Journal of Dairy Research (2007) **74** 262–268. © *Proprietors of Journal of Dairy Research 2007*
doi:10.1017/S0022029907002543 *First published online 30 April 2007 Printed in the United Kingdom*

Antibiotic survey of *Lactococcus lactis* strains to six antibiotics by Etest, and establishment of new susceptibility-resistance cut-off values

Ana Belén Flórez[1], Morten Danielsen[2], Jenni Korhonen[3], Joanna Zycka[4], Atte von Wright[3], Jacek Bardowski[4] and Baltasar Mayo[1]*

[1] Instituto de Productos Lácteos de Asturias (CSIC), Carretera de Infiesto s/n, 33300-Villaviciosa, Asturias, Spain
[2] Chr. Hansen A/S, Bøge Allé 10-12, DK-2970 Hørsholm, Denmark
[3] Institute of Applied Biotechnology, University of Kuopio, Bioteknia 2, PO Box 1627, Kuopio, Finland
[4] Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5a, 02-106 Warsaw, Poland

Received 6 March 2006 and accepted for publication 6 November 2006

In order to establish cut-off values for *Lactococcus lactis* to six antibiotics to distinguish susceptible and intrinsically resistant strains from those having acquired resistances, the minimum inhibitory concentration (MIC) of tetracycline, erythromycin, clindamycin, streptomycin, chloramphenicol and vancomycin was determined in 93 different *Lc. lactis* strains using the Etest. These bacterial strains were originally isolated from dairy and animal sources in widely separated geographical locations. Cut-offs were defined on the basis of the distribution of the MICs frequency of the studied antibiotics, which in the absence of acquired determinants should approach to a normal statistical distribution. In general, the new cut-off values proposed in this study are higher than previously defined (European Commission, 2005. The EFSA Journal 223, 1–12). Based on these new values, all the strains tested were susceptible to erythromycin, chloramphenicol and vancomycin, and 79 susceptible to all six antibiotics. However, 11 strains (around 12%) were considered resistant to tetracycline (six of which had been identified after screening of a large collection of lactococci strains for tetracycline resistance) and five (5·4%) resistant to streptomycin. Of these, two fish isolates proved to be resistance to both tetracycline and streptomycin. From the tetracycline resistant strains, *tet*(M) and mosaic *tet*(L/S) genes were amplified by PCR, demonstrating they harboured acquired antibiotic resistance determinants.

Keywords: *Lactococcus lactis*, susceptibility testing, minimum inhibitory concentration, Etest, antimicrobial resistance, tetracycline resistance.

Lactococcus lactis is a lactic acid bacterium commonly dominant in natural niches such as spontaneous milk fermentations, on cattle, and on plant material (Mundt, 1986). Recently, it has also been reported as a dominant microorganism in the intestinal tract of freshwater fish (Hagi et al. 2004; Ringo, 2004). Strains of this species are also the main component of starters used in the economically important fermentation of milk into cheese (Fox et al. 2000). Lactococci are also responsible for flavour formation through their proteolytic and amino acid conversion pathways (Smit et al. 2005). In addition, these bacteria have recently found a use in other biotechnological applications, such as the expression of heterologous proteins, the synthesis of food-grade additives and nutraceuticals, and in vaccine delivery (Hols et al. 1999; Nouaille et al. 2003).

Food and certain food supplements can introduce large numbers of live *Lc. lactis* into the human and animal gastrointestinal tract (GIT), a complex ecosystem containing hundreds of different types of resident and transient microorganisms including opportunistic and pathogenic bacteria (Guarner & Malagelada, 2003). There is therefore concern about the possibility of commensal bacteria acquiring antibiotic resistance genes from their environment and acting as a reservoir of such determinants (Teuber et al. 1999; European Commission, 2005). Resistance could ultimately be transferred to pathogenic species, which would hamper the treatment of infections

*For correspondence; e-mail: baltasar.mayo@ipla.csic.es

(Hamilton-Miller, 2004). Indeed, the transfer of resistance into food matrices and the GIT of mammals has been shown possible in a number of experiments (Netherwood et al. 1999).

Antibiotic resistant lactococci may be selected for in environments where they come into contact with antibiotics, such as the udder of antibiotic-treated cows and, therefore, the products derived from their raw milk (Bennish, 1999), or in the ponds for fish culturing (Chopra & Roberts, 2001). The need for new lactococcal strains to complement those currently used as starters (Klijn et al. 1995; Salama et al. 1995) demands that, as well technological characterization studies, antibiotic surveys be performed to avoid selecting those harbouring acquired, transferable resistance determinants (Teuber et al. 1999). This need is all the more evident since several antibiotic resistance genes have been successfully transferred to *Lc. lactis* from related bacteria under laboratory conditions (Gasson & Fitzgerald, 1994; Clewell et al. 1995), and since a multiple resistance plasmid has been discovered in an isolate from a raw-milk cheese (Perreten et al. 1997) Antibiotic resistance cut-off values for *Lc. lactis* have only been defined for strains used in animal feed (European Commission, 2005). These have been based on antibiotic surveys using different methodologies (microdilution, plate dilution, Etest), media and culture conditions, and sometimes a small number of related strains (Cogan, 1972; Orberg & Sandine, 1985; Elliot & Facklam, 1996; Teuber et al. 1999).

This paper reports the determination of the MICs of six antibiotics with activity against Gram positive bacteria, inhibiting either the synthesis of proteins (tetracycline, erythromycin, clindamycin, streptomycin, and chloramphenicol) or the synthesis of the cell wall (vancomycin) in a large collection of lactococcal strains. These strains were isolated from different geographical locations over many years (from 1950 to the present), and from different dairy products and animal environments.

Material and Methods

The following analyses were performed at the different laboratories involved in this research: CSIC, Instituto de Productos Lácteos de Asturias (CSIC), Villaviciosa, Spain; CH, Christian Hansen A/S, Hørsholm, Denmark; IBB, Institute of Biochemistry and Biophysics, Warsaw, Poland; and UKU, University of Kuopio, Kuopio, Finland.

Bacterial strains, growth media and culture conditions

Ninety three *Lc. lactis* isolates from industrial starter cultures (28), cows' milk (12), artisanal starter-free cheeses (41), cat (4) and fish (5) intestines, and culture collections (3) were surveyed for antimicrobial resistance. The isolates were geographically well separated, as they were collected in Spain (31), Denmark (15), Belgium (18), Poland (7), Finland (5) and other European countries (17). Most were isolated in the 1980s and 1990s (73), but several were collected before the generalized use of antibiotics (20), the so-called pre-antibiotic era (Teuber et al. 1999).

Enterococcus faecalis LMG 8222 (=ATTC 29212), *Lc. lactis* LMG 8520, and two well-known laboratory strains (*Lc. lactis* MG 1363; *Lc. lactis* IL 1403), all from the LMG collection of the Belgian Co-ordinated Collections of Microorganisms, BCCM^{TM}, University of Ghent, Belgium, were used as controls throughout the study.

Cryopreserved cultures of *Lc. lactis* strains in glycerol were first recovered on Mueller-Hinton agar (Oxoid, Basingstoke, UK). Isolated colonies were then streaked onto Mueller-Hinton plates and incubated for 16–18 h at 28 °C. The isolates from fish intestines, however, did not grow well at 28 °C; these incubations were therefore performed at 25 °C.

Classification and characterisation of isolates

The isolates were phenotypically grouped by their carbohydrate fermentation profiles using the API50 CHL system (bioMérieux, Marcy l'Etoile, France) and later identified by PCR using either universal primers (Young et al. 1991) or species-specific primers (Pu et al. 2002), all based on conserved sequences of 16S rRNA genes. Amplicons were then sequenced and the sequences compared to those in public databases. Genetic fingerprinting was performed by RAPD-PCR and/or by PFGE as previously described (Delgado & Mayo, 2004). Clustering calculations were performed using GelCompar II v. 2.5 software (Applied Maths BVBA, Sint-Martens-Latem, Belgium).

Minimum inhibitory concentrations determined by the Etest method

Individual colonies from the Mueller-Hinton plates were suspended in a sterile glass or plastic tube containing 2–5 ml of sterile saline (Oxoid) until a density corresponding to McFarland standard 1 or its spectrophotometric equivalent (corresponding to around 3×10^8 cfu/ml) was obtained.

A sterile cotton swab was dipped into the above McFarland suspension and used to inoculate the Mueller-Hinton agar plates. The agar surface was then allowed to dry for approximately 15 min before applying the Etest strips (AB Biodisk, Solna, Sweden). Readings were recorded after 48 h of incubation at 28 °C (or 25 °C for fish isolates). The MICs for the bacteriocidal antibiotics (streptomycin, chloramphenicol and vancomycin) studied were taken as the first value on the Etest strips at which growth did not occur, as per the manufacturer's recommendations. For the bacteriostatic antibiotics (tetracycline, erythromycin and clindamycin), the MIC was taken as the point where growth was significantly inhibited (around 80%, as estimated by visual inspection).

https://doi.org/10.1017/S0022029907002543 Published online by Cambridge University Press

Table 1. Minimum inhibitory concentrations (MICs) of the different antibiotics assayed against *Lactococcus lactis* isolates

LAB (no. of isolates)	Antimicrobial agent	MIC (μg/ml)¶		
		Range	50%	90%
§CSIC (66)	Tetracycline	0·064–96	0·125	0·38
	Streptomycin	0·19–32	8	24
	Erythromycin	0·023–0·5	0·094	0·25
	Clindamycin	<0·016–0·5	0·094	0·25
	Chloramphenicol	0·5–6	2	6
	Vancomycin	0·125–3	0·38	0·5
CH (15)	Tetracycline	0·094–0·19	0·125	0·19
	Streptomycin	1·5–24	12	24
	Erythromycin	0·094–0·19	0·125	0·19
	Clindamycin	0·047–0·19	0·094	0·125
	Chloramphenicol	2–4	3	3
	Vancomycin	0·25–0·5	0·38	0·5
IBB (7)	Tetracycline	0·25–256	—†	—
	Streptomycin	6–12	—	—
	Erythromycin	0·064–0·125	—	—
	Clindamycin	0·047–0·25	—	—
	Chloramphenicol	1·5–2	—	—
	Vancomycin	0·25–0·5	—	—
UKU (5)	Tetracycline	0·094–96	—	—
	Streptomycin	96–128	—	—
	Erythromycin	0·38–0·5	—	—
	Clindamycin	0·125–0·5	—	—
	Chloramphenicol	1–2	—	—
	Vancomycin	0·125–0·25	—	—
Total (93)	Tetracycline	0·064–⩾256	0·125	0·25‡
	Streptomycin	0·19–128	12	24
	Erythromycin	0·023–0·5	0·125	0·38
	Clindamycin	⩽0·016–0·5	0·125	0·25
	Chloramphenicol	0·5–6	2	4
	Vancomycin	0·125–3	0·38	0·5

§ Abbreviation key of collections and laboratories: CSIC, Instituto de Productos Lácteos de Asturias (CSIC), Villaviciosa, Spain; CH, Christian Hansen A/S, Hørsholm, Denmark; IBB, Institute of Biochemistry and Biophysics, Warsaw, Poland; and UKU, University of Kuopio, Kuopio, Finland
¶ 50% and 90%, MIC_{50} and MIC_{90}, respectively
† Too few isolates for percentual MIC determinations
‡ Excluding the six tetracycline resistant strains from IBB

Measurement of MICs by the standard NCCLS agar dilution method

To check the performance of the Etest compared with the standard plate agar dilution technique (CLSI, 2004), the MICs of these antibiotics for 25 selected strains were also determined by this last procedure using Mueller-Hinton agar.

PCR amplification of tetracycline resistance genes

Total DNA was isolated from tetracycline resistant strains by using a commercial kit (GenElute bacterial genomic DNA kit; Sigma-Altdrich Co., St. Louis, Mo., USA). DNA dilutions were used as template in PRC reactions with universal primers of ribosomal protection genes (DI, 5′-GA(T/C)ACICCIGGICA(T/C)(A/G)TIGA(T/C)TT-3′, and DII, 5′-GCCCA(A/G)(T/A)IGG(A/G)TTIGGIGGIAC(T/C)TC-3′) (Clermont et al. 1997). Following PCR conditions as described by Gevers et al. (2003), specific primers for *tet*(K) (TetK-FW1 5′-TTATGGTGGTTGTAGCTAGAAA-3′ and TetK-RV1 5′-AAAGGGTTAGAAACTCTTGAAA-3′), *tet*(L) (TetL-FW3 5′-GYMHYYHVHVHVYSYSYYVV-3′ and TetL-RV3 5′-GTGAAMGRWAGCCCACCTAA-3′), and *tet*(M) (DI and tetMR, 5′-CACCGAGCAGGGATTTCTC-CAC-3′) were also used. Amplicons obtained were purified using Microcon PCR filters (Millipore, Bedford, Ma., USA) and double-stranded sequenced in an ABI 370 Genetic Analyzer (Applied Biosystems, Warrington, UK). Sequences were then compared with others in the EMBL data library by using the online BLASTN resource (http://www.ncbi.nlm.nih.gov/blast/).

https://doi.org/10.1017/S0022029907002543 Published online by Cambridge University Press

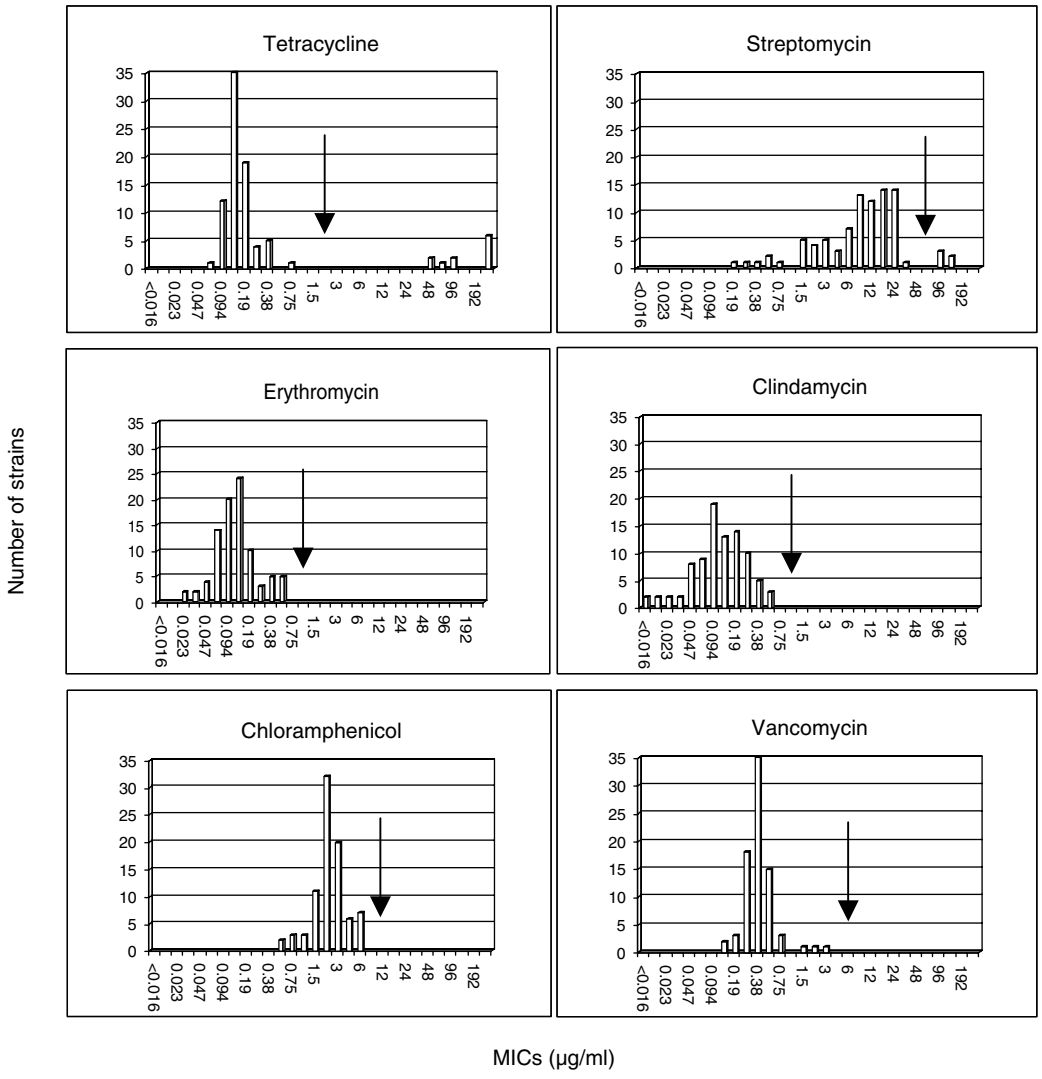

Fig. 1. Distribution of Minimum inhibitory concentrations (MICs) frequency of the *Lactococcus lactis* strains analysed for the six antibiotics. Arrows point to the new cut-off MIC values proposed in this study.

Results and Discussion

All strains grew well on plain Mueller-Hinton without the need for frequent additions of glucose and yeast extract (Katla et al. 2001) or lysed horse blood (Green et al. 1990; Elliot & Facklam, 1996), thus allowing the determination of MICs by the Etest method. Etest modal MIC values of the analysis performed in the different laboratories (in µg/ml) for the internal control strain *Ec. faecalis* ATCC 29212 of tetracycline (8), streptomycin (256), erythromycin (3), clindamycin (16), chloramphenicol (8), and vancomycin (2) compared well with the Etest manufacturer' guidelines.

MICs of the different antibiotics assayed

In this study, MICs of six antibiotics with action on Gram-positive bacteria were evaluated by the Etest method in 93 *Lc. lactis* isolates from different environments, locations and periods of time. Table 1 shows the range of MICs to the antibiotics analysed plus the MICs that inhibited the growth of 50 and 90% of the strains. Differences in ranges converged when the $MIC_{50\%}$ and $MIC_{90\%}$ values recorded at the different laboratories were compared. Moreover, differences were always within one or two dilutions of the range, the normal variation in inter-laboratory assays. Similar differences were observed for MICs obtained in the same strains by either Etest or the standard CLSI agar dilution method. Grouping the strains by their year of isolation, their geographical origin or their environment of origin appeared to have no influence on the distribution of the MICs.

Early antibiotic surveys involving *Lc. lactis* showed this species to have little in the way of antibiotic resistance (Cogan, 1972; Reinbold & Reddy, 1974; Orberg &

https://doi.org/10.1017/S0022029907002543 Published online by Cambridge University Press

Sandine, 1985). The present results agree well with those of other authors who found lactococcal species to be susceptible to broad and Gram positive spectrum antibiotics and β-lactams (Elliot & Facklam, 1996; Katla et al. 2001; Delgado et al. 2002; Flórez et al. 2005). Variations in the MIC for streptomycin similar to those obtained in the present work have been reported by other authors (Orberg & Sandine, 1985; Katla et al. 2001). In the present work, the standardised protocol probably reduced the differences that might arise in microdilution and the Etest assays through the use of different culture media (Huys et al. 2002) or inoculum sizes (M Egervärn & S Lindgren, unpublished). In addition to antibiotic resistance genes, differences in the activity of general detoxification systems such as those of multi-drug resistance (MDR) transporters might contribute towards enhancing the MICs of otherwise susceptible lactococcal strains (Perreten et al. 2001; Putman et al. 2001). The MICs for 79 lactococci strains showed them to be susceptible to all six antibiotics.

Distribution of MICs among the Lc. lactis *strains*

Figure 1 shows the distributions of the MIC values frequencies for the antibiotics over the range of strains. Most frequency distributions followed a rather normal curve; only tetracycline and streptomycin differed, which showed bimodal curves. The curves for tetracycline, chloramphenicol and vancomycin were very narrow, while those for erythromycin, clindamycin and streptomycin were wider. The distribution of some tetracycline and streptomycin MICs strongly suggests that certain isolates possess active resistance mechanisms.

Proposition of new microbiological susceptibility-resistance cut-off values

The term microbiological breakpoint was introduced to replace the concept of the clinical breakpoint for the purpose of identifying bacterial strains with acquired and potentially transferable resistance determinants (Olsson-Liljequist et al. 1997). In the present work, however, a microbiological term more related to risk assessment is used: the resistance-susceptibility cut-off value. The cut-off for each antibiotic was considered as the second MIC above the apparently normal range of the MICs (Fig. 1). The large number of strains examined allows the proposition of the resistance-susceptibility cut-offs shown in Table 2, which are based on both the MIC ranges and their unimodal or bimodal distribution. In the experimental conditions of this study, some of the cut-offs were quite different from previously published breakpoints, such us those of the European Commission Scientific Panel Committee on additives and products or substances used in animal feed (FEEDAP) (European Commission, 2005), and others, which are also summarized in the table for comparison. To validate the new cut-offs, analysis of resistant strains to all antibiotics will be necessary. Especially, a careful inspection of the strains contributing to the higher MIC levels of the curves will be needed to exclude the presence of dedicated (acquired) mechanisms of resistance.

Table 2. Microbiological susceptibility-resistance cut-off values proposed for *Lactococcus lactis* to the six antibiotics under assay

	Proposed susceptibility-resistance cut-off MICs (μg/ml)		
Antibiotic	Clinical breakpoints†	FEEDAP[A]	This work
Tetracycline	8	4	2
Streptomycin	—	16	64
Erythromycin	0·25	4	1
Clindamycin	1‡	4	1
Chloramphenicol	4	8	12
Vancomycin	1	4	6

† Breakpoints by the Clinical and Laboratory Standards Institute (CLSI, 2004), former NCCLS, as defined for *Streptococcus* spp. other than *Streptococcus pneumoniae*

[A] FEEDAP, Scientific Panel on additives and products or substances used in animal feed. Breakpoints as related on a recent report (European Commission, 2005)

‡ Breakpoint as defined by the Société Française de Microbiologie (http://sfm.asso.fr)

Strains with a MIC equal to or higher than the cut-off values were considered resistant. In this sense, 11 strains were found resistant to tetracycline (six of which had been selected by their tetracycline resistance phenotype after screening of a large collection of dairy lactococci for resistance to this antibiotic) (J Zycka, JB Lampkowska & J Bardowski, unpublished). Tetracyclines continue to be important as therapeutic antibiotics, and are still employed in stockbreeding and aquaculture in many countries (Chopra & Roberts, 2001), which may favour the selection of tetracycline resistant bacteria. Furthermore, five strains resistant to streptomycin were also encountered among the fish intestinal tract isolates; of these, two proved to be resistance to both tetracycline and streptomycin.

Antimicrobial resistance genes in Lc. lactis

Few antimicrobial resistance determinants have been found in strains of *Lc. lactis*, although plasmid-encoded genes have been described for tetracycline [*tet*(S)], macrolides [*mdt*(A) and *erm*(T)], chloramphenicol (*cat*) and streptomycin (*str*) (Perreten et al. 1997; Raha et al. 2002). The tetracycline resistant strains were screened for tetracycline resistance determinants by using universal primers for ribosome protection genes and primers specific for *tet*(K), *tet*(L), and *tet*M. An amplification product of around 1·5-kbp was obtained by using specific primers for *tet*(M) when DNA from two resistant strains from a farmhouse cheese (Spain) and a resistant strain from a cat tonsil (BCCM™, Belgium) was used as a template. The same

https://doi.org/10.1017/S0022029907002543 Published online by Cambridge University Press

gene was also amplified from the two tetracycline resistant fish isolates. The partial sequences of all these genes proved to be 100% identical to each other and also to the *tet*(M) gene from Tn*916* of *Ec. faecalis* (Su et al. 1992). Further, two different tetracycline resistance genes were detected among the Polish resistant strains, a mosaic *tet*(L/S) gene along with *tet*(M). This demonstrated the usefulness of the tetracycline resistance cut-offs and the presence of acquired tetracycline resistance determinants among the isolates of this work. The analysis of genes involved in streptomycin resistance is currently underway.

In conclusion the Etest is a convenient method that accurately determines MICs and suggests the presence of atypical resistances which may be encoded by acquired determinants. These added genes are considered to have the greatest risk for horizontal dissemination (European Commission, 2005). The analysis of strains from different ecotypes may be useful for precisely defining the cut-off values separating acquired resistances from all other types, while at the same time revealing acquired resistance that has already spread among the *Lc. lactis* populations. Owing to the current low incidence of resistance in *Lc. lactis*, in which multi-resistance is practically absent, this species could be useful for studying the acquisition and spread of antibiotic determinants among commensal organisms. In this work, a few strains harbouring tetracycline resistance genes identical to those present in other bacteria have been identified.

This work was supported by an EU project within the VI Frame Programme (ACE-ART, Reference CT-2003-506214). BCCM™, University of Gent, Gent, Belgium, and EFFCA, Paris, France, are acknowledged for providing some of the strains in this work.

References

Bennish ML 1999 Animals, humans, and antibiotics: implications of the veterinary use of antibiotics on human health. *Advances in Pediatric Infection Diseases* **14** 269–290

Charteris WP, Kelly PM, Morelli L & Collins JK 2001 Gradient diffusion antibiotic susceptibility testing of potentially probiotic lactobacilli. *Journal of Food Protection* **64** 2007–2014

Chopra I & Roberts M 2001 Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews* **66** 232–260

Clermont D, Chesneau O, De Cespédès G & Horaud T 1997 New tetracycline resistance determinants coding for ribosomal protection in streptococci and nucleotide sequence of *tet*(T) isolated from *Streptococcus pyogenes* A498. *Antimicrobial Agents and Chemotherapy* **41** 112–116

Clewell DB, Flannagan SE & Jaworsky DD 1995 Unconstrained bacterial promiscuity: the Tn*916*-Tn*1545* family of conjugative transposon. *Trends in Microbiology* **3** 229–236

CLSI (Clinical and Laboratory Standards Institute) 2004 Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria: Approved Standard-six edition. M11-A6, Vol. 24, n° 2. Wayne, Pennsylvania, USA.

Cogan TM 1972 Susceptibility of cheese and yogurt starter bacteria to antibiotics. *Applied Microbiology* **23** 960–965

Delgado S, Delgado T & Mayo B 2002 Technological performance of several *Lactococcus* and *Enterococcus* strains of dairy origin in milk. *Journal of Food Protection* **65** 1590–1596

Delgado S & Mayo B 2004 Phenotypic and genetic diversity of *Lactococcus lactis* and *Enterococcus* spp. strains isolated from Northern Spain starter-free farmhouse cheeses. *International Journal of Food Microbiology* **90** 309–319

Elliot JA & Facklam RR 1996 Antimicrobial susceptibilities of *Lactococcus lactis* and *Lactococcus garviae* and a proposed method to discriminate between them. *Journal of Clinical Microbiology* **34** 1296–1298

European Commission 2005 Opinion of the Scientific Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) on the updating of the criteria used in the assessment of bacteria for resistance to antibiotics of human and veterinary importance. *The EFSA Journal* **223** 1–12

Flórez AB, Delgado S & Mayo B 2005 Antimicrobial susceptibility of lactic acid bacteria isolated from a cheese environment. *Canadian Journal of Microbiology* **51** 51–58

Fox PF, Guinee TP, Cogan TM & McSweeney PLH 2000 Fundamentals of Cheese Science. AN Aspen Publishers Inc., Gaithersburg, Ma., USA

Gasson MJ & Fitzgerald GF 1994 Gene transfer systems and transposition. In *Genetics and Biotechnology of Lactic Acid Bacteria*, pp. 1–51 (Eds MJ Gasson & WM de Vos). London: Blackie Academic and Professional

Gevers D, Danielsen M, Huys G & Swings J 2003 Molecular characterization of *tet*(M) genes in *Lactobacillus* isolates from different types of fermented dry sausage. *Applied and Environmental Microbiology* **69** 1270–1275

Green M, Wadowsky RM & Barbadora K 1990 Recovery of vancomycin-resistant Gram-positive cocci from children. *Journal of Clinical Microbiology* **28** 484–488

Guarner F & Malagelada JR 2003 Gut flora in health and disease. *Lancet* **360** 512–519

Hagi T, Tanaka D, Iwamura Y & Hoshimo T 2004 Diversity and seasonal changes in lactic acid bacteria in the intestinal tract of cultured freshwater fish. *Aquaculture* **234** 335–346

Hamilton-Miller JM 2004 Antibiotic resistance from two perspectives: man and microbe. *International Journal of Antimicrobial Agents* **23** 209–212

Hols P, Kleerebezem M, Schanck AN, Ferain T, Hugenholtz J, Delcour J & de Vos WM 1999 Conversion of *Lactococcus lactis* from homolactic to homoalanine fermentation through metabolic engineering. *Nature Biotechnology* **17** 588–592

Huys G, D'Haene K & Swings J 2002 Influence of the culture medium on antibiotic susceptibility testing of food-associated lactic acid bacteria with the agar overlay disc diffusion method. *Letters in Applied Microbiology* **34** 402–406

Katla AK, Kruse H, Johnsen G & Herikstad H 2001 Antimicrobial susceptibility of starter culture bacteria used in Norwegian dairy products. *International Journal of Food Microbiology* **67** 147–152

Klijn N, Weerkamp AH & de Vos WM 1995 Detection and characterization of lactose-utilizing *Lactococcus* spp. in natural ecosystems. *Applied and Environmental Microbiology* **61** 788–792

Mundt JO 1986 Lactic acid streptococci. In *Bergey's Manual of Systematic Bacteriology*, Vol. 2, pp. 1065–1068 (Eds PHA Sneath, NS Mair, ME Sharpe & JG Holt). Baltimore, USA: Williams and Wilkins

Netherwood T, Bowden R, Harrosin P, O'Donnel AG, Parker DS & Gilbert HJ 1999 Gene transfer in the gastrointestinal tract. *Applied and Environmental Microbiology* **65** 5139–5141

Nouaille S, Ribeiro LA, Myoshi A, Pontes D, Le Loir Y, Oliveira SC, Langella P & Azevedo V 2003 Heterologous protein production and delivery systems for *Lactococcus lactis*. *Genetics and Molecular Research* **2** 102–111

Olsson-Liljequist B, Larsson P, Walder M & Miorner H 1997 Antimicrobial susceptibility testing in Sweden. III. Methodology for

susceptibility testing. Scand. *Journal of Infectious Diseases Supplement* **105** 13–23

Orberg PK & Sandine WE 1985 Survey of antimicrobial resistance in lactic streptococci. *Applied and Environmental Microbiology* **49** 538–542

Perreten V, Schwarz F, Cresta L, Boeglin M, Dasen G & Teuber M 1997 Antibiotic resistance spread in food. *Nature* **389** 801–802

Perreten V, Schwarz FV, Teuber M & Levy SB 2001 *Mdt*(A), a new efflux protein conferring multiple antibiotic resistance in *Lactococcus lactis* and *Escherichia coli*. *Antimicrobial Agents and Chemotheraphy* **45** 1109–1114

Pu ZY, Dobos M, Liwsowtin GK & Pobell IB 2002 Integrated polymerase chain reaction-based procedures for the detection and identification of species and subspecies of the Gram-positive bacterial genus *Lactococcus*. *Journal of Applied Microbiology* **93** 353–361

Putman M, van Veen HW, Degener JE & Konings WN 2001 The lactococcal secondary multidrug transporter LmrP confers resistance to lincosamides, macrolides, streptogramins and tetracyclines. *Microbiology* **147** 2873–2880

Raha AR, Ross E, Yusoff K, Manap MY & Ideris A 2002 Characterisation and molecular cloning of an erythromycin resistance plasmid of *Lactococcus lactis* isolated from chicken cecum. *Journal of Biochemistry, Molecular Biology and Biophysics* **6** 7–11

Reinbold GW & Reddy MS 1974 Sensitivity or resistance of dairy starter and associated microorganisms to selected antibiotics. *Journal of Milk and Food Technology* **37** 517–521

Ringo E 2004 Lactic acid bacteria in fish and fish farming. In *Lactic Acid Bacteria. Microbiological and Functional Aspects*, pp. 581–610 (Eds S Salminen, A von Wright & A Ouwehand). New York, USA: Marcel Decker Inc.

Salama MS, Musafa-Jeknic T, Sandine WE & Giovannioni SJ 1995 An ecological study of lactic acid bacteria: isolation of new strains of *Lactococcus* including *Lactococcus lactis* subspecies *cremoris*. *Journal of Dairy Science* **78** 1004–1017

Smit G, Smit BA & Engels WJM 2005 Flavour formation by lactic acid bacteria and biochemical flavours profiling of cheese products. *FEMS Microbiology Reviews* **29** 591–610

Su YA, He P & Clewell DB 1992 Characterization of the *tet*(M) determinant of Tn*916*: evidence for regulation by transcription attenuation. *Antimicrobials Agents and Chemotheraphy* **36** 769–778

Teuber M, Meile L & Schwarz F 1999 Acquired antibiotic resistance in lactic acid bacteria from food. *Antonie van Leeuwenhoek* **76** 115–137

Young JPW, Downer HL & Eardly BD 1991 Phylogeny of the prototrophic *Rhizobium* strain BTAil by polymerase chain reaction-based sequencing of a 16S rRNA segment. *Journal of Bacteriology* **173** 2271–2277