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Problems related to the safety assessment of lactic acid bacteria starter cultures and probiotics*

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Introduction

Empirical food fermentations, relying on experience gained by trial and error, have been common practice of food processing since ancient times and even up to about a century ago. Unknowingly, use has thus been made of (e.g.) lactic acid bacteria (LAB), the major microbial group associated with beneficial food fermentations, in the production of fermented foods for centuries (1). Excavations in Switzerland have shown that sourdough bread was part of a typical diet over 5000 years ago (2) and the 'leavening' of sourdough bread was already mentioned in the Bible (e.g., Matthew 13, 133). Reference to fermented dairy products (cheese, butter, yoghurt) is documented in archaic texts from Uruk/Warka (Iraq) dated about 3200 B.C. (3, 4).

A significant step in this development, however, has been the introduction of the first "pure" starter cultures for sour milk simultaneously by Storch in Copenhagen, Weigmann in Kiel, and Conn in the USA in 1890. Problems in maintaining the quality of undefined, multistrain cultures and the resulting products, especially in large production plants, urged companies to produce and maintain pure, defined cultures. Thereby, improved control of the fermentation process was achieved, and a constant and predictable quality of the final product could be maintained.

Early starter culture developments focused mainly on lactic acid bacteria (LAB) known to be associated with well accepted fermented foods, first the various fermented dairy products. Soon, also (at least technically) defined cultures were introduced for other products, e.g. for sourdough fermentation around 1910, and slightly later also for fermented meat products. Early developments in meat fermentations

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around 1919/1920 were directed towards the role of yeasts and their importance in "fleur du saucisson" (5). Presently, a large variety of lactic fermentations are employed at levels ranging from household to industrial scale involving raw products such as milk, vegetables, meat and cereals (1, 4, 6, 7). Early developments in the implementation of yeast strains, and particularly *Saccharomyces cerevisiae,* were especially focused on the beer brewing industry, on their use in baking, and later also on applications in wine fermentation.

Even when preservation and safeguarding of foods are still major objectives of fermentation, other aspects such as wholesomeness, acceptability and overall quality have become increasingly important and are features valued by consumers. Quality, safety and acceptability of fermented foods were significantly improved through the use of selected strains as starter cultures. Present approaches towards the implementation of multifunctional cultures, also take into account the probiotic concept and the prospective offered by carefully selected strains for improved health benefits.

LAB as starters for food fermentations

The basis for modern food biotechnology was laid during the second half of the 19th century with the first well-founded scientific developments in microbiology. These included, amongst others, the first description of the lactic acid fermentation by Louis Pasteur in 1857, and the development of the first bacterial pure culture *("Bacterium lactis",* Syn.: *Lactococcus lactis)* by Lister in 1873. The early 'starter' cultures were based on 'back-slopping' procedures, i.e. mixed culture isolates were obtained from earlier successful fermentations. Such former spontaneous fermentations were associated with the (desired) development of the autochthonous microbial population typical of the raw material. These were propagated and handled at the site of production (8). Such spontaneous fermentations were gradually optimised by different backslopping procedures, which comprise the inoculation of the raw material with a small quantity of the previous successful fermentation. Such backslopping assures that the dominant strains present in the successful fermentation are used to inoculate fresh raw material and again take over a new fermentation. Backslopping is still used in the production of numerous fermented foods such as sauerkraut, cucumbers and sourdough (4, 7), or for products for which the microbial ecology and the role of succession in the microbial population are not well known (7, 8). Today, backslopping and spontaneous fermentation still represent a cheap and reliable preservation technology in less developed countries, whereas in Western countries many fermentations are carried out on an industrial scale (7). However, even in Western countries, some manufacturers still rely on traditional technologies for processing e.g. cheese and fermented meat products without the use of starter cultures (4).

The important role of LAB in fermentation is illustrated by their contribution to rapid acidification of the raw material by production mainly of lactic acid. In addi-

tion, some strains produce acetic acid, ethanol, aroma compounds, bacteriocins, exopolysaccharides and enzymes (e.g., proteases) and thereby enhance the shelf life and microbial safety of the fermented product, in addition to improving the product texture and sensory characteristics (7).

LAB as probiotics

A beneficial association of LAB with the human host was suggested by *Metchnikoff* already in 1908 (9). He considered the longevity of Caucasian persons to be related to their high intake of fermented milk products. In contrast to modern perception, Metchnikoff suggested that gut microbes were detrimental rather than beneficial to human health. In addition, he suggested that the substitution of gut microbes with yoghurt bacteria may be beneficial. In this context LAB and their major metabolite of sugar fermentation, i.e. lactic acid, were suggested as health promoting. Originally defined as micro-organisms promoting the growth of other micro-organisms (10), probiotics have in recent years been defined as "mono- or mixed cultures of live micro-organisms which, when applied to animal or man, beneficially affect the host by improving the property of the indigenous flora" (11) while in relation with food, they are considered as "viable preparations in foods or dietary supplements to improve the health of humans and animals" (12). The suggested health improving properties are still not well understood but are commonly suggested to relate to pathogen interference, exclusion or antagonism; immunostimulation and immunomodulation; anticarcinogenic and antimutagenic activities; alleviation of symptoms of lactose intolerance; reduction in serum cholesterol; reduction in blood pressure; decreased incidence and duration of diarrhoea; prevention of vaginitis, and maintenance of mucosal integrity (13, 14, 15, 16, 17, 18, 19).

Lactic acid bacteria are the major representatives of probiotics both on the food and pharmaceutical market. As some strains are associated with the human body and occur in the oral cavity, gastrointestinal tract and vagina, this makes these bacteria ideal candidates for application as probiotics. *Reuter* (20, 21, 22) described the typical lactobacilli associated with the human gastrointestinal tract and these can be separated into three groups, i.e. the "*Lactobacillus acidophilus*-group (containing mostly the closely related species *L. acidophilus, L. gasseri, L. crispatus* and *L. johnsonii),* the *L. salivarius*-group and the *L. casei*-group (presently comprising *L. paracasei, L. zeae* and *L. rhamnosus).* In addition, Reuter identified *L. reuteri* and *L. fermentum* as the major heterofermentative lactobacilli associated with the human gastrointestinal tract. Today, viable probiotic strains supplied in the market mostly contain these LAB species, either as fermented food commodities or in lyophilised form as supplements or pharmaceutical preparations (17). Other microbial species which find application in probiotic products belong to the genus *Bifidobacterium (B. adolescentis, B. animalis, B. bifidum, B. infantis, B. breve, B. longum)* or *Enterococcus (E. faecium).* In addition, strains of *Bacillus cereus* (toyoi), *E. coli* Nissle, 1917, and *Saccharomyces cerevisiae* (boulardii) have also found application (17).

Impact of LAB on the food industry

LAB are consumed in enormous quantities, primarily through consumption of fermented foods. According to the latest statistics as published in bulletin No. 355[24] of the International Dairy Federation (IDF), the average annual consumption of fermented milk products is 22 kg per capita in Europe. In total, this amounts to about 8.5 billion kg fermented milk per year. With an average microbial content in these fermented products of 10^8 bacteria per gram (or ml), this amounts to a total of 8.5×10^{20} LAB. Assuming one bacterial cell weighs 4×10^{-12} gram, this means that 3400 tonnes of pure lactic acid bacterial cells are consumed every year in Europe. This figure does not take into account the LAB used in other food fermentations such as vegetable and meat fermentations, or especially the probiotic bacteria consumed as supplements or as pharmaceutical preparations. It can thus be expected to be far greater. Today, probiotic foods comprise between about 60 and 70 % of the total functional food market. A continued increase is observed among the dairy type probiotic foods, but is found even in the range of non-dairy probiotic food products such as fermented meats, and vegetable and fruit juices. Taking into account the wide range of potential (fermentable) substrates, and the different conditions under which LAB strains may be challenged for "functional performance", it can be expected that developments towards new food-based probiotics will even proceed further in the future (23).

LAB associated with human infections, and safety considerations

Cases of infection due to lactobacilli and bifidobacteria are rare and estimated to represent about 0.05 % to 0.4 % of cases of infective endocarditis or bacteremia (12, 24, 25, 26). Leuconostocs have been reported to cause <0.01 % of bacteremia cases, while enterococci are the major exception among the LAB (excluding the pathogenic streptococci), in that these are well known to be important agents of nosocomial disease causing 5–15 % of bacteremia cases (12, 24, 25, 27, 28, 29). A few rare cases of infection have been associated with LAB used in foods but, in most cases, a firm connection was not established (30). In one case of a liver abscess, the isolate was indeed closely related to a probiotic (31). In another case involving bacteremia, a connection with a combination of chewing a probiotic capsule and dental infection was suggested (32), even though the isolate was not typed at the molecular level (33). In another case of *L. fermentum* related endocarditis, large daily consumption of milk and dairy products was suggested to play a role, even though no connection with the illness could be proven and no proof of its use in dairy products in general has been shown (12, 34).

Most LAB which have caused infections in humans belong to the species *E. faecalis* and *E. faecium* (27, 35, 36, 37, 38), but other LAB species such as *L. rhamnosus, L. acidophilus, L. jensenii, L. paracasei, L. casei, L. curvatus, W. confusa, P. acidilactici* and *P. pentosaceus* have also been noted to be associated with human disease (Table 1). Generally, most patients from which LAB were isolated had serious

Table 1
Overview of reports of disease where lactic acid bacteria were isolated

Disease	*Causative agent*	*source of isolation*	*Reference*
Endocarditis	*L. rhamnosus*	Blood	87, 88, 89, 90, 91, 92, 93, 94
Endocarditis	*L. curvatus*	blood	95
Endocarditis	*L. acidophilus*	Blood	96
Endocarditis	different species of *Lactobacillus*	Blood	32
Endocarditis	*Pediococcus acidilactici*	Blood	97
Urinary tract infection	*P. acidilactici, L. gasseri*	Urine	97, 98
Septicemia	*P. acidilactici, P. pentosaceus*	Blood	97, 99, 100
Endocarditis	*L. rhamnosus*	Aortic valve	101
Pneumonia/ lung abscess	*L. rhamnosus*	Sputum	102
Empyema of the gallbladder	*L. rhamnosus*	Puss	99
Erysipeloid	*L. rhamnosus*	Lymphatic system	93
Bacteremia	*L. rhamnosus*	Blood	93, 25
Endocarditis	*L. casei*	Blood	104, 105
chest infection	*L. rhamnosus*	Sputum	106
Endocarditis	*L. paracasei*	Blood	107
Endocarditis	*Weissella confusa*	Blood	108
Caries	*L. paracasei, L. rhamnosus*	Sputum	109
Liver abscess	*L. acidophilus*	Liver	110
Liver abscess	*L. acidophilus*	Blood and Liver	111
Liver abscess	*L. rhamnosus*	Liver	31
Peritonitis	*L. rhamnosus*	Peritoneum	112
Various diseases	various species		28
Various disease	mainly *Lactobacillus* spp.		61, 113
Bacteremia	various species	Blood	25

underlying disease, which predisposed them to infection. Risk factors appear to include abnormal heart valves in the case of endocarditis and the presence of a catheter in cases of septicaemia (12, 39). Extremes of age and pregnancy appear not to be risk factors for LAB associated infection (40). For enterococci, endocarditis was often noted to occur in patients with underlying heart disease. Risk factors for endocarditis furthermore appear to include preceding genitourinary instrumentation, urinary tract infection, abortion, or urinary tract instrumentation (27, 41, 42). Risk factors associated with enterococcal bacteremia include underlying disease, presence of urethral or intravascular catheters, surgery, major burns, multiple trauma or prior antibiotic therapy (41, 42). For *Pediococcus* infections, risk factors appear to include underlying conditions or underlying malignancy such as diabetis, pulmonary or vascular disease, hyperparathyroidism, burns or trauma, previous antibiotic treatment, abdominal surgery, tube feeding, or in this case also pregnancy (43, 61). Immunocompromised individuals are generally assumed to be more at risk to infection with pathogens and are known to be afflicted with a high incidence of opportunistic infections. However, at least regarding the consumption of probiotic

lactobacilli or bifidobacteria, there is no evidence that consumption of probiotic preparations containing these bacteria leads to an increased risk of opportunistic infection among such individuals (26). Various clinical studies have been conducted to assess the safety of probiotics in small groups of specific immunocompromised patients (i.e., with HIV infection), and the findings of the studies support the safety of probiotics consumed by such groups (26, 44, 45, 46).

It is generally agreed that the sources from which the LAB associated with the infection may originate from, are the patient's own autochthonous microbes (12, 33). *Saxelin et al.* (26, 29) studied the prevalence of bacteremia with which lactobacilli were associated in southern Finland during a 4- and a 6-year period, and compared the characteristics of the blood culture isolates with the dairy strains. In both studies the lactobacilli isolated from blood did not correspond to a dairy strain. *Salminen et al.* (47) evaluated the possible effects of the increased use of *L. rhamnosus* GG on the occurrence of bacteraemia due to lactobacilli in Finland. The results of their investigation suggested that, despite wide application of this strain as a probiotic, this has not led to an increase in frequency of *Lactobacillus*-associated bacteraemia. Nevertheless, given the large scale use of these bacteria as starter cultures and as probiotics, there are quite valid safety concerns, especially in light of transfer of antibiotic resistances. Furthermore, as has been shown in the documented case of liver abscess caused by a strain of *L. rhamnosus* that was indistinguishable from a probiotic strain, certain LAB strains may become associated with human disease and thus the virulence potential of certain strains should be assessed.

Regulatory aspects

Internationally, the application of LAB in foods is regulated in different ways and under various categories. These may vary from country to country, and may not necessarily be related to the function of a strain. Functions may refer to its use either as a starter culture and/or biopreservative, or as a probiotic, or by a particular function of a metabolite other than lactic acid (e.g. aroma compounds, bacteriocins or exopolysaccharides). This categorisation has a direct influence on possible regulation or not in the EU, based on the fact that its classification as a processing aid would exempt such a strain from labelling required for food additives. Categorisation as an ingredient would demand its addition to the list of ingredients for a food commodity. Notification prior to marketing is required in Denmark for starter cultures defined as additives. In Finland and Sweden, no present legislation deals with starter cultures, whilst national guidelines are presently being prepared in France for documentation of a new strain (48).

Under the Food Drug and Cosmetic Act of 1958, microbial cultures used in the USA have been defined as "food additives", following pre-market approval of new uses on the basis of an established standard of safety. However, according to the FD & C Act Section 210 (s), certain classes of substances were explicitly excluded from requirement for FDA pre-market approval under Section 409. Among these, sub-

stances "generally recognised as safe" ("GRAS") have particularly been mentioned with reference to ...."their safety under the conditions of its intended use". A GRAS substance was distinguished from a food additive by (a) widely known information relevant to its safe use, and (b) consensus among qualified experts on the established safety of the GRAS substance for its intended use. The USA launched a new GRAS programme in 1997 with the objective of simplifying the GRAS concept and also to reduce the bureaucratic activities for substances considered to be relatively safe. Information on the present status of the GRAS notification procedure, etc., may be found on the website http://vm.cfsan.fda.gov/~dms/opa-noti.html. The Food and Drug Administration (FDA) and its Division of Biotechnology and GRAS Notice Review, have the responsibility of "GRAS" regulating of new microbial strains for foods in the USA. With two exceptions (Denmark and France), the use of LAB in foods is not regulated by any single harmonised legislation in the EU, with regard both to their use as starter cultures, as probiotic cultures or as protective culture and/or food supplements. In the other hand, probiotic cultures for animal feed are regulated by detailed and fully harmonised EU legislation since 1994. According the *Wessels et al.* (48), 7 EU laws may determine the use of new LAB strains in foods in the present situation, comprising:

- Regulation 258/97/EC on novel foods and novel food ingredients (European Parliament and Council, 1997);
- Directive 90/219/EEC on the contained use of genetically modified microorganisms (Council of the European Union, 1990);
- Directive 89/107/EEC on food additives (Council of the European Union, 1989);
- Directive 88/388/EEC on flavourings for use in foods (Council of the European Union, 1988);
- Directive 2002/46/EC on food supplements (European Parliament and Council, 2002);
- Directive 95/2/EC on food additives other than colours and sweeteners (European Parliament and Council, 1995); and
- Directive 94/40/EC on microorganisms as additives in animal feeding stuffs (European Commission, 1994).

At least since 1992, approaches towards safe biotechnology in industry were initiated in Germany by the "Berufsgenossenschaft der chemischen Industrie", when they published a document in which the presumed safety of biological agents was indicated by allocation to risk groups 1 to 4 (49). No bacteria are grouped into risk group 4 and very few (i.e. only the highly virulent species and types such as *Salmonella* Typhi, most rickettsiae, and *Brucella melitensis)* were grouped into category 3. Most bacteria were grouped into category 1, i.e. the category considered to be safe, which includes practically all lactobacilli except *Lactobacillus rhamnosus.* This list is not restricted only to food related organisms. It is issued as a booklet, and is actualised from time to time. EU directive 90/679/EC deals with the protection of the

worker against risks associated with biological working materials. This has been modified and extended by council directives 93/88/EC of 12th October 1993, 95/30/EC of 30th June 1995, 97/59/EC of 7th October 1997 and 97/65/EC of 26th November 1997. This has been transferred into German legislation "Verordnung zur Umsetzung von EG-Richtlinien über den Schutz der Beschäftigten gegen Gefährdung durch biologische Arbeitsstoffe bei der Arbeit" (so-called "Biostoffverordnung") on 27th January 1999 (BGBl. I, page 50), and modified further by the regulation of 18th October 1999 and of 25th November 2003 (BGBl. Page 2304). An actualised list with the classification of bacteria into risk groups refers (amongst others) to *Lb. gasseri* and *Lb. johnsonii,* although classified in risk group 1, of which strains in isolated cases have been presumed responsible for infections. Similar approaches towards safe biotechnology in the industry have been taken by the "Berufsgenossenschaft der chemischen Industrie" (49), mentioned before.

EU legislation covering the implementation of microbial strains in foods does not exist at this stage. In anticipation of such an announcement, SCAN initiated a position paper, issued by the European Commission in 2002, under the title "Safety assessment and regulatory aspects of micro-organisms in feed and food applications". In this paper, a list of micro-organisms with a history of safe use has been proposed on the basis of a qualified presumption of safety (QPS). A more elaborate "working paper open for comment" on a QPS system was issued by the European Commission in 2003 under the title "On a generic approach to the safety assessment of micro-organisms used in feed/food and feed/food production". In this document, the assessment of the suitability for QPS of a microbial strain or culture was suggested to be based on a decision tree approach. On the expiry of the mandate of SCAN early in 2003, EFSA took over the task of handling developments of the QPS concept within a EU regulatory framework.

Antibiotic resistance of lactic acid bacteria

Antibiotic resistance of LAB isolated from human infections

Among the LAB strains that were isolated from human infections, strains of the genus *Enterococcus* are particularly known to contain multiple antibiotic resistances (50). Enterococci are either intrinsically resistant and resistance genes are located on the chromosome, or they possess acquired resistance determinants which are located on plasmids and transposons (27, 51, 50). Intrinsic antibiotic resistances include resistance to cephalosporins, β-lactams, sulphonamides, and low levels of clindamycin and aminoglycosides, while examples of acquired resistances include resistance to chloramphenicol, erythromycin, high levels of clindamycin and aminoglycosides, tetracycline, high levels of β-lactams, fluoroquinolones, and glycopeptides such as vancomycin (27, 52, 50). Vancomycin resistance is of special concern because this antibiotic was considered as a last resort for treatment of multiple resistant enterococci infections. In the mid 1990's, the source of vancomycin-resis-

tant enterococci (VRE) in Europe was shown to be most likely the farm animals as a result of ergotropic use of avoparcin, a glycopeptide antibiotic (53, 54). VRE have indeed been isolated from a wide variety of farm animals, and these constitute an important reservoir of VRE that could be transmitted to the hospital environment via contaminated meat (50, 53, 54, 55, 56). These findings strongly suggested that food transmission occurred and, as a result, the use of avoparcin in animal husbandry was banned in the European Union in 1997 (57).

All pediococci isolated from human infections share the characteristic that they have intrinsic and high level resistance to vancomycin and teicoplanin (58, 59) and are thus often isolated after vancomycin treatment, together with other vancomycin-resistant LAB (58, 60). In addition, such pediococci appear to be resistant to quinolone antibiotics as well as tetracycline (59, 61) but they are generally sensitive towards antibiotics such as penicillin, ampicillin, and aminoglycosides and moderately sensitive to chloramphenicol (58, 59, 60, 62). The majority of strains appear to be sensitive also to erythromycin (59, 63).

Not all isolates of *Lactobacillus* spp. are vancomycin resistant, and *Husni et al.* (64) showed that 73 % of isolates showed resistance towards this antibiotic. The species *L. casei* and *L. rhamnosus,* which are the most common *Lactobacillus* species associated with endocarditis and bacteremia, tended to be most sensitive to erythromycin and clindamycin, and resistant to vancomycin (65). The species most sensitive to vancomycin was *Lactobacillus acidophilus* (65). In lactobacilli, glycopeptide resistance is considered a natural trait of some species (e.g., *L. reuteri, L. casei, L. rhamnosus, L. paracasei)* and the absence of transferable resistance determinants such as those encoding the *van*A, *van*B and *van*C-types was confirmed by *Klein et al.* (66). *Leuconostoc* spp. and *Weissella confusa* strains are also known to be naturally resistant to vancomycin (67, 68, 69). Leuconostocs, pediococci and lactobacilli from human infections were shown to be generally susceptible to penicillin, clindamycin, gentamicin, chloramphenicol and erythromycin (58). Moreover, *Swenson et al.* (58) noted an interesting difference in resistance spectra between the three different genera: while *Pediococcus* species were mostly susceptible to ciprofloxacin and resistant to imipenem, leuconostocs were mostly ciprofloxacin resistant and susceptible to imipenem. Lactobacilli, on the other hand, appeared to be generally susceptible to ciprofloxacin and imipenem.

Antibiotic resistances of LAB from foods and probiotic strains

Because of the intensive application of LAB in food fermentations and their increasing use as probiotics on the one hand, and the (albeit low) association of LAB with human disease on the other, researchers in the field have recently turned their attention towards studying the antibiotic resistance of LAB associated with food or used as antibiotics. In our studies (70), we investigated the antibiotic resistance of enterococci from foods and showed that resistance towards one or more antibiotics was a common phenomenon among *E. faecium* and *E. faecalis* strains (Fig. 1). *Ente-*

rococcus faecium strains were mostly (56.3 % of strains) resistant to ciprofloxacin, followed by penicillin (45.8 %), erythromycin (27.1 %), chloramphenicol (10.4 %), and tetracycline, streptomycin, gentamicin and vancomycin at an incidence of <10 % (Fig. 1). *Enterococcus faecalis* strains, on the other hand, were mostly resistant to chloramphenicol (63.8 %), followed by streptomycin (46.8 %), tetracycline (44.7 %), erythromycin (31.9 %), ciprofloxacin (27.7 %), gentamicin (25.5 %), penicillin (12.8 %), and ampicillin (2.1 %) (70). *Teuber et al.* (71) also reported high incidences of gentamicin, tetracycline, chloramphenicol and erythromycin resistant enterococci from foods such as cheeses or fermented meats. *Huys et al.* (72) showed that 24 % of enterococci from cheeses were resistant to tetracycline. *Giraffa* (50) also reported that enterococci, mainly belonging to the species *E. faecalis* and *E. faecium,* isolated from European cheeses and meat products, showed resistances in different proportions to antibiotics such as penicillin, tetracycline, bacitracin, chloramphenicol, erythromycin, gentamicin, streptomycin, lincomycin, rifampicin, fusidic acid and vancomycin.

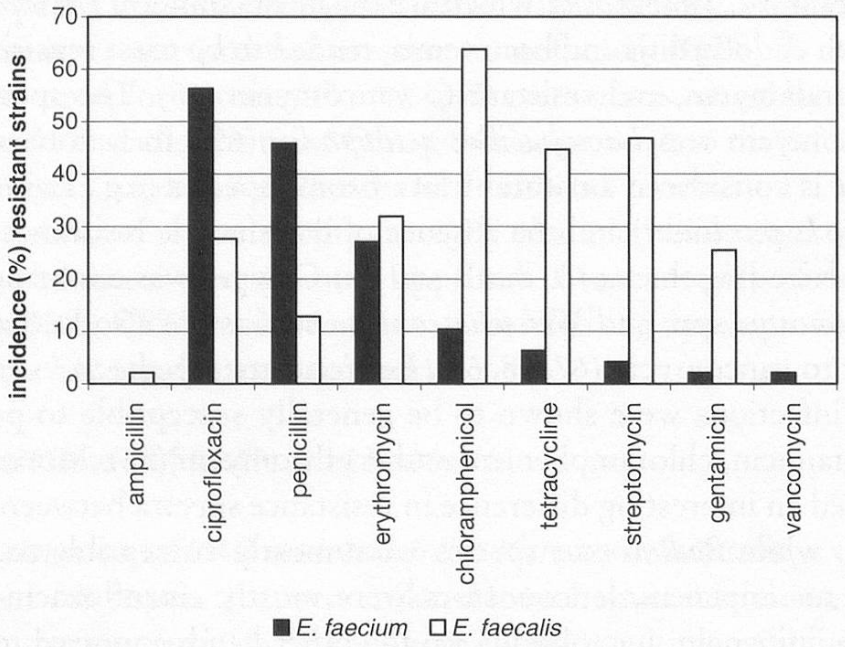

Figure 1 **Incidence (%) of antibiotic resistant *Enterococcus* strains isolated from foods**

Gevers et al. (73) studied the occurrence of tetracycline-resistant LAB associated with different dry fermented sausage types and showed that different *Lactobacillus* spp., including strains of *L. plantarum, L. sakei* subsp. *carnosus, L. sakei* subsp. *sakei, L. curvatus* and *L. alimentarius,* could possess the tetracycline resistance phenotype. Moreover, *Gevers et al.* (74) also studied the incidence of tetracycline-resistant LAB along the process line of fermented dry sausages and showed that in the raw meat starting material, tetracycline-resistant lactococci, lactobacilli,

streptococci and enterococci occurred. Interestingly, this diversity of tetracycline-resistant LAB decreased with fermentation to only the lactobacilli which persisted till the end of fermentation (74). These result suggested that these food commodities, for example, can serve as vehicles for antibiotic resistant LAB which were present in the process already in the raw materials.

To determine the safety of starter culture bacteria with respect to antibiotic resistance, *Katla et al.* (75) investigated the occurrence of antibiotic resistances among LAB starter cultures used in the production of Norwegian dairy products. Almost 200 isolates belonging to the genera *Lactobacillus, Lactococcus, Leuconostoc* and *Streptococcus* were screened for resistances towards 14 antibiotics. Only one strain, a *Lactobacillus,* was classified as high-level streptomycin resistant (75). Fourteen other *Lactobacillus* strains were shown to have high resistance to aminoglycosides, which was above the normal distribution. However, *Katla et al.* (75) did not classify these strains as resistant because they argued that LAB, in particular *Lactobacillus* strains, have a natural reduced susceptibility to aminoglycosides. In addition, a few isolates were also shown to possess MIC values just above the breakpoint of bacitracin, erythromycin, chloramphenicol and tetracycline, and the authors suggested that these may reflect a natural variation in susceptibility among the LAB to these antimicrobial agents. Vancomycin-resistance was noted especially for *Leuconostoc* spp. and was considered to constitute an intrinsic resistance, as this resistance is well-known to occur for this genus. None of the strains from the genera *Lactobacillus, Lactococcus* and *Streptococcus* were classified as resistant to vancomycin (75).

Danielsen and Wind (76) could also show that high levels of aminoglycoside resistances were common among lactobacilli. Furthermore, these authors showed that most strains of *Lactobacillus* species investigated (species of the *L. acidophilus*-group, *L. casei*-group, and *L. sakei, L. curvatus, L. plantarum* and *L. pentosus)* were naturally resistant towards the antibiotics bacitracin, cefoxitin, ciprofloxacin, fusidic acid, kanamycin, metronidazole, nitrofurantoin, norfloxacin, streptomycin, sulphadiazine, teicoplanin, trimethroprim/sulphamethoxazole and vancomycin. Some strains of *Lactobacillus* with unusually high resistances to chloramphenicol, erythromycin and tetracycline were also identified (76), suggesting an acquired resistance. One very important aspect of their study was that the resistance breakpoints should be defined for the different *Lactobacillus* species, as susceptibilities were species-specific.

Similarly, *Temmerman et al.* (77), investigated antibiotic resistances of LAB strains (lactobacilli, pediococci, bifidobacteria, *S. thermophilus* and *E. faecium* strains), and also showed that resistances to antibiotics such as vancomycin, tetracycline, erythromycin, penicillin and chloramphenicol did occur. Among the lactobacilli, 29.5 % of the strains were resistant to tetracycline, 8.5 % were resistant to chloramphenicol and 12 % to erythromycin. In addition, they showed that more than 68 % of their isolates exhibited resistance to two or more antibiotics (77).

We investigated the antibiotic resistance of 46 commercial LAB starter cultures used in the production of cheese, yoghurt and fermented meats and used the E-test to test for resistance to the antibiotics erythromycin, chloramphenicol, gentamicin, streptomycin, tetracycline, ciprofloxacin, ampicillin and penicillin on three different nutrient media. Only few (less than 10% of strains) were resistant towards the antibiotics ampicillin, penicillin, erythromycin and tetracycline. Most strains, especially lactobacilli, were resistant towards the aminoglycoside antibiotics, confirming the results of *Katla et al.* (75) and *Danielsen and Wind* (76).

Among 18 strains of *Bifidobacterium* tested for susceptibility to a range of antibiotics, 14 were found to be resistant to more than 10 antibiotics (77). Also, the role of the commensal microbial population in the development of antibiotic resistance among pathogens appears difficult to asses; however, *Andremont* (78) has shown that this population may play a key role in the spreading of antibiotic resistance.

Problems associated with determination of LAB antibiotic resistances

Choice of media

The choice of media can greatly influence the MIC values obtained in resistance testing. Media used for testing antibiotic resistances are firstly chosen to provide an optimal nutritional environment to support the growth of the test strain. Secondly, it should provide a suitable gel matrix to allow reproducible and uniform diffusion of the test antibiotic. Thirdly, possible interactions between (un)defined medium components and the antibiotic gradient should be at minimum (79). LAB have complex nutrient requirements and thus do not grow easily on antibiotic test media (e.g., Mueller-Hinton, Iso-Sensitest Agar) which are often used for other, nutritionally less demanding, bacteria for clinical antimicrobial resistance testing. From our own experience, Mueller-Hinton agar can be used for enterococci, but only poorly supports the growth (if growth occurs at all) of certain *Lactobacillus* spp., leuconostocs, streptococci and lactococci. *Herra et al.* (80) showed that Wilkens-Chalgren Agar, a medium recommended by the National Committee for Clinical Laboratory Standards (NCCLS) for use when determining antibiotic resistances of anaerobic bacteria, did not support the growth of lactobacilli. However, if Wilkens-Chalgren was supplemented with 5% horse blood, good growth of lactobacilli resulted (80).

Although MRS agar does support the growth of a wide variety of food associated LAB, the interaction of medium components with antibiotic gradients are little known (76, 79). Furthermore, LAB are a very heterogeneous group of bacteria many of which do not grow or do not grow well on MRS medium. For example, carnobacteria will not grow on MRS agar, and many streptococci and lactococci also do not or do not grow well on this medium. Thus, MRS agar is not a universal growth medium suitable for the optimal growth of all LAB. *Huys et al.* (79) reported that from 270 LAB isolates belonging to the genera *Lactobacillus, Entero-*

coccus, Lactococcus, Pediococcus and *Streptococcus,* more than 20 % did not grow on Iso-Sensitest Agar (ISA) and it was therefore not recommended for routine antibiotic resistance testing of LAB.

Apart from problems with growth of LAB on various media, different media formulations also appear to have an effect on MIC-values and thus resistance determinations. *Huys et al.* (79) moreover reported that such differences in MIC-values were also largely dependent on the antibiotic tested. For example, for ampicillin the inhibition zones were consistently larger (i.e. lower MIC values) on MRS agar than on ISA medium, while an opposite effect was noted for gentamicin, bacitracin and erythromycin. For tetracycline, inhibition zones (and thus MIC values) corresponded well between the two media (79).

In our study on the antibiotic resistances of the 46 commercial LAB starter cultures, we determined the MIC values under anaerobic conditions on three different test media: MRS Agar (Heipha, Heidelberg), Brain Heart Infusion (BHI; Viva Diagnostika, Cologne) and Antibiotic Agar No. 12 (AB12; Difco, Heidelberg). Streptococci and lactobacilli used as starters for yoghurt grew poorly on all these three media and resistance was therefore assessed also on M17 Agar (Difco, Heidelberg) medium. As already observed by *Huys et al.* (79) MIC-values could differ greatly between the media and were dependent on the antibiotic compound tested. Thus for ampicillin and chloramphenicol, MIC values for *Lactobacillus, Leuconostoc* and *Lactococcus* strains were in most cases higher on BHI and AB12 medium when compared to MRS (Fig. 2a and b). For *S. thermophilus* strains, the MIC values for ampicillin and chloramphenicol were generally higher when tested on M17 medium as compared to MRS medium (result not shown). For erythromycin and tetracycline resistance on the other hand, the MIC-values were generally highest on AB12 medium or BHI medium, respectively (Fig. 2c and d). For ciprofloxacin, streptomycin or gentamicin antibiotic resistances were generally high and because of this and inter-species fluctuation in MIC values, no obvious medium effect could be observed (results not shown). The reasons for these observed differences in MIC-values measured on the different media are not clear. Possibly, they may be connected to different growth of the bacteria on the media, interaction with medium components, differences in diffusion rates and establishment of a concentration gradient, as well as spontaneous breakdown of the antibiotic upon prolonged anaerobic incubation. Nevertheless, the problems encountered clearly point towards an urgent need for standardisation of methods which should specifically address the question of choice of media.

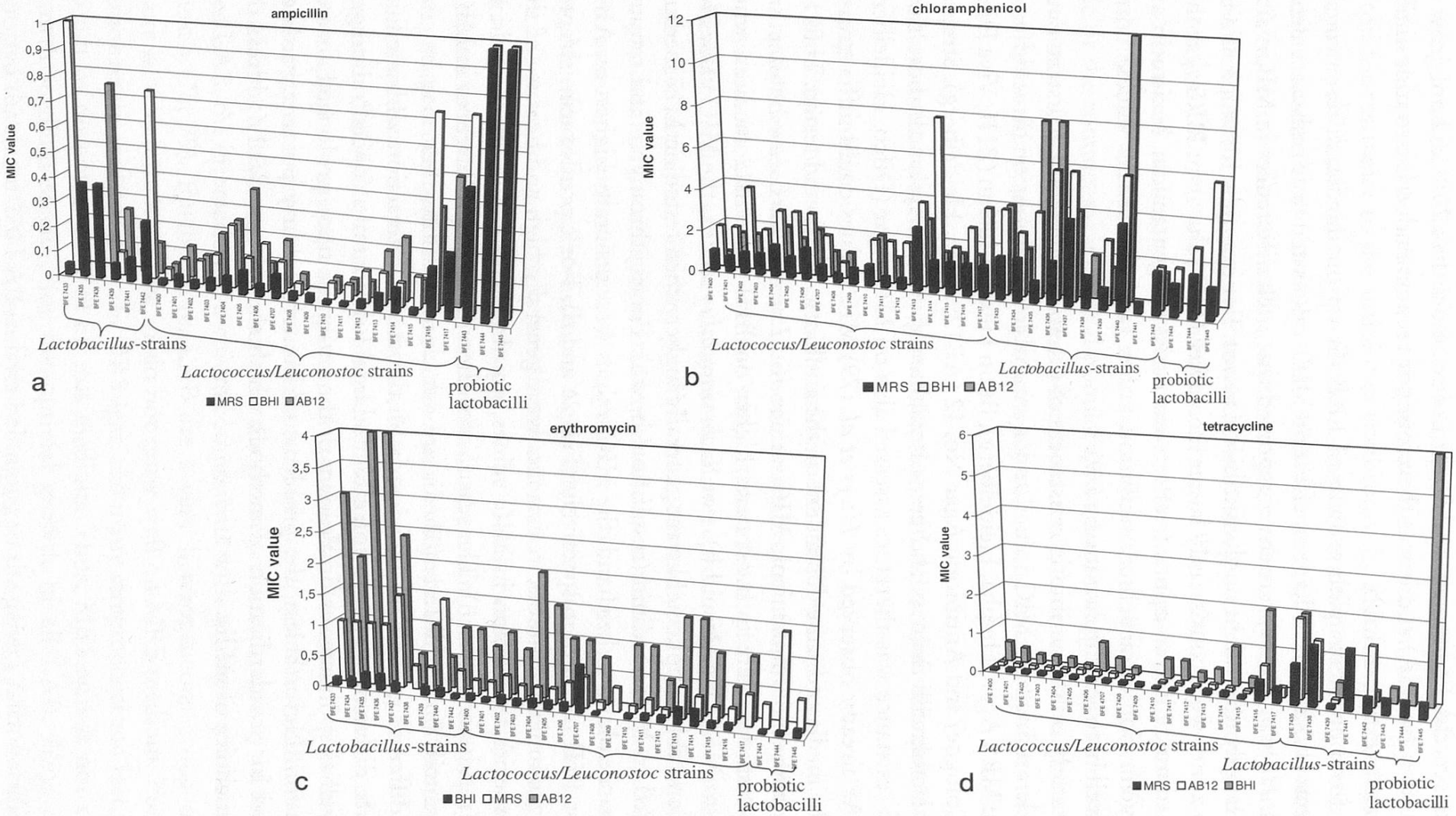

Figure 2 **Minimum inhibitory concentration values (µg/ml) for lactic acid bacteria starter strains using the antibiotics ampicillin (A), chloramphenicol (B), erythromycin (C) and tetracycline (D) as determined on MRS agar (MRS), brain heart infusion agar (BHI) and antibiotic agar no. 12 (AB12)**

Specification of breakpoint values

Because LAB are such a diverse group of LAB with many genera, each containing many species (e.g. the genus *Lactobacillus* currently comprises about 80 species) it is difficult to specify breakpoints for MIC values which separate the resistant from the susceptible strains. Clearly, some of such breakpoints may be defined at the genus level, when most of the species are known to be resistant or sensitive towards an antibiotic. However, for many LAB genera this view may be too simplistic, as the different species may vary considerably in their susceptibilities towards various antibiotics. First of all, the exact conditions for determination of antibiotic resistance regarding especially choice of medium as well as incubation temperatures, inoculum densities and method for analysis, should be standardised *(vide supra)* for such a definition to make sense. Currently, standards and guidelines for the media preparation, incubation parameters and the interpretation of results for disc diffusion, broth dilution and agar dilution methods are provided for example by the United States NCCLS and are available for selected aerobic and anaerobic bacteria (81). Although breakpoints for clinical enterococci are included, such breakpoints for other LAB can only be inferred from these or from suggested breakpoints of other Gram-positive bacteria such as *S. aureus.* The European Commissions Scientific Committee on Animal Nutrition (82) has subsequently supplied a list of breakpoint values for LAB other than enterococci, which is also limited because the breakpoint values given relate to the genera *Pediococcus* and *Lactobacillus,* and do not take inter-species deviations into account. *Danielsen and Wind* (76) recognised the shortcoming of this approach when investigating the antibiotic resistances of LAB starter cultures, as for some species a natural resistance would require the delineation of a higher breakpoint value when compared to more susceptible strains. Thus, while SCAN, for example, gives an MIC breakpoint value of 1 µg/ml for gentamicin, Danielsen and Wind recognised that this was far too low and suggested a breakpoint value of 128 µg/ml for *L. paracasei, L. plantarum, L. pentosus, L. rhamnosus, L. sakei* and *L. curvatus,* and an even higher breakpoint of 256 µg/ml for *L. acidophilus* for this antibiotic. Similarly, the breakpoint MIC value for streptomycin suggested by SCAN at 16 µg/ml was considered too low by *Danielsen and Wind* (76), who suggested a value of >256 µg/ml for all LAB. On the other hand, while SCAN recommended an MIC breakpoint value of 4 µg/ml for erythromycin, *Danielsen and Wind* (76) found this too high to account for observed natural resistances of some *Lactobacillus* spp., and suggested breakpoint values of 1 µg/ml for *L. acidophilus, L. sakei* and *L. curvatus,* 2 µg/ml for *L. paracasei* and *L. rhamnosus* and 4 µg/ml for *L. plantarum* and *L. pentosus.*

In our studies we found all LAB starters investigated to be resistant to gentamicin when considering the SCAN MIC breakpoint of 1 µg/ml (Fig. 3). When using the 128 µg/ml MIC breakpoint value suggested by *Danielsen and Wind* (76), still more than 40 % of *Lactobacillus, Pediococcus* and *Leuconostoc* strains investigated showed a resistance phenotype, indicating that this breakpoint value may still be set

too low. Similarly, when using the MIC breakpoint value for streptomycin as suggested by SCAN (16 µg/ml), more than 60% of strains of all genera tested were resistant (Fig. 3), indicating that indeed this breakpoint value was far too low. Even when considering the MIC value suggested by Danielsen and Wind at >256 µg/ml, more than 60% of *Lactobacillus* strains, 40% of the *Pediococcus* and *Leuconostoc* strains and 30% of the *Lactococcus* strains showed a resistance phenotype (Fig. 3), indicating that at least for the lactobacilli this breakpoint value is still too low.

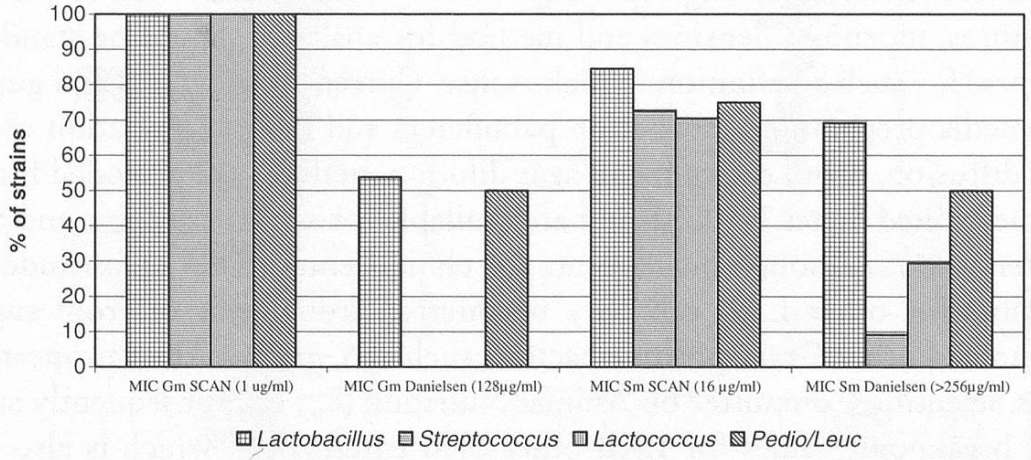

Figure 3 **Percentage of LAB starter strains classified as resistant to the antibiotics gentamicin and streptomycin when using the MIC breakpoint values supplied by SCAN (82) and *Danielsen and Wind* (76)**

The problematic of specifying meaningful breakpoint values will be the most challenging to solve in the area of LAB safety research. It clearly seems that is many cases the approach of defining an MIC breakpoint applicable to a genus is too simplistic. On the other hand, although the approach of defining such breakpoints for each species makes sense, it will create a lot of confusion and debate because the extent of inter-strain variation will still need to be clarified and a consensus for breakpoints needs to be found. One approach which could greatly aid in the specification of such breakpoints will be to correlate the resistance phenotype to a resistance mechanism and a genetic marker. Is such a mechanism identified, the genetic basis known and possible genetic transfer mechanism are elucidated, this will aid in breakpoint value specification as well as in discerning between natural (intrinsic) or acquired resistance.

Lactic acid bacteria 'virulence'

Given the long, thousands of years safe association of LAB with foods and the extremely low incidences of association with human disease (excluding *Enterococcus* and *Streptococcus* strains), one really hesitates when using the terms 'virulence' or

'virulence factors' or 'pathogenic' in association with these bacteria. In the absence of better coined terms which may imply factors that can explain why some LAB can cause disease and allow these bacteria to become associated with an infection, the terms 'virulence' and 'virulence factors' will be used in the following discussion. Even for the enterococci, virulence factors were long thought to be much more subtle than those of well-recognised Gram-positive pathogens such as the food associated *C. botulinum, S. aureus, L. monocytogenes* and *B. cereus.* Considerable progress has, however, been made in the last few years and factors associated with specific stages of enterococcal infection have been well described (38, 42). *Johnson* (36) described specific stages for a pathogenic bacterium to cause an infection i.e., the strain should be able to colonise host tissue, resist host specific and unspecific defence mechanisms and produce pathological changes either directly, by producing toxin, or indirectly by causing inflammation. Virulence factors of enterococci which are associated with all these four stages have been studied and identified. These virulence factors are summarised in Table 2 and their (possible) roles in the infection causing process are reviewed by *Franz et al.* (38) and *Franz and Holzapfel* (42). Interestingly, enterococci isolated from foods were shown to harbour either single or multiple virulence factors (70, 83). *Eaton and Gasson* (83), however, showed that the incidences of virulence factors among probiotic enterococci strains were noticeably lower than for food strains. The fact that enterococci from foods often carry virulence determinants, may imply that the association of these bacteria with foods constitutes a safety risk, especially for persons with underlying disease (38, 42). As mentioned above, it has been noted that VRE transmission in Europe from the community to the hospitals probably occurred via the food chain. This emphasises the importance of the food route for transmission of enterococci risk strains which may carry antibiotic resistance or virulence determinants (42).

Table 2
Enterococcal virulence factors and their association with stage of virulence or suggested role in disease (42)

Virulence determinant	*(suggested) association with stage of virulence*
Aggregation substance (AS)	Adhesion to eukaryotic cells (adhesin)/promotes colonisation Invasion of eukaryotic cells (invasin) Adhesion to extracellular matrix proteins (may promote translocation) Increases survival in immune cells (evasion of host immune response)
Cytolysin (Cyl)	Eukaryotic cell toxin Lyses immune cells (evasion of host immune response)
Gelatinase (Gel)	Can hydrolyse various biological peptides e.g., collagens and fibrin (role in translocation?) Can hydrolyse antibacterial peptides (evasion of host innate immune response)
Enterococcal surface protein (Esp_{fs} and Esp_{fm})	Adhesin, promotes colonisation Exhibits characteristics of MSCRAMM[a]'s – role in evasion of immune response?
Adhesin to collagen of *E. faecalis* (Ace) or *E. faecium* (Acm)	Adhesion to extracellular matrix proteins (may promote translocation) Exhibits MSCRAMM characteristics: role in evasion of immune response?
Endocarditis antigen from *E. faecalis* or *E. faecium* ($EfaA_{fs}$)	Adhesin: role in endocarditis
Hyaluronidase	Degrades hyaluronic acid,a major extracellular matrix constituent: role in translocation?
Capsule	Evasion of host immune response

[a] MSCRAMM: microbial surface component recognising adhesive matrix molecules

Problems related to determining LAB 'virulence' factors

Because LAB (excluding enterococci and pathogenic streptococci) have a long term history of safety with healthy humans and clearly possess an extremely low virulence potential, one is hard stressed to find 'virulence factors' among these bacteria. Although it is known that some *Weissella confusa* strains possess an α-haemolytic phenotype (69) and a dairy strain of *Lactococcus lactis* of which the chromosome was sequenced was shown to harbour a gene for a haemolysin III (84), the impact of these can be described as negligible because such haemolytic characteristics have never been reported to play a major role in establishment or contribution to an infection. According to *Salminen et al.* (12) three approaches can be used to assess the safety of a probiotic strain: i) studies on the intrinsic properties of the strain, ii) studies on the pharmacokinetics of the strain (survival, activity in the intestine, dose response relationships, faecal and mucosal recovery and iii) studies searching for the interactions between the strain and the host.

Intrinsic properties of LAB strains related to safety

Metabolic activities of the LAB strains may be important safety criteria. For example, *Lactobacillus* spp. are largely responsible for the D-lactic acidosis frequently observed in patients with short bowel syndrome and consumption of *Lactobacillus* spp. tablets has been associated with D-lactic acidosis in such patients (33), indicating that probiotic strains should be screened for their ability to produce D-lactate (26). The formation of biogenic amines may also have safety implications. Tyrosine decarboxylation is quite common in lactobacilli and has been observed to varying degrees in different species. Many LAB possess bile salt deconjugase activity and excessive deconjugation in the small intestine can lead to impaired digestion or absorption of fats. In addition, further metabolism of primary (deconjugated) bile salts to secondary bile salts by bacteria with 7-hydroxylase activity is a further unwanted effect. Secondary bile salts may exhibit carcinogenicity by acting on mucus-secreting cells and promoting their proliferation, or they may act as promoters of carcinogenesis (26, 85). Enzymes such as azoreductase, nitroreductase, β-glucuronidase and various glycosidases may be produced by LAB and may play a role in undesirable toxicological effects, especially with respect to carcinogen activation (26). LAB vary in their potential and extent to produce such enzymes and this should be assessed when tested for their safety. In preliminary studies, we investigated the potential of *Lactobacillus* strains to degrade hyaluronic acid, and did find a few strains which were capable of this trait. The implications of hyaluronidase activity among lactobacilli, however, is not clear as this has not been reported within the context of virulence and pathogencity yet. Platelet aggregation activity has also been considered to be a required test in the assessment of safety. Although such in vitro tests can give some information on the perceived safety of the strains, these are generally not good predictors of activity in vivo (26).

Pharmacokinetics of LAB strains and their relationship to safety

The survival aspects of probiotics in pharmacokinetic studies can be measured in vivo using a faecal collection of intestinal intubation and colonic biopsy techniques (12). Dose response studies may give an indication of safety, although the concept of a minimum infective dose is difficult because of the large number of microbial and host factors (12). *Salminen and Marteau* (86) proposed translocation and colonisation properties for pharmacokinetics studies to assess the safety of probiotics. Colonisation itself, however, is not a good marker for safety investigations. Adhesion is indeed considered to be virulence factors when pathogens are studied. However, mucosal adhesion and other colonisation factors are considered functional properties of most probiotic strains. It is largely believed, that probiotic strains should not invade host cells and this invasive potential should be studies in cell culture using intestinal cells. It may also be speculated, that lesions in the digestive tract and immunodeficieny may favour translocation of probiotics and other bacteria from the gut lumen.

Although bacterial translocation does not occur readily in healthy, specific pathogen free animals, it is known to occur for a long duration in germ-free mice, a phenomenon which is caused by an immature intestinal barrier and underdeveloped immunity of the lymphocytic system (85). Using germ-free animals as test system, translocation of LAB such as *Lactobacillus* spp. and enterococci has been observed. Nevertheless, it is quite surprising than to note that studies with hamsters with *Clostridium difficile* colitis demonstrated that an adhesive probiotic enhanced the mucosal barrier and prevented translocation of intestinal microbes. Also, *Lactobacillus* strains have been administered during clinical trials to premature children and patients with Crohn's disease or diarrhoeal diseases with no side-effects. Moreover, these results are in agreement with those reported in the case of lethal irradiation and immuno-compromosed mice (12, 33).

Studies on the interaction between the LAB strain and the host

The safety of LAB used as probiotics should be confirmed in studies of humans. Studies in healthy volunteers and in clinical trials should demonstrate the effectiveness and safety of the probiotic strain. Indeed, extensive short term clinical trials with healthy volunteers have demonstrated the safety of probiotics (12). The safety can be confirmed in studies by providing non-invasive measurements such as measurement of body weight or blood pressure, as well as parameters of hematologic analysis ands of serum/plasma chemical analysis (26).

Thus, for LAB to cause disease, both the bacterial factors and the host factors need to be involved. Clearly, the bacterial factors are subtle and not easily identified. Given the background of the low incidence of LAB association with human infections, and their historical safety background, one can only conclude that the host factors are the determining parameters of the equation of infection with LAB. This makes such a safety evaluation even more difficult.

Summary

Thanks to their association with numerous traditional fermented foods, most lactic acid bacteria (LAB) are generally presumed and accepted as safe for human consumption. Exceptions are most species of the genus *Streptococcus* and, with some limitations, some species of the genus *Enterococcus.* Still, some strains of the genera *Lactobacillus, Pediococcus* and *Leuconostoc* have, in rare cases, been either identified or presumed to be infectious. In view of possible EU regulation of microbial strains for technical uses, including starter, protective and probiotic cultures, relevant information is supplied and discussed with regard to the technical application and to possible risks related to their applications. Special reference is made to the lack of standardisation of antibiotic resistance testing for most LAB (exceptions being the genera *Enterococcus* and *Streptococcus).* Thereby, a clear distinction should be made between transferable constitutive (intrinsic) resistence, also by the application of genetic methods. Another problem is related to the question on "typ-

ical" virulence factors which have been extensively studied for the enterococci, whereas none has been found thus far for any *Lactobacillus.*

Zusammenfassung «Problematik der Sicherheitsuntersuchung von Starterkulturen mit Schwerpunkt der Milchsäurebakterien»

Dank ihrer Assoziation mit vielen traditionellen Lebensmittelfermentationen gelten die meisten Milchsäurebakterien generell als unbedenklich. Ausnahmen bildet vor allem die Gattung *Streptococcus* und bedingt auch die Gattung *Enterococcus.* Dennoch wurden Vertreter der Gattungen *Lactobacillus, Pediococcus* und *Leuconostoc* in Einzelfällen entweder als Krankheitserreger nachgewiesen oder vermutet. Gegen den Hintergrund einer möglichen EU-Regulierung von Nutzorganismen, einschliesslich Starterkulturen, bei Lebensmitteln, werden aktuelle Informationen zum technischen Einsatz und Fragen zu möglichen Risiken bei deren Verwendung, entweder als Starter- oder Schutzkulturen oder als Probiotika, dargestellt und diskutiert. Es wird u.a. auch auf die derzeit noch fehlende Standardisierung bei der Bestimmung von Antibiotikaresistenzen bei Milchsäurebakterien (ausser *Enterococcus* und *Streptococcus)* eingegangen, wobei die Unterscheidung zwischen übertragbaren und konstitutiven Resistenzen unter Einsatz genetischer Methoden von grosser Bedeutung ist. Problematisch ist nach wie vor die Frage bzgl. «echter» Virulenzfaktoren, die z.B. bei den Enterokokken recht ausführlich untersucht worden sind, bei Laktobazillen aber bisher unbekannt sind.

Résumé

Du fait de leur implication dans la fabrication de nombreux aliments fermentés traditionnels, la plupart des bactéries lactiques sont généralement considérées et reconnues sécuritaires pour la consommation humaine. Parmi les exceptions à cette règle, on retrouve la plupart des espèces du genre *Streptococcus* et, avec certaines limitations, quelques espèces du genre *Enterococcus.* Cependant, certaines souches appartenant aux genres *Lactobacillus, Pediococcus* et *Leuconostoc* ont été dans de rares cas impliquées dans des infections ou présumées infectieuses. Dans la perspective d'une réglementation possible des souches microbiennes pour des usages techniques en alimentation, incluant les cultures starters, protectrices et probiotiques, nous présentons et discutons les informations pertinentes relatives à ces applications et aux risques possibles associés. En particulier le manque de standardisation pour les tests de résistance aux antibiotiques est souligné pour la plupart des bactéries lactiques (à l'exception des genres *Enterococcus* et *Streptococcus).* Ainsi, une distinction claire devrait être faite pour une résistance constitutive (intrinsèque) et transférable, aussi en utilisant des méthodes génétiques. Un autre problème est celui des facteurs de virulence « typiques » qui ont été largement étudiés pour les entérocoques, alors qu'aucun tel facteur n'a été à ce jour démontré chez les lactobacilles.

Key words
lactic starters, safety, probiotics, antibiotic resistance, virulence

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