Antibiotic resistance in probiotic bacteria

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INTRODUCTION

One of the most important selection criteria for bacterial strains intended for use in the food industry is concern for their safety. In present in a candidate microorganism should be determined prior to approval for QPS status. Therefore, antibiotic resistance per se is not a safety issue; it only becomes such when the risk of resistance transfer is present.

Those probiotics belonging to species included in the EFSA QPS list (EFSA, 2012) have excellent safety records, and detrimental effects produced as a consequence of their ingestion are very scarce (Gosier et al., 2012). Undoubtedly, a full safety assessment begins with a proper identification of the strain and an in vitro evaluation of the potential risks. In this regard, the presence of antibiotic resistance determinants, and their potential mobilility, deserves special attention. 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.

Most probiotics are common members of the human intestinal tract, and they are ingested in large amounts in functional foods, and the presence of antibiotic resistance determinants in their genome must be systematically screened. For instance, the bifidobacterial population in the human gut can be as high as 10^10 cells/g of intestinal content, and even if the presence of the resistance genes are not a threat when they are present in bifidobacterial cells due to their lack of infectivity, these cells can constitute a reservoir from which genes could be transmitted to pathogenic bacteria. Thus, it is of great interest to investigate whether these determinants can be transferred in the food and gut environment present in a candidate microorganism should be determined prior to approval for QPS status. Therefore, antibiotic resistance per se is not a safety issue; it only becomes such when the risk of resistance transfer is present. 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.

In this review, we summarize the current knowledge on antibiotic resistance mechanisms in lactobacilli and bifidobacteria, as well as in other potential probiotic candidates, such as Bacillus strains. We did not consider enterococci because of the high prevalence of antibiotic resistance determinants in this genus and the obvious safety concerns.

ANTIBIOTIC RESISTANCE DETERMINANTS IN LACTOBACILLUS

The genus Lactobacillus is the largest group among the lactic acid bacteria (LAB) and likely the most widely used as a probiotic in a variety of foods, mainly meat and fermented dairy products. To date, 182 species have been described within the genus (list of prokaryotic names with standing in nomenclature; www.bacterio.cict.fr/), giving an idea of its complexity. With regard to antibiotic resistance, the vancomycin-resistant phenotype of some lactobacilli is perhaps the best characterized intrinsic resistance in LAB. Vancomycin comes into contact with the peptidoglycan precursors on the cell wall side of the cytoplasmic membrane and binds to the D-alanine/D-alanine terminus of the pentapeptide, preventing polymerization of peptidoglycan precursors. In several species of LAB, the terminal D-alanine residue is replaced by D-lactate or D-serine in the muramylpentapeptide, preventing vancomycin binding (Delsore et al., 1999) and therefore becoming resistant to the antibiotic. In addition, chromosomal mutations leading to antibiotic resistance phenotypes have also been described in lactobacilli. Hóez et al. (2007) identified a single mutation in the 23S rRNA gene reducing the affinity
of erythromycin for the ribosome. This mutation conferred macrolide resistance in a strain of *L. rhamnosus*. In this respect, the transfer risk is considered to be very low for intrinsic resistance, or acquired resistance due to chromosome mutation(s). Nevertheless, knowledge of the antibiotic resistance phenotypes may still be important, even in the absence of transferable resistance; since lactobacilli are commonly used in food and feed products, intrinsic resistance might still be relevant for the treatment of *Lactobacillus*-related bacteremias (Cannon et al., 2005).

Horizontally transferred antibiotic resistance genes, particularly those carried within mobile genetic elements, are the most likely to be transmitted 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 a representative number of different strains from each species. Although some effort has been made to this end, work has only been carried out for some antibiotics and particular *Lactobacillus* species. These include the most commonly used probiotic species such as *L. casei*, *L. acidophilus*, *L. reuteri*, or *L. rhamnosus*, among others, or the yogurt starter bacteria *L. delbrueckii* (Ammor et al., 2008b; Korhoen et al., 2008; Mayrhofer et al., 2010). However, given the taxonomic complexity of this microbial genus, there is still a lack of agreement on the resistance susceptibility breakpoint values for most antibiotics. The use of molecular methods, such as microarray analysis and various PCR techniques is being extremely helpful in determining the genetic basis of the acquired resistance phenotypes. Moreover, the increasing availability of genome sequences and the cost reduction of genome sequencing facilities offer new possibilities for the screening of antimicrobial resistance genes (Benenson et al., 2011).

With regard to specific antibiotics, lactobacilli are usually sensitive to the cell wall-targeting penicillin and β-lactamase, but are more resistant to cephalosporins. As previously mentioned, many *Lactobacillus* species show a high level of resistance to vancomycin. Also, most inhibitors of nucleic acid synthesis seem to be involved in bile resistance in *Lactobacillus* (Hummel et al., 2007), and *L. delbrueckii* (Ammor et al., 2008b; Korhoen et al., 2008; Mayrhofer et al., 2010). However, given the taxonomic complexity of this microbial genus, there is still a lack of agreement on the resistance susceptibility breakpoint values for most antibiotics. The use of molecular methods, such as microarray analysis and various PCR techniques is being extremely helpful in determining the genetic basis of the acquired resistance phenotypes. Moreover, the increasing availability of genome sequences and the cost reduction of genome sequencing facilities offer new possibilities for the screening of antimicrobial resistance genes (Benenson et al., 2011).

Several genes responsible for atypical antibiotic resistance properties among lactobacilli have been reported (Table 1). Chloramphenicol resistance genes (*cat*, chloramphenicol acetyltransferases) have been identified in *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus* (Hummel et al., 2007), and *L. johnsonii* (Mayrhofer et al., 2010) 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 streptogramins (MLS) resistance phenotype, have been identified in several *Lactobacillus* species; the *erm* (B) gene, which encodes a 23S rRNA methylase acting on the 23S ribosomal subunit, is the most frequently found of such genes, but others such as *erm*(A)*, erm*(C)*, or *erm*(T) have also been detected (van Hoek et al., 2008c; Mayrhofer et al., 2010). The presence of genes coding for macrolide efflux pumps, such as *mef*(A), genes for lincosamide transferase
Table 1 | Antibiotic resistance determinants identified and characterized in lactobacilli, bifidobacteria, and probiotic Bacillus strains.

<table>
<thead>
<tr>
<th>Gene(s)</th>
<th>Resistance Mechanism</th>
<th>Location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>blaZ</td>
<td>β-Lactams</td>
<td>Antibiotic hydrolysis</td>
<td>Aquilanti et al. (2007)</td>
</tr>
<tr>
<td>vanE</td>
<td>Quinupristin–dalfopristin</td>
<td>Antibiotic acetylation</td>
<td>Mayrhofer et al. (2010)</td>
</tr>
<tr>
<td>Cat</td>
<td>Chloramphenicol</td>
<td>Antibiotic acetylation</td>
<td>Plasmid</td>
</tr>
<tr>
<td>mraC</td>
<td>MLS</td>
<td>Efflux</td>
<td>Ahn et al. (1992); Lin et al. (1996); Hummel et al. (2007); Mayrhofer et al. (2010)</td>
</tr>
<tr>
<td>marA</td>
<td>MarC</td>
<td>Efflux</td>
<td>Cauwerts et al. (2006)</td>
</tr>
<tr>
<td>aac(6′)-aph(2′′), armB, armC, armT</td>
<td>Aminoglycoside</td>
<td>Enzymatic modification</td>
<td>Ropo-Bezares et al. (2006)</td>
</tr>
<tr>
<td>armB, armC, armT, armL, armD, armA</td>
<td>MLS</td>
<td>Plasmid, transposon,</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>chromosome</td>
<td></td>
</tr>
<tr>
<td>tet(W), tet(V), tet(S), tet(O), tet(O), tet(O), tet(O), tet(O), tet(O), tet(O), tet(W/O)</td>
<td>Tetracycline</td>
<td>Ribosomal protection</td>
<td>Aquilanti et al. (2007); Klare et al. (2007); Ammor et al. (2008b); van Hoek et al. (2008b)</td>
</tr>
<tr>
<td>tet(W/O), tet(W/O/O), tet(W/O/O/O/O)</td>
<td>Tetracycline</td>
<td>Chromosome</td>
<td></td>
</tr>
<tr>
<td>tet(K/tet(L)</td>
<td>Tetracycline</td>
<td>Efflux</td>
<td>Plasmid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>armO</td>
<td>MLS</td>
<td>Ribosomal methylation</td>
<td>Transposon</td>
</tr>
<tr>
<td>tet(W), tet(V), tet(S), tet(O), tet(O), tet(O), tet(O), tet(O), tet(O), tet(O), tet(O), tet(W/O)</td>
<td>Tetracycline</td>
<td>Ribosomal protection</td>
<td>Föllner et al. (2006); Kasimierzczak et al. (2006); Ammor et al. (2008a); van Hoek et al. (2008b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chromosome</td>
<td></td>
</tr>
<tr>
<td>Bacillus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aac(3′)</td>
<td>Aminoglycoside</td>
<td>Efflux</td>
<td>Chromosome</td>
</tr>
<tr>
<td>arm(D)</td>
<td>MLS</td>
<td>Antibiotic adenylation</td>
<td>Chromosome</td>
</tr>
<tr>
<td>BC1</td>
<td>pI-lactams</td>
<td>Antibiotic hydrolysis</td>
<td>Chromosome</td>
</tr>
<tr>
<td>cat(D)</td>
<td>Chloramphenicol</td>
<td>Antibiotic acetylation</td>
<td>Chromosome</td>
</tr>
</tbody>
</table>

Bifidobacterium with QPS status (*B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, and *B. longum*; EFSA, 2012) have not been linked to any infective processes in healthy individuals. However, several strains displaying antibiotic resistance phenotypes have been characterized, and in many cases the phenotype has been linked to specific antibiotic resistance genes, representing a potential risk of transfer to other bacteria in the intestinal ecosystem (Ammor et al., 2008b). Bifidobacteria are intrinsically resistant to mupirocin, an antibiotic that is being used in selective media for this genus. Mupirocin competes with isoleucine as a substrate for isoleucyl-tRNA synthetase, thus affecting protein synthesis. The resistance phenotype of bifidobacteria is a consequence of the synthesis of an atypical isoleucyl-tRNA synthetase that contains key amino acid residues responsible for the high level of mupirocin resistance (Serafini et al., 2011). Furthermore, they are not susceptible to high concentrations of aminoglycosides, most likely as a consequence of the lack of cytochrome-mediated drug transport (Mayrhofer et al., 2011). On the contrary, low concentrations of macrolides, vancomycin, chloramphenicol, beta-lactams, rifampicin, and spectinomycin, normally inhibit their growth (Zhou et al., 2005; Lahtinen et al., 2009). However, it is worth mentioning that a few streptomycin resistant strains have been characterized, leading to the conclusion that the resistance phenotype in these strains is due to chromosomal mutations, and not to the acquisition of specific antibiotic resistance genes, and therefore do not represent a potential risk of transferability. Thus, a high resistance to streptomycin was correlated with a mutation on the rpsL gene for ribosomal protein S12 in *B. bifidum* and *B. breve* (Kiwaki and Sato, 2009; Sato and Iino, 2010). Also, a
B. bifidum strain displaying low erythromycin susceptibility was found to possess mutated 23S ribosomal RNA gene copies, likely to be responsible for the observed phenotype (Sato and Iino, 2010).

Data on antibiotic resistance determinants in bifidobacteria are relatively scarce, and are limited to tetracycline and macrolide antibiotics (Table 1). MDR proteins have been described in B. longum and B. breve. The transporters are able to confer low resistance levels to erythromycin, although their contribution to the MLS phenotype remains to be determined (Margolles et al., 2005; Price et al., 2006). Also, a gene coding for a ribosomal protection protein, erm(X), was identified in B. animalis subsp. lactis and B. thermophilum, as a part of the transposon Tn532 (van Hoek et al., 2008a).

Tetracycline resistance in Bifidobacterium deserves special attention. We have known for more than a decade, that proteins that protect the ribosome from the action of tetracyclines, the so-called tet genes, are commonly found in this genus (Scott et al., 2008; Guermonde et al., 2010). The genes tet(W), tet(M), tet(O), tet(W)/32(O), and tet(O)/W have been detected in several Bifidobacterium species, including B. longum subsp. infantis and subsp. longum, B. breve, B. animalis subsp. lactis, B. bifidum, B. pseudocatenulatum, and B. thermophilum (Flórez et al., 2006; Kazimierczak et al., 2006; Aires et al., 2007, 2009; Ammor et al., 2008a; van Hoek et al., 2008b; Guermonde et al., 2010). The gene tet(W) is especially ubiquitous; it has been detected at high frequencies in B. longum strains (Aires et al., 2007; Ammor et al., 2008a), and in all B. animalis subsp. lactis strains analyzed until now (Aires et al., 2007; Guermonde et al., 2010). This last fact is very relevant, taking into account that B. animalis subsp. lactis strains are extensively used in the functional food industry, especially in fermented dairy products (Masco et al., 2005). The tet(W) gene in Bifidobacterium seems to be integrated in the chromosome and its surrounding regions vary depending on the strain, but very often the gene is flanked by transposase target sequences or genes coding for transposases, enzymes that catalyze the movement of DNA fragments between different locations by recognizing specific target sequences, suggesting that, under adequate conditions, the gene could be transferred (Kazimierczak et al., 2006; Ammor et al., 2008a; van Hoek et al., 2008a,b; Guermonde et al., 2010). In fact, a tet(W) gene of B. longum, containing a transposase located in the conserved upstream region of the gene, and flanked by imperfect direct repeats, is transferable, at low frequencies, between B. longum and B. adolescentis under in vitro conditions (Kazimierczak et al., 2006). This suggests the potential of bifidobacteria to transfer antibiotic resistance genes to closely related bacteria. However, although attempts have been made to demonstrate the donor capacity of bifidobacteria to other enteric bacteria, to the best of our knowledge this has not been experimentally proved yet.

**ANTIBIOTIC RESISTANCE GENES IN OTHER PROBIOTIC STRAINS**

Members of the genus Bacillus are aerobic or facultative aerobic, endospore-forming and rod-shaped Gram-positive bacteria, which inhabit a wide range of habitats, mostly soil and sediments. These bacteria do not belong to the commensal microflora of the gastrointestinal tract, but some strains of the genus are included in food supplements and used in human nutrition as probiotics, notably Bacillus clausii (Cifio, 1984). Furthermore, several Bacillus species have been employed for centuries in the manufacture of traditional, fermented dishes in Africa and Asia (Sarkar et al., 2002). Nowadays, certain Bacillus strains are used as feed additives, plant production products, biomass for animal feed, or enzyme/vitamin production (SCAN, 2000; Hong et al., 2003), and several species are included on the EFSA QPS list (EFSA, 2012). Many characteristics of probiotic Bacillus strains differ from those of other probiotic bacteria, including its ability to sporulate and the mechanisms of interaction with the human intestinal mucosa (Sánchez et al., 2009).

Regarding the presence of antibiotic-resistance mechanisms in Bacillus, macrolide-resistance genes present on extra-chromosomal elements have been identified in mobile elements, such as the plasmid-encoded erm(C) from Bacillus subtilis (Monod et al., 1986). Tetracycline resistance determinants have also been found in mobile elements, including the plasmid-encoded tetr(L) gene from Bacillus subtilis (Pöldän et al., 2011), and the tet(M) gene, contained within the conjugative transposon Tn5397 of Bacillus subtilis (Roberto et al., 1999). Other tetracycline resistance genes, such as tet(K), have been observed in some Bacillus isolates (Neela et al., 2009). Recently, the presence of cfr-like genes in several Bacillus species has been reported. Cfr genes encode ribosome methyltransferases providing resistance to several classes of antibiotics including phenicols, oxazolidinone, lincomycin, pleuromutilins, and streptogramin A (Dai et al., 2010). However, these genes are not apparently expressed in the species assayed, in spite of being fully functional in other bacterial hosts (Hansen et al., 2012). In this regard, it is worth noting the presence of specific antibiotic resistance mechanisms in certain Bacillus clausii strains, which have been used as probiotics in humans, especially for the prevention of infectious bacterial diarrhea. For instance, the erm(34) gene has been identified in the probiotic Bacillus clausii DSM7874 strain (Bozdogan et al., 2004). Probiotic Bacillus clausii strains also harbor specific antibiotic defense mechanisms, such as an aminoglycoside resistance gene (aadD2), a chloramphenicol acetyltransferase gene, cat(Bcl) or a β-lactamase (BCL-4; Bozdogan et al., 2003; Guérich et al., 2007; Galopin et al., 2009).

**CONCLUSION**

Bacteria naturally present in foods or food supplements, or deliberately added to them, including probiotic bacteria, constitute a potential source of antibiotic resistance determinants. Especially some fermented foods, such as dairy products, possess an extremely high bacterial density, mostly composed of LAB, quantitatively comparable with the microbial population found in some parts of the human intestine. This microbial population represents a huge reservoir of antibiotic resistance genes whose ingestion could influence the presence, establishment, and dynamics of antibiotic resistance bacteria in our body.

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Mayo, B. (2008a). Analysis of tetra-cycline resistance mediated by tet(W), tet(M), and tet(O) genes of Bacillus clausii species.


Consecutive human bifidobacteria isolates from broilers show antibiotic resistance to antibiotics of human and veterinary relevance.


Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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