HAL
open science

Assessment of the antimicrobial wild-type minimum inhibitory concentration distributions of species of the Lactobacillus delbrueckii group

Morten Danielsen, Sigrid Mayrhofer, Konrad Johann Domig, Ernst Amtmann, Helmut Karl Mayer, Ana Belén Flórez, Baltasar Mayo, Jenni Korhonen, Lorenzo Tosi

To cite this version:

Morten Danielsen, Sigrid Mayrhofer, Konrad Johann Domig, Ernst Amtmann, Helmut Karl Mayer, et al.. Assessment of the antimicrobial wild-type minimum inhibitory concentration distributions of species of the Lactobacillus delbrueckii group. Dairy Science & Technology, 2008, 88 (2), pp.183-191. hal-00895675

HAL Id: hal-00895675

https://hal.science/hal-00895675

Submitted on 11 May 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Dairy Sci. Technol. 88 (2008) 183–191
© INRA, EDP Sciences, 2008
DOI: 10.1051/dst:2007002

Available online at:
www.dairy-journal.org

Original article

Assessment of the antimicrobial wild-type minimum inhibitory concentration distributions of species of the *Lactobacillus delbrueckii* group

Morten DANIELSEN[1]*, Sigrid MAYRHOFER[2], Konrad Johann DOMIG[2], Ernst AMTMANN[3], Helmut Karl MAYER[3], Ana Belén FLÓREZ[4], Baltasar MAYO[4], Jenni KORHONEN[5], Lorenzo TOSI[6]

[1] Chr. Hansen A/S, Bøge Allé 10-12, 2970 Hørsholm, Denmark
[2] Division of Food Microbiology and Hygiene, Department of Food Science and Technology, BOKU – University of Natural Resources and Applied Life Sciences, Vienna, Austria
[3] Division of Food Chemistry, Department of Food Science and Technology, BOKU – University of Natural Resources and Applied Life Sciences, Vienna, Austria
[4] Instituto de Productos Lácteos de Asturias (CSIC), Villaviciosa, Spain
[5] Institute of Applied Biotechnology, University of Kuopio, Finland
[6] Institute of Microbiology, Università Cattolica del Sacro Cuore, Piacenza, Italy

Received 22 June 2007 – Accepted 12 September 2007

Abstract – Determination of antimicrobial wild-type minimum inhibitory concentration (MIC) distributions is a prerequisite before differentiating susceptible bacteria from bacteria with acquired resistance. The antimicrobial susceptibility was determined for 190 strains of eight species of the *Lactobacillus delbrueckii* group. These were *L. acidophilus, L. amylovorus, L. crispatus, L. delbrueckii, L. helveticus, L. gallinarum, L. gasseri* and *L. johnsonii*. For most antimicrobial agents a clear distinction between susceptible and resistant bacteria was observed. Tetracycline resistance was abundant among *L. johnsonii* and *L. amylovorus* strains isolated from animals, while the *L. delbrueckii* and *L. helveticus* strains isolated from dairy products, and *L. gasseri* isolated from humans, rarely contained acquired resistance.

***Lactobacillus delbrueckii* / *Lactobacillus acidophilus* / susceptibility testing / food safety / antimicrobial resistance**

摘要 – 野生型德氏乳杆菌菌株抗菌素最低抑菌浓度分布的评价。确定野生型菌株的抗菌素最低抑菌浓度 (MIC) 分布是区别敏感菌与获得耐药性菌的先决条件。来自德氏乳杆菌群中的 8 个种的 190 株菌被用于抗菌素敏感性检测。菌株涵盖了嗜酸乳 (*L. acidophilus*) 、食淀粉乳杆菌 (*L. amylovorus*)、卷曲乳杆菌 (*L. crispatus*)、德氏乳杆菌 (*L. delbrueckii*)、瑞士乳杆菌 (*L. helveticus*) 、鸡乳杆菌 (*L. gallinarum*)、加氏乳杆菌 (*L. gasseri*) 和约氏乳杆菌 (*L. johnsonii*) 等 8个种。研究表明, 对于大多数抗菌素而言, 敏感菌和抗性菌具有明显的差异。源于自动物的约氏乳杆菌 (*L. johnsonii*) 和食淀粉乳杆菌 (*L. amylovorus*) 菌株多数具有四环素抗性, 而源于乳品的德氏乳杆菌 (*L. delbrueckii*) 和瑞士乳杆菌 (*L. helveticus*) 以及源于人的加氏乳杆菌 (*L. gasseri*) 菌株对四环素敏感 ,罕有获得性抗性。

德氏乳杆菌 / 嗜酸乳杆菌 / 敏感性试验 / 食品安全 / 抗菌剂的抗性

* Corresponding author (通讯作者): dkmda@chr-hansen.com

Article published by EDP Sciences and available at http://www.dairy-journal.org
or http://dx.doi.org/10.1051/dst:2007002

Résumé – Estimation de la distribution des concentrations minimales inhibitrices d'antibiotiques pour des souches sauvages au sein du groupe *Lactobacillus delbrueckii*. La détermination de la distribution des concentrations minimales inhibitrices d'antibiotiques sur des souches sauvages est un préalable pour différencier les bactéries sensibles de celles qui ont acquis une résistance. La sensibilité aux antibiotiques a été déterminée chez 190 souches de 8 espèces du groupe *Lactobacillus delbrueckii* : *L. acidophilus*, *L. amylovorus*, *L. crispatus*, *L. delbrueckii*, *L. helveticus*, *L. gallinarum*, *L. gasseri* et *L. johnsonii*. Pour la plupart des antibiotiques, une distinction claire entre bactéries sensibles et bactéries résistantes était observée. La résistance à la tétracycline était abondante parmi les souches de *L. johnsonii* et de *L. amylovorus* isolées d'animaux, alors que la résistance acquise des souches de *L. delbrueckii* et *L. helveticus* isolées de produits laitiers, et de *L. gasseri* isolées d'humains, était rare.

***Lactobacillus delbrueckii* / *Lactobacillus acidophilus* / test de sensibilité / sécurité alimentaire / résistance aux antibiotiques**

1. INTRODUCTION

The *Lactobacillus delbrueckii* group includes at least 19 species [18] and several of these species are widely used in food fermentations and as probiotic bacteria [11]. *Lactobacillus helveticus* and *L. delbrueckii* subsp. *lactis* are starter components in Swiss-type cheeses, and *L. delbrueckii* subsp. *bulgaricus* together with *Streptococcus thermophilus* are used as yoghurt starters. Strains of *L. acidophilus, L. johnsonii* and *L. gasseri* are used as probiotics. *Lactobacillus crispatus* and *L. gasseri* are commonly isolated from humans [8, 17, 19], while *L. gallinarum* and *L. amylovorus* are most often isolated from animals [8]. *Lactobacillus johnsonii* strains have been isolated both from humans and from animals [8, 16].

Bacteria for feed applications for animals within the EU must be characterized with regards to the absence of acquired genes conferring resistance to antimicrobial agents [6]. Bacteria used as probiotics and as starter cultures should also be screened for transferable resistance genes [11]. Wild-type minimum inhibitory concentration (MIC) distributions can be used to separate the susceptible (wild-type) strains from the resistant strains [21]. Species of the *L. delbrueckii* group have not been investigated closely and often the papers investigate only a limited number of isolates [5, 7] or include isolation of bacteria from dairy products from a limited geographic area [13], and the wild-type MIC distributions for these species have so far not been established.

Susceptibility testing of species of the *L. delbrueckii* group has been limited by the lack of a standard method [5, 11]. These fastidious bacteria grow poorly on standard test media such as Mueller-Hinton or ISO-Sensitest, and the MRS medium normally used for growing lactobacilli is not standardized, and has furthermore been shown to inhibit the action of several classes of antimicrobial agents such as aminoglycosides and trimethoprim [4, 12].

In this study we report the collection of 190 strains of eight species from the *L. delbrueckii* group. The strains of each species were collected from different locations or environments in order to achieve the most diverse set of strains. These 190 strains were susceptibility tested on a recently developed Lactic acid bacteria Susceptibility test Medium (LSM) [14] in order to contribute to the determination of the wild-type MIC distributions.

2. MATERIALS AND METHODS

2.1. Strain collection

The 190 strains tested in this project are summarized by species and origin in Table I. The primary sources for

Table I. The number of strains of each species and the origin of the strains.

Species	No. of strains	Pig	Poultry	Other animals	Human	Dairy	Other
L. acidophilus	11			2	8	1	
L. amylovorus	31	24		1	2	1	3
L. crispatus	7		2	1	4		
L. delbrueckii	44				4	39	1
L. helveticus	27					27	
L. gallinarum	8		8				
L. gasseri	36				35		1
L. johnsonii	26	14	2	6	2	1	1
Total	190	38	12	10	55	69	6

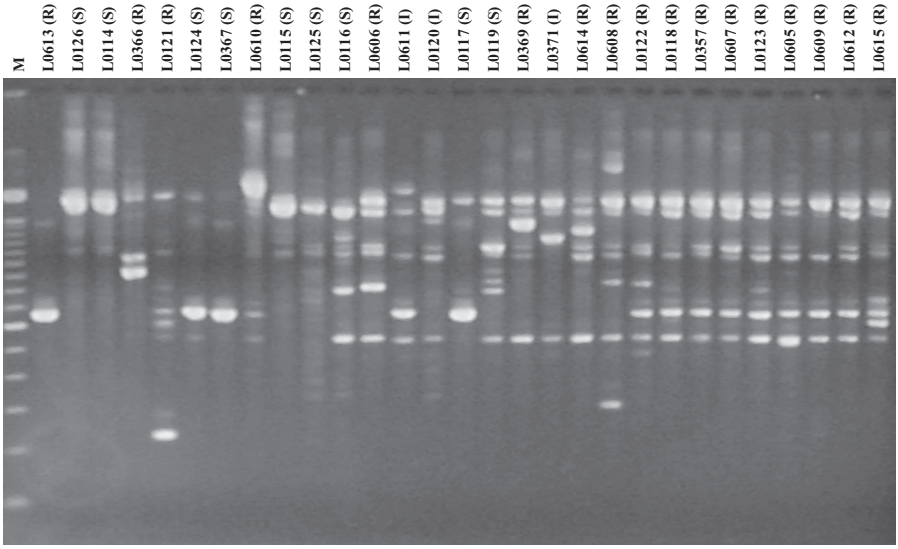

Figure 1. RAPD fingerprints of *L. amylovorus* strains (primer P3). M:100 bp ladder (Invitrogen/Gibco®, Carlsbad, CA, USA); (S): tetracycline MIC < 2 μg·mL^{-1}; (I): tetracycline MIC from 8–64 μg·mL^{-1}; (R): tetracycline MIC > 64 μg·mL^{-1}.

collection of the strains were environmental samples and 46 strains of various origins from the BCCM™/LMG bacteria collection, Belgium (LMG). The sources for each species were (no. of strains): *L. acidophilus*: LMG (8), human fecal samples (3); *L. amylovorus*: LMG (13), Austrian pigs (17), dairy (1); *L. crispatus*: human sample (4), animal: (3); *L. delbrueckii*: Egyptian fermented milk (22), Italian whey (4), dairy (13), Spanish fecal samples (4), grain (1); *L. gallinarum*: LMG (8); *L. gasseri*: LMG (10), human fecal samples Spain (3), Austria (16), Denmark (7); *L. helveticus*: Argentinean (7) and Italian (10) whey, Italian (7) and Finnish (2) cheese starters, dairy (1); *L. johnsonii*: LMG (8), Austrian (12) and Finnish (2) pigs, other animals (4). Of the 190 strains a total of 22 strains were deposited before 1980 (i.e. *L. acidophilus* (3), *L. delbrueckii*

(4), *L. gasseri* (1) and *L. helveticus* (14) strains). Only one strain from each sample was included in the study unless different genotypes had been observed.

2.2. Identification at species level

Different methods were used to identify the eight species at species level. PCR using species-specific primer pairs was applied for: *L. acidophilus*: Aci 16SI/16SII [22] and Laci-1/23-10C [19]; *L. gasseri*: Lgas-3/Lgas-2 [19]; *L. johnsonii*: Joh 16SI/16SII [22] and *L. amylovorus*: Cri 16SI/16SII [22], which gives an amplicon for both *L. amylovorus* and *L. crispatus*; as well as Lcri-3/Lcri-2 [19], which is specific for *L. crispatus*. All *L. gallinarum* strains were from the BCCM™/LMG bacteria collection and species identity was confirmed by ARDRA (Amplified Ribosomal DNA Restriction Analysis) [9]: primers Lb16a/23-1B were used to get an amplicon, which was cut by *Mse*I, and *L. gallinarum* shows a unique profile within species of the *L. acidophilus* group. *Lactobacillus delbrueckii* was identified with partial sequencing of the 16S rRNA gene. Fluorescent in situ hybridization (FISH) was applied to identify strains of *L. helveticus* [10].

2.3. Identification at strain level

Only strains with unique fingerprints or different origin were used for antimicrobial susceptibility testing. RAPD fingerprinting was not only applied to distinguish between isolates of the same species but also to investigate the genetic similarity of resistant and susceptible strains. Genomic DNA was isolated according to a standard protocol [1]. Several different arbitrary primers were used for screening purposes, and seven primers were used for strain differentiation. The primer P3 (5′-CTGCTGGGAC-3′) was used for the RAPD presented in Figure 1 [15]. The reaction mixture contained a total volume of 25 μL containing 50 ng of DNA, 25 pmol of primer and 1 unit of Dynazyme™ DNA Polymerase (Finnzymes OY, Espoo, Finland). DNA amplification was performed in a Thermal Cycler (Hybaid PCR Sprint, Mandel Scientific Inc., Ontario, Canada) as follows: initial denaturation for 5 min at 95 °C, followed by 45 cycles of denaturation for 1 min at 95 °C, annealing for 1 min at 36 °C, and extension for 2 min at 72 °C. Reactions were finished with an 8-min elongation period at 72 °C, followed by cooling to 4 °C. Electrophoretic separation of amplified PCR products was performed in 2% agarose gels in 0.5 X TBE buffer.

2.4. Susceptibility testing

The Etest (ABBiodisk, Solna, Sweden) was used as described previously to determine the minimum inhibitory concentrations (MICs) [5]. Lactic acid bacteria Susceptibility test Medium (LSM) agar plates were used for susceptibility testing [14]. The agar plates were incubated for 48 h at 37 °C under anaerobic conditions. All strains were susceptibility tested with seven antimicrobial agents (i.e. ampicillin, clindamycin, erythromycin, gentamicin, streptomycin, tetracycline and vancomycin). *Enterococcus faecalis* ATCC 29212 was included as a control strain for reproducible performance of the test method (medium, etc.).

3. RESULTS

A total of 190 strains was susceptibility tested. A few strains of *L. amylovorus, L. crispatus, L. delbrueckii* and *L. helveticus* did not grow well on LSM, but all

Table II. Susceptibility levels ($\mu g \cdot mL^{-1}$) of species of the *L. delbrueckii* group[a].

Species	AMP	CLI	ERY	GEN	STR	TET[b]	VAN
L. acidophilus	0.125–1	0.064–8	< 0.5	0.5–8	2–16	0.25–2	0.5–2
(n = 11)	(11)	(10)	(10)	(11)	(11)	(10)	(11)
L. amylovorus	0.064–2	< 0.016–2	< 0.5	0.5–16	2–16	0.5–1[b]	0.5–1
(n = 31)	(30)	(27)	(27)	(31)	(21)	(10)	(31)
L. crispatus	0.5–1	< 0.016–2	< 0.5	1–16	0.5–16	0.25–1	1–2
(n = 7)	(7)	(7)	(7)	(7)	(7)	(6)	(7)
L. delbrueckii	< 0.016–0.25	< 0.016–2	< 1	0.5–8	0.5–32	0.032–2[b]	0.25–2
(n = 44)	(44)	(44)	(43)	(44)	(44)	(43)	(44)
L. gallinarum	0.5–2	0.032–1	< 0.125	0.5–4	2–16	0.5	1–2
(n = 8)	(8)	(8)	(8)	(8)	(5)	(1)	(8)
L. gasseri	0.125–0.5	0.125–16	< 1	2–16	1–32	0.25–4	2–4
(n = 36)	(36)	(25)	(35)	(36)	(36)	(36)	(36)
L. helveticus	< 0.016–0.25	< 0.016–4	< 1	0.5–8	0.25–32	0.032–1	0.25–1
(n = 27)	(27)	(27)	(27)	(27)	(27)	(27)	(27)
L. johnsonii	0.125–1	0.25–16	< 1	2–16	2–32	0.5–4[b]	1–2
(n = 26)	(26)	(21)	(22)	(26)	(25)	(11)	(26)

(Number in brackets indicates number of strains within this susceptibility level.)
[a]The resistant strains in Table III are not included in this table.
[b]Three *L. johnsonii* strains with MICs = 8, 32 and 64 $\mu g \cdot mL^{-1}$, one *L. delbrueckii* MIC = 8 $\mu g \cdot mL^{-1}$, three *L. amylovorus* strains with MICs = 8 (two strains) and 64 $\mu g \cdot mL^{-1}$.
AMP: ampicillin; CLI: clindamycin; ERY: erythromycin; GEN: gentamicin; STR: streptomycin; TET: tetracycline; VAN: vancomycin.

strains grew sufficiently for reading of the Etest strips. All results reported here are after 48 h of growth.

In Table II the MIC ranges of the species are reported.

All tested strains were susceptible to ampicillin, except one *L. amylovorus* strain of porcine origin with a MIC > 256 $\mu g \cdot mL^{-1}$. No differences were observed between the species.

For erythromycin all the strains except two were either susceptible with a MIC < 1 $\mu g \cdot mL^{-1}$ or highly resistant with a MIC > 256 $\mu g \cdot mL^{-1}$. The highly resistant strains were: one *L. acidophilus*, one *L. gasseri,* three *L. amylovorus* and four *L. johnsonii* strains. Of these strains, one *L. gasseri* and one *L. johnsonii* strain showed isolated resistant colonies within the inhibition zone after an incubation of 48 h. All these resistant strains were also resistant to clindamycin with a MIC > 256 $\mu g \cdot mL^{-1}$, whereas only isolated resistant colonies could be detected for the *L. gasseri* strain after an incubation time of 48 h. Two strains had an erythromycin MIC of 4 $\mu g \cdot mL^{-1}$ (one *L. amylovorus* and one *L. delbrueckii*). The clindamycin MICs of the two strains were > 256 $\mu g \cdot mL^{-1}$ (the *L. amylovorus* strain) and 0.032 $\mu g \cdot mL^{-1}$ (the *L. delbrueckii* strain).

The MIC range for clindamycin was larger than for erythromycin. The MICs were either < 32 $\mu g \cdot mL^{-1}$ or

Table III. Number of resistant strains[a].

	Origin	Year[b]	AMP	CLI	ERY	GEN	STR	TET	VAN
L. acidophilus	Human	2001	–	1	1	–	–	1	–
L. amylovorus	Animal	1982	1	4	4	–	10	18	–
L. crispatus	Chicken	1986	–	–	–	–	–	1	–
L. delbrueckii	Dairy	2003	–	–	1	–	–	–	–
L. gallinarum	Chicken	1983	–	–	–	–	3	7	–
L. gasseri	Human[c]	1989	–	11	1	–	–	–	–
L. helveticus			–	–	–	–	–	–	–
L. johnsonii	Animal	1993	–	5	4	–	1	12	–
Total			1	21	11	–	14	39	–

[a]AMP: ampicillin MIC > 2 $\mu g \cdot mL^{-1}$; CLI: clindamycin MIC > 16 $\mu g \cdot mL^{-1}$; ERY: erythromycin MIC > 1 $\mu g \cdot mL^{-1}$; GEN: gentamicin MIC > 16 $\mu g \cdot mL^{-1}$; TET: tetracycline MIC > 64 $\mu g \cdot mL^{-1}$; STR: streptomycin MIC > 32 $\mu g \cdot mL^{-1}$; VAN: vancomycin MIC > 4 $\mu g \cdot mL^{-1}$.
[b]Earliest deposit of resistant strain.
[c]One resistant strain isolated from wine.

> 256 $\mu g \cdot mL^{-1}$. Additional to the above-mentioned erythromycin- and clindamycin-resistant strains, ten strains of *L. gasseri* and a single strain of *L. johnsonii* had clindamycin MICs > 256 $\mu g \cdot mL^{-1}$ due to isolated resistant colonies. This phenotype required 48 h of growth to be detected.

A total of 183 out of the 190 strains had tetracycline MICs that were either < 8 $\mu g \cdot mL^{-1}$ or > 64 $\mu g \cdot mL^{-1}$. Three strains of *L. johnsonii*, three strains of *L. amylovorus* and one strain of *L. delbrueckii* had MICs in the intermediate range 8–64 $\mu g \cdot mL^{-1}$.

Gentamicin MICs were between 0.5 $\mu g \cdot mL^{-1}$ and 16 $\mu g \cdot mL^{-1}$ for all strains. For streptomycin, the other aminoglycoside tested, the MICs were more unevenly distributed. Most strains (176 out of 190) had MICs < 64 $\mu g \cdot mL^{-1}$, but for one *L. johnsonii*, three *L. gallinarum* and 10 *L. amylovorus* strains the MICs were above 64 $\mu g \cdot mL^{-1}$.

All strains had vancomycin MICs < 8 $\mu g \cdot mL^{-1}$.

Strains with MICs that were clearly not within a normal wild-type distribution were identified based on the MIC being > 2 $\mu g \cdot mL^{-1}$ for ampicillin, > 16 $\mu g \cdot mL^{-1}$ for clindamycin, > 1 $\mu g \cdot mL^{-1}$ for erythromycin, > 16 $\mu g \cdot mL^{-1}$ for gentamicin, > 64 $\mu g \cdot mL^{-1}$ for tetracycline, > 32 $\mu g \cdot mL^{-1}$ for streptomycin and > 4 $\mu g \cdot mL^{-1}$ for vancomycin. These strains are labeled "highly resistant" and are summarized in Table III. No resistant strains isolated before 1982 were observed. Only tetracycline-resistant *L. johnsonii* (12 out of 26) and *L. amylovorus* (18 out of 31) were present in sufficient numbers to allow a cluster analysis based on the RAPD. For both species the resistant strains could not be clustered and only for a few strains could a correlation between susceptibility to antimicrobial agents and RAPD fingerprint be observed.

4. DISCUSSION

Susceptibility testing of species of the *L. delbrueckii* group has previously mainly been done in order to separate susceptible and resistant strains for use in food, especially fermented milk, cheese or as probiotics [5, 13]. However, the fastidious nature of these species and the lack of studies providing wild-type MIC distributions have made susceptibility testing and the

interpretation of these tests difficult. With the new medium for susceptibility testing (LSM) and the large number of strains collected in this study, determining the wild-type MIC distributions should become more feasible.

For the selection of the 190 strains for this project a lot of effort was made in order to find strains with the most diverse origin. Typing of the strains with RAPD was used to confirm the variability of the strains; however, it was not possible to collect these numerous strains without duplicates with respect to DNA fingerprinting. Therefore, it was decided within the European project ACE-ART that isolates with the same fingerprint but different origin (year, geographical location or type of sample) could also be used for antimicrobial susceptibility testing to achieve a clear picture of the susceptibility pattern of a bacterial species. We find this argument especially valid when investigating the prevalence of acquired resistance to antimicrobial agents, since the genes in virtually all cases would have been acquired after the use of the antimicrobial agents began after World War II. This means that strains with the same DNA fingerprint might easily have acquired different genes without changing the RAPD fingerprint. When determining wild-type MIC distributions a large number of strains are desired for statistical significance. In this study we reached 190 strains, representing eight species. Even though eight species are represented, these species are phenotypically very similar and in most cases they can only be identified to species level by using genotypic methods. The MIC ranges presented in Table II and analysis of the MICs as outlined below indicate that in most cases the species investigated in this project can be grouped and wild-type MIC distributions might thus be defined.

For some of the seven antimicrobial agents tested it appears that differentiating the susceptible and resistant strains would be straightforward. Ampicillin, gentamicin and vancomycin MICs were all distributed within a narrow range and comparable with susceptibility levels of other Gram-positive bacteria [2].

Erythromycin and tetracycline MICs were in most cases clearly distinguishable with either very low or very high MICs. However, one *L. amylovorus* and one *L. delbrueckii* strain had an erythromycin MIC of 4 $\mu g \cdot mL^{-1}$, and in total seven strains of *L. johnsonii, L. amylovorus* and *L. delbrueckii* had tetracycline MICs that were neither clearly susceptible nor resistant. Erythromycin resistance has previously been reported in strains of *L. crispatus* and *L. johnsonii* [16, 20].

The interpretation of the clindamycin MICs is even more complex. For clindamycin it appears that besides the strains with low MICs (< 32 $\mu g \cdot mL^{-1}$) and the resistant strains (MIC > 256 $\mu g \cdot mL^{-1}$), there is a subgroup with high resistance (MIC > 256 $\mu g \cdot mL^{-1}$) being expressed only after 48 h by the presence of isolated resistant colonies. This type of resistance was only observed in strains of *L. gasseri* and in one strain of the closely related species *L. johnsonii*. Higher levels of clindamycin resistance in *L. gasseri* than in other species of the *L. delbrueckii* group have been reported previously [5]. Although most strains had streptomycin MICs < 64 $\mu g \cdot mL^{-1}$ there was no clear distinction between susceptible and resistant strains. Streptomycin-resistant *L. delbrueckii* strains have previously been described [13]. Strains with a higher MIC might be resistant either due to acquired genes or due to mutations. Lactobacilli have been reported to have a high mutation rate to streptomycin resistance [3].

The prevalence of resistance phenotypes with respect to species, year of isolation or biological origin (e.g. pigs or dairy products) was also to be investigated by this work. However, the species isolated

from various environments were different. From dairy almost all isolates were *L. delbrueckii* or *L. helveticus*, all *L. gallinarum* originated from chickens, most *L. johnsonii* and *L. amylovorus* originated from pigs or other animals, and finally, almost all *L. gasseri* were isolated from human samples. From Table III it is clear that significantly more *L. amylovorus, L. gallinarum* and *L. johnsonii* are resistant to tetracycline than strains of the other species. None of the strains of these species were isolated before 1980 and an analysis of the prevalence of resistance based on year of isolation cannot be made. These three species were mainly isolated from animal sources. The presence of antimicrobial resistance in several unrelated strains implies that the resistance was acquired on many unique occasions and not through clonal expansion (Fig. 1). Although the tetracycline resistance levels might be due to the high use of tetracycline in animal husbandry we cannot, based on the results here, conclude whether this is actually the case or whether these three species acquire genes at a higher frequency than the other investigated species of the *L. delbrueckii* group. However, it can, on the other hand, be stated that acquired resistance in *L. gasseri* isolated from humans and *L. delbrueckii* and *L. helveticus* isolated from dairy products is very rare. In fact, the single *L. gasseri* strain with erythromycin and clindamycin resistance, due to isolated resistant colonies, was the only *L. gasseri* not isolated from a human source (the strain was isolated from wine).

In this study we collected a large number of strains from species of the *L. delbrueckii* group and investigated the resistance to seven antimicrobial agents. We show that in most cases strains will be either clearly susceptible or clearly resistant, using the breakpoints defined in Table III. However, in some cases this differentiation was more difficult, as was the case with the strains with intermediate resistance to tetracycline. Genotypic investigations are planned and it is hoped that the phenotype of the species with the MICs in the range between susceptible and resistant strains can be explained, so a complete safety evaluation of potential industrial strains can be carried out with the greatest safety for consumers combined with the clearest demands for the documentation efforts of the industrial producers.

Acknowledgements: This work was performed as part of the EU project "ACE-ART" (CT-2003-506214). The financial support of the European Commision, 6th Framework Program is acknowledged. G. Huys is appreciated for supplying strains to the project. W. Kneifel, A. Wind and A. von Wright are appreciated for good advice and helpful discussions.

REFERENCES

[1] Anonymous, Preparation and analysis of DNA, in: Ausubel F.M., Brent R., Kingston R.E., Moore D.D., Seidman J.G., Smith J.A., Struhl K. (Eds.), Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley-Interscience, New York, USA, 1990, pp. 2.4.1–2.4.5.

[2] Clinical and Laboratory Standards Institute/NCCLS, Performance Standards for Antimicrobial Susceptibility Testing: Fifteenth Informational Supplement, CLSI/NCCLS document M100-S15, Pennsylvania, USA, 2005.

[3] Curragh H.J., Collins M.A., High levels of spontaneous drug resistance in *Lactobacillus*, J. Appl. Bacteriol. 73 (1992) 31–36.

[4] Danielsen M., Andersen H.S., Wind A., Use of folic acid casei medium reveals trimethoprim susceptibility of *Lactobacillus* species, Lett. Appl. Microbiol. 38 (2004) 206–210.

[5] Danielsen M., Wind A., Susceptibility of *Lactobacillus* spp. to antimicrobial agents, Int. J. Food Microbiol. 82 (2003) 1–11.

[6] FEEDAP Panel, Opinion of the scientific committee on a request from EFSA related to a generic approach to the safety assessment by EFSA of microorganisms used in food/feed and the production of food/feed additives, EFSA J. 226 (2005) 1–12.

[7] Felten A., Barreau C., Bizet C., Lagrange P.H., Philippon A., *Lactobacillus* species identification, H_2O_2 production, and antibiotic resistance and correlation with human clinical status, J. Clin. Microbiol. 37 (1999) 729–733.

[8] Fujisawa T., Benno Y., Yaeshima T., Mitsuoka T., Taxonomic study of the *Lactobacillus acidophilus* group, with recognition of *Lactobacillus gallinarum* sp. nov. and *Lactobacillus johnsonii* sp. nov. and synonymy of *Lactobacillus acidophilus* group A3 (Johnson et al. 1980) with the type strain of *Lactobacillus amylovorus* (Nakamura 1981), Int. J. Syst. Bacteriol. 42 (1992) 487–491.

[9] Guan L.L., Hagen K.E., Tannock G.W., Korver D.R., Fasenko G.M., Allison G.E., Detection and identification of *Lactobacillus* species in crops of broilers of different ages by using PCR-denaturing gradient gel electrophoresis and amplified ribosomal DNA restriction analysis, Appl. Environ. Microbiol. 69 (2003) 6750–6757.

[10] Hertel C., Ludwig W., Pot B., Kerster K., Schleifer K.H., Differentiation of lactobacilli occurring in fermented milk products by using oligonucleotide probes and electrophoretic protein profiles, Syst. Appl. Microbiol. 16 (1993) 453–467.

[11] Hummel A.S., Hertel C., Holzapfel W.H., Franz C.M., Antibiotic resistances of starter and probiotic strains of lactic acid bacteria, Appl. Environ. Microbiol. 73 (2007) 730–739.

[12] Huys G., D'Haene K., Swings J., Influence of the culture medium on antibiotic susceptibility testing of food-associated lactic acid bacteria with the agar overlay disc diffusion method, Lett. Appl. Microbiol. 34 (2002) 402–406.

[13] Katla A.K., Kruse H., Johnsen G., Herikstad H., Antimicrobial susceptibility of starter culture bacteria used in Norwegian dairy products, Int. J. Food Microbiol. 67 (2001) 147–152.

[14] Klare I., Konstabel C., Müller-Bertling S., Reissbrodt R., Huys G., Vancanneyt M., Swings J., Goosens H., Witte W., Evaluation of new broth media for microdilution antibiotic susceptibility testing of lactobacilli, lactococci, pediococci and bifidobacteria, Appl. Environ. Microbiol. 71 (2005) 8982–8986.

[15] Mangin I., Corroler D., Reinhardt A., Gueguen M., Genetic diversity among dairy lactococcal strains investigated by polymerase chain reaction with three arbitrary primers, J. Appl. Microbiol. 86 (1999) 514–520.

[16] Martel A., Meulenaere V., Devriese L.A., Decostere A., Haesebrouck F., Macrolide and lincosamide resistance in the gram-positive nasal and tonsillar flora of pigs, Microb. Drug Resist. 9 (2003) 293–297.

[17] Pavlova S.I., Kilic A.O., Kilic S.S., So J.S., Nader-Macias M.E., Simoes J.A., Tao L., Genetic diversity of vaginal lactobacilli from women in different countries based on 16S rRNA gene sequences, J. Appl. Microbiol. 92 (2002) 451–459.

[18] Roos S., Engstrand L., Jonsson H., *Lactobacillus gastricus* sp. nov., *Lactobacillus antri* sp. nov., *Lactobacillus kalixensis* sp. nov. and *Lactobacillus ultunensis* sp. nov., isolated from human stomach mucosa, Int. J. Syst. Evol. Microbiol. 55 (2005) 77–82.

[19] Song Y., Kato N., Liu C., Matsumiya Y., Kato H., Watanabe K., Rapid identification of 11 human intestinal *Lactobacillus* species by multiplex PCR assays using group- and species-specific primers derived from the 16S-23S rRNA intergenic spacer region and its flanking 23S rRNA, FEMS Microbiol. Lett. 187 (2000) 167–173.

[20] Stroman P., Muller C.C., Sorensen K.I., Heat shock treatment increases the frequency of loss of an erythromycin resistance-encoding transposable element from the chromosome of *Lactobacillus crispatus* CHCC3692, Appl. Environ. Microbiol. 69 (2003) 7173–7180.

[21] Turnidge J., Jorgensen J.H., Antimicrobial susceptibility testing: General considerations, in: Murray P.R. (Ed.), Manual of Clinical Microbiology, 7th edn., ASM Press, Washington, USA, 1999, pp. 1505–1525.

[22] Walter J., Tannock G.W., Tilsala-Timisjarvi A., Rodtong S., Loach D.M., Munro K., Alatossava T., Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers, Appl. Environ. Microbiol. 66 (2000) 297–303.