infections, and occasionally from patients with meningitis, endocarditis, respiratory tract infections or neonatal sepsis. Many of these diseases are polymicrobial, with enterococci being only part of the responsible microbial flora. The overwhelming number of enterococcal infections occur in patients with underlying conditions representing a wide spectrum of severity of illness and immune modulation. It is not surprising, therefore, that enterococci rank second to third in frequency as causes of nosocomial infections.

Because enterococci are part of the normal gut flora of humans, it was previously thought that infections were endogenously acquired from the patient's own flora. A rather new concept that has emerged is that enterococcal disease is a two-stage process. There is an initial, usually asymptomatic colonization of the gastrointestinal tract by enterococcal strains possessing virulence traits and/or antibiotic resistances. Subsequently, this population is expanded, often facilitated by antibiotic elimination of competitors. For a select number of patients, there is subsequent tissue invasion from the gastrointestinal tract reservoir. From this concept it can be deduced that enterococcal strains without virulence traits and antibiotic resistances exogenously transferred into the human gut via food products or probiotics will not present any risk for immunocompetent individuals. Because of the sophistication of support currently available to prolong the life of very severely immunocompromised patients, a risk for enterococcal disease by commensal strains without virulence traits and antibiotic resistance cannot be completely excluded.

## Occurrence and spread of antibiotic resistances in *Enterococcus faecium*

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Enterococci are the second to third important bacterial genus in hospital infections. Especially *E. faecium* possesses a broad spectrum of natural and acquired antibiotic resistances. From medical point of view the transferable resistances to glycopeptides (e. g., vancomycin, VAN, or teicoplanin, TPL) and streptogramins (e. g., quinupristin/dalfopristin, Q/D) in enterococci are of special interest.

The VanA type of enterococcal glycopeptide resistance is the most important one (VAN-r, TPL-r); its main reservoir is *E. faecium*. Glycopeptide-resistant *E. faecium* (GRE) can be found in hospitals and outside of them, namely in European animal husbandry in which the glycopeptide avoparcin (AVO) was used as "growth promoter" in the past. Across the food chain (by GRE-contaminated meat products) these multiply resistant bacteria (or their *vanA* gene cluster) reach the humans. There are identical *vanA* gene clusters of enterococci from different ecological origins (faecal samples of animals, animal foodstuffs, patients in hospitals, persons in the community, waste water samples).

In hospital infections wide spread epidemic-virulent *E. faecium* isolates of the same clonal group with or without glycopeptide resistance plasmids can occur; these strains often harbour different plasmids. This means that epidemic-virulent strains have picked up the *vanA* gene cluster from different sources *after* they were already widely spread. The *vanA* gene cluster can originate from the enterococcal reservoir of human gut flora. GREF strains from fattening animals can be involved in the development of this resistance reservoir in the human intestine.

The streptogramin virginiamycin was used in commercial animal husbandry of Europe for more than 20 years. In 1998/99 streptogramin-resistant *E. faecium* (SRE) could be isolated in Germany from waste water of sewage treatment plants and from faecal samples and meat products of animals that were fed by virginiamycin (cross resistance to Q/D). Additionally in this time SRE could be found in stools of humans in the community and in clinical *E. faecium* isolates, that means in a time *before* the streptogramin combination Q/D was introduced for therapeutic purposes in German hospitals in May 2000. This is a strong indication that the transferable streptogramin resistance types in enterococci from hospitals originate from animal origin(s).

## Molecular structure and evolution of the conjugative multiresistance plasmid pRE25 of *Enterococcus faecalis* isolated from a raw fermented sausage

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Plasmid pRE25 was detected in an *E. faecalis* strain present in a raw fermented sausage at about  $10^2$  colony forming units per gram. The strain was resistant towards the following antibiotics: kanamycin, neomycin, streptomycin, clindamycin, lincomycin, azithromycin, clarithromycin, erythromycin, roxithromycin, tylosin, chloramphenicol, nourseothricin sulfate, and tetracycline. All resistances except that against tetracycline were transferrable by conjugation *in vitro* to *E. faecalis* JH2-2, *Lactococcus lactis* Bu2 and *Listeria innocua* L19.

The nucleotide sequence revealed a size of the circular, double stranded DNA of pRE25 of 50237 base pairs, being presently the largest conjugative multiresistance plasmid of enterococci with a known nucleotide sequence (Schwarz et al., Plasmid 46: 170-187, 2001).

The gene for chloramphenicol resistance (*cat*) was identified as a chloramphenicol acetyl transferase identical to the one of plasmid pIP501 of *Streptococcus agalactiae*. Erythromycin resistance is due to a 23S ribosomal RNA methyl transferase, again as found in pIP501 (*ermB*). The aminoglycoside resistance genes are present in a tandem package as previously reported in the transposon *Tn5405* of *Staphylococcus aureus*. It is composed of the genes for an aminoglycoside 6-adenyltransferase, a streptothricin acetyltransferase, and an aminoglycoside phosphotransferase, representing all three known chemical mechanisms of aminoglycoside antibiotic inactivation.

The molecular analysis reveals that pRE25 is composed of a 30.5- kbp segment very similar to pIP501, including the segment responsible for conjugative transfer. Of the 15 genes involved, ten code for putative transmembrane proteins (e.g. *traB*, *traC*, *traF*, *traJ*, and *traL*).

The enterococcal part of pRE25 is joined into the pIP501 derived part by insertion elements like IS1216V of *E. faecium* *Tn1545* (three copies) , and homologues of IS1062 (*E. faecalis*) and IS1485 (*E. faecium*). IS1485 is inserted into another IS-element, namely the one of plasmid pEF1 of *E. faecium*.

The putative 49 open reading frames of pRE25 contain antibiotic resistance genes known from human pathogens like *Streptococcus pyogenes*, *Str. agalactiae*, *Staphylococcus aureus*, *Campylobacter coli*, *Clostridium perfringens* and *C. difficile*.

This molecule is evidence that enterococci detectable in fermented food do participate in the molecular communication between Gram-positive and Gram-negative bacteria of the human and animal microflora (mouth and intestine).

Food containing enterococci with mobile genetic elements like conjugative plasmids and transposons therefore contribute to the dissemination of antibiotic resistance genes in the human population if consumed with food (Teuber et al., Antonie van Leeuwenhoek 76: 115-137, 1999). Since we know that animal enterococci enter fermented food from the raw material (meat or milk) and do multiply during food processing, such food items (raw milk cheeses and raw meat sausages, and the raw milk and meat itself) are a direct link between the animal and human microflora.

If we use food technologies developed before the age of antibiotic resistance, we must expect resistant bacteria in the traditional products. It seems necessary to discuss this new dimension in food microbiology, and at the same time to develop new technologies to avoid this new threat.

## Gene transfer of antibiotic resistance and virulence determinants among enterococci in food

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Enterococci compose the microbial association of a variety of fermented foods such as cheese, fermented sausages and fermented vegetables. However members of the genus *Enterococcus* have distinguished themselves from other lactic acid bacteria by their role in human infection, harboring a number of identified virulence factors, and for their acquired resistance to antibiotics. The purpose of the present study was to assess the frequency of transfer of two mobile genetic elements coding for virulence determinants and antibiotic resistance factors, into food associated enterococci during a fermentation process. First, the transfer of the pheromone-inducible pCF10 plasmid, carrying tetracycline resistance and AS virulence, factor between virulent and food strains of *Enterococcus faecalis* was investigated in models of cheese and fermented sausage. The experiments demonstrated that even in the absence of selective tetracycline pressure, plasmid pCF10 was transferred from *E. faecalis* OG1rf cells to other *E. faecalis* organisms present in food and that the plasmid subsequently persisted in these environments. Very high frequency of transfer were observed in sausage ( $10^{-3}$ /recipient) if compared to cheese ( $10^{-6}$ ) and plate mating ( $10^{-4}$ ). Transconjugants were subsequently verified by PCR. The second transmissible element was the plasmid harbouring the Tn1546, a transposon coding for the vancomycin resistance (VanA phenotype). The transfer of this antibiotic resistance to a food strain of *E. faecalis* was studied in vitro and in food models. Although the transfer of Tn1546 was achieved in all the environments, the highest conjugation frequencies were observed during the ripening of fermented sausages, reaching  $10^{-3}$  transconjugants/recipient cell. PCR allowed to confirm the transfer of VanA genotype into food associated enterococci. The effect of physicochemical attributes of food, pH and temperature, on the growth of enterococcal strains and on the frequency of plasmid transfer was evaluated.

This study showed that even in the absence of selective pressure, mobile genetic elements carrying antibiotic resistance and virulence determinants can be transferred at high frequency to food associated enterococci during cheese and sausage fermentation

## Species distribution and antibiotic resistance patterns of enterococci Isolated from food of animal origin in Germany

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Nowadays enterococci take the third place of bacterial pathogenics of nosocomial infections after staphylococci and *Escherichia coli*. Especially the resistances of enterococci against many available antibiotics are dreaded. We tried to figure out which species of enterococci can be found in food of animal origin and their meaning according to their antibiotic resistances for human beings.

From November 2000 until February 2002 we examined 93 food samples of animal origin: 27 samples of sausages, 21 of minced meat, 19 of ham, 26 of cheese.

From these food samples we isolated 270 strains of enterococci. The most frequent species was *E. faecalis* (203 strains), furthermore we found *E. durans* and *E. hirae* (17 strains), *E. faecium* (31 strains), *E. casseliflavus* (14 strains) and *E. gallinarum* (5 strains).

We investigated the resistance pattern of 120 selected strains against 16 antimicrobial active agents (ampicillin, amoxicillin/clavulanic acid, avilamycin, bacitracin, chloramphenicol, enrofloxacin, erythromycin, flavomycin, gentamicin, penicillin, trimethoprim/sulfamethoxazole, quinupristin/dalfopristin, teicoplanin, tetracyclin, tylosin tartrate and vancomycin).

The situation of antibiotic resistance against the examined antimicrobial agents is not threatening (from the clinical point of view).

The investigated strains were sensitive to ampicillin and amoxicillin/clavulanic acid. These antibiotics are (in combination with an aminoglycoside, for example gentamicin) drugs of the first choice for the treatment of enterococcal infections in human medicine.

Only two strains were resistant to penicillin, no strain showed resistance to trimethoprim/sulfamethoxazole and to the glycopeptide-antibiotics vancomycin and teicoplanin (except the both natural low-level-resistant species *E. casseliflavus* and *E. gallinarum*).

A critical situation exists with regard to the resistance against the antibiotics tetracycline and quinupristin/dalfopristin. 28% of the investigated strains were resistant to tetracyclines and 20% of the *E. faecium*-strains showed resistance to the streptogramin quinupristin/dalfopristin, a new antimicrobial agent in the human medicine for the treatment of infections with vancomycin-resistant *E. faecium*.

## Detection of species and resistance genes in enterococci of animal origin by multiplex PCR

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Enterococci are regarded as one of the leading causes of nosocomial infections. They have a large spectrum of antibiotic resistances. Especially their resistance towards glycopeptide antibiotics such as vancomycin and teicoplanin is threatening because these antibiotics are used in the human medicine for the treatment of severe infections. Avoparcin which belongs to the same group of antibiotics had been used as a growth promotor in the animal husbandry. Many reports led to the conclusion that the use of this glycopeptide was responsible for the resistance development in enterococci strains isolated from food. Considering these warnings avoparcin was banned in the Federal Republic of Germany in June 1996.

Our research project deals with the statistical analysis of the findings of enterococci in food, especially those strains isolated from food of animal origin (broilers, cattle, pigs). Apart from the biochemical differentiation of these enterococcal strains we use the PCR, a simple method of choice, to detect vancomycin resistance genes (*vanA*, *vanB*, *vanC1*, *vanC2*) and to identify the species (*E. faecium*, *E. faecalis*, *E. gallinarum*, *E. casseliflavus*). This procedure helps us to underline or to correct the biochemical results.

DNA-isolation method and the essential primers for the PCR are described by Klare et al. (1997), Dutka-Malen et al. (1995) and Cheng et al. (1997). Our goal was to investigate the routine use of this procedure.

367 enterococcal strains which had been isolated from food samples in the years 2000 and 2001 all over Germany had been chosen out of an extensive sample collection. They had been examined for the existence of the mentioned genes and gene fragments, respectively.

According to our PCR results we found out that apart from the 21 strains isolated from samples of broilers origin possessing the high-level *vanA*-gene the two *vanC*-genes were dominant in the three examined sources. The *vanB*-gene could not be detected in any strain. By the use of the multiplex-PCR we tried to optimize a rather rapid method for the detection of resistance genes and the species at the same time. For the majority of the investigated strains this kind of procedure was very helpful and successful but we had to mention that there were also false negative and "unclear" results in all sources. Accidentally we found out that a small number of *E. faecalis* strains was also specific to the used *E. faecium* specific primers described by Cheng et al. (1997).

In the sum the multiplex PCR offers us a rapid method to detect the examined genes and is suitable for routine use. False-negative and unclear results might occur but they can be easily cleared up by repetition of the whole PCR procedure and by additional biochemical tests, respectively.

## **Epidemiology and ecology of enterococci, with special reference to antibiotic resistant strains, in animals, humans and the environment \***

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Some results from a project within the European research programme.

The aim of this European project was to study the population structure among enterococci in different geographical regions of the EU and in different links of the food chain. Materials and methods. A total number of 2868 samples were collected from different countries within the EU (Sweden, Denmark, UK and Spain), and from different habitats, selected to contain enterococcal populations of human as well as animal origin (humans, water, sewage, pig farms, carcasses in slaughterhouses, soil, manure). The samples were characterized with regard to presence of enterococci and of vancomycin resistant strains. More than 20.000 isolates were phenotyped and preliminary species identified with the automated PhenePlate (PhP) system. Resistance to vancomycin, ampicillin, erythromycin, virginiamycin, tetracycline and avilamycin was determined for selected isolates by the VetMic™ system.

Results. A majority of the samples (77%) showed growth of presumed enterococci. The most common species found were *E.faecium* (33%), *E.faecalis* (29%), and *E.hirae* (24%). In 8.3% of the samples there was growth of presumed enterococci in the presence of 20 mg/l of vancomycin (PEV20). The PEV20 were most common in urban sewage samples (54%), hospital sewage (16%) and in pig manure (in 21%), but rare in samples from slaughterhouses and samples from farmland and crop with and without manure. There was also a clear difference in the frequencies of PEV20 isolated from animals between the different countries. Thus, in Sweden only 1 % of the samples of animal origin contained PEV20, whereas in Denmark 3%, Spain 9% and in the UK 12% of such samples contained PEV20.

Resistance to tetracycline and/or erythromycin were most common (35% and 21% of all isolates tested) and found frequently in all sample types except in calves from Denmark and Sweden. Resistance to ampicillin was more frequent among human than among animal isolates (7% versus 3%). Avilamycin-resistant strains were almost exclusively isolated from broilers and pigs in all countries except Sweden.

Most PEV20 were confirmed as VRE. A much higher proportion of the VRE were resistant to at least one of the other antibiotics tested (97%) than the VSE (41%). Ten percent of the VRE were resistant to all antibiotics tested. These isolates were mainly of human (urban sewage) origin. The phenotypic diversity according to PhP typing among all VRE isolates was as high as 0.99, indicating that the VRE belonged to a wide variety of different clones. Only three cases with identical PhP types in human and animal VRE were found. Most VRE were *E.faecium*, but 16 out of the 148 confirmed VRE were *E.faecalis*. Notably 12 of these were from Sweden, mostly from urban and hospital sewage.

Conclusions. There are currently two different populations of VRE in Europe, one associated with animal production, and possibly due to previous use of avoparcin as a growth promoter, and another population that may be derived from antibiotic use in hospitals, that is also spreading to the environment via sewage, but is yet uncommon in animals. Thus it seems that today the danger of spreading multi-resistant enterococci in humans is mainly associated to use of antibiotics in the human population. However, since there is an environmental reservoir of such bacteria in all countries caused by use of antibiotics in both the human and animal populations, precautions must be taken in both these fields to limit the release of antibiotic-resistant enterococci into the environment as much as possible.

\* further results with the same title will be presented during the poster presentation

## The effect of raw-milk cheese consumption on the enterococcal faecal flora

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Enterococci are one of the major facultative anaerobic bacterial groups that reside in the human gastrointestinal tract. They also have been used as silage inoculants, probiotics, antilisterial bacteriocin producing starter cultures and they are naturally present in raw-milk cheeses. Nevertheless they have become the focus of attention as one of the major causes of nosocomial infections and their increasing resistance to microbial agents is a major concern. As it is believed that some nosocomial enterococcal infections originate in the intestinal tract it is important to investigate the dynamic of the passage through the GI tract. In the present study the composition of the enterococcal faecal flora in healthy humans was analysed before, during and after the daily consumption of ~125g of a raw-milk Cheddar cheese containing  $3.2 \times 10^4$  enterococci/g cheese.

Enterococcal counts per gram faeces varied within subjects and from week to week and were between  $1.4 \times 10^2$  and  $2.5 \times 10^8$  cfu/g. The cheese contained mainly *E. casseliflavus* and a small population of *E. faecalis*. One-dimensional whole-cell protein electrophoresis (SDS-PAGE) and Pulsed Field Gel Electrophoresis (PFGE) were used to identify cheese and human isolates at the species level and type them to strain level, respectively. Prior to and after the consumption of cheese, the microflora of human faecal samples was dominated by *E. faecium*. Clones of *E. faecium* were repeatedly found to be residents of the faecal microflora and persisted over long periods of time. During consumption of the cheese one particular transient clone of *E. faecalis* originating from the cheese largely dominated the samples.

# **Abstracts of Posters**

## Session 1: Ecology, Taxonomy and Physiology of Enterococci

- 1 **Genetic characterization of multiple bacteriocin production by *Enterococcus faecium* L50**  
R. Criado, J. Gutiérrez, D.B. Diep, A. Aakra, R. Citti, C. Herranz, I.F. Nes, P.E. Hernández, and L.M. Cintas
- 2 **Identification of a new operon, *as-48EFGH* from the *as-48* gene cluster**  
Marta Diaz, Eva Valdivia, Manuel Martínez-Bueno, Matilde Fernández, Andrés Santos Soler-González, Hilario Ramírez-Rodrigo and Mercedes Maqueda
- 3 **Methods for the official control of enterococci used as feed additives: Molecular biological identification of enterococci**  
K.J. Domig, H.K. Mayer and W. Kneifel
- 4 **Cloning, production, secretion and purification of the bacteriocin Enterocin P in *Escherichia coli***  
J. Gutiérrez, R. Criado, A. Oliveira, R. Citti, M. Martín, C. Herranz, M.F. Fernández, L.M. Cintas and P.E. Hernández
- 5 **Characterization and identification of *Enterococcus faecium* strains of food and human origin with probiotic potential, and differentiation from other lactic acid bacteria by PCR and Dot-Blot**  
R. Martín, S. Langa, A. Fernández, A. Zabala, L. Fernández and J.M. Rodríguez
- 6 **Plant-associated enterococci: Identification, antagonistic activity and antibiotic resistance**  
Thomas Müller, Edda-Maria Ott, Andreas Ulrich and Marina Müller
- 7 **The protein sequence and genetic determinants of Enterocin EJ97**  
M. Sánchez-Hidalgo, M. Maqueda, A. Gálvez, H. Abriouel, E. Valdivia and M. Martínez-Bueno
- 8 **Phylogenetic relationship of enterococci and their differentiation by *RecA* gene sequence analysis**  
Sandra Torriani, Giovanna E. Felis, Edo Knijff and Franco Dellaglio
- 9 **Citrate metabolism by *Enterococcus faecalis* FAIR-E 229**  
Panagiotis Sarantinopoulos, Evi Rodi, George Kalantzopoulos and Effie Tsakalidou
- 10 **Partial purification and characterization of a bacteriocin produced by *Enterococcus faecium* FAIR-E 406, a probiotic strain**  
Erika Van den Berghe and Luc De Vuyst
- 11 **Prevalences of different enterococcal species in faecal samples of cattle, pigs and broilers**  
H. Wichmann-Schauer, J. Peters, K. Mac and L. Ellerbroek

## Session 2: Functionality of Enterococci in Food

- 12 **Enterococci – the prevailing microflora of autochthonous ewe's cheese produced in Slovenia**  
Bojana Bogovič Matijašić, Vesna Katana, Irena Rogelj, Bogdan Perko, William Kelly and Lawrence J. H. Ward
- 13 Enterococci isolated from Spanish foods: Technological and safety aspects  
A. Castro, N. Ben Omar, R. Lucas, H. Abriouel, N.M.K. Yousif, C.M.A.P. Franz, W.H. Holzapfel, R. Pérez, M. Martínez-Cañamero and A. Gálvez.
- 14 **Use of enterococci as microbial adjuncts for making Tetilla cows' milk cheese (NW Spain)**  
J. A. Centeno and J. M. Izco
- 15 **Molecular identification and technological characterization of lactococci and enterococci strains isolated from starter-free cheeses**  
Susana Delgado and Baltasar Mayo
- 16 **Inhibitory effect of *Enterococcus faecalis* Fair-E 171 on *Listeria innocua* during manufacture and ripening of an experimental Caciotta cheese**  
Edo Knijff, Christian Andrighetto, Sandra Torriani, Angiolella Lombardi and Franco Dellaglio
- 17 **An antagonistic effect of Enterocin P in Gombasek sausage experimentally inoculated by *Listeria innocua***  
A. Lauková, P. Turek and M. Mareková
- 18 **Influence of the aloe vera additive on the enterococci growth during lactic acid curd formation**  
Izabela Steinka
- 19 **Effect of *Enterococcus faecium* on microbiological, physicochemical and sensory characteristics of Greek feta cheese**  
Panagiotis Sarantinopoulos, George Kalantzopoulos and Effie Tsakalidou
- 20 **Technological and safety aspects of enterococci isolated from 'Hussuwa', a Sudanese fermented sorghum product**  
N.M.K. Yousif, H.A. Dirar, W.H. Holzapfel, C.M.A.P. Franz

### Session 3: Functionality of Enterococci in the GI Tract

- 21 **The enhancement of biological activity of *Enterococcus faecium* M-74 by selenium**  
L. Ebringer, A. Belicová, M. Boča, J. Dobias, I. Ďuriš, M. Ferenčík, E. Jahnová, J. Krajčovič, L. Križková, Z. Mikeš, J. Rovenský and M. Seman
- 22 **Inhibitory effect of Enterocin A against *Salmonella* in gnotobiotic Japanese quails**  
A. Lauková, P. Guba, R. Nemcová and M. Mareková
- 23 **Selection of enterococci for dog probiotic adjuncts**  
V. Stropfová and A. Lauková
- 24 **Recovery of an *Enterococcus faecium* probiotic strain in faeces after gastrointestinal transit in human**  
Bodil Lund and Charlotta Edlund
- 25 **Homemade regional cheeses as resources for the isolation of probiotic *Enterococcus faecium* strains**  
María Pía Taranto, Lucila Saavedra, Fernando Sesma and Graciela Font de Valdez
- 26 **Influence of probiotic *Enterococcus faecium* strains on microbiological parameters in the gastrointestinal tract of poultry**  
W. Vahjen, A. Jadamus and O. Simon
- 27 **Antimutagenic and reactivation activity of *Enterococcus faecalis***  
Lena I. Vorobjeva and Andrey I. Shestakov
- 28 **Genetic characterization of the bile salt hydrolase gene from *Enterococcus faecium* E-345 and chromosomal location of BSH genes among enterococci**  
Agus Wijaya, Anette Herrmann, Hikmate Abriouel, Nuha M.K. Yousif, Wilhelm H. Holzapfel, and Charles M.A.P. Franz

## Session 4: Safety Aspects of Enterococci

- 29 **Plasmid associated resistance in food enterococci**  
Ana Eugénio, Maria de Fátima Silva Lopes and Maria Teresa Barreto Crespo
- 30 **High prevalence of vancomycin resistant enterococci in urban sewage and in hospital sewage, but not in pig samples, in Sweden**  
Aina Iversen, Inger Kühn, Anders Franklin and Roland Möllby
- 31 **Diffusion of combined vancomycin and gentamycin resistance in enterococcal strains of food and clinical origin**  
Lucia Rizzotti, Edo Knijff, Michele Gastaldelli, Sandra Torriani and Franco Dellaglio
- 32 **Dissemination of *Tn917* and *ermB* in dairy and clinical enterococci**  
Maria de Fátima Silva Lopes , Paula Alves, Rogério Tenreiro and Maria Teresa Barreto Crespo
- 33 **Molecular and physiological comparison of *Enterococcus faecium* antibiotic resistance and virulence determinants between dairy, faecal and clinical isolates**  
L. Mannu, E. Daga, A. Paba, R. Comunian, S. Zanetti, I. Duprè and L. A. Sechi
- 34 **Ocurrence of glycopeptide-resistant enterococci (GRE) in foods of animal origin**  
J. Peters, H. Wichmann-Schauer, L. Ellerbroek and G. Klein
- 35 **Characterization of gentamicin resistance in dairy and clinical *Enterococcus* spp.**  
Tânia Ribeiro, Maria de Fátima Silva Lopes, Maria Paula Martins, Rogério Tenreiro, Maria Teresa Barreto Crespo
- 36 **Multiple virulence factors and multiple antibiotic resistances in *Enterococcus faecalis* isolated from food**  
Franziska Rossi, Gottfried Dasen, Leo Meile and Michael Teuber
- 37 **Epidemiology and ecology of enterococci, with special reference to antibiotic resistant strains, in animals, humans and the environment**  
I. Kühn, A. Iversen, L.G. Burman, B. Olsson-Liljequist, A. Franklin, M. Finn, F. Aarestrup, A-M. Seyfarth, A.R. Blanch, H. Taylor, J. Caplin, M.A. Moreno, L. Dominguez, I. Herrero and R. Möllby

# **Ecology, Taxonomy and Physiology of Enterococci**

# 1

## Genetic characterization of multiple bacteriocin production by *Enterococcus faecium* L50

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*Enterococcus faecium* L50, a naturally occurring strain isolated from a Spanish dry-fermented sausage, displays a broad antimicrobial spectrum, which includes food-borne pathogens, such as *Listeria monocytogenes*, *Clostridium perfringens* and *C. botulinum*, and human and animal clinical pathogens, such as *Streptococcus pneumoniae*, *S. mitis*, *S. oralis*, *S. parasanguis* and *S. agalactiae*. *E. faecium* L50 produces three bacteriocins (four peptides) under temperature regulation: enterocin L50 (EntL50), consisting of EntL50A and EntL50B, enterocin Q (EntQ) and enterocin P (EntP). EntL50 and EntQ are unmodified non-pediocin-like peptides synthesized without an N-terminal leader sequence or signal peptide, while EntP is a sec-dependent pediocin-like bacteriocin.

*E. faecium* L50 harbours two plasmids: pCIZ1 (50 kbp) and pCIZ2 (7,382 bp). Southern blot analyses using specific PCR-derived probes for each enterocin structural gene showed that EntL50 and EntQ are encoded by pCIZ1 and pCIZ2, respectively, while EntP is a chromosomally-encoded bacteriocin. Curing experiments of *E. faecium* L50 with novobiocin at concentrations of 3 µg/ml yielded two type of mutants: EntL50<sup>+</sup>-Imm<sup>+</sup>/EntQ<sup>-</sup>-Imm<sup>-</sup> (frequency 7.3%) and EntL50<sup>-</sup>-Imm<sup>-</sup>/EntQ<sup>-</sup>-Imm<sup>-</sup> (frequency 13.3%), revealing the plasmid-linkage of bacteriocin production and immunity phenotypes.

The transferability of EntL50 and EntQ production and immunity was tested by conjugation with *Enterococcus faecalis* JH2-2 (rifampicin and fusidic acid resistant, bacteriocin susceptible, non-bacteriocin producer) as the recipient strain. Recipient cells were further selected onto brain heart infusion (BHI) agar plates supplemented with rifampicin (100 µg/ml) and fusidic acid (20 µg/ml) and, after incubation at 37°C for 48h, the existence of Bac<sup>+</sup> transconjugants was analyzed by overlying the plates with a melted soft BHI agar inoculated with *Enterococcus faecalis* JH2-2 (1x10<sup>5</sup> cfu/ml). EntL50 and/or EntQ-production by the Bac<sup>+</sup> transconjugants was then evaluated by a stab-on-agar test using *Pediococcus acidilactici* 347 (EntL50<sup>s</sup>-EntQ<sup>r</sup>) and *E. faecium* P13 (EntL50<sup>s</sup>-EntQ<sup>s</sup>) as indicator microorganisms.

The enterocin Q structural gene (*entQ*) and the putative immunity/transport gene (*entiQ*) were cloned jointly and separately into the vectors pELS200 and pMG36e, and the recombinant vectors were transformed and stabilized in *Escherichia coli* JM109. The recombinant vectors were further electroporated into selected lactobacilli and enterococci strains in order to investigate the acquisition of EntQ<sup>+</sup> and/or Imm<sup>+</sup> phenotypes.

## 2

### Identification of a new operon *as-48EFGH* from the *as-48* gene cluster

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In recent years, there has been renewed interest in the search for bacteriocins produced by lactic acid bacteria (LAB) due to high efficiency in controlling the growth of pathogenic or harmful microorganisms which makes them attractive as food preservatives. Considerable progress has been made towards a better genetic characterization of these substances, but to date, the use of bacteriocins, other than nisin and pediocin, is still at an experimental stage. Nevertheless, they may have a future application in enhancing the safety and extending the shelf life of many inherently perishable foods.

Enterocin AS-48, produced by *Enterococcus faecalis* S-48, is a cyclic peptide of cationic nature, very stable in a wide range of pH and temperatures. This enterocin has a bactericidal action mechanism against a wide range of Gram-positive bacteria as well as some Gram-negative bacteria, offering promising perspectives for biotechnological applications.

We previously proposed that the basal expression of AS-48 requires the presence of the six genes (*as-48A*, *as-48B*, *as-48C*, *as-48C<sub>1</sub>*, *as-48D* and *as-48D<sub>1</sub>*) located in a 7.8-kb from pMB2 plasmid. This does not preclude the possibility that the AS-48 trait could comprise an additional transcription unit, *as-48EFGH*, that has been cloned and sequenced from 6,6 kb *Bgl*II fragment B from pMB2. When *as-48ABCC<sub>1</sub>DD<sub>1</sub>EFGH* were expressed in JH2-2 (pAM401-81 plasmid, 25 kb), the transformants became producers and resistant to wild level.

*as-48EFGH* encodes a new ABC transporter that could be involved in producer self-protection. On the basis of the observed similarities, As-48G would be the ATP-binding domain, the deduced amino acid sequence of As-48E and As-48H could be assigned as transmembrane subunits and As-48F, with an N-terminal transmembrane segment and a coiled-coil domain, strongly resembles the structure of some known ABC transporter accessory proteins, which would stimulate the ATPase activity of As-48G, accelerating the release of ADP and allowing As-48G to recycle more efficiently.

Phenotype of mutants with Tn5 insertions into *as-48G* or *as-48H* genes indicated that they were still capable of producing enterocin although in lesser amounts, suggesting that they are not directly involved in AS-48 biosynthesis. However, the immunity level of these mutants was clearly reduced (as far as 16,6%).

This cluster of genes is expressed by two polycistronic mRNA, T<sub>2</sub> (*as-48C<sub>1</sub>DD<sub>1</sub>EFGH*) and T<sub>3</sub> (*as-48EFGH*) in coordinate expression in JH2-2(pAM401-81) transformants. However, when *as-48EFGH* was cloned in JH2-2 (pAM401<sub>EH</sub>) T<sub>3</sub> was no detected, suggesting that these genes do not by themselves confer immunity.

### 3

#### **Methods for the official control of enterococci used as feed additives: Molecular biological identification of enterococci**

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The identification of probiotic enterococci strains has become an important item within quality control of probiotic animal feeds. As an important part of the EU project SMT-CT98-2235, molecular biological methods based on PCR and on Pulsed Field Gel Electrophoresis (PFGE) techniques were examined with 74 strains and isolates of *Enterococcus*. For the isolation of the DNA, a commonly used standard protocol was applied. In addition, an alternative rapid procedure was included and adapted. Randomly Amplified Polymorphic DNA (RAPD) involving a set of 39 RAPD primers was tested. This method showed good discriminatory power, but it is difficult to reach an acceptable level of reproducibility between laboratories. Other typing methods like Amplified Ribosomal DNA-Restriction Analysis (ARDRA), Internally Transcribed Spacer PCR (ITS-PCR) and Repetitive Element sequence-based PCR (REP) were also checked for their suitability and performance to differentiate among *Enterococcus* species and strains.

Due to the fact that even other members of lactic acid bacteria (LAB) gave wrong-positive results when using a PCR technique with Genus-specific PCR primers as published, this method cannot be applied for molecular identification of enterococci. However, using Species-specific PCR (*E. faecalis*, *E. faecium*) involving primers as published was able to discriminate among the test strains considered. In another test series, all enterococcal strains were screened with primers specific for vanA, vanB, vanC1, VanC2, and vanC3 genes as proposed by several authors. The important issue of vancomycin resistance (especially VanA) was included in the studies, since vancomycin resistance and its transferability cause severe problems in human medicine. In contrast to the other typing methods, which vary regarding their reproducibility and discriminatory properties, PFGE is currently considered to be the "gold standard" for sub-typing enterococci. The strain collection established during the EU project was typed using a proven PFGE protocol, which was slightly modified and examined with nine restriction endonucleases. It could be demonstrated that this method shows a pronounced efficacy. The PFGE protocol was brought into ISO format and proposed as a standard method which is capable of checking the identity of enterococcal strains used as probiotic feed additives.

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## 4

### Cloning, production, secretion and purification of the bacteriocin Enterocin P in *Escherichia coli*

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The cloning and secretion of bacteriocins produced by Lactic Acid Bacteria (LAB) in other microbial hosts is of a great scientific and industrially applied interest, since such hosts may produce bacteriocins in larger quantities, may facilitate their purification to homogeneity or may be considered as safer organisms when used as starter cultures or as producers of bacteriocins as natural antimicrobial agents in foods. In this context, enterocin P (EntP) produced by *Enterococcus faecium* P13 (Cintas *et al.*, 1997) and other *E. faecium* strains (Herranz *et al.*, 1999; Cintas *et al.*, 2000), is a pediocin-like bacteriocin synthesized as a prepeptide (PreEntP) and it seems to be processed and secreted by a *sec*-dependent system (GTS) (Cintas *et al.*, 1997) instead of a dedicated ABC-transport system (DTS).

The cloning, production, secretion and purification of enterocin P produced by *E. faecium* P13 has been evaluated in *Escherichia coli*. Total DNA from *E. faecium* P13 was used as target DNA for PCR amplification of gene *entP* (fragment P) and genes *entP* and *orf2* (fragment PI). Fragments P and PI were cloned in the blunt-end plasmid pETBlue-1 and the resulting plasmids transformed in *E. coli* Novablue hosts. The plasmids with the fragments in the correct orientation, pJG01 (fragment P) and pJG02 (fragment PI), were purified and transformed into *E. coli* Tuner(DE3)pLacI cells. Before transformation, the correct nucleotide sequence of the fragments was confirmed. Production and quantification of EntP was evaluated in *E. coli* Tuner (DE3)pLacI (pJG01) and *E. coli* Tuner (DE3)pLacI (pJG02) cells, grown at 37°C and induced with different concentrations of IPTG. The direct antimicrobial activity of the isolates was visualized in LB agar plates against the indicator organism *E. faecium* T136, and its quantification was determined by the use of anti-peptide specific antibodies against the C-terminal fragment of EntP and a NCI-ELISA plate format. Briefly, the presence of EntP in the LB-culture supernatants (CS) of the induced cells increased with higher concentrations of IPTG, it was fairly constant in the cellular soluble protein fraction (CSP) and was related to the concentration of the inducer in the inclusion bodies (IB).

The secretion and further characterization of enterocin P was evaluated in the supernatants of *E. coli* Tuner (DE3)pLacI (pJG01) cultures grown at 37°C in the complex LB media and in the M9 minimal media, after induction with 1mM IPTG. The EntP in the cell-free culture supernatants of cultures grown in LB broth was recovered by immunoaffinity chromatography with purified IgG antibodies with specificity for enterocin P. When a sample of EntP recovered by immunoaffinity chromatography was subjected to electrophoresis in a Tricine-SDS-Polyacrylamide gel, a protein electrophoretic behaviour similar to that of purified to homogeneity EntP produced by *E. faecium* P13 was observed. The cell-free culture supernatants of cultures grown in M9 broth was also purified by a simplified two-step procedure, involving adsorption chromatography on an Amberlite XAD-16 hydrophobic polycarbonate resin and reverse phase chromatography on a PepRPC HR5/5 column integrated in a FPLC system. The purification procedure for EntP was evaluated determining the antimicrobial activity of the eluted fractions by a microtiter plate assay and the specific presence of EntP by a NCI-ELISA. The production of EntP by *E. coli* grown in the LB complex and in the M9 minimal media was lower than the produced by *E. faecium* P13 grown in MRS at 32°C, but the recovery of purified bacteriocin was very effective in *E. coli* cultures either grown in the LB complex or the M9 minimal media.

## 5

### Characterization and identification of *Enterococcus faecium* strains of food and human origin with probiotic potential, and differentiation from other lactic acid bacteria by PCR and Dot-Blot

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Initially, 36 lactic acid bacteria (LAB) were randomly isolated from sausage, cheese and human faeces samples and tested by MTT assay for stimulation or inhibition of the proliferation of Vero and myeloma cells (Zabala *et al.*, 2001a; 2001b). Isolates HN1 (from breast-fed baby faeces), HA1 (from healthy adult faeces) and CH3 (from sausage) were further selected because they showed a strong inhibition of myeloma cell proliferation but did not significantly affect the proliferation of Vero cells.

Subsequently, they were examined by phase-contrast microscopy to determine cell morphology and Gram-staining reaction, and tested for oxidase and catalase activities. The strains were further characterized by a variety of tests, which included arginine dehydrolase activity, Voges-Proskauer reaction, urease production and haemolysis on 5% calf blood agar (Oxoid). Growth in MRS broth (aerobic and anaerobic conditions), Brilliant Green Bile broth (2%) and UHT skim milk under different conditions was also evaluated. Fermentation patterns were obtained with API Rapid CH fermentation strips (BioMérieux) in Lactobacillus Identification Medium (CHL broth, API 50 CHL) as specified by the manufacturer. Antibiotic susceptibility was determined semi-quantitatively by a disc diffusion assay. The antimicrobial susceptibility discs were obtained from BioMérieux or Difco, and included erythromycin (15 µg), penicillin G (10 units), cefoxitin (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), chloramphenicol (30 µg), ampicillin (10 µg), tetracycline (30 µg), vancomycin (30 µg) and trimethoprim (1.25 µg) combined with sulfamethoxazole (23.75 µg). The three isolates were Gram-positive, catalase- and oxidase-negative, facultatively anaerobic, non-haemolytic cocci with the ability to grow in all the media tested (including milk) at temperatures ranging from 8 to 45°C, at pH 4.5-9.5, and in the presence of 6.5% NaCl, 2% bile or 0.04% sodium azide.

Identification of the isolates was performed by SDS-PAGE protein profiling at the BCCM/LMG Culture Collection (University of Ghent, Belgium). The normalized and digitized protein patterns were analysed numerically and clustered with the reference profiles existing in the LAB database of the Culture Collection. DNA sequencing of PCR products containing 16S rRNA sequences of the selected strains was performed at the Department of Food Functionality, NIZO Food Research (Ede, The Netherlands) to confirm strain identification. By both methods, isolates HN1, HA1 and CH3 were unambiguously identified as *Enterococcus faecium*. *E. faecium* HN1 displayed vancomycin resistance, a property that may preclude its use as a probiotic strain. In contrast, *E. faecium* HA1 and CH3 were sensitive to this antibiotic.

Finally, since probiotic LAB tend to be mixed with *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* for the production of probiotic dairy products, PCR and dot-blot procedures based on 16S-23S rRNA spacer region were successfully developed to differentiate the yogurt starter cultures from *E. faecium* strains.

#### References

Zabala, A., Martín, R., Haza, A., Fernández, L., Rodríguez, J.M. and Morales, P. (2001a). Antiproliferative effect of two lactic acid bacteria strains of human origin on the growth of a myeloma cell line. *Lett. Appl. Microbiol.*, **32**: 287-292

## 6

### Plant-associated enterococci: Identification, antagonistic activity and antibiotic resistance

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While research on enterococci has focused mainly on isolates of clinical or food origin, less attention has been given to enterococcal colonisation of leaf surfaces. In our study, enterococci could be detected on above-ground plant parts of grass throughout the growing season with high continuity but low cell numbers ( $2.60 \times 10^1 - 6.16 \times 10^4$  CFU g<sup>-1</sup> FM).

A total of 750 *Enterococcus* isolates were subjected to taxonomical investigations and tested for their capability to produce bacteriocins and for antibiotic resistance.

The identification procedure included phenotypic characterizations, whole-cell protein profile analyses, restriction analyses of PCR-amplified 16S rDNA, and 16S rDNA sequence analyses. The isolates were identified as *Ent. faecalis* (7.9 %), *Ent. mundtii* (7.9 %), *Ent. casseliflavus* (5.5 %), *Ent. faecium* (5.2 %) and *Ent. sulfureus* (0.1 %). However, the majority of isolates (69.7 %) differed distinctly in their restriction patterns from those of known species. They formed a group of a homogeneous 16S rDNA genotype (VI). The 16S rDNA sequence of a representative isolate revealed the closest relationship to the species *Ent. faecalis* (similarity of 97.4 %).

A screening for antagonistic activity using an agar spot test revealed that 18.4 % of all enterococcal isolates were potential antagonists. Partially purified proteins extracted from cell-free culture supernatants of six strains of various species were characterised as pH- and heat-stable bacteriocins active against a wide range of lactic acid bacteria, clostridia and *Listeria*. Attempts to detect known enterocin genes in total enterococcal DNA by means of PCR amplifications with specific primers revealed that only one *Ent. faecium*-strain carries the genes of both enterocins A and B, whereas no further PCR products were obtained from DNA of the other five bacteriocin-producers. Nevertheless, the producing strains were antagonistically active even on “phylloplane agar” at temperatures between 4 and 37 °C.

Agar diffusion tests were performed to detect antibiotic resistance. All of the 204 isolates tested were sensitive to vancomycin, but almost all were resistant to gentamycin and streptomycin.

The results provide new information on the distribution, species diversity, antagonistic potential, and antibiotic resistance of enterococci in the phyllosphere of forage grass. The taxonomical investigations suggest that the isolates of the 16S rDNA genotype VI represent a new plant-associated *Enterococcus* species. Antibiotic resistance seems to be also very common in plant enterococci. However, the suggestion that vancomycin resistance is ubiquitous in enterococci could not be supported in this study.

## 7

### The protein sequence and genetic determinants of Enterocin EJ97

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Production of anti-listerial bacteriocins is widely spread among enterococci, a trait that adds interest to this bacterial group in food preservation. Nevertheless, before planning the use of a given bacteriocin or bacteriocinogenic strain as a biopreservative, several aspects including its antimicrobial spectrum, mode of action, chemical structure and genetic determinants must be elucidated.

*Enterococcus faecalis* EJ97 produces a cationic bacteriocin (enterocin EJ97) which is active on Gram-positive bacteria, including enterococci and species of *Bacillus*, *Listeria* and *Staphylococcus*. EJ97 has been purified and characterized previously and its N-terminal sequence has been elucidated (Gálvez *et al.*, 1998).

Enterocin EJ97 was digested with endoproteinase Glu-C or trypsin, and the amino acid sequence of the N-terminal region of each individual resulting peptide was determined by automated microsequencing. The complete amino-acid sequence of EJ97 allowed us to calculate a molecular mass of 5323, closely matching the value of 5327.7 previously determined by MALDI-TOF and confirming its primary structure. A search of protein sequence databases revealed no polypeptides with homology to EJ97. The hydrophobicity plot of enterocin EJ97 indicates that it is a hydrophobic and basic peptide with up to 48 % of hydrophobic amino acids, and a predicted pI of 10.8.

Results from hybridization with DNA probes derived from the peptide sequence suggested that the 60-kb conjugative plasmid pEJ97 carries the genetic determinants (production and immunity). The structural gene *ej97A* and three additional ORFs (Ej97B, Ej97C and Ej97D) have been located in a 14-kb *Bgl*II fragment and also a putative transposase-resolvase module (ORF1 and ORF6) immediately upstream and downstream of the *ej97* genes. Analysis of *ej97A* gene revealed a 132-nucleotide-residue that is translated as a 44 amino-acid mature protein, lacking the leader peptide sequence found in other bacteriocins. This fact suggests that EJ97 could represent a new class of bacteriocins with a novel secretion mechanism.

On the basis of the deduced amino acid sequences, most of the gene products involved in EJ97 production were predicted to be predominantly basic and hydrophobic proteins, as deduce from hydropathy and amino acid composition. Except for Ej97B, none of these proteins showed significant homology/similarity to known proteins after comparison with the current protein databases. The deduced translation product of *ej97B* (Ej97B) revealed an extensive hydrophobic stretch (residues 1-300) able to form six putative transmembrane domains, and also a strong homology of its C-terminal domain with the superfamily of bacterial ABC transporters. The ORFs products Ej97C and Ej97D could be proteins with 71 and 64 residues, respectively, of unknown functions.

Furthermore, ORF1 and ORF6 have been identified as a transposon-like structure (*tnp*). The ORF1 potentially codes for a 229-residue protein showing similarities to transposase of the *Lactococcus lactis* element ISS1 and being up to 50% identical to that of IS1216. This is flanked by two inverted repeats (IRs) with sizes of 18 bp that are almost identical (94%) to those of ISS1 and IS1216. The ORF6 (named *revEJ97*) encoded a putative protein of 213 amino acid residues, which has a strong homology (97.2 % identity) with the resolvase of plasmid pAM373, and up to 40-50 % homology with the recombinase of several multiresistant plasmids and transposons from *Staphylococcus aureus* and *E. faecalis*.

## 8

### Phylogenetic relationships of enterococci and their differentiation by *RecA* gene sequence analysis

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The need for more stable bacterial classification schemes has led to the widespread application of phylogenetic analysis which integrates phenotypic, genotypic and biochemical data in a polyphasic approach. Classical phylogenetic analysis relies on the comparison of rRNA sequences or their coding genes (rDNA) because of their ubiquity, constant function and relatively high conservation. However, the 16S rRNA-based phylogenies suffer from some disadvantages, such as the identity of sequences from distinct but closely related species and horizontal gene transfer. Thus, in order to give stable evolutionary relationships among bacteria, a phylogenetically integrated approach should be implemented, by taking into consideration other molecular sequences. Several studies have established the utility of using alternative markers, such as HSP60, EF-Tu, GroEL and RecA protein sequences, to define bacterial phylogenetic relationships. These molecules can be considered “bi-functional”: the amino acid sequences can be used to infer phylogeny between distant taxa, preventing the GC-bias introduced by 16S rDNA analysis, while the nucleotide sequences can be analysed to separate closely related species, as they bear a greater number of mutations in respect to 16S rDNAs.

Comparative phylogenetic analysis based on *recA* and 16S rRNA gene sequences of various bacterial species, including lactic acid bacteria, have demonstrated highly similar branching patterns, indicating the suitability of the *recA* gene for use in molecular systematic studies and species differentiation. However, the *recA* genes of *Enterococcus* species have not been studied yet.

In this research we investigated the usefulness of *recA* gene as a phylogenetic marker for inferring phylogeny and differentiating the species actually assigned to the genus *Enterococcus*. For these purposes, new degenerate PCR primers were designed to amplify a 655-bp *recA* gene fragment. A database of partial *recA* gene sequences for the majority of the validly described species of enterococci was generated. These sequences were compared and analysed by three different bioinformatic methods, i.e. maximum parsimony, distance matrix and maximum likelihood; phylogenetic trees were constructed by using different outgroup sequences, as these factors are known to influence the analysis. The robustness of each topology was assessed by bootstrap.

We discussed phylogenetic relationships among enterococcal species on the basis of comparison of partial *recA* gene and 16S rDNA sequences, and also of other available or newly sequenced genes from the species under investigation.

A general agreement was recognised among the different obtained classification schemes, however partial *recA* gene sequences showed a major discriminative power in respect to 16S rDNA. This higher level of divergence between the *recA* sequences of different enterococcal species was explored as the basis for their differentiation. New species-specific PCR primers were designed to distinguish enterococci of more relevant food and clinical interest. Moreover, an ARDRA-like identification technique was developed to differentiate enterococcal species. This procedure, which was called ARGRA (Amplified RecA Gene Restriction Analysis) consisted of amplification and restriction of *recA* gene amplicons.

In conclusion, the use of partial *recA* sequences appears to be a powerful complementation for a more accurate taxonomy of enterococci.

## 9

### Citrate metabolism by *Enterococcus faecalis* FAIR-E 229

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Citrate metabolism by *Enterococcus faecalis* FAIR-E 229 was studied in various growth media, containing citrate either in the presence of glucose or lactose, or as the sole carbon source. In skimmed milk (lactose 133 mM, citrate 8 mM), co-metabolism of citrate and lactose was observed. Lactose was stoichiometrically converted to lactate, while citrate to acetate, formate and ethanol. When MRS broth (glucose 111 mM) or MRS broth containing lactose (28 mM) instead of glucose were used, *E. faecalis* FAIR-E 229 catabolized only the carbohydrate, and lactate was the sole end product (99%). Increasing concentrations of citrate (10, 40, 70 and 100 mM) added to the normal MRS broth, enhanced both growth rate of *E. faecalis* and glucose catabolism, although citrate itself was not catabolized. Glucose was converted stoichiometrically to lactate, while small amounts of ethanol were also produced. Finally, when citrate was used as the sole carbon source in increasing concentrations (10, 40, 70 and 100 mM), the main end products were acetate (65%) and formate (22%), and only small amounts of lactate, ethanol and acetoin were detected.

## 10

### Partial purification and characterization of a bacteriocin produced by *Enterococcus faecium* FAIR-E 406, a probiotic strain

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Lactic acid bacteria (LAB) used as probiotics are defined as viable microorganisms that improve the intestinal microbial balance and beneficially affect the host when ingested. The production of antimicrobial compounds, such as bacteriocins, for instance to control intestinal infections, may contribute to this health-promoting effect of LAB. Nowadays, the use of gut-associated LAB to combat food-borne pathogens in the gastro-intestinal tract is gaining interest. From this point of view, a bacteriocin produced by *Enterococcus faecium* FAIR-E 406 was partially purified and characterized.

The antimicrobial compound produced by *E. faecium* FAIR-E 406 was isolated and partially purified from de Man-Rogosa-Sharpe (MRS) broth by a two-step method consisting of a salt precipitation and a solvent extraction step. The cell-free culture supernatant was shown to be antagonistic towards enterococci, *Listeria innocua*, *Lactobacillus sakei* subsp. *sakei* LMG 13558<sup>T</sup> and *Pediococcus pentosaceus* LMG 13560. The partially purified fraction showed also low activity against the food-borne pathogens *Clostridium perfringens* and *Clostridium sporogenes*; no activity was found against *Escherichia coli*.

Partially purified enterocin FAIR-E 406 was not affected by a heat treatment at 100 °C during 1 h, but was sensitive to autoclaving at 121 °C for 20 min. It was completely inactivated by proteolytic enzymes indicating that the active moiety of the inhibitor was proteinaceous. This heat-stable enterocin was not active against the producer strain.

Partially purified enterocin FAIR-E 406 possessed a molecular mass of approximately 6400 Da through SDS-PAGE analysis and was active over a wide pH range (pH 2.2 – pH 12.0).

Enterocin FAIR-E 406 showed a concentration-dependent bactericidal and bacteriolytic action against the indicator strain *Listeria innocua* LMG 13568. After 30 min of incubation, the cell count was reduced with 3.6, 3.9 and 4.1 log units in the presence of 50, 400 and 1200 AU/ml, respectively. During further incubation, re-growth of resistant *Listeria* cells was observed. The cell count increased with 1.4 log units, during a period of 270 min, for all applied concentrations indicating that the rate of re-growth was independent of the applied bacteriocin concentration. The bacteriolytic action was clearly slower than the bactericidal effect. After 5 h of incubation, almost no cell lysis was observed in the presence of 50 AU/ml, while the optical density was reduced with 0.11 and 0.19 absorbance units in the presence of 400 and 1200 AU/ml, respectively.

The bacteriocin production kinetics of *E. faecium* FAIR-E 406 was studied in MRS broth at 37 °C and at free pH. Enterocin FAIR-E 406 was produced during the exponential growth phase indicating primary metabolite kinetics. The maximum activity (1200 AU/ml) was reached at the end of the exponential growth phase and remained constant during the stationary phase.

## 11

### Prevalences of different enterococcal species in faecal samples of cattle, pigs and broilers

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In the framework of a research study on the antibiotic resistance of enterococci faecal samples of food animals were collected at slaughter in different parts of Germany. One faecal sample per herd respectively flock was examined for enterococci using selective media containing 0.04% azide. Enterococci could be isolated from every sampled broiler flock (n=38) and from 34 of 40 sampled cattle herds (85%). In pigs they were found less frequently. Only 45 of 59 sampled pig herds (76%) were positive for enterococci.

Up to ten isolates per sample were included in the series. In total, 503 strains were investigated: 148 from cattle, 158 from pigs and 197 from broilers. The strains were identified to the genus *Enterococcus* and differentiated to the species by cultural and biochemical tests (catalase-reaction, growth in 6.5% NaCl broth, pyrrolidonyl arylamidase, haemolysis, motility, pigmentation, arginine dehydrolase, utilization of pyruvate and acid production from ribose, L-arabinose, raffinose, mannitol, melibiose and methyl- $\alpha$ -D-glucopyranoside). In addition, PCR analysis was used for strains which could not clearly be identified by conventional tests. Nevertheless, the identification scheme allows no accurate distinction between *E. durans* and *E. hirae*.

The most frequent enterococcal species in faecal samples of pigs and broilers was *E. faecalis* (~33%). *E. faecium* was detected in 26.1 percent of the examined broiler samples and in 17.9 percent of the pig samples. *E. durans* / *hirae* were found in samples of 21 broiler flocks (23.9%) and samples of 18 pig herds (26.9%). *E. gallinarum* was present in 13.4 percent of the pig samples and 10.2 percent of the broiler samples. *E. casseliflavus* could be isolated out of six broiler samples (6.8%) and five pig samples (7.5%).

In contrast, the dominant enterococcal species in cattle samples was *E. casseliflavus* (39.7%). Moreover, *E. faecium* was found very rare (1.7%). *E. faecalis* and *E. durans* / *hirae* were detected less frequently in faecal samples of cattle (29.3% and 17.2% respectively). Six cattle samples contained *E. gallinarum* (10.3%).

Only two *E. avium*-strains were isolated out of pig and cattle faeces.

# **Functionality of Enterococci in Foods**

## 12

### Enterococci – the prevailing microflora of autochthonous ewe's cheese produced in Slovenia

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The preservation of biological diversity is of worldwide interest and importance. Traditional dairy products, produced without the use of selected starter cultures, especially those made from raw milk, represent a valuable source of different strains which can be used for the development of new dairy starters. One such product is an autochthonous Slovenian cheese, »Kraški ovčji sir«, a hard-type cheese made from raw ewe's milk derived from Karst, the limestone region of Slovenia. The aim of this work was to characterise the dominant cocci microflora of Kraški cheese.

Two hundred colonies isolated from 8-weeks old cheese by plating on M17 media were examined morphologically and by phenotypic and genotypic tests. Phenotypic tests included the presence of catalase, growth at different temperatures (10°C, 40°C and 45°C) and in the presence of NaCl (2%, 4% and 6%) and bile (40%) and growth on Kanamycin Aesculin Azide agar. Fermentation profiles were determined by API 20STREP. Genotypic analysis included determination of the plasmids and PFGE profiles.

All the isolates studied were Gram positive cocci and grew at temperatures from 10°C to 45°C, with bile, and with 2% and 4% NaCl. Sugar fermentation patterns most closely resembled those for *Enterococcus faecalis*. The growth and production of typical black colonies on Kanamycin Aesculin Azide Agar indicated that most of the isolates examined (90%) were enterococci.

The strains could be divided into two groups on the basis of their PFGE patterns when the genomic DNA was digested with *Sma* I or *I-Ceu* I. Four different but closely related patterns were observed inside the first group, and one inside the second. Differences in the *I-Ceu* I patterns between the two groups suggested that they belong to different species, with the pattern for group 2 matching that previously reported for *Enterococcus faecalis*. Isolates were divided into eight groups by their plasmid profiles, although isolates with the same PFGE pattern did not have always completely the same plasmid pattern.

The use of species specific probes for different species of enterococci (*E. faecalis*, *E. faecium*, *E. avium*, *E. hirae*, *E. durans*, *E. malodoratus*) and dot-blot hybridisation confirmed that *E. faecalis* species prevailed among the isolates from cheese.

Future studies of the autochthonous ewe's milk cheese will examine the microflora at different stages of cheese production, to get an insight into the development and composition of the bacterial population during cheese making and ripening.

## 13

### Enterococci isolated from Spanish foods: Technological and safety aspects

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The molecular diversity, functional and safety aspects of fifty enterococcal strains isolated from different Spanish fermented and non-fermented food samples were investigated. Typing of the strains was done by Randomly Amplified Polymorphic DNA (RAPD)-PCR analysis and the isolates were clustered into two main groups, one group contained *Enterococcus faecium* which represent 66% of the total enterococci studied and the other group contained *Enterococcus faecalis* (34%).

The functional properties of enterococci strains were investigated, as well as their safety aspects and probable role in food preservation. Nineteen of the strains produced antimicrobial activity against *Listeria monocytogenes*, and many of them tested positive upon PCR amplification of structural genes for enterocins A, B & P. Other interesting properties were their ability to degrade antinutritive factors such as stachyose (18 strains) and raffinose (14 strains), and to produce bile salt hydrolase (30 strains). However, only 3 strains produced hydrogen peroxide, a compound known for its antimicrobial activity but also for its interference with the organoleptic properties of fermented meats. The incidence of virulence traits in enterococci strains was investigated with the objective of safety assurance of the particular fermented products. The results obtained showed that **none** of the 50 strains revealed potential virulence traits such as the production of gelatinase and proteinase activity, DNAase activity, cytolysin production, enterococcal surface protein (Esp) and enterococcal collagen adhesin (Ace).

These results suggest that the enterococci isolated in this study may play a role in fermentation processes and confer interesting functional properties which encourage their incorporation as starter cultures in other fermentations, and also as functional protective cultures in foods and foodstuffs. However, further investigations are required as sound basis for the selection of safe and technically valuable enterococcal strains for practical application.

## 14

### Use of enterococci as microbial adjuncts for making Tetilla cows' milk cheese (NW Spain)

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Tetilla is a soft, creamy cheese, with a thin yellowish rind and a conical or convex-conical shape, weighing 0.5-1.5 kg. It is traditionally manufactured from raw cow's milk in Galicia (NW Spain). The annual production is about 350.000 kg and the cheese is regulated under an Appellation of Origin, which has been protected since 1996 by the European Union. Dominant microbial groups among the microbiota present in Tetilla raw-milk cheeses are lactococci and enterococci.

In the present study, six batches of Tetilla were prepared from pasteurized milk in two different trials. In each trial, one control batch (CB) was made with an acid-aromatic starter containing skim milk cultures of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *lactis* var. *diacetylactis*, and two batches (EB) were made with the lactococcal starter plus one of two *Enterococcus faecalis* cultures. All the strains employed had been previously isolated from raw-milk cheeses, the enterococcal strains being selected according to the absence of some pathogenic properties and on the basis of their low degradation of  $\kappa$ -casein and moderate lipolytic activity in pasteurized milk, besides the absence of antibacterial activity against the strains of the lactococcal starter. Cheeses were ripened for 21 days at  $8\pm 2$  °C and  $80\pm 3$  % relative humidity.

Mean pH value for the EB cheeses was slightly higher than that determined for the CB cheeses. Both  $\kappa$ -casein and  $\kappa_{s1}$ -casein broke to a greater extent in the EB than in the CB. The mean contents of nitrogen-soluble fractions were also higher in the EB than in the CB. In all the batches made with enterococci, volatile free fatty acids and long chain free fatty acids contents exceeded those determined in the control batches, though all the values were too much lower than those previously observed for Tetilla raw-milk cheeses. The highest values for diacetyl-acetoin content was determined for one of the enterococcal batches.

Bitter taste was not significantly higher in the EB than in the CB. EB cheeses were defined as more rancid in flavor and more elastic and less firm in texture than CB cheeses. Batches manufactured with one of the *Enterococcus faecalis* strains showed organoleptic characteristics similar to those found in raw-milk cheeses, according to some panelists who were connoisseurs of traditional cheeses. These batches were characterized by the highest lipolytic indexes. It is concluded that these microorganisms could be used as microbial adjuncts in a positive way for Tetilla manufacture, in order to get a higher lipolysis level and perhaps a more specific proteolysis of  $\kappa_{s1}$ -casein, though these bacteria should be exhaustively characterized from a healthy point of view before being employed in cheesemaking.

## 15

### Molecular identification and technological characterization of lactococci and enterococci strains isolated from starter-free cheeses

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Members of the genus *Lactococcus* are used worldwide as starters for the manufacture of cheese and other dairy products. They also constitute the dominant populations in most starter-free farmhouse cheeses. Owing to the recognised need for the complementing and replacing the strains of lactococci currently used, a growing interest exists in the study and characterization of wild strains from natural fermented products. There is also a need for strains with novel properties, which might be used to manufacture new products or permit new processes. Besides the lactococci in most cheeses made from unpasteurized milk, there is a highly variable population of enterococci. Enterococci strains display diverse enzymatic activities that may have a desirable role in ripening. Consequently, strains of enterococci have been repeatedly proposed, or even used, as starters or starter adjuncts in many traditional cheeses. However, their presence in food systems is still a matter of controversy owing to their pathogenic potential.

In order to evaluate a previous phenotypic classification of a set of lactococci from dairy origin and to study their technological application, 39 strains isolated from five different artisanal cheeses from Northern Spain were identified by molecular techniques and subjected to a complete technological characterization.

Partial ARDRA of a region of the 16S rDNA gene was applied to our isolates with the enzymes *Mbo* II and *Hha* I. Four different digestion profiles were obtained which divided the strains into four distinctive groups. Sequencing of representative amplicons identified 29 isolates as belonging to *Lactococcus lactis* subsp. *lactis* (24) and *Lactococcus lactis* subsp. *cremoris* (5). The remaining 10 isolates were shown to be *Enterococcus durans* (8) and *Enterococcus faecalis* (2), which had been misclassified by traditional tests.

All strains were assayed for lactic acid production, proteolytic activity and production of organic acids and volatile components from milk. Several strains of both lactococci and enterococci produced lactic acid at a rate and a final concentration suitable for cheesemaking. In general terms, lactococcal isolates appeared to be more acidifying than enterococcal ones. Proteolytic activity correlated well with acidification in all strains; the more proteolytic being the more acidifiers. The levels of organic acids were in the normal range of those described for lactic acid bacteria. With few exceptions, enterococci isolates produced more formic and acetic acid than did lactococci. The volatile compounds profiles obtained by all the isolates were rather simple.

Antibiotic resistance and other determinants of virulence were analysed in all isolates. In general, enterococci strains were resistant to more antibiotics than lactococci. Nevertheless, particular strains of lactococci were resistant to antibiotics such as bacitracin, cephalothin, clindamycin, streptomycin or tetracycline.

## 16

### Inhibitory effect of *Enterococcus faecalis* FAIR-E 171 on *Listeria innocua* during manufacture and ripening of an experimental Caciotta cheese

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Cheeses might be supplemented with chemical preservatives in order to protect them from the growth of spoilage and disease causing bacteria.

Some bacteriocin producing bacteria are naturally capable of inhibiting the growth of these unwanted microorganisms and can be used as added starters for cheese production, thus making these chemical additives obsolete. Strains of enterococci, producing enterocins, have been found to inhibit *in vitro* the growth of *Clostridium* spp, *Listeria* spp, *Bacillus* spp. and *Staphylococcus aureus*. However, few studies have been conducted on their efficiency when used in cheese-making experiments in real dairy systems.

During the characterisation of the enterococcal strains of the FAIR-E collection, *Enterococcus faecalis* strain FAIR-E 171 was found to have *in vitro* a strong activity against the *Listeria innocua* strains tested. In order to evaluate its potential as a protective culture, an experimental production of Caciotta (a typical Italian cheese) was performed following a standard cheese-making protocol.

Two batches of pasteurised cows' milk were prepared and rennet, salt and starter (*Streptococcus thermophilus*) were added. Both batches were inoculated with *L. innocua* LMG 1312 to simulate a contamination. A culture of *E. faecalis* FAIR-E 171 was used as adjunct starter to one batch (experimental cheese). The cheeses were ripened at 13°C for 50 days. Samples were drawn every 5 days and analysed on selective media for the presence of listeria (both cheeses) and enterococci (experimental cheese).

The initial number of the listeria was  $5 \times 10^3$  CFU/g in both cheeses. In the control cheese, the number of listeria increased dramatically, reaching  $5 \times 10^8$  CFU/g after 5 days and remained constant during the entire ripening process. In the experimental cheese, the enterococci were present at  $1 \times 10^8$  CFU/g throughout the ripening process, while an initial increase (although lower than in the control:  $1 \times 10^7$  CFU/g after 5 days) of the level of listeria was noticed. The counts of listeria continued to decrease gradually, to reach  $2 \times 10^5$  CFU/g after 50 days.

In the complex chemico-physical conditions of cheese the listeria cells could survive in contrast with the strong inhibition observed during *in vitro* trials. Such behaviour might be connected to various factors of which the selective pressure of the enterocin was investigated. Representative clones of listeria were isolated from the experimental cheese and tested for their resistance towards the enterocin. The majority of the clones resulted to be susceptible, indicating that other factors are involved in the survival.

In conclusion, the enterocin-producing *E. faecalis* FAIR-E 171 had a limited inhibitory effect on the listeria strain used in the experimental cheese manufacture. Further studies on the interactions of the enterocin with the compounds of the food matrix and on its production rate and effective concentration in the cheese environment have to be carried out to evaluate the potential use of this enterocin as a biopreservative.

## 17

### An antagonistic effect of Enterocin P in Gombasek sausage experimentally inoculated by *Listeria innocua*

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*Listeria* spp. are widely distributed organisms which has been also isolated from a variety of foods, meat products including. The ingestion of the products contaminated with these microorganisms may potentially threaten health of population especially children, pregnant women and/or elderly people. Within the last decade, biopreservation has received increased attention as a new preservation method to control pathogenic and spoilage bacteria in foods. Biopreservation includes the use of bacteriocin producing lactic acid bacteria or addition of their bacteriocins. Many of bacteriocins were already experimentally applied into different food systems (meat involving) to reduce contamination by *Listeria* spp. However, only a few information were reported concerning enterocins (bacteriocins produced by some enterococci which are known to be predominant inhibitors of listeriae) in association with their application in different meat products. In our study an inhibitory effect of enterocin P (ent P) produced by our own isolate *Enterococcus faecium* AL41 strain against *Listeria innocua* Li1 strain in Gombasek sausage was tested. *Listeria innocua* Li1 (0.1 % inoculum, Hans Blom, Matforsk, Norway) was used to inoculate experimental (A) as well as control trials (B). Moreover, reference sausage (C) without ent P was manufactured too. Gombasek sasusage was manufactured according to standard technological norm CSN 576099/PNMP 105/84 for this product. Ent P was applied in a concentration 3200 AU/ml into the trial A. Sampling was provided at the beginning of the experiment, on day 2, after 1 week and after 2 weeks. The initial number of Li1 strain in the trials A and B was  $10^6$  cfu/g. On day 2, a reduction of Li1 strain with difference 2 orders of magnitude was noticed between trial A and B (trial B- $5.6 \times 10^7$  cfu/g; trial A- $1.1 \times 10^5$  cfu/g). The same difference between trials was found also after one week, although in trial A slight re-growth of Li1 strain was determined (B- $5.5 \times 10^9$  cfu/g; A- $1.5 \times 10^7$  cfu/g). Up to two weeks, the re-growth of Li1 strain in both trials was noted. Despite of the fact, that finally the re-growth of Li1 strain was found, ent P showed an inhibitory effect during sausage manufacture. It looks, that its effect was bacteriostatic only in the system experimented. Although, bacteriocin activity of ent P was not analytically detected by agar spot test in the sausage, its inhibitory effect was clearly done. Therefore, to continue in this kind of the experiments means to develop the possibilities of bacteriocins in their applying with preventive effect in meat ecosystem.

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## 18

### Influence of the aloe vera additive on the enterococci growth during lactic acid curd formation

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The research aiming to assess the contamination of lactic acid cheese by enterococci shows a considerable growth of these microorganisms while storage of vacuum-packaged cheese and in low temperature conditions.

It was observed that the number of enterococci in vacuum packed cheese ranged from 3,78 log cfu/g to 5,36 log cfu/g after 7 days of storage and to 5,68 log cfu/g after 14 days refrigeration storage ( Steinka at al. 2001 ).

The growth of enterococci in vacuum packaged lactic acid cheese stored for 14 days in low temperatures can be circumscribed by mathematical model:

$$E_{14} = 28,88M_0 + 34674,5pH_0 + 0,0646Pt_0 + 0,4559Y_0 - Scp_0$$

where:

E- enterococci from vacuum – packed lactic acid cheese under refrigeration storage 14<sup>th</sup> days

pH<sub>0</sub> – lactic acid cheese acidity after production

Pt<sub>0</sub> – psychrotrophs after lactic acid cheese production

Y<sub>0</sub> – yeasts after lactic acid cheese production

M<sub>0</sub> – moulds after lactic acid production

Scp<sub>0</sub> – coagulase-positive staphylococci after production

As there is a threat of growth of enterococci and other facultative anaerobes there were trials to apply phytochemicals from Aloe vera in order to inhibit growth of these microorganisms in lactic acid cheese.

According to our earlier research Aloe vera preparation showed efficiency in inhibition of staphylococci growth present in lactic acid cheese curd ( Steinka at al. 2000 )

To assess the ability to inhibit growth of enterococci as well, there were trials to apply Aloe vera additive.

To assess the sensitivity of enterococci to the action of additive 1, 2, 5, and 10 percent of Aloe vera additive, was added to broth, milk and curd.

Lactic acid cheese curd was produced on the basis of milk containing 2% fat content, where lyophilised cheese cultures manufactured by Biolacta - Texel were used as starters. Lactic acid curd was produced in model conditions for a long period of time and was acidified for 20 hours at a temperature 24 ± 2 °C. Milk (1 dm<sup>3</sup>) was pasteurised at 72 °C for 15" and treated with starter. The obtained curd was heated and gently stirred for about 2 hours at 32- 38 °C.

The results of the research under model conditions show lack of efficiency of preparation's action when enterococci occur with large number of Candida sp.

There was no inhibition of growth of enterococci in the time of phytochemicals from Aloe vera action when it was added under model condition.

There was also no considerable inhibition of growth of enterococci when the preparation was added to milk used to production of curd.

The results of the research enabled to assess that an effective dose of preparation is 5 and 10 percent and the preparation is effective when its addition is made at the proper stage of process of the production of curd.

It is probable that if enterococci occur with yeasts then automatically the action, both of lactic acid bacteria and phytochemicals against enterococci is not effective.

#### References

1. Steinka I., 2001, Med.Wet., **57**,10,757-761;
2. Steinka I., Stankiewicz J., Proceedings 14<sup>th</sup> Forum for Appl. Biotechnol. 539-542.

## 19

### **Effect of *Enterococcus faecium* on microbiological, physicochemical and sensory characteristics of Greek feta cheese**

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Greek Feta cheese was prepared using as adjunct starter cultures *Enterococcus faecium* FAIR-E 198, *Enterococcus faecium* FAIR-E 243, and their combination. Numbers of enterococci in the control and in the batches containing *E. faecium* strains as adjunct starters rapidly increased until day 15 of ripening, and then remained constant. Both *E. faecium* strains positively affected the counts of non-starter lactic acid bacteria (NSLAB), micrococci and coliforms, while thermophilic cocci were not influenced. Moreover, *E. faecium* FAIR-E 243 enhanced the growth of mesophilic cocci and thermophilic bacilli. Physicochemical characteristics, such as pH, moisture, ash, salt in moisture and fat in dry matter were not influenced by the addition of the *E. faecium* strains. The most pronounced effect was observed in the case of proteolysis. Both *E. faecium* strains, either as sole adjunct starter or in combination, increased the proteolytic index and the free amino groups concentration, and enhanced degradation of  $\alpha_{s1}$ - and  $\beta$ -caseins in comparison to the control. Furthermore, the reverse-phase (RP)-HPLC peptide profiles of the water soluble nitrogen (WSN) fractions were significantly affected by the addition of enterococci. The main volatile compounds produced were ethanol, acetate, acetone, acetaldehyde, acetoin and diacetyl, with highest amounts determined for ethanol, followed by acetate. Both *E. faecium* strains positively affected taste, aroma, colour and structure of the full-ripened cheeses, as well as the overall sensory profile. The present work emphasizes the technological significance of *Enterococcus faecium* strains and supports their use as adjunct cultures in the manufacture of Feta cheese.

## 20

### Technological and safety aspects of enterococci isolated from 'Hussuwa', a Sudanese fermented sorghum product

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In an ecological study, 262 Gram-positive bacteria were isolated during production of 'Hussuwa', a fermented sorghum product from Sudan. From these, 22 strains were identified as enterococci. Strains were typed using RAPD-PCR analysis and RFLP of the 16S/23S intergenic spacer region. The 16S rDNA gene from selected strains, representative of specific clusters as determined in typing experiments, were sequenced to confirm species identification. All of the enterococci strains isolated from Hussuwa could be identified as *E. faecium* strains.

Genotypic analysis showed that our strains and selected reference strains clustered into two major groups, which confirms earlier observations that two intraspecies groups occur within *E. faecium*.

The enterococci strains were further investigated regarding their safety aspects and involvement in the fermentation. The investigations included bacteriocin production, antibiotic resistance, and incidence of potential virulence factors such as production of haemolysin/cytolysin (Cyl), gelatinase, DNase and adhesins including enterococcal surface protein (Esp) and adhesin of collagen from enterococci (Ace). Six of the *Enterococcus* strains produced bacteriocins, five of which produced enterocin P, while 6 strains produced enterocin L50 A&B. Three strains were vancomycin resistant, three resistant to penicillin and three were resistant to ciprofloxacin. Only one strain was resistant to erythromycin. However, all strains were susceptible to the clinically relevant anti-enterococcal antibiotics ampicillin, gentamycin and streptomycin. In addition, all strains were susceptible to chloramphenicol and tetracycline. None of the strains produced the potential virulence factors aggregation substance and gelatinase. However, two strains produced Esp, while one strain produced Ace. In addition, two strains produced beta-haemolysin.

The results show that enterococci may play a functional role in the fermentation of Hussuwa and that bacteriocin production, a common characteristic among investigated strains, may be important to stabilise the product and/or to enable stronger competition with related strains within this food ecosystem. However, some strains from this product also produced potential virulence factors. Therefore, should enterococci be considered for incorporation into a starter culture preparation for Hussawa, each individual strain should be investigated for the presence of potential virulence traits before final selection.

# **Functionality of Enterococci in the GI Tract**

## 21

### The enhancement of biological activity of *Enterococcus faecium* M-74 by selenium

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Selenomethionine along with other amino acids is produced by *Enterococcus faecium* M-74 when it is grown in selenite-containing media. The lyophilized product of this selenized strain contains  $2-3 \times 10^{11}$  CFU per gram enriched with 1000 µg of organic selenium. The product is manufactured by **IVAX-CR, Opava-Komárov**, Czech Republic of the trade mark „**Enterococcus forte + selén**“ (each capsule contains 50 µg of the probiotic bacteria and 50 µg of selenium).

The biological activity of the selenized strain differs from the standard one in several ways: its viability is prolonged, it exerts higher antimutagenic activity, etc. Besides preserved probiotic activity it represents a supply of selenium for macroorganisms. There is no difference in susceptibility to a wide range of antibiotics.

It was observed that the bacteria (one capsule daily) colonized the host intestine within 7–10 days and its excretion with stool persisted for 5–6 weeks after the last dose. Immunostimulatory (mainly potentiation of phagocytic activity and antibody formation) and other health beneficial effects including the lowered mean level of serum cholesterol as well as activity of pro-carcinogenic enzyme β-D-glucuronidase in stools were observed.

The preliminary results have encouraged us to organise some clinical trials: a) the treatment of hepatic encephalopathy (very positive results), b) the study of the prevention of recurrence of colorectal polyps (to be evaluated next year), c) at present a one year clinical trial in a group of 48 volunteers in two parallel groups (double blind, randomized, placebo controlled) is still running.

A therapeutic effect of low dosage of methotrexate therapy in the combination with *Enterococcus faecium* M-74 plus selenium on the adjuvant arthritis in rats was observed as well.

Lyophilized *Enterococcus faecium* M-74 plus selenium was introduced in both Slovak and Czech Republic to several types of functional foods: **I.D.C. Holding, Bratislava** manufactures wafer bars with cocoa or vanilla filling plus the lyophilized probiotic culture but without selenium. **NIKA Company, Považská Bystrica** produces the traditional Slovak sheep cheese named **Bryndza** fortified with *Enterococcus faecium* M-74 plus selenium. Natural product **Bryndza** contains *E. faecium* and *E. faecalis* besides other lactic acid bacteria which play an important role in its organoleptic properties. The above fortification of **Bryndza** improves its both organoleptic and health beneficial properties.

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## 22

### Inhibitory effect of Enterocin A against *Salmonella* in gnotobiotic Japanese quails

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Effect of enterocin A (entA) produced by *Enterococcus faecium* EK13 strain against *Salmonella duesseldorf* SA31 was checked on the model of gnotobiotic Japanese quails. Twenty-one 3 days old gnotobiotic birds were divided into 3 groups (each group involved 7 birds). The quails were infected by SA31 strain (200  $\mu$ l x  $10^7$  cfu/ml). Ent A was applied with the two principal aims; to control its protective effect (ENT 1) and to follow its therapeutic effect (ENT2). Sampling of feces was provided after 8 h, 24 h, 48 h as well as after 168 h. At the end of the experiments, quails were killed and the content of caecum and ileum was screened by plate count of an appropriate dilutions on Brilliant green agar plates. After 8 h from ent A application in ENT1, first reduction of SA31 strain in feces was detected ( $P < 0.001$ ). There a difference of 1.37 log cycle was noticed comparing control group (CG) and ENT1. In opposite, SA31 grew very well in ENT2 group ( $\log 5.07 \pm 0.07$  cfu/ml). However, after 24 h a significant difference ( $P < 0.001$ ) was indicated between CG and ENT2 (in group to follow a therapeutic effect of ent A). After 48h, reduction in SA31 counts was found in both ES with a significance  $P < 0.01$ ,  $P < 0.001$ , respectively. This effect was prolonged up to end of the experiment (after 168 h). In the content of caecum a reduction of SA31 strain 2.44 log cycle was found ( $P < 0.001$ ) comparing CG and ENT2 groups as well as in ileum (3.16 log,  $P < 0.001$ ). To compare CG and ENT1 groups nor in caceum, nor in ileum significance was noted. It can be concluded that under our conditions, ent A showed better therapeutic effect than protective effect. However, in both cases ent A has an antagonistic influence on SA31 strain.

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## 23

### Selection of enterococci for dog probiotic adjuncts

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The optimal flora balance in the intestinal tract is important for keeping the animal health. This delicate equilibrium can be affected by such factors as diet, antibiotic therapy, weaning, environmental and emotional stress, many of which resulting in clinical signs like diarrhoea, stomach upsets and tiredness. In order to prevent these gastrointestinal disorders dogs including, live microbial cultures - probiotics, can be orally administered to strengthen the barrier function of the gut microflora and/or for a non-specific stimulation of the immune system. Enterococcal strains are normal inhabitants of the GI-tract and they can be used as well as the other lactic acid bacteria for development of probiotic preparations. In our study probiotic properties of enterococci isolated from feces of ten healthy dogs were examined by *in vitro* testing. Forty strains of fecal enterococci were tested by their ability to tolerate bile, by antibiotic sensitivity and antagonistic activity towards intestinal, environmental and food-contaminating Gram-positive and Gram-negative microorganisms with the aim to select the most suitable strain for further application *in vivo* as dog probiotic adjunct. The selected strain *Enterococcus* sp. AD4 appears to be the most promising strain through a good chance to survive in the GI-tract and its antimicrobial activity.

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## 24

### Recovery of an *Enterococcus faecium* probiotic strain in faeces after gastrointestinal transit in human

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**Objectives.** The objective of this study was to determine if an *Enterococcus faecium* probiotic strain could survive gastrointestinal transit in humans, and if so how large amount of the total recovered intestinal *E. faecium* consists of the ingested strain. A further aim was to determine how simultaneous vancomycin administration affected the survival of the probiotic strain and the persistence of the endogenous *E. faecium* strains of the subjects.

**Methods.** Twenty healthy volunteers were given 150 ml of a probiotic product containing the Causido® culture, which consists of two *Streptococcus thermophilus* strains and one *E. faecium* strain, once daily for 10 days. This intake corresponds to a daily dose of  $4.5 \times 10^9$  to  $7.5 \times 10^9$  colony-forming units (CFU) *E. faecium*. Ten of the volunteers were simultaneously per os given vancomycin 125 mg q.i.d. for 10 days. Faecal samples were collected before intake started, day 0, directly after intake stop, day 10, and 3 weeks after ceased intake, day 31. Isolates of *E. faecium* were further genotyped with pulsed-field gel electrophoresis (PFGE) or phenotyped with the PhenePlate™ system.

**Results.** The probiotic strain was recovered from faeces on day 10 in eight of the ten subjects given only the probiotic product but in none 3 weeks after intake stop. The number of detected probiotic *E. faecium* in this group ranged from  $1.2 \times 10^3$  to  $4.2 \times 10^6$  CFU/g faeces, median value  $2.8 \times 10^4$  CFU/g faeces. The total number of *E. faecium* on day 10 ranged from  $9.5 \times 10^3$  to  $8.0 \times 10^6$  CFU/g faeces, median value  $2.7 \times 10^5$  CFU/g faeces. Simultaneous vancomycin intake prevented faecal recovery of the food strain and endogenous *E. faecium* strains on day 10. The *E. faecium* strains recovered 3 weeks after ceased intake of the probiotic and vancomycin displayed no phenotypic relationship to the food strain or the endogenous *E. faecium* strains detected on day 0.

**Conclusions.** The Causido® derived *E. faecium* strain can survive gastrointestinal transit in humans. A continuous intake of the fermented milk-product seems necessary to maintain detectable levels of the probiotic *E. faecium* in faeces. The probiotic *E. faecium* strain may, after intake, become a substantial part of the total intestinal *E. faecium* population in some individuals. Reoccurrence of *E. faecium* 3 weeks after simultaneous vancomycin intake seems to occur with strains not related to the probiotic strain or endogenous strains harboured before treatment.

## 25

### Homemade regional cheeses as resources for the isolation of probiotic *Enterococcus faecium* strains

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Probiotics are traditionally defined as viable microorganisms that have a beneficial effect in the prevention and treatment of specific pathologic conditions as well as in maintaining the health of the gastrointestinal tract (GIT). Most studies were performed with lactobacilli and bifidobacteria, although other lactic acid bacteria (LAB) of enteric origin such as enterococci might also be considered as potential probiotics.

Amongst the probiotic effects attributed to LAB, the assimilation of cholesterol would be of particular interest for reducing the absorption of dietary cholesterol from the digestive system into the blood. Several studies indicated that the phenomenon is related to the ability of the strains to deconjugate bile salts. The synthesis of bacteriocins (antimicrobial compounds) is another property of interest. It is well known that enterococci are able to produce various bacteriocins, the so-called enterocins. Although these compounds are usually effective against closely related species, some of them can display a fairly broad inhibitory spectra to food-borne gram-positive pathogenic bacteria including *Listeria monocytogenes*.

The enterococci are natural flora of fermented foods and they contribute to the bouquet and particular flavour of different kind of regional cheeses. However, they are recognised as nosocomial pathogens causing bacteraemia, endocarditis, and other infections. From these considerations, it becomes outmost important to determine the presence of virulence determinants in strains of enterococci that might be used as probiotics.

In this study, thirteen strains of *Enterococcus* belonging to the “*faecium*” group isolated from Tafí cheese (a homemade cheese made in the mountain region in Northwest Argentina) were evaluated according to the following selection criteria: bile tolerance, cholesterol reduction, bile salts deconjugation, bacteriocin production, and their potential pathogenesis through the presence of virulence determinants.

Bile tolerance (measured as growth delay [D]) showed broad variations among the cultures indicating the heterogeneity of this property while a high bile salt hydrolase activity (HSB) and cholesterol remotion were observed in nine of the strains tested.

The bacteriocin production was determined by the spot-on-lawn method. No inhibitory activity against *Listeria innocua* 7 and *Listeria monocytogenes* Sott A was observed. Nevertheless, a PCR product of approximately 126 bp (confirmed by sequence) was obtained from the DNA of six strains using specific primers for the structural enterocin A gene while negative results were obtained with the enterocin P and enterocin L50AB primers. The presence of silent *EntA* genes in these Bac<sup>-</sup> strains will be the subject of future studies.

The potential pathogenesis of the selected strains was evaluated according to the adherence to host tissue by using PCR with specific primers encoding cell wall adhesines (primers *efaAfm* and *efaAfs*). Results obtained indicated the absence of this virulent factor in the enterococci strains tested.

## 26

### Influence of probiotic *Enterococcus faecium* strains on microbiological parameters in the gastrointestinal tract of poultry

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*Enterococcus faecium* strains are widely used as probiotic feed additives in animal nutrition. Contrary to human nutrition, desired effects on weight gain and feed conversion in the range of 1 – 5 % can be directly measured. However, it has also been shown that many feeding trials fail to show statistical significances due to varying response of individual animals. The modes of action of probiotics in animal nutrition have not been clarified yet. In order to investigate possible influences of probiotic products, the analysis of microbiological parameters in the gastrointestinal tract seems important.

A number of feeding trials with probiotic *E. faecium* strains and poultry have been carried out at our institute. Several microbiological parameters were monitored to evaluate the influence of probiotic *E. faecium* strains on intestinal microbial communities. It could be shown that the supplementation of broiler and turkey feed with *E. faecium* strains significantly increase lactate concentrations in the small intestine, accompanied with a drop in pH in the muscle stomach, but not in other small intestinal compartments. Colony forming units (CFU) of total enterococci also increased after feed supplementation. CFU of enterobacteria were reduced in feeding trials with broiler chicken, but not in a poultry trial. In an overdose feeding trial ( $10^9$ ,  $10^{10}$  and  $10^{12}$  CFU *E. faecium*/ kg feed) with broiler chicken, no differences in enterococci CFU could be observed between  $10^{10}$  and  $10^{12}$  CFU *E. faecium*/ kg feed, but all trial groups showed increased enterococci CFU in the proximal small intestine compared to the control group.

Metabolic activity of total enterococci in the poultry trial, as measured with a specific 16S rRNA probe, was not significantly different from the control group except in the first week of life. However, metabolic activity of lactobacilli increased from day 7 to 21, while total eubacterial metabolic activity was reduced on day 7 and 14.

Our studies show that probiotic *Enterococcus* spp. strains may be already active in the crop and muscle stomach of poultry, but metabolic activity in the small intestine may not be sufficient to directly influence digestive processes. Secondary effects on microbial communities, such as an increased activity of lactobacilli, could play a mayor role in the mode of action of probiotic enterococci in poultry nutrition.

**Antimutagenic and reactivation activity of *Enterococcus faecalis***

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*Enterococcus faecalis* represents normal microflora of humans, cattle, birds and fishes. Enterococci are the dominant streptococci in the human intestine.

We have shown that *E. faecalis* synthesizes and excretes into the growth medium substances possessing antimutagenic (AM) activity against mutagenicity induced by 4-nitroquinoline-1-oxide (4-NQO, base-pair substitutions) and 2-nitro-fluorene (2-NF, frame-shift mutations). AM activity was determined by a modified Ames method using *Samonella typhimurium* TA100 and TA98 as test cultures.

It was shown that AM activity is localized in the fraction of heat stable peptides with molecular mass less than 12 kDa, which act in a low ( $0.1 \mu\text{g}\cdot\text{ml}^{-1}$ ) concentration. The maximum accumulation of antimutagens in the medium was observed within 16-24 h of growth and corresponds to the maximum of accumulation of thiol compounds in the medium. Antimutagenicity was associated both with extracellular factors interacting with mutagens (dismutagenicity) and with factors affecting intracellular processes of mutagen biotransformation and mutation induction (bioantimutagenicity).

An extracellular proteinaceous factor exerted a reactivation activity in bacteria subjected to UV-irradiation: the survival of irradiated cells after incubation with the supernatant of culture liquid was increased three-fold in comparison with irradiated but not incubated with the supernatant cells. It is essential that supernatant of *E. faecalis* had a positive effect in UV-irradiated *Luteococcus casei* culture - phylogenetically remote strain, increasing its survival rate by a factor 3. The cells of *E. faecalis* possess also AM properties whose action was not limited to the cell absorption of 4NQO, since the cells could be induced to antimutagenicity by reinoculations in medium containing 4-NQO. Dismutagenic activity of 4-NQO-adapted cells was higher than those of control ones. AM and antistress activity of *E. faecalis* point to a new useful properties of these bacteria as probiotics: their capability to inactivate mutagenic and cancerogenic ingredients of foods, mutagens, synthesized by some intestine bacteria and to reactivate inactivated intestine microflora.

**Genetic characterization of the bile salt hydrolase gene from *Enterococcus faecium* E-345 and chromosomal location of BSH genes among enterococci**

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Enterococci are well adapted to the gastro-intestinal tract of humans and warm-blooded animals. This may explain the generally common property of their bile salt hydrolase activity. However, the BSH-gene from a BSH-positive *Enterococcus* strain has not been studied further characterised yet.

The chromosomally-encoded bile salt hydrolase (BSH) gene from *Enterococcus faecalis* E-345 was cloned and sequenced. Analysis of the DNA sequence from 3-kb cloned chromosomal DNA fragment showed that this gene encoded a protein of 324 amino acids with an isoelectric point of 4.877. The deduced amino acid sequence showed homology to BSH genes from *Lactobacillus plantarum*, *Lactobacillus gasseri*, *Clostridium perfringens*, *Bifidobacterium longum* and *Lactobacillus johnsonii*. A gene probe was prepared from the cloned *bsh* gene and was used for probing plasmid and total genomic DNA of BSH positive enterococci isolated from food, in order to determine the genomic location of this gene. From a total of 47 BSH-positive enterococci strains, 27 were determined to harbour plasmid DNA; however, the *bsh* gene could not be detected on plasmid DNA from any of these strains. The chromosomal location of *bsh* genes among food enterococci suggests that transfer of this trait by conjugation is unlikely.

# **Safety Aspects of Enterococci**

## 29

### Plasmid associated resistance in food enterococci

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Enterococci constitute, at the present moment, a subject of great concern, due to their increasing resistance to the majority of antibiotics used in the health care system. The emergence of multiple resistant strains are, in part, a consequence of enterococci capability to exchange genetic material, namely plasmids and transposons, both horizontal and vertically. However, studies conducted in the last decades have been concerned mostly with clinical strains. The environmental contribution to this serious antibiotic resistance problem has been neglected. Therefore, the present work aims to study the mobile genetic elements, namely plasmids, that transport antibiotic resistance traits, of enterococci isolated from traditional dairy products, namely from four different types of cheese and the corresponding raw eye's milk. Several assays of plasmid curing were performed using acridine orange as the curing agent. Thirteen isolates cured of resistance to erythromycin were obtained and further studied in order to identify the plasmids carrying this antibiotic resistance trait. Antimicrobial susceptibility disk diffusion method was used for 26 antibiotics in order to understand if other antibiotic resistances were associated with the same plasmid carrying erythromycin resistance.

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## 30

### High prevalence of vancomycin resistant enterococci in urban sewage and in hospital sewage, but not in pig samples, in Sweden

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#### Objectives

Enterococci are members of the normal gut flora of animals and humans, and are thus released into the environment directly or via sewage outlets, where they can survive for long time periods. Their role in nosocomial infections has increased due to their ability to acquire high-level resistance to antimicrobial agents that make them difficult to treat. In Europe the use of the growth promoter avoparcin is considered to have selected for vancomycin resistant enterococci (VRE). Sweden ceased using avoparcin 1986 and only occasional cases of clinical VRE have been reported since 1995. Within the framework of a European study FAIR-CT97-3709, samples from hospital sewage, urban raw sewage, treated sewage, surface water, and fecal samples and manure from pigs in Sweden, were screened for VRE.

#### Methods

Isolation of VRE from sewage and water samples was done through membrane filtration followed by growth on mEnterococcus agar supplemented with 8 µg /mL vancomycin. Faecal samples and manure were diluted in PBS and then spread on agar with vancomycin. An aliquot of 10 mL of all samples was added to an enrichment broth supplemented with vancomycin. The recovered VRE were phenotyped with the PhenePlate™ rapid screening system and genotyped using PFGE. All isolates were subject to antimicrobial susceptibility testing. Detection of the resistance genotypes *vanA* and *vanB* and identification to the species level of *E. faecalis* and *E. faecium* were done by PCR.

#### Results

VRE were isolated from 21 of 35 (60%) untreated sewage samples, from 5 of 14 (36%) hospital sewage samples, from 6 of 32 (19%) treated sewage samples, from 1 of 37 surface water samples, from 1 of 54 samples from pig manure and in none of the samples from pig feces. Thirty-five isolates from 33 samples were characterized with the specified methods. Most isolates (30 of 35) carried the *vanA* gene and the majority (24 of 35) of the isolates were *Enterococcus faecium*. Most of the VRE were multi-resistant. The typing revealed a high diversity for the isolates. However, one major cluster with seven identical or similar isolates was found. These isolates came from three different sewage treatment plants and were collected at different occasions during one year. All VRE from hospital sewage originated from one of the two hospitals studied. That hospital also had a consumption of vancomycin ten-fold that of the other.

#### Conclusions

We conclude that VRE were commonly found in sewage samples and rarely found in pig feces and manure in Sweden. The origin of the VRE found might be both healthy individuals in the society and individuals in hospitals. Possibly, antimicrobial drugs or chemicals released into the sewage system may sustain VRE in the system.

## 31

### Diffusion of combined vancomycin and gentamicin resistance in enterococcal strains of food and clinical origin

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During the last decades there has been a growing interest by scientists and policy makers in the presence of antibiotic resistances in food-borne microorganisms due to their clinical impact.

Even though enterococci have been found in several types of food products, they are not considered GRAS (generally recognised as safe) organisms. The number of antibiotic resistances observed in these bacteria is increasing and this, in combination with several virulence factors, has made them feared infectious agents in hospitals. Especially their latest acquisition, resistance against vancomycin, is worrisome.

Infections with vancomycin resistant enterococci (VRE) can be treated with aminoglycosides, such as gentamicin. Hence, a combined resistance is very difficult to treat and VRE with an acquired gentamicin resistance (VGRE) have to be kept under surveillance. Furthermore, enterococci are able to rapidly transfer their resistance genes to other members of the genus and potentially to other bacteria of clinical importance.

There are several types of acquired vancomycin resistance in enterococci, of which the high-level *vanA* phenotype (coded by the genecluster *vanA*) is the most diffused, followed by *vanB*. Cases with other forms of vancomycin resistances are rare. Resistances to gentamicin are linked to the genes *aac* (6')-*Ie-aph* (2'')-*Ia* (high level resistance and most found), *aph* (2'')-*Id* (high level), *aph* (2'')-*Ib* (high level in *Enterococcus faecium*) and *aph* (2'')-*Ic* (mid level).

In this research, the spread of the combined resistance against vancomycin and gentamicin has been investigated in strains of enterococci isolated from two main food sources of enterococci (i.e. meat and cheese). In order to have a more complete view of the VGRE diffusion, also clinical isolates were included. For this purpose, PCR assays, employing primers targeting the above-mentioned genes were applied and the Minimal Inhibition Concentration (MIC) was determined.

VRE were isolated from a large number of Italian meat products and cheeses. An enrichment step in broth containing vancomycin was performed prior to the isolation on KAA with vancomycin. The isolated VRE were then tested for their resistance against gentamicin. Also 56 well-characterised clinical isolates, originating from the FAIR Project CT97-3078, were included.

VRE containing the *vanA* gene were found in 75% (17 *E. faecium* and 3 *E. faecalis* strains) of the meat samples which contained enterococci and in 10% (3 *E. faecium* strains) of the cheese samples. In 26.8% (13 *E. faecium* and 2 *E. faecalis* strains) of the clinical isolates the presence of the gene *vanA* was established. The results of the MIC assays confirmed those of the molecular methods, as only the strains in which the *vanA* gene was found, grew in the presence of high concentrations of vancomycin.

PCRs with primers targeted to the gentamicin resistance genes were executed on the VRE strains. The gene *aac* (6')-*Ie-aph* (2'')-*Ia* was found in four VRE, all of clinical origin (26.6% of clinical VRE). Three of these showed a high level resistance to gentamicin, as demonstrated by the MIC results, while strain FAIR-E 41 did not grow at high concentration of this aminoglycoside. This finding needs to be deeper studied.

From this research it emerges that VRE are broadly diffused in common foods like meat products and to a lesser extent in cheeses. No VGRE were detected in these food systems. Even if a low number of strains of clinical origin harboured a combined vancomycin/gentamicin resistance, their retrieval should be considered a health threat.

**Dissemination of *Tn917* and *ermB* in dairy and clinical enterococci**

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Enterococci are part of the commensal microbiota of humans and animals, and are present in the environment, namely in some food products, like milk and cheese. Some enterococci are opportunistic pathogens. In recent years they have become a subject of increasing concern due to their increasing antibiotic resistance. Previous studies support the hypothesis that enterococci carried on foods are exchanging antibiotic resistance genes with enterococci and other pathogenic and commensal bacteria that colonize humans and may constitute the main source of antibiotic resistance genes. One way genes are transferred between bacteria is through transposons. One of the transposons found in *Enterococcus* is *Tn917*, which carries the macrolide-lincosamide-streptogramin B (MLS) resistance. Macrolides are used for treatment of humans, with erythromycin as first choice, and also as a substitute for penicillin in cases patients are allergic to penicillin. *Tn917* is already sequenced. Therefore, primers designed for the methylase (*ermB*) and resolvase genes, both encoded in *Tn917*, were used in PCR, both with genomic and plasmid DNA, to screen for this transposon in dairy and clinical enterococci. Resistance of these enterococci to clindamycin, lincomycin, erythromycin and spiramycin (MLS antibiotics) is already determined. In this way, dissemination of this transposon and *ermB* gene, in both foodborne and clinical enterococci, will be determined.

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### **Molecular and physiological comparison of *Enterococcus faecium* antibiotic resistance and virulence determinants between dairy, faecal and clinical isolates**

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Enterococci are part of the dominant microflora of several dairy products. They are also present in the gut of humans and animals. Their presence in traditional raw milk cheeses is probably due to faecal contamination of milk during milking. However enterococci are of increased significance as a cause of nosocomial infection which are becoming more and more difficult to treat due to the development of antibiotic resistance. In the present work, 30 *Enterococcus faecium* strains isolated from dairy products, 30 *E. faecium* isolated from ewes' faeces and 30 clinical isolates of the same species were studied for the antibiotic resistance and for the presence of virulence determinants. The resistance to 12 different antibiotics commonly used in the treatment of human infections was tested by the broth microdilution method as described by the NCCLS. In addition, the presence of vancomycin resistance genes was tested by using PCR. The presence of the aggregation substance (AS) gene, the surface protein gene *esp*, the accessory colonization factor *ace*, the *E. faecalis* endocarditis antigen *efaA* and the gelatinase *gel-E* gene, involved in the virulence of enterococci were investigated by PCR and by means of *in vitro* physiological tests. The aim of this investigation was to compare the potential virulence activity of *Enterococcus faecium* isolated from different ecological habitats and to establish if strains isolated from dairy products should actually be considered as potential pathogens.

## 34

### **Occurrence of glycopeptide-resistant enterococci (GRE) in foods of animal origin**

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Enterococci possess a large amount of natural and acquired resistances against many antibiotics. Resistance against the glycopeptide-antibiotics such as vancomycin and teicoplanin is especially critical and dangerous because infections with such glycopeptide-resistant enterococci (GRE) in hospitals can nearly not be treated. At the end of the 1980s the first GRE-strains appeared in english and french hospitals. The source of these strains could be the hospital itself, where these glycopeptides were used, but also the modern animal husbandry, where a special glycopeptid, avoparcin, was used as a growth promoter until it was banned in 1996. The aim of our investigations was to find out the burdening of food of animal origin with GRE five years after the ban of avoparcin.

From November 2000 until February 2002, 93 food samples of animal origin were examined with regard to the occurrence of enterococci in general and for GRE in particular.

For this reason we bought food, which might contain GRE, in the retail trade in different states of the Federal Republic of Germany. In detail we chose minced meat, ham, raw sausages and soft cheese.

From these samples we isolated 270 enterococci-strains and determined the species of each strain. For selected strains we investigated the resistance-pattern against 16 antimicrobial active agents including vancomycin and teicoplanin in accordance with NCCLS-resistance-guidelines.

Our investigations showed, that none of the food samples contained GRE. This result is in correspondence with topical results of other research scientists (A. Baumgartner et al., 2001).

**Characterisation of gentamicin resistance in dairy and clinical *Enterococcus* spp.**

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Enterococci are ubiquitous bacteria, which frequently occur, in large numbers in dairy and other foods. Along with approximately 450 other taxa of anaerobic and aerobic bacteria, *Enterococcus* are part of the normal intestinal microbiota. However, in recent years the prevalence of enterococci among nosocomial pathogens has increased, which is mostly due to their acquisition of multiple antimicrobial resistances.

One of the antibiotics of major concern is gentamicin, an aminoglycoside. In fact, the appearance of an increasing number of high-level gentamicin resistant enterococci makes it difficult to treat severe cases of endocarditis because synergism with cell-wall active agents (like ampicillin and penicillin G) no longer works. Due to the high number of enterococci in milk and cheese, it is important to ascertain if they constitute any threat in terms of gentamicin resistance. Therefore, gentamicin resistance was screened for in enterococci isolated from these dairy products. For comparison, clinical strains of enterococci were also studied. Classification of these enterococci as susceptible or resistant was done based on results of a disk diffusion method, with different gentamicin concentrations, and on MIC determination. Detection of some aminoglycoside modifying enzyme genes was done using PCR. The presence of synergism with ampicillin and penicillin G was also searched for.

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## 36

### **Multiple virulence factors and multiple antibiotic resistances in *Enterococcus faecalis* isolated from food**

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Multiple antibiotic resistances are now common in enterococci isolated from food which has been prepared from animal raw materials like milk and meat (Teuber et al., Antonie van Leeuwenhoek 76: 115-137, 1999). An additional worrisome dimension is the presence of different virulence factors recently described in enterococci from fermented and other food (Eaton & Gasson, Appl. Environ. Microbiol. 67: 1628-1635, 2001; Franz et al., Appl. Environ. Microbiol. 67: 4385-4389, 2001).

We have investigated the presence of virulence factors (hemolysis, gelatinase, aggregation substance, enterococcal surface protein) in all enterococcal isolates previously characterized by their multiple antibiotic resistances (Teuber et al., Lebensmittel-Technologie 29: 182-197, 1996). The strains had been mainly isolated from fermented sausages and raw milk cheeses purchased on the Swiss market. Phenotypic and genotypic methods used by Eaton & Gasson (2001) and by Franz et al. (2001) were used to determine the named virulence factors.

150 strains of *E. faecalis*, *E. faecium*, *E. durans*, *E. casseliflavus* and *E. avium* were screened. Virulence factors were only detected in *E. faecalis* and in one *E. avium* strain. Out of 63 *E. faecalis* strains, 30 expressed hemolysis, and 29 gelatinase activities. 15 strains each had the genes for aggregation substance and enterococcal surface protein (eps). 5 strains carried all the 4 virulence factors, 16 strains carried 3 determinants. Hemolysis and gelatinase activity were coupled in 29 strains. Overall, 48% of the *E. faecalis* isolates carried one or more virulence factors. This level is higher than the one reported in faecal isolates, but lower than that of isolates from human clinical material.

Since certain cheeses and fermented raw sausages may contain between  $10^5$  and  $10^7$  living multiple antibiotic resistant and virulence factor-positive enterococci per gram, and since it has been shown that enterococci do survive the passage of the stomach and may transiently establish themselves in the human intestine, a meal of 100g of cheese or sausage presents a load of  $10^7$  to  $10^9$  living potentially pathogenic enterococci to the consumer. It is our opinion that food microbiologist and food technologist must develop tools and procedures to avoid such a high load of undesirable bacteria in otherwise wholesome food items.

## Epidemiology and ecology of enterococci, with special reference to antibiotic resistant strains, in animals, humans and the environment

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Some results from a project within the European research programme.

The aim of this European project was to study the population structure among enterococci in different geographical regions of the EU and in different links of the food chain. Materials and methods. A total number of 2868 samples were collected from different countries within the EU (Sweden, Denmark, UK and Spain), and from different habitats, selected to contain enterococcal populations of human as well as animal origin (humans, water, sewage, pig farms, carcasses in slaughterhouses, soil, manure). The samples were characterized with regard to presence of enterococci and of vancomycin resistant strains. More than 20.000 isolates were phenotyped and preliminary species identified with the automated PhenePlate (PhP) system. Resistance to vancomycin, ampicillin, erythromycin, virginiamycin, tetracycline and avilamycin was determined for selected isolates by the VetMic™ system.

Results. A majority of the samples (77%) showed growth of presumed enterococci. The most common species found were *E.faecium* (33%), *E.faecalis* (29%), and *E.hirae* (24%). In 8.3% of the samples there was growth of presumed enterococci in the presence of 20 mg/l of vancomycin (PEV20). The PEV20 were most common in urban sewage samples (54%), hospital sewage (16%) and in pig manure (in 21%), but rare in samples from slaughterhouses and samples from farmland and crop with and without manure. There was also a clear difference in the frequencies of PEV20 isolated from animals between the different countries. Thus, in Sweden only 1 % of the samples of animal origin contained PEV20, whereas in Denmark 3%, Spain 9% and in the UK 12% of such samples contained PEV20.

Resistance to tetracycline and/or erythromycin were most common (35% and 21% of all isolates tested) and found frequently in all sample types except in calves from Denmark and Sweden. Resistance to ampicillin was more frequent among human than among animal isolates (7% versus 3%). Avilamycin-resistant strains were almost exclusive isolated from broilers and pigs in all countries except Sweden.

Most PEV20 were confirmed as VRE. A much higher proportion of the VRE were resistant to at least one of the other antibiotics tested (97%) than the VSE (41%). Ten percent of the VRE were resistant to all antibiotics tested. These isolates were mainly of human (urban sewage) origin. The phenotypic diversity according to PhP typing among all VRE isolates was as high as 0.99, indicating that the VRE belonged to a wide variety of different clones. Only three cases with identical PhP types in human and animal VRE were found. Most VRE were *E.faecium*, but 16 out of the 148 confirmed VRE were *E.faecalis*. Notably 12 of these were from Sweden, mostly from urban and hospital sewage.

Conclusions. There are currently two different populations of VRE in Europe, one associated with animal production, and possibly due to previous use of avoparcin as a growth promoter, and another population that may be derived from antibiotic use in hospitals, that is also spreading to the environment via sewage, but is yet uncommon in animals. Thus it seems that today the danger of spreading multi-resistant enterococci in humans is mainly associated to use of antibiotics in the human population. However, since there is an environmental reservoir of such bacteria in all countries caused by use of antibiotics in both the human and animal populations, precautions must be taken in both these fields to limit the release of antibiotic-resistant enterococci into the environment as much as possible.

