31 Safety Assessment of Probiotics

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31.1 Introduction

Viable microbes have been a natural part of human diet throughout the history of mankind. Today, different fermented foods and other foods containing live microbes are consumed around the world, including industrialized countries, where the diet has become increasingly sterile during the last decades. By definition, probiotics are viable microbes with documented beneficial effects on host health. Probiotics have an excellent safety record, both in humans and in animals. Despite the wide and continuously increasing consumption of probiotics, adverse events related to probiotic use are extremely rare. Many popular probiotic strains such as lactobacilli and bifidobacteria can be considered as components of normal healthy intestinal microbiota, and thus are not thought to pose a risk for the host health – in contrast, beneficial effects on health are commonly reported. Nevertheless, the safety of probiotics is an important issue, in particular in the case of new potential probiotics which do not have a long history of safe use, and of probiotics belonging to species for which general assumption of safety cannot be made. Furthermore, safety of probiotics in high-risk populations such as critically ill patients and immunocompromized subjects deserves particular attention, as virtually all reported cases of bacteremia and fungemia associated with probiotic use, involve subjects with underlying diseases, compromised immune system or compromised intestinal integrity.

Several approaches to the evaluation of the safety of probiotics have been applied. Assessment of the safety of a probiotic begins with the correct identification of the strain. Laboratory tests applied in the safety assessment of probiotics include *in vitro* assays assessing different intrinsic properties of the strains such as resistance to antibiotics or production of toxic metabolites, and different animal models, which can be used to evaluate the potential of

probiotics to translocate from the host's gut into the host's bloodstream and tissues, or assess the infectivity of the probiotics in different disease models. In addition, the safety of probiotics may be evaluated in clinical trials. In this review, the different approaches for the safety evaluation of probiotics are reviewed. In addition, the adverse events associated with probiotic use to date are outlined, and the factors affecting the likelihood of adverse events are discussed.

31.2 Taxonomy and Identification as the Basis of Safety Evaluation

The study of taxonomy comprises of different sub-disciplines including classification, identification and nomenclature. Classification assigns microorganisms to a known taxonomic group (taxa) according to the similarity between the microorganism and other members of the taxa, allowing the prediction of the properties of the microorganism based on what is already know on the taxa. Reliable identification confirms the identity of a microorganism, for example a strain isolated from fermented milk. Nomenclature, which includes assigning names to taxonomic groups and specific microorganisms, allows not only scientific communication but also proper labeling of products containing probiotic microorganisms (Felis and Dellaglio, 2007). Reliable labeling of probiotic products requires both correct identification of the bacterial species and strain used and use of up-to-date nomenclature. Establishing the identity of microorganisms constitutes the first step for the assessment of their safety. In fact, the Qualified Presumption of Safety (QPS) approach, recently established by the European Food Safety Agency (EFSA), considers identification the first pillar of the safety assessment of microorganisms (EFSA, 2007). In this respect, a FAO-WHO expert group recommended that phenotypic tests should be conducted first, followed by genetic identification, using methods such as DNA/DNA hybridization, 16S RNA sequencing or other well-established methods (FAO/WHO, 2006). The availability of such methods makes the improper identification and labeling of probiotics unacceptable. Failure to properly identify the strains may lead to the inclusion of potentially harmful microorganism in the food chain.

Many different microorganisms are being used as probiotics, including both gram-positive and gram-negative bacteria. Most of the currently used probiotics belong to the genera *Lactobacillus* and *Bifidobacterium,* which are two genera of gram-positive, non-sporeforming microorganisms. Lactobacilli are generally

aerotolerant whilst bifidobacteria are anaerobes. The genus *Bifidobacterium* shares phenotypic features and habitat with many lactobacilli and other lactic acid bacteria (LAB), and for practical reasons some authors have considered this genus to be part of the LAB. However, they are phylogenetically distinct, with bifidobacteria having DNA with high guanine and cytosine (G + C) content (55–67%) and belonging to the phylum *Actinobacteria*, whilst LAB form part of the so-called *Clostridium* branch of the phylum *Firmicutes*, and are characterized by a low G + C content. In addition, bifidobacteria possess a particular metabolic pathway for hexose fermentation, characterized by the fructose-6-phosphate phosphoketolase (F6PPK) enzyme activity. Determination of F6PPK constitutes a reliable test for the identification of the family *Bifidobacteriaceae* (Felis and Dellaglio, 2007). The F6PPK pathway leads to the production of acetic and lactic acid in a ratio of 3:2, whilst in LAB the major end product of sugar fermentation is lactic acid (Felis and Dellaglio, 2007).

Proper strain identification constitutes a critical starting point for probiotic studies. Special attention should be paid to the strain identification, as a number of studies have reported that the identity of microorganisms isolated from probiotic products often does not correspond to the information stated on the product label (Gueimonde et al., 2004; Hamilton-Miller et al., 1999). In fact, a recent EU-funded project showed that 28% of the commercial probiotic cultures were misidentified already by their manufacturers or distributors, which may partly explain the disagreements observed between the label information and the true identity of the isolated microorganisms in many products (Huys et al., 2006). Accurate and reliable identification of probiotic strains is thus necessary to evaluate both the documented health benefits and the safety of probiotic products, and to avoid the inclusion of potentially pathogenic microorganisms in commercial products. Pathogenic microorganisms can be found all around the domain Bacteria, indicating the lack of common "pathogenicity" determinants and making the identification of all the potentially pathogenic microorganisms difficult. It is therefore important to clearly identify the pathogenicity traits associated with a specific microorganism. In some studies similar properties have been found between clinical isolates and commercial probiotic strains (Ouwehand et al., 2004a, b), indicating that not only bacterial factors, but also factors associated with the host play a role in pathogenicity. In this context, it is necessary to clearly identify the possible risks associated with each probiotic strain, as different strains can possess different characteristics. The first step in identifying the possible risks is the proper identification of the strain, which allows the preliminary establishment of the potential risks of the strain based

on the previous knowledge on the corresponding taxonomical unit. An example of misidentification and possible deliberate mislabeling of a probiotic is that of *Bacillus coagulans*, which in some products may be labeled according to old and long outdated nomenclature as *Lactobacillus sporogenes*. It is widely known that the correct identification of this strain is *Bacillus coagulans*, but despite this, the old and incorrect nomenclature of *L. sporogenes* is continuously used in many products sold as "probiotics." Given the long and good safety record of *Lactobacillus* and the lack of safety assessments of *Bacillus coagulans*, it is possible that the incorrect nomenclature is sometimes used on purpose to benefit from the safety and efficacy status of members of the genus *Lactobacillus* (De Vecchi and Drago, 2006). This example highlights the importance of proper identification and labeling of probiotic products.

Traditional phenotypic identification of probiotic bacteria can be tedious and not always reliable, since certain species cannot be distinguished by these methods. Molecular techniques have emerged in recent years to replace or complement the traditional phenotypic tests for the identification and comparison of strains of probiotic bacteria. Two strains are considered to belong to the same species if their DNA-DNA relatedness is 70% or higher. The DNA-DNA hybridization method has become the gold standard for the determination of bacterial identity. However, this method is laborious and difficult to perform and hence expensive, and therefore not suitable for large scale typing. Phylogenetic approaches such as comparison of DNA sequences have therefore become commonly used frequently techniques in bacterial identification. Amongst the sequence-based methodologies, sequence analysis of the 16S rRNA gene and the 16–23S internally transcribed spacer regions have proven to be useful tools for bacterial identification. In general, if two microorganisms share a 16S rRNA gene homology higher than 97%, they are considered to belong to the same species. Nevertheless, it is important to underline that in some cases the 16S rDNA sequencing has limited resolution and is not enough for discrimination of closely related species, some of which are frequently used in probiotic preparations (Felis and Dellaglio, 2007; Vankerckhoven et al., 2008b). Among lactobacilli, the most complex groups to identify are the *Lactobacillus delbrueckii*, the *L. casei*, and the *L. plantarum* groups. Within the genus *Bifidobacterium* the most challenging groups are *B. animalis* and *B. longum*. The 16S rDNA sequences do not allow proper identification within these groups, and therefore complementary information may be required by using other molecular methods. For example, the sequencing of certain protein-encoding genes may be of help in the development of standardized methods for identification.

In the future, the increasing availability of genome sequences will allow genome-wide and/or multilocus phylogenetic analysis. It is important to point out that when comparing a gene sequence with sequences found in the databases, the quality (number, accuracy and proper identification of the microorganisms) of the sequences deposited in the database has a great impact on the accuracy of the identification. To this regard, the EU-funded project PROSAFE concluded that biochemical tests should not be used as a stand-alone approach for identification of probiotic cultures. The use of 16S rRNA gene sequence analysis was considered the best tool for routine determinations but it was also underlined that public sequence databases contain unreliable, poorly documented or incomplete sequence entries, and the need for a list of validated complete 16S rRNA gene sequences for the purposes of identification was recognized. Moreover, the use of sequence-based methods was encouraged given the high reproducibility and data exchangeability of these techniques (Vankerckhoven et al., 2008b).

Correct identification of the probiotic species used is of critical importance but it is very important to keep on mind that the safety aspects of probiotics are often strain-specific. Highly discriminatory molecular methods, such as randomly amplified polymorphic DNA (RAPD), amplified rDNA restriction analysis (ARDRA), repetitive DNA element-PCR (rep-PCR), or pulsed field gel electrophoresis of macrorestriction fragments (PFGE) among others, are also available for strain characterization (genetic typing) (Huys et al., 2006). DNA macrorestriction followed by PFGE is considered to be the gold standard (FAO/WHO, 2006) and has been used for differentiating commercial probiotic strains (Gueimonde et al., 2004). Moreover, it is widely recognized that the comparison of the results obtained by using different molecular methodologies (polyphasic approach) is the best way to establish strain identity.

It is clear that strains used by the food industry and scientists should be identified using molecular methods and up-to-date taxonomical nomenclature. Importantly, the manufacturers of probiotic products have the responsibility on the product composition. It is also important to make all relevant strains easily available in international culture collections to all research groups participating in the assessment of the health effects, the safety and the mechanisms of probiotics. The FAO-WHO working group on probiotics strongly urged for the deposit of probiotic strains in internationally recognized culture collections (FAO/WHO, 2006). Even today, many scientific articles are published with no access data for the tested strains or sometimes even without mentioning the strain designation, which hampers the progress of scientific development in this area.

31.3 *In Vitro* Safety Assessments of Probiotics

In vitro assessments offer means to investigate the safety of probiotics based on the intrinsic properties of the strains. *In vitro* safety assessments should always precede the use of potential probiotic strains in animals and in humans. Assessment of the antibiotic resistance determinants of potential and established probiotic strains has received much interest, but also other *in vitro* assessments targeting the safety aspects of probiotics have been proposed. It should be noted however that classical risk assessments commonly used for pathogens may not always be directly applicable for probiotic strains such as lactobacilli and bifidobacteria, which become members of the normal healthy intestinal microbiota soon after birth and are also components of normal human diet (Borriello et al., 2003). In pathogens, pathogenicity is normally a consequence of several properties of the strain. The presence of such a property in a strain of low infective potential and low clinical significance does not necessarily imply that the strain has pathogenic potential or poses a risk to health under certain conditions (Borriello et al., 2003). An example of this is the ability to adhere to human mucosa, which is a virulence factor in the case of true pathogens, but is also an essential feature of many commensal microbes with very low pathogenic potential. To date, no clear virulence factors similar to those associated with pathogenic microorganisms have been identified for lactobacilli (Vesterlund et al., 2007) or bifidobacteria (Ouwehand et al., 2004a). Screening for the presence of such virulence factors is more applicable for genus such as *Enterococcus* and *Bacillus*, which include known pathogenic organisms but also some strains which have been proposed as probiotics (Eaton and Gasson, 2001). Here, the *in vitro* assessments used in the safety assessments of probiotics are reviewed. Examples of such assessments are listed in *Table 31.1*.

31.3.1 Antibiotic Resistance of Probiotics

One of the main targets of the *in vitro* safety assessments of existing and potential probiotic strains is the determination of antibiotic resistance properties. Resistance of a probiotic strain to a certain antibiotic is clinically relevant only in the case of infections, and infections related to probiotics are extremely rare. The presence of antibiotic resistance genes in the probiotic genomic content is not a safety concern in itself, as long as the genes are not mobilized and transferred to other bacteria. Theoretically, probiotics possessing antibiotic resistance genes

Table 31.1

Proposed *in vitro* safety assessments of probiotics

Assessment	Notes
Presence of antibiotic resistance genes	Commonly used safety assessment
Mobility of antibiotic resistance genes	Commonly used assessment; particularly relevant for *Enterococcus*
Adhesion to host tissues	Not recommended as part of safety assessment (Vankerckhoven et al., 2008b)
Resistance to host defense mechanisms	Commonly used safety assessment (Vesterlund et al., 2007)
Presence of virulence genes and toxic metabolites	Particularly relevant for *Bacillus* and *Enterococcus* (Tompkins et al., 2008)
Hemolysis	Very rare among probiotics (Vesterlund et al., 2007)
Bile salt deconjugation	Irrelevant as safety measurement (Vankerckhoven et al., 2008b)
Presence of macrocapsules	Rarely used safety assessment (Baumgartner et al., 1998)

could serve as a reservoir of resistance for potential pathogens. Therefore, microorganisms intended for use as probiotics have to be systematically screened for antibiotic resistance susceptibility in order to avoid the transfer of antibiotic resistance genes, since the ability of these determinants to transfer in the food and gut environment has been demonstrated. However, the current methodologies may not always unequivocally demonstrate the absence of transfer, and it should be noted that the transfer rates can be completely different under *in vitro* and *in vivo* conditions. Thus, it is of great interest to investigate whether probiotics can act as reservoirs for antibiotic resistance genes, from which they could be spread to opportunistic or pathogenic bacteria.

The EFSA considers that the nature of any antibiotic resistance determinant present in a candidate microorganism for QPS status evaluation needs to be determined. However, antibiotic resistance per se is not a safety issue; it only becomes a safety issue when horizontal transfer is concerned (EFSA, 2008). Currently, it is generally accepted that the possibility of transfer is related to the genetic basis of the resistance mechanism, i.e., whether the resistance is intrinsic, acquired as a result of a chromosomal mutation(s), or acquired by horizontal gene transfer. Intrinsic (or natural) resistance is inherent to a bacterial species or genus. Such is the case of the vancomycin resistant phenotype of some lactobacilli, the best characterized intrinsic resistance among LAB. In certain

Lactobacillus species, such as *L. casei, L. rhamnosus* and *L. plantarum,* the terminal D-alanine residue of the muramyl pentapeptide in the cell wall is replaced by D-lactate, thus preventing vancomycin binding (Delcour et al., 1999). For probiotic use, intrinsic resistance might be clinically relevant in some cases of *Lactobacillus*-related bacteremia (Cannon et al., 2005). In addition, chromosomal mutations leading to antibiotic resistance phenotypes have been described in lactobacilli. A single A-to-G transition mutation in the 23S rRNA gene reduces drastically the affinity of erythromycin for the ribosome. Such mutation has been suggested as the most plausible cause of macrolide resistance in a strain of *Lactobacillus rhamnosus* (Florez et al., 2007). In this respect, the transfer risk is considered to be very low for intrinsic resistance or acquired resistance due to chromosomal mutation(s).

Horizontally transferred antibiotic resistance genes, particularly those carried within mobile genetic elements, are the most likely to be transmitted between different microbes and thus deserve particular attention. A major step in the differentiation between the intrinsic and the acquired antibiotic resistance in probiotic bacteria is the determination and the comparison of antibiotic susceptibility patterns of representative numbers of different strains from each species. Unfortunately there is still a lack of agreement on the resistance susceptibility breakpoints for most antibiotics in lactobacilli and bifidobacteria. This is mainly due to the multiplicity of methods used, which include antimicrobial gradient strips, agar dilutions, disc diffusions, microbroth cultures, and others, and to the lack of standardized guidelines. However, major advances in this field have been achieved during recent years in order to harmonize methods for antimicrobial susceptibility testing in probiotics, and new susceptibility breakpoints for some species of *Lactobacillus* and *Bifidobacterium* have been proposed (Florez et al., 2008b; Klare et al., 2007; Mättö et al., 2007). Also, with the help of new molecular biology methods, such as microarray analysis and various PCR techniques, the genetic basis responsible for the acquired resistance phenotypes is beginning to be elucidated. The essay reviews the current evidence on antibiotic resistance determinants of probiotics, and their potential importance in the safety assessment of probiotic bacteria used in human and animal feed.

31.3.1.1 Antibiotic Resistance in *Lactobacillus*

In regard to antibiotics acting on cell wall, lactobacilli are usually sensitive to penicillin and β-lactamase inhibitors, but more resistant to cephalosporins. Many

Lactobacillus species show a high level of resistance to vancomycin, as previously mentioned. Also, most inhibitors of nucleic acid synthesis seem to have a low inhibitory effect among the majority of *Lactobacillus* species. On the other hand, lactobacilli are generally susceptible to low concentrations of many inhibitors of protein synthesis, such as chloramphenicol, macrolides, lincosamides, and tetracycline, but their resistance to aminoglycosides is often higher. Resistance to other antibiotics varies greatly among lactobacilli.

Several genes responsible for atypical antibiotic resistance properties among lactobacilli have been identified. Chloramphenicol resistance genes (*cat*; chloramphenicol acetyltransferases) have been identified in *L. acidophilus, L. delbrueckii* subsp. *bulgaricus* (Hummel et al., 2007) as well as in *L. reuteri* (Lin et al., 1996) and *L. plantarum* (Ahn et al., 1992). In addition, erythromycin resistance genes, responsible for the macrolides, lincosamides, and streptrogramins (MLS) resistance phenotype, have been identified in several *Lactobacillus* species (› *Table 31.2*), with the *erm*(B) gene, which encodes a rRNA methylase acting on the 23S ribosomal subunit, being the most frequent of such genes. The presence of genes coding for macrolide efflux pumps, such as *mef*(A), has also been reported (Cauwerts et al., 2006), as well as genes for lincosamide transferase (*lnu*(A)) (Cauwerts et al., 2006) and streptogramin A acetyltransferases (*vat*(E)) (Gfeller et al., 2003). However, the most common resistance determinants found in lactobacilli are the tetracycline resistance genes, and to date at least 11 different tetracycline resistance genes have been detected among lactobacilli, including genes coding for ribosomal protection proteins (*tet*(W), *tet*(M), *tet*(S), *tet*(O), *tet*(Q), *tet*(36), *tet*(Z), *tet*(O/W/32/O/W/O), *tet*(W/O)) and tetracycline efflux pumps (*tet*(K) and *tet*(L)) (› *Table 31.2*). Some strains were even found to harbor various tetracycline resistance determinants (Ammor et al., 2008b). On the other hand, aminoglycoside resistance genes, such as *aac*(6′)-*aph*(2″), *ant*(6), and *aph*(3′)-IIIa, *aph*(E) or *sat*(3), and β-lactam resistance genes (*blaZ*) were found much less frequently in lactobacilli (Aquilanti et al., 2007; Rojo-Bezares et al., 2006). It is important to point out that many of the genetic determinants mentioned above are sometimes found in potentially mobile elements, such as transposons and plasmids, which may spread the antibiotic resistance genes mainly by conjugation mechanisms. The localization of these genes within the genome, the nucleotide content, and the analysis of the flanking regions surrounding the antibiotic resistance genes may yield important clues to the acquisition process of these determinants, and their source or origin (Aquilanti et al., 2007; Florez et al., 2006; van Hoek et al., 2008a). Remarkably, some of these genes have been found to be transferred *in vitro* between strains of *Lactobacillus* but also

Table 31.2

Examples of the main antibiotic resistance determinants identified and characterized in lactobacilli and bifidobacteria

Gene(s)	Resistance phenotype	Mechanism of action	Location (when studied)	References
Lactobacillus				
erm(B)/*erm*(C)/*erm* (T) *erm*(LF)/*erm* (GT)	MLS	Ribosomal methylation	Plasmid transposon chromosome	Ammor et al. (2008a), Aquilanti et al. (2007), Cauwerts et al. (2006), Gfeller et al. (2003), Hummel et al. (2007), Klare et al. (2007), and Tannock et al. (1994)
mef(A)	Macrolide	Efflux	–	Cauwerts et al. (2006)
Cat	Chloramphenicol	Antibiotic acetylation	Plasmid	Ahn et al. (1992), Hummel et al. (2007), and Lin et al. (1996)
tet(W)/*tet*(M)/*tet*(S) *tet*(O)/*tet*(Q)/*tet* (36) *tet*(Z)/*tet* (W/O) *tet*(O/W/32/O/W/O)	Tetracycline	Ribosomal protection	Plasmid transposon chromosome	Ammor et al. (2008a), Ammor et al. (2008b), Aquilanti et al. (2007), Klare et al. (2007), and van Hoek et al. (2008b)
tet(K)/*tet* (L)	Tetracycline	Efflux	Plasmid	Ammor et al. (2008b), Aquilanti et al. (2007)
Bifidobacterium				
tet(W)/*tet*(M)/*tet* (O) *tet*(W/32/O)/*tet* (O/W)	Tetracycline	Ribosomal protection	Chromosome	Ammor et al. (2008a), Florez et al. (2006), Kazimierczak et al. (2006), van Hoek et al. (2008b)
tet(L)	Tetracycline	Efflux	Chromosome	van Hoek et al. (2008b)
erm(X)	MLS	Ribosomal methylation	Transposon	van Hoek et al. (2008a)

from lactobacilli to different Gram-positive bacteria, including food pathogens, such as *Staphylococcus* (Tannock et al., 1994). On the other hand, lactobacilli may be able to acquire antibiotic determinants from other Gram-positive bacteria (Vescovo et al., 1983). In addition to *in vitro* studies, the potential risks associated with lactobacilli carrying transferable antibiotic have also been demonstrated in experimental animal modes (Mater et al., 2008). The transfer of these

determinants may be enhanced in the presence of antibiotic selective pressure (Feld et al., 2008). Taken together, these results support the hypothesis of the resistance gene reservoir within intestinal bacteria, and their role as traffickers in antibiotic resistance genes.

31.3.1.2 Antibiotic Resistance in *Bifidobacterium*

Most *Bifidobacterium* species are resistant to aminoglycosides, metronidazole and Gram-negative spectrum antibiotics. They are also intrinsically resistant to mupirocin, an antibiotic that is being used in the selective isolation of this genus. In contrast, bifidobacteria are very susceptible to macrolides/lincosamides, vancomycin, rifampicin, spectinomycin, chloramphenicol, and β-lactams. The susceptibility to tetracyclines and cephalosporins varies widely among strains (Zhou et al., 2005a). Compared to lactobacilli, the data on antibiotic resistance determinants in bifidobacteria are much scarcer. In the case of the macrolide resistance determinants, the presence of the gene *erm*(X) has been described in *B. animalis* subsp. *lactis* and in *B. thermophilum*. This resistance determinant was part of transposon Tn*5432* that has been detected in several opportunistic pathogens (van Hoek et al., 2008a). Also, multidrug resistance transporters able to confer erythromycin resistance have been described in *B. longum* and *B. breve*, although their contribution to a macrolide resistance phenotype is supposed to be very limited (Margolles et al., 2005). Tetracycline resistance in this genus deserves a separate mention. Current knowledge suggests that a potential concern for the safe use of *Bifidobacterium* probiotic strains is the presence of tetracycline resistance genes, especially *tet*(W), although other genes, such as *tet*(M), *tet*(O), *tet*(L), *tet*(W/32/O), and *tet*(O/W) have been detected, albeit much less frequently (Ammor et al., 2008a; Florez et al., 2006; Kazimierczak et al., 2006; van Hoek et al., 2008b). Several studies have shown a high frequency of positive isolates of these determinants in human isolates, with some strains containing up to three different *tet* genes (Florez et al., 2006; van Hoek et al., 2008b). In bifidobacteria *tet* genes seem to be integrated in the chromosome, and thus far they have not been found to be associated with transposons or plasmids, but they are very often flanked by putative transposase genes (Florez et al., 2006; Kazimierczak et al., 2006; van Hoek et al., 2008b). Transposases are enzymes that catalyze the movement of DNA segments among different locations by recognizing insertion sequences in the DNA, and they are thought to be involved in the mobilization of *tet*(W) genes in bifidobacteria. In fact, chromosomically encoded *tet*(W) have

been shown to transfer at low frequency from *B. longum* to *B. adolescentis in vitro*, and the site of chromosomal insertion in the *B. adolescentis* transconjugant was shown to be identical to that of the donor strain, consistent with a transposase-mediated site-site specific insertion event (Kazimierczak et al., 2006). However, transfer within the genus *Bifidobacterium* is not a safety concern; more concerning would be transfer to other genera or even worse to pathogens, but currently there are no indications that such transfer is likely to occur.

31.3.1.3 Antibiotic Resistance in Other Probiotic Species

Currently, different species of bifidobacteria and lactobacilli are the most commonly used probiotics, but other bacteria as well as the probiotic yeast *Saccharomyces boulardii* (*Saccharomyces cerevisiae*) are also used as probiotics. *Saccharomyces* is a member of the domain Eukaryota, and hence naturally resistant to all antibiotics. In the case of *Saccharomyces*, resistance to fungicides is more relevant. *Lactococcus* and *Pediococcus* are LAB present in the commensal intestinal flora of humans and animals. Strains of these genera are frequently used as large-scale starter cultures in the food industry (Klare et al., 2007), and have also been proposed as potential probiotics. Genes conferring resistance for chloramphenicol, tetracycline, erythromycin and streptomycin have been found in different *Pediococcus* species (Danielsen et al., 2007; Hummel et al., 2007; O'Connor et al., 2007; Rojo-Bezares et al., 2006). Remarkably, a plasmid from *P. acidilactici* encoding resistance to clindamycin, erythromycin [*erm*(B)] and streptomycin (*aadE*) has been shown to be able to replicate in *Lactococcus* and *Lactobacillus* species. Moreover, the gene *aadE* was 100% identical to an *aadE* gene found in a *Campylobacter jejuni* plasmid, suggesting a recent horizontal gene transfer event between Gram-positive and Gram-negative intestinal bacteria (O'Connor et al., 2007). Relating to *Lactococcus* strains, in Perreten et al. (1997) described a *Lactococcus lactis* strain resistant to streptomycin, tetracycline and chloramphenicol isolated from a raw-milk cheese. The three resistances were encoded by three different genes, located in a multi-antibiotic resistance plasmid, and these genes were almost identical to others previously found in *Staphylococcus aureus* and *Listeria monocytogenes*. This was the first strong evidence that antibiotic resistance can be spread in a food environment (Perreten et al., 1997). Since then, many genes coding for proteins conferring resistance to several antibiotics, mainly tetracycline and erythromycin, have been described in *Lactococcus lactis* (Ammor et al., 2008a;

Aquilanti et al., 2007), and transfer from *Lactococcus* to other bacteria, including Gram-positive pathogens, as well as the acquisition of resistance genes, has been demonstrated (Florez et al., 2008a). Probiotic strains of different *Bacillus* species have been proposed (Duc et al., 2004), and antibiotic susceptibility patterns of potential probiotic *Bacillus* strains have been determined (Sorokulova et al., 2008; Tompkins et al., 2008). Antibiotic resistance of *Bacillus clausii* to certain antibiotics has been shown to be chromosome-encoded and not linked to transferable genetic elements (Girlich et al., 2007), thus suggesting a low transfer possibility. Antibiotic resistance assessment has also been applied to study the safety of a probiotic strain of *Streptococcus salivarius*, intended for an application in the oral cavity (Burton et al., 2006).

A major issue of concern is the safety of cultures containing enterococci. These LAB constitute a significant percentage of probiotics in the worldwide market. However, in recent years, the genus *Enterococcus* has become increasingly relevant clinically, due to its increasing incidence as a cause of diseases, mainly in nosocomial infections. Further concerns on the safety of *Enterococcus* have been raised because of the widespread distribution of transferable virulence factors among the genus, and because antibiotic therapies are being compromised by evolving antibiotic resistance, and therefore the antibiotic susceptibility profiles in enterococci have been extensively studied and numerous resistance determinants have been identified (Eaton and Gasson, 2001; SCAN, 2003; Vankerckhoven et al., 2008b). Enterococci are able to acquire high-level drug resistance through horizontal gene transfer. Examples of acquired resistance genes by enterococci include those that confer resistance to tetracycline, aminoglycoside, macrolide, streptogramin and chloramphenicol, with resistance to vancomycin being the most clinically relevant (Florez et al., 2008a; SCAN, 2003). Often, these resistance genes are mobilized via transposons or plasmids. Furthermore, transfer among *Enterococcus* strains, and from *Enterococcus* to other Gram-positive pathogens has been reported (Lester et al., 2006), stressing the need for a careful and rigorous examination of the antibiotic resistance of *Enterococcus* strains intended to be used as probiotics.

31.3.1.4 Summary of Antibiotic Resistance of Probiotics

In summary, the potential ability of probiotic strains to transfer antibiotic resistances to pathogenic bacteria in the food and gut environment should be taken into account in the safety assessment of probiotics. Bacterial products

intended for use as food and feed additives must be examined to determine the susceptibility of the strain(s) to a relevant range of antimicrobials, starting from appropriate *in vitro* tests. The detection of minimal inhibitory concentrations above the breakpoint requires further investigations to make the distinction between acquired and intrinsic resistance. When a strain of a typically susceptible species is resistant to given antibiotic, the presence of acquired resistance determinants is indicated, and clearly the presence of these genes in mobile genetic elements presents the highest risk for lateral spread (EFSA, 2008; SCAN, 2003). In this respect, the scientific community must provide clear and convincing evidence to establish a risk assessment of antibiotic resistant probiotics. Currently, many open questions regarding the antibiotic resistance of probiotics remain. Numerous discrepancies have been encountered between the available phenotypic data and the genetic basis of the resistance. For example, in susceptible strains, antibiotic resistance determinants are sometimes detected. On the other hand, in atypically resistant strains, such determinants are not always detected. This suggests the existence of novel resistance genes which have escaped the detection, or the presence of silent genes that may be activated under specific conditions. In fact, recent studies have shown that gastrointestinal conditions may induce the appearance of antibiotic resistance (Noriega et al., 2005), and a higher proportion of tetracycline-resistant bifidobacteria has been detected during antibiotic/probiotic intervention in humans (Saarela et al., 2007). Also, it appears that the gastrointestinal tract may comprise a more favorable environment for antibiotic resistance transfer than conditions provided *in vitro* (Feld et al., 2008). Thus, future *in vivo* experiments should shed some light on the transfer events occurring from, via, or to probiotics. However, to put the risks associated with antibiotic resistance of probiotics into context, it should be noted that antibiotic resistance is not a property of probiotic strains alone and for example wild-type strains of lactobacilli and bifidobacteria also carry antibiotic resistance genes similar to those of the probiotic isolates. Therefore, probiotic strains do not pose any more risk in this respect than the lactobacilli and bifidobacteria occurring naturally in the human intestine.

31.3.2 Virulence Genes and Toxic Metabolite Production

The potential presence of virulence genes may raise concerns on the safety of certain microorganisms used as probiotics. For example, species belonging to the genus *Enterococcus* often harbor such genes (Vankerckhoven et al., 2008b).

Generally, *Enterococcus faecium* strains found in foods and used as probiotics are free from virulence determinants (Eaton and Gasson, 2001; Vankerckhoven et al., 2008a), while strains of *E. faecalis* typically possess multiple determinants (Eaton and Gasson, 2001). Tompkins et al. (2008) detected no virulence genes in a strain of *E. faecium* marketed as a probiotic. They did however detect a PCR product for the adhesion factor $efaA_{fm}$, but the role of this factor in virulence has not been clearly demonstrated. The potential presence of virulence determinants in *Enterococcus* strains proposed as probiotics is a potential risk factor and the safety of these strains requires critical evaluation. Certain strains of *Bacillus* are also being marketed as probiotics. Duc et al. (2004) demonstrated that three strains of *Bacillus cereus*, marketed as probiotics, produced enterotoxins, making them unsafe for human use. Toxin producers can be also found among strains of *Bacillus subtilis* (From et al., 2005), although not all *Bacillus subtilis* carry toxin genes (Tompkins et al., 2008). The presence of virulence factors has also been used to demonstrate the safety of other probiotic species. For example, Ouwehand et al. (2004a) detected no virulence factors in strains of *Bifidobacterium*, and Burton et al. (2006) detected no streptococcal virulence genes in a probiotic strain of *Streptococcus salivarius*.

Since many *Lactobacillus* species produce both L-lactic acid and D-lactic acid as their metabolic products, and since excessive D-lactic acid may cause D-lactic acidosis in certain high risk populations such as children with short-bowel syndrome, the safety of D-lactic acid producing probiotics in infant formulas has raised concerns. However, current evidence suggests that the D-lactic acid producing probiotics are safe to use also in infant formulas (Connolly and Lönnerdal, 2004). D-lactic acid is being effectively metabolized by humans, but in fact only little of the D-lactic acid produced in the gastrointestinal tract is absorbed by the host, as other bacteria in the gut quickly consume lactic acid to produce e.g., butyrate. Many of the naturally occurring microbes in the gut produce both D- and L-lactic acid, also in infants. The risk of D-lactic acidosis is limited to children with short-bowel syndrome, and no data suggest that the ingestion of DL-producing lactobacilli by healthy infants is by any account harmful (Connolly and Lönnerdal, 2004).

31.3.3 Adhesion of Probiotics to Host Tissues

Adhesion is considered an important mechanism for probiotic action, as it contributes to the ability of the beneficial strains to interact with the host,

remain temporarily colonized, and displace potential pathogens (Collado et al., 2007, 2008). However, in true pathogenic bacteria, adhesion is a negative trait which may be associated with the ability of the bacteria to translocate and to cause infection. Strains of *Lactobacillus* and *Bifidobacterium* are also known to adhere to human tissues, and adhesion to different host tissues has been proposed to be included in the *in vitro* safety assessment of these microorganisms (Harty et al., 1994). Many probiotic strains have good adherence to host mucus and intestinal epithelial cell lines as well as adhesion to extracellular matrix proteins such as fibronectin, fibrinogen and collagen (Schillinger et al., 2005). No difference was observed in the adhesion properties to host extracellular matrix proteins between fecal, blood and probiotic isolates of *Lactobacillus* (Vesterlund et al., 2007). However, blood isolates were more adherent to mucus compared to probiotic isolates. Blood culture isolates of *Lactobacillus* spp. have been reported to adhere to intestinal mucus in greater numbers than isolates from human feces or dairy products (Apostolou et al., 2001). However, adherence of bacteremia-associated *Lactobacillus* strains varies significantly between the isolates, suggesting that adhesion to mucus is not a prerequisite to *Lactobacillus* bacteremia and does not serve as a good marker of potential of *Lactobacillus* strain to cause bacteremia (Kirjavainen et al., 1999). Moreover, many widely used probiotic strains such as *B. animalis* subsp. *lactis* and *L. acidophilus* show good adhesion to host mucus *in vitro*, but have not been associated with cases of probiotic sepsis.

Vankerkhoven et al. (2007) found no differences in adhesion to fibrinogen, fibronectin, collagen and laminin between endocarditis and probiotic *L. rhamnosus* and *L. paracasei* isolates. Apart from one fecal isolate of *L. paracasei*, all tested lactobacilli adhered only weakly to immobilized host matrix proteins. Apart from strains of *Lactobacillus*, adhesion properties have also been included in the *in vitro* safety assessments of other strains such as bifidobacteria (Ouwehand et al., 2004a) as well as strains of *Enterococcus* and *Bacillus* (Tompkins et al., 2008). The ability of translocated bacteria to bind to fibrinogen may be more relevant in relation to the risk of endocarditis than binding to fibronectin (Vankerckhoven et al., 2007), but current evidence does not suggest that the adhesion to any of the host extracellular matrix proteins provides a good marker for the potential of *Lactobacillus* strains to cause bacteremia. The recent EU-PROSAFE project (Vankerckhoven et al., 2008b) concluded that currently, adhesion assays are not recommended as part of safety assessment of probiotics.

31.3.4 Platelet Aggregation

The ability to aggregate human platelets is considered a pathogenic trait among true pathogens. Platelet aggregation may be a relatively common trait among genus *Lactobacillus* (Harty et al., 1994). Some bacteremia-associated strains of *Lactobacillus* are able to aggregate platelets, while others are not, suggesting that platelet aggregation is neither a prerequisite of *Lactobacillus* bacteremia nor a good marker for the ability of these strains to cause bacteremia (Kirjavainen et al., 1999). The inability to induce human platelet aggregation has been used to demonstrate the safety of certain specific probiotic strains, including *L. rhamnosus* HN001 and *B. lactis* HN019 (Zhou et al., 2005b).

31.3.5 Hemolysis

Hemolysis is a known virulence factor among pathogenic microorganisms. Assessment of hemolytic activity has also been used in the *in vitro* evaluation of probiotic safety (Baumgartner et al., 1998). No evidence of hemolytic activity was found in fecal, blood and probiotic *Lactobacillus* strains (Vesterlund et al., 2007). Similarly, no hemolytic activity could be detected among strains of *Bifidobacterium* (Ouwehand et al., 2004a) or *L. rhamnosus* (Ouwehand et al., 2004b). However, some strains of lactobacilli express α-hemolysin (Baumgartner et al., 1998).

31.3.6 Resistance to Host Defense Mechanisms

Resistance to host defense mechanisms may enhance the survival of translocated microbes and increase the risk of infections, and *in vitro* assessments of host defense resistance have been applied in the safety assessment of probiotics. Probiotic lactobacilli have been found to be less resistant to intracellular killing by macrophages in cell culture than the clinical *Lactobacillus* isolates (Asahara et al., 2003). Moreover, similar differences were observed in the sensitivity of the strains to nitric oxide, a compound which plays a role in the killing of bacteria by macrophages. Notably, the study also suggested that differences in the sensitivity to host defense mechanisms exist between the different probiotic strains (Asahara et al., 2003). Vesterlund et al. (2007) assessed the ability to avoid the induction

of respiratory burst in peripheral blood mononucleocytes and the resistance to human serum of fecal, blood and probiotic isolates of lactobacilli. Probiotic *Lactobacillus* strains induced a lower respiratory burst in comparison to clinical *Lactobacillus* isolates, and tended to survive better in human serum in comparison to fecal isolates. Similar results were reported for strains of *L. rhamnosus* by Ouwehand et al. (2004b). Vankerckhoven et al. (2007) did not find differences between endocarditis and probiotic isolates in susceptibility to platelet microbicidal proteins. The above-mentioned factors and their relevance to the safety of probiotics may require further investigation. Resistance to serum by *L. rhamnosus* strains was earlier demonstrated by Baumgartner et al. (1998). Ouwehand et al. (2004a) investigated the resistance to the bactericidal effect of human serum and the induction of respiratory burst of strains of bifidobacteria, and concluded that these are unlikely risk factors for the genus *Bifidobacterium*.

31.3.7 Bile Salt Deconjugation

The role of bile salt deconjugation ability in the safety assessment of probiotics is controversial. While there are some implications that free bile acids may affect tumor promotion, there is insufficient evidence for the suggested harmful effects of free bile acids in general and no evidence suggesting that bile salt deconjugation by probiotics is harmful in humans (Vankerckhoven et al., 2008b). In fact, it has been suggested that bile salt deconjugation activity of probiotics may have beneficial effects on human health by lowering serum cholesterol. The EU-PROSAFE project concluded that bile salt deconjugation activity is irrelevant for safety assessment of probiotics (Vankerckhoven et al., 2008b).

31.3.8 Summary of *In Vitro* Assessment of Probiotic Safety

Several different *in vitro* approaches have been used in the safety assessment of probiotics (*Table 31.1*). *In vitro* tests assessing the resistance to antibiotics and the presence of mobile antibiotic resistance genes are common. Several studies have attempted to identify relevant virulence determinants for bacterial species used commonly as probiotics. However, to date such determinants have not been identified. Certain properties which are considered to be virulence

factors for true pathogens may be present in probiotic bacteria, but the presence of such factors (e.g., adhesion to host tissues) does not correlate with the infective potential of the probiotics. This is likely to result from the minimal infectivity of the probiotics in general. Nevertheless, certain *in vitro* measurements may be relevant in probiotic research, even in the case of organisms which are generally considered as safe. Certain microorganisms belonging to bacterial groups for which the general assumption of safety cannot be applied have also been suggested as potential probiotics. For such microorganisms, extensive *in vitro* safety evaluation is required, e.g., to determine the presence of virulent genes or transferable antibiotic resistance.

31.4 Animal Models in the Safety Assessment of Probiotics

31.4.1 Animal Models in Probiotic Research

Preclinical laboratory testing of the safety and efficacy of probiotics can be carried out using *in vivo* animal models. In contrast to *in vitro* assays, the *in vivo* models are dynamic systems in which the complex interactions between the administered probiotics and the host can be assessed in physiological environment. For scientific, regulatory and ethical reasons, studies using animal models should only be carried out following prior *in vitro* tests have been completed. *In vivo* testing of a probiotic strain is essential for scientific and regulatory purposes before the strain can be accepted for widespread use in humans or animals. *In vivo* models are important for studies in which different interactions between probiotics and the host, such as effects on host metabolism and immune system as well as distribution of probiotics following administration are investigated. Moreover, *in vivo* models are essential for safety studies, which may include studies on toxicity, bacterial translocation, and effects of probiotics in seriously ill and immunocompromized hosts. It is important to emphasize that experiments using animals are rigorously regulated by legislation, and they must be conducted humanely and only when similar results cannot be obtained by alternative methods. Similarly, *in vivo* testing should be conducted in animals with lowest degree of neurophysiologic sensitivity, and the lowest number of animals should be employed. Animal welfare and experimental procedures should be improved as much as possible to minimize animal distress and suffering and also to achieve good scientific practices.

Various animal models have been shown to contribute to our knowledge on the function of probiotics, and they have proved particularly useful in the investigation of mechanisms of action, effects on health, and safety of probiotics. The *in vivo* models used in probiotic research typically involve vertebrate laboratory animals, most commonly mice and rats. Also other animals such as pets, livestock or fish have been used in probiotic research, particularly in the field of veterinary science.

The choice and suitability of the animal models is influenced by numerous factors (and their interactions), including the animal species and strain used, the animal genotype and phenotype, the possible use of specific disease model, the number of animals required, the target outcomes, the choice of probiotic strains, and the quantity and quality of biological samples collected. When choosing animal models for probiotic research, the biological, physiological and genetic similarity between the model animal and the ultimately targeted host (e.g., human) should be evaluated. However, phylogenetic closeness is not always a guarantee for a valid model. The use of animal models to assess the safety of probiotics focuses on the microbe-host interactions. Microbiological factors, such as the microbiological quality of the animal facilities and feed are one of the most important factors to be considered by the scientists working with probiotics, because of these factors have the potential to confound and invalidate results and conclusions drawn from the animal experiments (Nicklas et al., 1999, 2002). Therefore, the use of *in vivo* models in probiotic research requires the selection of animals with high standards of microbiological quality. In order to achieve reliable, valid and reproducible experimental results, it is crucial that the microbiological status of the laboratory animal model is defined and free from unwanted microbial agents, such as viruses, mycoplasmas, bacteria, fungi and parasites specific for the animal species (i.e., SPF or specific pathogen free). Likewise, it is important to remark that most rodent infections are latent and do not to cause overt clinical symptoms, but are nevertheless capable of causing various degrees of abnormalities in the experimental results and increase biological variation. This may lead to the need to increase the number of animals used in order to counter the increased variation, which in turn has effects on the project cost as well as on the animal welfare.

The relevance and the extrapolation of the results obtained from animal studies to the ultimate target of probiotic use (e.g., human) depends on the choice of the animal model as well as other exogenous factors including the quality of the work, the targeted outcomes and the microbiological factors. At times, the data generated from animal models are not directly applicable

to the target host and *vice versa*. This is particularly true in microbiological models, where factors such as the high variability in species-specific responses and the differences between the compositions of the commensal microbiota of different species may not always allow the direct extrapolation of the results. Nonetheless, there are many published examples on probiotic research where health data generated from an animal model has been associated with similar outcomes in the ultimate target of probiotic use. However, careful assessment of animal experiment data and the relevancy to the targeted host is needed before the results may be extrapolated.

31.4.2 Examples of Probiotic Safety Assessments Using Animal Models

Various animal models have been used to assess the safety of probiotics (› *Table 31.3*). For the most commonly used probiotics, in particular lactobacilli and bifidobacteria, no clear virulence determinants have been identified, indicating general lack of pathogenicity. This makes the selection of *in vivo* models for safety assessment of probiotics challenging. Probiotic bacteria have a good safety record, but in rare cases these microorganisms have been isolated from infections in subjects with severe underlying diseases (Boyle et al., 2006). For this reason, most of the models used in probiotic safety assessment correspond to different

Table 31.3

Examples of animal models used in the safety assessment of probiotics

Animal model	Example
Healthy	Zhou et al. (2000b)
Neonatal	Lee et al. (2000)
Colitis	Daniel et al. (2006)
Infective endocarditis	Asahara et al. (2003)
Immunodeficient (congenital)	Wagner et al. (1997)
Immunodeficient (induced)	Zhou and Gill (2005)
Liver injury	Osman et al. (2005)
Acute pancreatitis	van Minnen et al. (2007)
Helminthic infections	Dea-Ayuela et al. (2008)
Intestinal resection	Mogilner et al. (2007)

disease models, both induced and spontaneous, and microbial translocation and/or organ colonization have been frequently used as the outcomes of these studies.

The risk of translocation associated with gut barrier disturbance, for example in the case of intestinal inflammation, has been studied extensively by using different models of induced colitis (Daniel et al., 2006; Pavan et al., 2003). In addition, healthy animals have been used in this respect, for example in the safety evaluation of the probiotic strains *L. rhamnosus* HN001, *L. acidophilus* HN017 and *B. lactis* HN019 (Zhou et al., 2000a, b). Also acute oral toxicity tests, using very high doses of probiotics, have been carried out (Kabeir et al., 2008; Tompkins et al., 2008; Zhou et al., 2000b). Immunocompromized animal models, of both adult and young animals, have also been used to evaluate the safety of probiotics. Congenitally immunodeficient animals (Wagner et al., 1997, 1998), and induced immunocompromized animal models (Dandekar et al., 2003; Zhou and Gill, 2005) are available. Many of the animal models applied in the safety assessment of probiotics were originally developed to study pathogenic microorganisms in which virulence traits are present and the infections caused by these pathogens, and therefore they may not be optimal for studying translocation of non-virulent microorganisms such as probiotics. Moreover, some of these models have been found to be resistant to translocation of ingested probiotics (Vankerckhoven et al., 2008b). The translocation ability of probiotics has also been assessed using neonatal animal models (Lee et al., 2000; McVay et al., 2008). Neonate animals may be considered to be immunocompromized due to the lack of properly established gut barrier function. Therefore, they may offer a good model for the determination of the safety of early probiotic intervention. In the light of the certain reports of probiotic sepsis in humans, it should be noted that the potential for probiotics to cause sepsis has also been observed in animal models. Wagner et al. (1997) colonized athymic mice with probiotic strains *L. reuteri, L. acidophilus* NCFM, *B. lactis* Bi-07 or *L. rhamnosus* GG (LGG). While athymic adult mice were not adversely affected by the probiotics, colonization with the probiotics *L. reuteri* and LGG did lead to death in some athymic neonatal mice, suggesting that the neonates with immune deficiency may be at elevated risk of probiotic sepsis.

Some cases of bacterial endocarditis due to lactobacilli have been reported in the literature (Salminen et al., 2004). This has drawn the attention of researchers to the identification of traits related with the ability to colonize heart valves. Animal models of induced experimental endocarditis are currently available (Gibson et al., 2007). Using one of these models it has been shown that lactobacilli are 100- to 10,000-fold less infective than the most common endocarditis pathogens;

Staphylococcus or *Streptococcus* strains. Most strains were even less infective than the non-pathogenic control *Lactococcus lactis* (Vankerckhoven et al., 2008b). Asahara et al. (2003) used rabbit experimental infective endocarditis model to demonstrate the safety of two probiotic strains *L. casei* Shirota and LGG, which were compared to endocarditis clinical isolates of *Staphylococcus*, *Streptococcus* and *Lactobacillus*. LGG was found to be more infective in the mouse model than *L. casei* Shirota, and this correlated with the *in vitro* ability of these strains to resist inactivation by host innate defense mechanisms. The colitis, immunocompromized and endocarditis models are the most commonly used disease models used in probiotic research, but many other models including induced acute liver injury models (Osman et al., 2005), a model for susceptibility to helminthic infections (Dea-Ayuela et al., 2008), models of interleukin deficient animals (Pena et al., 2005), or models of intestinal resection (Mogilner et al., 2007) have also been used. Animal models have also been used to investigate the possible transfer of antibiotic resistance genes between lactobacilli and other bacteria *in vivo* (Mater et al., 2008). Good results in animal models do not always correlate with good results in human clinical trials. Van Minnen et al. (2007) demonstrated the safety and the efficacy of a probiotic mix in a rat model of acute pancreatitis. The same probiotic mix was subsequently used in a human clinical trial assessing the efficacy of probiotics on severe acute pancreatitis. In the human study, compared to the placebo group, the rate of mortality was found to be higher in the group administered with probiotics (Besselink et al., 2008). This demonstrates that caution must be used when extrapolating the results obtained from animal disease models to humans. Moreover, it is important that the animal models used in the safety assessment reflect the real-life situations. In the case of the severe acute pancreatitis study, the animal model involved rats which were administered intragastrically with probiotics before the onset of pancreatitis (van Minnen et al., 2007), while in the human trial, the probiotic mixture was administered through nasojejunal tube to already gravely ill patients with severe complications (Besselink et al., 2008) (e.g., the organ failure rate was already high prior to the treatment in the probiotic group).

31.4.3 Concluding Remarks on Animal Models in the Safety Assessment of Probiotics

Animal models provide the opportunity to investigate many different aspects safety of probiotics (*Table 31.3*). Animal models are used to investigate

scientific questions which can not be answered by using *in vitro* or human trials. Examples of this include the studies focusing on the translocation of bacteria into host tissues, and acute toxicity tests. It is known that the extremely rare but clinically significant adverse effects related to probiotic use in humans are practically always associated with severe underlying diseases and compromised immune system of the host (Salminen et al., 2006). Animal models offer a way to study the safety of probiotics in severely ill hosts. When using the currently available animal models it should be keep on mind that most of them were developed to study virulence traits of pathogenic microorganisms, and caution is required when drawing conclusions from the studies with non-pathogenic microorganism, such as probiotics, in which the potential mechanisms of both beneficial and possible adverse effects but also the potential risks are different from pathogens. To date, the use of animal models has not revealed any specific virulent or pathogenic determinants among the different probiotic microorganisms studied, demonstrating the general safety of probiotics. Animal models designed specifically to assess the safety of probiotics should further be developed, to allow improved safety assessment of new and existing probiotic organisms.

31.5 Human Interventions in the Safety Assessment of Probiotics

Clinical trials assessing the safety of probiotics, along with the widespread and safe use of probiotics worldwide, constitute the most compelling evidence of the safety of probiotics. Clinical safety trials enable the *in vivo* evaluation of the effects of probiotics in humans in a controlled manner, with a special focus on attributes relevant to the safety of the administered probiotic and the factors contributing to possible adverse events. Clinical trials assessing the safety of new probiotics should be carried out following appropriate *in vitro* and animal model safety assessments, but preferably prior to introducing products containing the probiotic to market. However, many organisms belonging to *Lactobacillus* and *Bifidobacterium* are generally regarded as safe (EFSA, 2007), and therefore extensive studies on the safety of these strains are not always carried out. Outcome measures of clinical probiotic safety studies often include stool consistency, defecation frequency and gastrointestinal complaints (Mäkeläinen et al., 2003), as well as serum and immune markers and the frequency of adverse events. Apart from trials specifically designed for assessing the safety of the probiotic

administration, the clinical trials assessing the efficacy of probiotic treatments also contribute to the clinical evidence of probiotic safety. Although the main target outcomes in these probiotic intervention trials focus on the health benefits of probiotics, the safety of the probiotic administration and the potential adverse events are often reported as a secondary outcome (Kajander et al., 2008; Peng and Hsu, 2005; Rautava et al., 2002). In particular, safety aspects are of interest in trials involving diseased patients and other potential risk groups.

Clinical investigations of the safety of probiotics are commonly carried out with healthy volunteers. No adverse effects on gastrointestinal health were seen during probiotic administration of *Bifidobacterium longum* 46 and *B. longum* 2C to healthy volunteers (Mäkeläinen et al., 2003). Safety of the administration of high dose of *L. reuteri* ATCC 55730 (1×10^{11} cells/day) to healthy volunteers has been demonstrated (Wolf et al., 1995). Clinical trials have been conducted to demonstrate the safety of the strain *Streptococcus salivarius* K12 strain used as a probiotic targeted at oral health (Burton et al., 2006), and the lack of tetracycline resistance gene transfer during concomitant ingestion of *L. acidophilus* LA-CH5, *B. animalis* subsp. *lactis* Bb-12 and antibiotics (Saarela et al., 2007). Long term safety studies of probiotics are rare. Laitinen et al. (2005) assessed the effects of perinatal administration of LGG on the subsequent growth of children, and found the early probiotic administration to be safe.

Safety of the probiotics may be of particular interest in specific age groups, such as neonates, who have compromised immune system. In neonates and low-birth-weight infants, successful clinical interventions have been carried out (Agarwal et al., 2003; Hoyos, 1999) but serious adverse events have not been reported. Clinical trials suggest that probiotics are safe to use in follow-up formulas and growing-up milks (Haschke et al., 1998). Clinical evaluation of probiotics in elderly populations is of special interest, since elderly subjects commonly have health related problems including infections and gastrointestinal problems, and may also have altered dietary habits and gut microbiota composition compared to healthy adults. For the very same reasons, the elderly subjects in particular may benefit from the use of probiotics. The safety and the lack of adverse events following the consumption of *B. longum* 46 and 2C (Pitkälä et al., 2007) as well as other strains including *B. lactis* HN0019 and *L. rhamnosus* HN001 (Gill et al., 2001) by elderly subjects has been demonstrated.

As expected, the commonly used probiotics such as strains belonging to *Lactobacillus* and *Bifidobacterium* perform well in studies involving the safety assessment of probiotics in healthy volunteers. Clinical intervention studies and the widespread and long-term consumption of fermented foods and probiotic

products clearly demonstrate the safety of probiotic administration in general population. However, the possible risks associated with probiotics may be elevated in certain high risk populations, and the demonstration of the safety of a certain probiotic strain in general population does not necessarily imply that the administration of the same strain is equally safe in high risk populations. A limited number of clinical safety assessments of probiotics have been conducted in high risk populations. In a clinical trial to assess the safety of enteral administration of *L. casei* Shirota to critically ill children, no evidence was found of bacteremia or colonization of the probiotic in surface swabs from various sites, endotracheal aspirates, sputum, blood, urine, cerebrospinal fluid, sterile body fluid samples or tip cultures of arterial and long venous line catheters (Srinivasan et al., 2006). Probiotics have also been used successfully in patients with necrotizing enterocolitis (Bin-Nun et al., 2005). Immunocompromized patients have an increased risk for translocation and infections, and are therefore a group of special interest for clinical safety assessment of probiotics. In a small placebo-controlled trial in patients infected with the Human Immunodeficiency Virus (HIV) designed to assess the safety of probiotics in this patient group, no changes were found in safety parameters such as serum chemistry, hematology, immune profile, urinalysis, gastrointestinal tolerance, fecal microbiota and physical examination parameters (Wolf et al., 1998), suggesting that the administration of *L. reuteri* ATCC 55730 was safe in this population. Several other probiotic intervention studies have also been conducted in this patient group. Clinical trials involving severely ill patients are associated with an elevated risk of adverse events. Careful preclinical safety assessment is required before such intervention trials are conducted. Even then, the potential for adverse events may remain high, as demonstrated by the recent study carried out in severely ill patients with acute pancreatitis (Besselink et al., 2008). In this study, adverse events were observed in the probiotic group, despite that earlier data from an animal model (van Minnen et al., 2007) and from clinical intervention trials (Olah et al., 2002) suggested the safety of the probiotic intervention.

Taken together, clinical safety trials of probiotics provide valuable information on the effects of these organisms *in vivo*. The importance of such trials is underlined by the fact that the results of *in vitro* safety assessments and animal models cannot be directly extrapolated to humans. The current evidence strongly suggests that probiotics are extremely safe for general population. However, for certain high-risk populations, more thorough safety evaluation may be required to confirm the safety of probiotic use.

31.6 Adverse Events and Potential Risks of Probiotics

Probiotics overall have an excellent safety record in humans. In clinical studies, probiotics have also been fed to in particular high risk populations without significant adverse effects, including subjects infected with HIV (Heiser et al., 2004) and premature infants suffering from necrotizing enterocolitis (Bin-Nun et al., 2005). In Finland there has been a marked increase in the use of the probiotic LGG since its introduction into the country in 1990, but during this period no significant increase in *Lactobacillus* bacteremia or bacteremia attributable to probiotic strains was observed by Salminen et al. (2002). When 47 *Lactobacillus* bacteremia isolates from Finland were species-characterized, 53% of the isolates were identified as *L. rhamnosus* and furthermore, in 23% of the cases the isolate was indistinguishable by PFGE from LGG (Salminen et al., 2004). In a survey from Sweden, lactobacilli were found to represent less than 1% of the total number of bacteremia cases each year, and commonly used probiotic strains were not identified among the clinical isolates (Sullivan and Nord, 2006). Although commercially available probiotic strains are widely regarded as safe, there are concerns with respect to safety in particular high-risk populations.

31.6.1 Sepsis Related to Probiotic Use

The most commonly reported serious adverse event from probiotic treatment is sepsis. In the absence of probiotic supplementation, *Lactobacillus* species are a known, albeit rare, cause of endocarditis in adults and other forms of sepsis in children. Certain reports have directly linked cases of sepsis to the ingestion of probiotic supplements (*Tables 31.4* and *31.5*). A case of a 74 year old diabetic woman who developed LGG liver abscess (isolate indistinguishable from the commercial strain using PFGE of chromosomal DNA restriction fragments) and pneumonia 4 months after commencing daily LGG supplements has been reported (Rautio et al., 1999). *L. rhamnosus* endocarditis after a dental extraction in a 67 year old man with mitral regurgitation who was taking daily probiotic capsules has been reported (Mackay et al., 1999). No differences between the probiotic and the infective *L. rhamnosus* were found using standard API 50 CH biochemical analysis and pyrolysis mass spectrometry. Although highly suggestive of probiotic supplement related sepsis, the aforementioned reports do not conclusively prove that the infectious agents were indeed originating from the

probiotic products, as bacterial strains seemingly indistinguishable from probiotic strains may sometimes be found in the intestinal microbiota of healthy humans (Presterl et al., 2001).

Table 31.4

Cases of bacterial sepsis in humans temporally related to probiotic use (*Cont'd* p. 1221)

Study	Age	Risk factors	Probiotic	Method of identification	Form of sepsis
Rautio et al. (1999)	74	Diabetes mellitus	LGG	API 50 CH; PFGE of DNA restriction fragments	Liver abscess
Mackay et al. (1999)	67	Mitral regurgitation Dental extraction	*L. rhamnosus* 3×10^9 cfu/day	API 50 CH; Pyrolysis mass spectrometry	Endocarditis
Kunz et al. (2004)	3 months	Prematurity Short gut syndrome	LGG	No confirmatory typing	Bacteremia
	10 weeks	Prematurity; Inflamed intestine; Short gut syndrome;	LGG	PFGE of DNA restriction fragments	Bacteremia
De Groote et al. (2005)	11 months	Prematurity; Gastrostomy; Short gut syndrome; CVC; Parenteral nutrition; Rotavirus diarrhea	LGG ¼ capsule/day	rRNA sequencing	Bacteremia
Land et al. (2005)	4 months	Cardiac surgery; Antibiotic diarrhea	LGG 10^{10} cfu/day	Repetitive element sequence-based PCR DNA fingerprinting	Endocarditis
		Cerebral palsy; Jejunostomy feeding; CVC; Antibiotic diarrhea	LGG 10^{10} cfu/day	Repetitive element sequence-based PCR DNA fingerprinting	Bacteremia

Table 31.4

Study	Age	Risk factors	Probiotic	Method of identification	Form of sepsis
Richard et al. (1988)	47	Not stated	*B. subtilis* 8×10^9 spores/day	Antibiotic susceptibility	Bacteremia
	25	Not stated	*B. subtilis* 8×10^9 spores/day	Antibiotic susceptibility	Bacteremia
	63	Neoplastic disease	*B. subtilis* 8×10^9 spores/day	Antibiotic susceptibility	Bacteremia
	79	Not stated	*B. subtilis* 8×10^9 spores/day	Antibiotic susceptibility	Bacteremia
Oggioni et al. (1998) and Spinosa et al. (2000)	73	Chronic lymphocytic leukemia	*B. subtilis* 10^9 spores / day	16S rRNA sequencing	Bacteremia

CVC, Central venous catheter. Adapted from Boyle et al. (2006)

In children, cases of bacterial sepsis related to probiotic use and short gut syndrome have been reported. Two premature infants with short gut syndrome who were fed via gastrostomy or jejunostomy developed *Lactobacillus* bacteremia while taking LGG supplements (Kunz et al., 2004). Similarly, catheter-related LGG bacteremia has been reported in an 11-month-old patient (De Groote et al., 2005). In both cases, the bacteremic strain and probiotic strain were found to be indistinguishable. Cases of probiotic sepsis have been seen in two severely ill children with antibiotic-related diarrhea, related to cardiac surgery or cerebral palsy, due to enteral administration of LGG (Land et al., 2005). Cases of bacteremia associated to other probiotic strains have also been reported. *Bacillus subtilis* bacteremia and cholangitis have been described in three reports (Oggioni et al., 1998; Richard et al., 1988; Spinosa et al., 2000), of which one included confirmation of the strain homology between the probiotic and pathogenic bacteria by molecular typing. Several cases of *Saccharomyces boulardii* fungemia in subjects taking *S. boulardii* supplements have also been described (*Table 31.5*). Molecular typing was used to demonstrate the homology between the probiotic and infective organisms in many cases. Significant sepsis due to *S. boulardii* administered to a neighboring patient, but not the patient developing

sepsis, has been reported in two cases (Cassone et al., 2003; Perapoch et al., 2000). Such cases may have been due to contaminated vascular catheters (Hennequin et al., 2000). Despite of the widespread use of the genus *Bifidobacterium* and in particular the species *B. animalis* subsp. *lactis* in commercial probiotic products,

Table 31.5

Cases of fungal sepsis in humans temporally related to probiotic use (*Cont'd* p. 1223)

Study	Age	Risk factors	Probiotic	Method of identification	Sepsis
Hennequin et al. (2000)	30 months	Cystic fibrosis; CVC; Poor nutritional state; Intestinal surgery; HIV infection; CVC; Diarrhea	*S. boulardii* 750 mg/day	PFGE of mitochondrial DNA restriction fragments	Fungemia
	36	Antibiotic diarrhea; Upper gastrointestinal surgery for malignancy	*S. boulardii* 1.5 g/day	PFGE of mitochondrial DNA restriction fragments	Fungemia
	47	Peptic ulcer; Chronic Renal Failure;	*S. boulardii* 2 g/day	PFGE of mitochondrial DNA restriction fragments	Septic shock
	78	Pneumonia/COPD	*S. boulardii* 1.5 g/day	PFGE of mitochondrial DNA restriction fragments	Fungemia
Cassone et al. (2003)	34	CVC; Intensive care unit	No direct treatment	PFGE of undigested chromosomal DNA	Fungemia
	48	CVC; Intensive care unit	No direct treatment	PFGE of undigested chromosomal DNA	Fungemia
	75	CVC; Intensive care unit	No direct treatment	PFGE of undigested chromosomal DNA	CVC colonization
	35	Intensive care unit	Unclear	PFGE of undigested chromosomal DNA	Fungemia

Table 31.5 (*Cont'd* p. 1124)

Study	Age	Risk factors	Probiotic	Method of identification	Sepsis
Perapoch et al. (2000)	3 months	CVC; Diarrhea; Parenteral nutrition	*S. boulardii* 100 mg/day	PFGE of mitochondrial DNA restriction fragments PFGE of undigested chromosomal DNA	Fungemia
		Short bowel syndrome; CVC	Not received directly (in cot next to first patient)	PFGE of mitochondrial DNA restriction fragments	Fungemia
	Infant	Parenteral nutrition		PFGE of undigested chromosomal DNA	
Lherm et al. (2002)	50–82	Acutely unwell on intensive care unit with respiratory failure; CVC	*S. boulardii* 1.5–3 g/day	PFGE of nuclear and mitochondrial DNA restriction fragments	Fungemia
Bassetti et al. (1998)	51	Immunosuppression; *C. difficile* diarrhea; CVC	*S. boulardii* 1 g/day	PFGE of DNA restriction fragments	Fungemia
Riquelme et al. (2003)	42	Kidney/Pancreas transplant; Immunosuppression; *C. difficile* diarrhea	*S. boulardii* 1 g/day	PFGE of DNA restriction fragments	Fungemia
	41	HIV; Diarrhea	*S. boulardii* 750 mg/day	PFGE of DNA restriction fragments	Fungemia
Fredenucci et al. (1998)	49	Antibiotic diarrhea; Immunosuppressed	*S. boulardii* 200 mg/day	API 32C PFGE of undigested chromosomal DNA	Fungemia
Cesaro et al. (2000)	8 months	Acute myeloid leukemia; CVC; Neutropenia	*S. boulardii*	API 32C	Fungemia
Cherifi et al. (2004)	89	*C. difficile* colitis; Gastrostomy	*S. boulardii* 300 mg/day	No formal identification described	Fungemia

Table 31.5

Study	Age	Risk factors	Probiotic	Method of identification	Sepsis
Henry et al. (2004)	65	Malignancy; Immune compromise; Mucositis; Diarrhea; Parenteral Nutrition	*S. boulardii*	No formal identification described	Fungemia
Niault et al. (1999)	78	Antibiotic diarrhea; Intensive Care unit; Intragastric feeding	*S. boulardii* 1.5 g/day	No formal identification described	Fungemia
Viggiano et al. (1995)	14 months	Burns; Diarrhea; Gastrostomy	*S. boulardii* 200 mg/day	No formal identification described	Fungemic shock
Zunic et al. (1991)	33	Inflammatory bowel disease; Intensive care unit; Parenteral nutrition	*S. boulardii* 1.5 g/day	No formal identification described	Fungemia
Pletincx et al. (1995)	1	Parenteral nutrition; Antibiotic diarrhea; CVC	*S. boulardii* 600 mg/day	No formal identification described	Septicemia
Rijnders et al. (2000)	74	Colitis; Nasogastric feeding	*S. boulardii* 600 mg/day	No formal identification described	Fungemia
Lestin et al. (2003)	48	Diabetes; *C. difficile* diarrhea	*S. boulardii* 150 mg/day	API 32C	Fatal fungemia

CVC, Central venous catheter; *COPD*, Chronic obstructive pulmonary disease. Adapted from Boyle et al. (2006)

these probiotics have never been related to sepsis associated with probiotic use, demonstrating the extremely low pathogenic potential of bifidobacteria.

31.6.2 Gastrointestinal Symptoms Related to Probiotic Use

It is clear from dose ranging studies that high dose probiotic treatment can lead to increased frequency and softening of feces (Larsen et al., 2006). Gastrointestinal adverse events such as vomiting and diarrhea are also rarely seen in probiotic treatment trials. In particular, one study would suggest that there is an increased risk of such adverse events associated with the use of heat-inactivated rather than viable probiotics. Kirjavainen et al. (2003) were forced to terminate their study of

the probiotic LGG in allergic infants due to adverse gastrointestinal symptoms and diarrhea in those treated with a heat-killed form of the probiotic, something which they did not find in those infants treated with viable LGG. However, bacteriological data of the study indicated that high numbers of *Clostridium* and *Bacteroides* may have been a predisposing factor for the observed side effects. The group treated with heat-killed LGG had higher numbers of both *Clostridium* (11%) and *Bacteroides* (12%) already before the treatment than did the group treated with live LGG (4 and 5%, respectively), which may also explain why the adverse gastrointestinal were more common in the group receiving heat-inactivated probiotics.

31.6.3 Other Adverse Events Related to Probiotic Use

A recent clinical trial identified the most severe potential risk of probiotic treatment reported to date – that of fatal bowel ischemia. Besselink and colleagues (Besselink et al., 2008) investigated the effects of a probiotic mix (*L. acidophilus, L. casei, L. salivarius, Lactococcus lactis, B. bifidum* and *B. infantis*) at a total dose of 10^{10} CFU/day in a randomized placebo-controlled trial of 296 adults with a first episode of high risk acute pancreatitis. The probiotic mix was administered by nasojejunal feeding tube, and had been specifically designed to inhibit the growth of pathogens important in pancreatic necrosis. Preliminary data in rats (van Minnen et al., 2007) and humans with less severe illnesses (Besselink et al., 2008) suggested that this probiotic mix would be safe and efficacious in the prevention of infectious complications of pancreatitis. The authors found a 2.53-fold increase in mortality risk in probiotic treated participants, and nine cases of bowel ischemia (eight fatal) in the probiotic group, with no bowel ischemia in the placebo group (Besselink et al., 2008). The bowel ischemia occurred after a median of 3 days probiotic treatment (range 2–11 days). It is known that small bowel ischemia, increased intestinal permeability and increased bacterial translocation are all associated with acute pancreatitis. In these cases the direct application of probiotic bacteria to an already damaged small intestinal mucosa may have precipitated a local inflammatory response leading to increased risk of small bowel ischemia. However, it is not known why exactly mortality was higher in the treatment group, and although the mortality was associated with randomization, this does not necessary implicate that the probiotic itself was the causative factor. Indeed, the observed results may have (at least partly) been due to unsuccessful randomization, because the rate of organ failure, a consequence of hemodynamic

disturbance parallel to bowel ischemia, was significantly higher in the probiotic group (n = 20) compared to the placebo group (n = 7) already at the day of randomization (Reid et al., 2008).

Probiotics have been proposed to have a role in the management of childhood allergies. In general, prevention of the occurrence of allergies appears to be more effective than the treatment of allergies by probiotics. Despite the promising results in this area, there are also some indications of adverse effects of probiotics in this respect. A recent systematic review demonstrated that the probiotic LGG leads to a minor worsening of disease severity when used to treat eczema in young children, whereas subgroup analysis of probiotics other than LGG suggested significant improvement in eczema severity (Boyle et al., 2008). In a 7-year follow-up of a study assessing the efficacy of administration of LGG during infancy in the prevention of atopic eczema, it was observed that children who received LGG during infancy had lower rate of eczema at 7 years of age compared to children who received placebo, but the rate of respiratory allergies and asthma tended to be higher in the probiotic group (Kalliomäki et al., 2007). Kopp et al. (2008) reported no reduction in the rate of atopic dermatitis in children following administration of LGG during late pregnancy and early infancy. Instead, increased frequency of recurrent episodes of wheezing bronchitis was observed in the probiotic group. Finally, Taylor et al. (2007) reported that postnatal administration of *L. acidophilus* LAVRI-A1 to infants was associated with increased allergen sensitization at 12 months of age. Despite these reports, the role of probiotics in the management of allergies appears to be beneficial in general, but the indications of the possible adverse effects following early probiotic administration deserve further attention in the future.

31.6.4 Factors Affecting the Adverse Effects Associated with Probiotics

31.6.4.1 Underlying Diseases and Treatments

To date, there have been no reports of sepsis related to probiotic use in otherwise healthy individuals. All reported cases of probiotic bacteremia or fungemia have occurred in patients with underlying chronic disease, or immune compromised or debilitated state. In most cases, probiotic sepsis has resolved with antimicrobial therapy, but in some cases patients have developed septic shock (Hennequin et al., 2000). In some cases the outcome has been fatal, but apart from a case of

a 48 year old diabetic patient with diarrhea attributable to *Clostridium difficile* who died from multiple organ failure and septic shock in association with a toxic megacolon and probiotic fungemia (Lestin et al., 2003), these fatalities have been related to underlying disease rather than being directly attributable to probiotic sepsis (Boyle et al., 2006). In the report by Lestin et al. (2003), the only case suggestive of fatal probiotic sepsis, molecular methods were not used to confirm homology between the probiotic and pathogenic fungi. Treatment of diarrhea and short bowel syndrome are common targets of probiotic therapies, but these pre-existing intestinal pathologies may also potentially increase the risk of probiotic translocation through the intestinal mucosa. Ongoing antibiotic treatment may increase the risk of *Lactobacillus* bacteremia, in particular in the case of *L. rhamnosus*. Salminen et al. (2006) reported that in approximately half of the 85 cases, the patient had received antimicrobial treatment prior to *Lactobacillus* bacteremia. Administration of probiotics via jejunostomy tube, bypassing the effect of gastric acid and the dilutional capacity of both stomach and duodenum, may increase the numbers of viable probiotic bacteria that reach the intestine. Some cases of adverse events in patients administered with probiotics via jejunostomy tube have been reported. Central venous catheter, a common finding in cases of probiotic sepsis, may serve as a possible source of sepsis (Hennequin et al., 2000). Premature infants and patients who are debilitated or have compromised immune function are overrepresented in the cases of sepsis associated with probiotics (› *Tables 31.4* and › *31.5*). In the case of lactobacilli, cardiac valvular disease may be a risk factor, as certain species of *Lactobacillus* may in rare cases colonize heart valve and cause endocarditis. In the light of the recent results reported by Besselink et al. (2008), enteral administration of probiotics to patients with severe acute pancreatitis or patients in high risk of developing bowel ischemia may be particularly risky.

31.6.4.2 Probiotic Strain Selection and Characteristics

The beneficial health effects of one probiotic cannot be assumed for another probiotic species, or even for different strains of the same species. The same applies for the rare adverse events associated with probiotics. The case reports of probiotic sepsis published to date suggest that *Saccharomyces boulardii,* LGG and *Bacillus subtilis* may be probiotics that carry a higher risk of sepsis than other strains (› *Tables 31.4* and › *31.5*). When 85 blood isolates from cases of *Lactobacillus* bacteremia in Finland were examined, 46 isolates were identified as

L. rhamnosus, 12 isolates of both *L. casei* and *L. fermentum*, and three isolates each of *L. jensenii*, *L. salivarius* and *L. gasseri* were identified (Salminen et al., 2006). Of the *L. rhamnosus* isolates, 22 (48%) were judged to be indistinguishable from LGG by pulsed-field gel electrophoresis, but it is important to note that it is not known whether the patients in this study had actually consumed LGG. In addition, phenotypic differences have later been demonstrated between some of the above-mentioned *L. rhamnosus* isolates and the probiotic strain LGG (Ouwehand et al., 2004b). Certain probiotic strains such as LGG and *S. boulardii* may be more frequently administered to subjects with underlying diseases, including antibiotic associated diarrhea, than other strains. This may partly explain the association of these strains with the reported cases of probiotic sepsis, but the differences in the intrinsic properties of probiotic strains, such as the properties increasing the potential of bacterial translocation, clearly also affect the probability of certain probiotic strains to cause sepsis. Results from animal model experiments also suggest that certain *Lactobacillus* strains have higher infectivity than others (Vesterlund et al., 2007). Other groups such as probiotics in the genus *Bifidobacterium* appear to have less pathogenic features and are underrepresented in case reports of probiotic sepsis (Boyle et al., 2006).

For probiotic sepsis, bacterial translocation appears to be a key event. Good adherence to host mucus and epithelium is thought to play a role in many of the beneficial effects of probiotics. However, theoretically, strong adherence to epithelial layer may also increase the likelihood of bacterial translocation, in particular in subjects with disturbed gut permeability, immune deficiency and intestinal immaturity (Boyle et al., 2006). Adherence alone should not be considered a risk factor for strains of commencal microbes and strains with low infectivity. Properties other than adherence are also required for increased likelihood of translocation and sepsis, as demonstrated by the fact that certain probiotic species such as *B. animalis* subsp. *lactis* and *L. acidophilus* are characterized with good adhering properties, but are not associated with the cases of probiotic sepsis, despite their widespread use as probiotics. Moreover, some clinical isolates of *Lactobacillus* exhibit only low level of adhesion (Apostolou et al., 2001).

The intestinal microbiota is important in stimulating normal immune development, particularly the development of the gut associated lymphoid tissue. The crucial role of the intestinal microbiota in normal immune development and function suggests that manipulations designed to alter this microbiota may have significant immunomodulatory effects. Immune modulation is thought to be one of the key mechanisms of the beneficial effects of probiotics. Although currently there is no evidence linking immune modulation of probiotics to adverse events, such effects remain a possibility (Boyle et al., 2006). For example,

administration of the probiotic LGG to infants with atopic eczema has been shown to induce systemically detectable low grade inflammatory responses characterized by increased levels of c-reactive protein and interleukin-6 (Viljanen et al., 2005). While these effects may serve as a mechanism for the beneficial effects of probiotics on atopic eczema, the potential for adverse events linked with systemic proinflammatory effects cannot be ruled out. At present there is little support for the hypothesis that probiotics lead to adverse immune development from empirical studies, but this is an area that warrants further investigation.

Potential pathogenic determinants to be taken into account in the safety assessment of probiotics, including transferable antibiotic resistance, hemolysis, platelet aggregation, production of deleterious metabolites and resistance to host defense mechanisms, are reviewed elsewhere in this chapter. In addition to the characteristics of administered probiotic strain, the administration dose should be taken into account when evaluating the safety of probiotic regimens. Since high dose regimens of probiotics may be associated with looser stools (Larsen et al., 2006), the dosing of probiotics may also have effect on certain potential side-effects, although it should be noted that in general, even very high doses of probiotics are well-tolerated.

31.7 Conclusion

The current evidence strongly suggests that probiotics are safe to use in general population. Several approaches for the safety evaluation of current and potential new probiotics are available, ranging from *in vitro* assessments to randomized, controlled clinical trials. Most commonly used probiotics are considered to be generally safe, and in Europe such microorganisms have been granted a QPS status (EFSA, 2007). Safety of probiotics, similar to the beneficial effects of probiotics, is a strain-dependent feature, and differences exist between different probiotics. For example, in the case of transferable antibiotic resistance elements, strains of *Enterococcus* are particularly problematic. The problems associated with the potential production of toxic compounds are especially evident among strains belonging to genus *Bacillus*. In regard to the potential of probiotics to cause sepsis, the currently reported cases suggest that strains of *Saccharomyces*, *L. rhamnosus* and *Bacillus* may posses higher risk of adverse events than other probiotics. Notably, among *L. rhamnosus*, clear differences exist between the infective potential of the strains belonging to this species (Vankerckhoven et al., 2007).

Despite the excellent overall safety record of probiotics, they should be used with caution in certain specific patient groups – particularly critically patients, those with immune deficiency and patient groups with increased risk for bacterial translocation due to disturbed intestinal mucosal barrier function. The risk of adverse events is likely to differ with each probiotic strain, and published literature has highlighted some strains which may carry higher risks than others. The dose and mode of administration are also important, with higher dose regimens being associated with gastrointestinal symptoms. Careful consideration should be given to these issues before using probiotic supplementation in high risk populations.

Taken together, the beneficial effects of probiotics clearly outweigh the possible risks of probiotic use. Probiotics provide a variety of health benefits for humans, from healthy subjects to patients with many different diseases.

31.8 Summary

- Probiotics have an excellent overall safety record.
- Several *in vitro* assessments of probiotics are available, but clear markers for potential infectivity have not been identified.
- Animal models allow the safety assessments focusing on bacterial translocation and underlying diseases.
- Numerous clinical trials assessing the safety of probiotic contribute to the safety of probiotics.
- In certain high-risk populations such as critically ill patients, the use of probiotics should be carefully considered.

List of Abbreviations

ARDRA	amplified rDNA restriction analysis
COPD	chronic obstructive pulmonary disease
CVC	central venous catheter
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization
HIV	human immunodeficiency virus

LAB	lactic acid bacteria
LGG	*Lactobacillus rhamnosus* strain GG
MLS	macrolide, lincosamide and streptrogramin
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
QPS	qualified presumption of safety
RAPD	randomly amplified polymorphic dNA
Rep-PCR	repetitive DNA element-PCR
SPF	specific pathogen free
WHO	World Health Organization

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