



International Scientific Association for Probiotics and Prebiotics

*An international non-profit association of scientists dedicated to advancing the
fundamental and applied science of probiotics and prebiotics*

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Report from ISAPP Meeting on Antibiotic Resistance in Probiotic Bacteria Held December 13, 2007 Paris

Date of report: January 30, 2008

Present at meeting:

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8. Artem Khlebnikov (local host), Danone Paris FRANCE



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Executive Summary

Understanding antibiotic resistance expression and transferability is an important component of the safety assessment of bacteria used as probiotics. In the US and elsewhere there is not a harmonized approach to this issue. The ACE-ART, PROSAFE (Vankerckhoven et al. 2008), and other research projects (Klare et al. 2005, 2007; Kastner et al. 2006) have addressed these topics. Because of the increase in safety discussions about probiotics in the USA, the leadership within ISAPP considered it important to convene an informal meeting to discuss the status of recommendations on antibiotic resistance and safety generated from the ACE-ART project, which then could be communicated to interested parties. Toward this end, a meeting was held in Paris on December 13, 2007 among specialists in this area and other interested scientists. Drs. Lorenzo Morelli and Wolfgang Kneifel presented a summary of the wealth of data generated from the ACE ART project.

A collection of 1,579 strains (not all found to be unique) was amassed from species of *Lactobacillus* (843), *Bifidobacterium* (467), *Lactococcus* (178) and *Streptococcus thermophilus* (91). Methodology was shown to be of paramount relevance (medium, inoculum size, incubation time) and methods for all four bacterial groups were developed. ISO is evaluating the ACE ART recommended procedures to determine if they should be transformed into an ISO standard. It was found that breakpoints for phenotypic antibiotic resistance assessment must be obtained at species level (or closely related species). A limited number of antibiotics were tested (vancomycin only for *Bifidobacterium*; tetracycline, erythromycin and streptomycin for *Lactobacillus*; ampicillin, chloramphenicol, clindamycin, erythromycin, gentamycin, streptomycin, tetracycline and vancomycin for *Lactococcus*; ampicillin, clindamycin, erythromycin, gentamycin, streptomycin and tetracycline for *S. thermophilus*). When available, 50 strains of each species should be used to establish the species breakpoints. However, not all tested species were represented by this number of strains. Findings indicated that genetic analysis must be done when the phenotype for a particular strain falls outside the breakpoint for the species. In some cases, resistance greater than the norm for the species is due to mutations in intrinsic genes and is not due to transferable resistance genes. Tet(O), tet(W), and erm(X) were detected in some *Bifidobacterium* species.

Although the ACE ART project has made significant progress toward developing the needed construct for generating and interpreting information on antibiotic resistance among bacteria intended for human food use, much remains to be done. Some additional study is needed before final recommendations can be made are:

- The range of antibiotics being tested must be expanded to include all those relevant to human clinical applications. Currently, only ampicillin, chloramphenicol, clindamycin, erythromycin, gentamycin, streptomycin, tetracycline and vancomycin have been tested, although not for all species.
- Not enough strains are available for some species to reliably establish the norm for inherent resistances for a species. Additional strains should be acquired if possible.

- The risk of transfer of antibiotic resistance determinants among commensal microbiota *in vivo* has not been calculated experimentally. However, it should be emphasized that an antibiotic resistant strain of *Lactobacillus*, *Bifidobacterium* or *S. thermophilus* is not a pathogen.
- It has been suggested (Borriello, et al. 2002) that probiotics for human use should be sensitive to two clinically relevant antibiotics. However, clinical sensitivity is not always predicted by *in vitro* test, so this recommendation might be difficult to meet. The link between *in vitro* antibiotic sensitivity and *in vivo* sensitivity must be better understood.

There is great value to the field in general to continue and expand this project. Focus on development of appropriate methods, sharing breakpoint data, providing reference strains to other laboratories, and establishment of a central, dynamic database would be useful. Laboratories using correct methods and comparative reference strains could submit data to the database, enabling expansion of the database.

Key recommendations for current assessment and interpretation of antibiotic resistance in probiotics for human use:

- Recommendations from this project only pertain to *Lactobacillus*, *Bifidobacterium*, *Lactococcus* and *Streptococcus thermophilus*. Other bacteria sometimes used as probiotics (e.g., *Bacillus* species, *Clostridium butyricum*, *Enterococcus* species and *E. coli*) are not included in this study.
- Phenotypic antibiotic resistance should be conducted on all strains destined for human use. The media found to be suitable for this purpose was Iso-Sensitest as the baseline medium, supplemented with 10% of the ideal media for each genus. For example, for *Streptococcus thermophilus* SSM (Iso-Sensitest 90% v/v plus M17 10% v/v plus lactose 0.5% w/v) was used (Tosi et al. 2007).
- The microdilution method is the preferred method for establishing phenotypic resistance (Domig, et al. 2007), although Etest was also acceptable (Matto et al. 2006).
- Phenotypic breakpoints have been determined for certain species of the following groups, with the following antibiotics:
 - Vancomycin for *Bifidobacterium*. (No vancomycin resistance has been found in *Bifidobacterium*)
 - Tetracycline, erythromycin and streptomycin for *Lactobacillus*
 - Ampicillin, chloramphenicol, clindimycin, erythromycin, gentamycin, streptomycin, tetracycline and vancomycin for *Lactococcus*
 - Ampicillin, clindimycin, erythromycin, gentamycin, streptomycin and tetracycline for *S. thermophilus*
- Results for phenotypic resistance for a new strain should be compared to the breakpoint established for the species (e.g., Figure 1 which shows breakpoints for *L. plantarum*). (Control strains for comparisons during analysis can be provided by ACE ART project leaders.) If the breakpoint is not exceeded, no further testing on antibiotic resistance need be conducted.
- Phenotypic breakpoints have been established for the following species (for antibiotics indicated above):
 - *Bifidobacterium: adolescentis, animalis, bifidum, longum, pseudolongum* and *thermophilum*
 - *Lactobacillus: amylovorus, brevis, casei, delbrueckii, fermentum, gasseri, helveticus, johnsonii, paracasei, plantarum, reuteri, rhamnosus* and *sakei*
 - *Lactococcus lactis* subsp. *lactis*
 - *Streptococcus thermophilus*
- Genetic analysis for acquired, transferable antibiotic resistance genes must be done when the phenotype for a particular strain falls outside the breakpoint for the species.

- Tools for identification of antibiotic resistant genes in Gram-positive bacteria are currently available. Perreten et al. (2005) developed a microchip which includes 90 antibiotic resistance genes from Gram positive bacteria (Kastner et al. 2006).
- Assurance of “no concern” with regard to antibiotic resistance can be improved by documenting the absence of known transferable resistance genes [(Tet(L), Tet(O), Tet(W) (van Hoek et al. 2008; Mayrhofer et al. 2007; Florez et al. 2006), Lmr(A, P, C and D) (Lincomycin) (Florez et al. 2006), erm(X) (Mayrhofer et al. 2007)]
- Conversely, if an antibiotic resistance gene is detected (during chromosomal sequencing, for example), the likelihood of gene transfer can be surmised by evaluating if the gene is transcribed, if it is flanked on both sides with transposable elements, if it resides on a conjugative plasmid, or if the complete gene is present. Furthermore, even if an antibiotic resistance gene can be transferred, it might not be of concern if the antibiotic is not clinically relevant or if transfer does not contribute to the reservoir of antibiotic resistance (as would be the case with resistance genes that are already broadly distributed). However, the concept of “clinical relevance” may need to be considered carefully, as co-selection of antibiotic resistant genes may occur. For example, tetracycline co-selects for erythromycin and cloramphenicol, which are present in the same cassette as the tetracycline resistance gene. This co-selection process occurs when the antibiotic resistance genes are nearby or adjacent. Therefore even antibiotics that are not clinically relevant may lead to selection of antibiotic resistance genes of clinical relevance.
- Accumulated antibiotic resistances within one strain must be considered carefully as this may result in limitation of the number of treatment options in the rare case of an infection.

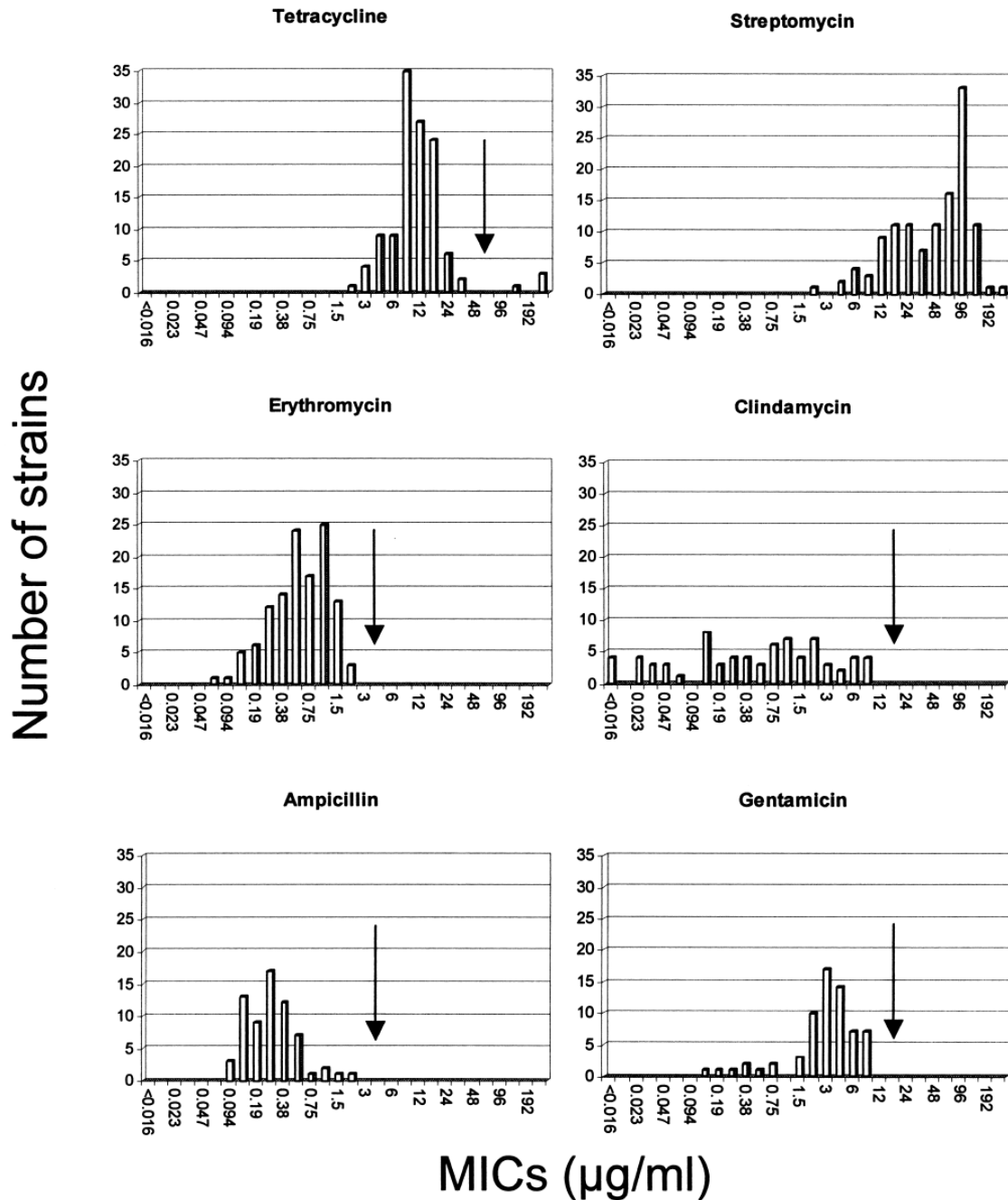


FIG. 1. Distribution of minimum inhibitory concentrations (MICs) among the *Lactobacillus plantarum* strains for the six antibiotics in this study. The real Etest MIC distribution values are indicated, while data were grouped in classes in Table 1. Arrowheads point to the susceptibility-resistance cutoff MIC values proposed in this work.

Cutoffs determined by Etest for *L. plantarum*

Figure 1. From Flórez AB, Egervärn M, Danielsen M, Tosi L, Morelli L, Lindgren S, Mayo B. Susceptibility of *Lactobacillus plantarum* strains to six antibiotics and definition of new susceptibility-resistance cutoff values. Microb Drug Resist. 2006 Winter;12(4):252-6.

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Additional Detail from Discussion

General facts about ACE ART

- ACE-ART (Assessment and Critical Evaluation of Antibiotic Resistance Transferability in Food Chain) is an EU project focused on the assessment of drug resistance in non pathogenic, food related bacteria. The focus of this project is on the importance of *Lactobacillus*, *Bifidobacterium*, *Lactococcus* and *Streptococcus thermophilus* as an environmental source of antibiotic resistance genes
- Background to this effort: Resistance to antimicrobial agents may be the result of many different mechanisms including inherent (natural) and acquired mechanisms. Acquired resistance may result from the acquisition of exogenous DNA or mutation of indigenous genes. From a safety point of view, it is important to differentiate between the intrinsic and acquired mechanisms of resistance and the transferability. But there are no official methods, no MICs, no microbiological breakpoints for phenotypic assessment of resistance in non pathogenic, food related bacteria. Other projects addressing antibiotic resistance have focused on pathogens; this project focused on food microbes.
- Specific scientific areas were addressed:
 - Phenotypic assessment to provide ecological breakpoints for each antibiotic and each species, including media/method development (work package 1)
 - Characterization of atypical phenotypic resistances that were identified in work package 1; Transmission of resistance in the environment and in the animal and human gut (work package 2)
 - Genetic mechanisms of antibiotic transfer within these microbes (work package 3)

ACE ART results

- Relevant strains (172 total) were amassed into a culture collection and identified to species and strain level (PFGE patterns of each strain were generated into a database)
- A test protocol was developed that enables comparison of MIC-values between laboratories for each bacterial group. Methods were specific to genus or to *S. thermophilus*.
 - Microdilution method was found to be most reliable (E-test also acceptable)
- *Lactobacillus* species represented in the collection (number of strains in each species)

<i>L. acidophilus</i> (17)	<i>L. farcimins</i> (2)	<i>L. plantarum</i> (156)
<i>L. agilis</i> (4)	<i>L. fermentum</i> (66)	<i>L. reuteri</i> (49)
<i>L. amylovorus</i> (31)	<i>L. galinarum</i> (7)	<i>L. rhamnosus</i> (93)
<i>L. bovis</i> (1)	<i>L. gasserii</i> (46)	<i>L. ruminis</i> (2)
<i>L. brevis</i> (26)	<i>L. helveticus</i> (32)	<i>L. sakei</i> (59)
<i>L. buchneri</i> (1)	<i>L. johnsonii</i> (34)	<i>L. salivarius</i> (6)
<i>L. casei</i> (27)	<i>L. lactis</i> (1)	<i>L. vaginalis</i> (2)
<i>L. coryneformis</i> (1)	<i>L. mucosae</i> (7)	<i>L. zeae - casei - paracasei</i> (4)
<i>L. cripatus</i> (6)	<i>L. murinus</i> (1)	
<i>L. delbrueckii</i> (9)	<i>L. parabuchneri</i> (1)	
<i>L. delbrueckii</i> (ssp. <i>bulgaricus</i> , <i>delbrueckii</i> and <i>lactis</i>) (51)	<i>L. paracasei</i> (91)	
	<i>L. paraplantarum</i> (4)	
	<i>L. pentosus</i> (15)	
- *Bifidobacterium* species represented in collection (number of strains of each species)

<i>B. adolescentis</i> (47)	<i>B. pseudocatenulatum</i> (16)
<i>B. adolescentis / ruminantium</i> (4)	<i>B. dentium related</i> (3)
<i>B. angenulatum</i> (4)	<i>B. longum</i> (127)
<i>B. animalis</i> (incl. ssp. <i>lactis</i>) (30)	<i>B. longum - infantis</i> (1)
<i>B. bifidum</i> (39)	<i>B. pseudolongum</i> (77)
<i>B. breve</i> (14)	<i>B. pseudolongum ssp. globosum</i> (2)
<i>B. catenulatum</i> (14)	

B. pseudolongum ssp. *pseudolongum*
(3)

B. ruminantium (2)
B. thermophilum (84)

- *Lactococcus* (178 total strains, 144 of these were *Lactococcus lactis* ssp. *lactis*)
 - *Lactococcus lactis* and *Lactococcus piscium* species were represented
 - Antibiotics tested:
 - i. Amp:
 - ii. Chlor:
 - iii. Clin:
 - iv. Ery:
 - v. Gent:
 - vi. Strept:
 - vii. Tet:
 - viii. Van:
- *Streptococcus thermophilus*: adequate number of strains, good media and good cutoffs for the 6 antibiotics tested
 - ix. 64 different strains
 - x. SSM (Iso-Sensitest 90% v/v plus M17 10% v/v plus lactose 0.5% w/v) was used to perform the antibiotic susceptibility test
 - xi. Cutoff values for tested antibiotics (microdilution method)
 - 1. Ery: 1 µg/ml
 - 2. Str: 64 µg/ml
 - 3. Tet: 2 µg/ml
 - 4. Clin: 0.25 µg/ml
 - 5. Gent: 32 µg/ml
 - 6. Amp: 1 µg/ml