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Intra- and Interlaboratory Performances of Two Commercial Antimicrobial Susceptibility Testing Methods for Bifidobacteria and Nonenterococcal Lactic Acid Bacteria▽†

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In a small-scale harmonization study involving nine laboratories in eight European countries, the intra- and interlaboratory performances of two commercially available systems, i.e., the VetMIC microplate system and Etest, for antimicrobial susceptibility testing of nonenterococcal lactic acid bacteria (NELAB) and bifidobacteria were analyzed. In addition, one laboratory also performed standard broth microdilution as a reference method. MICs of tetracycline, erythromycin, ampicillin, gentamicin, clindamycin, and streptomycin for the type strains of 25 species of NELAB and bifidobacteria and MICs of vancomycin for a selection of relevant taxa were determined. The previously described lactic acid bacterium susceptibility test medium (LSM) and related mixed-medium formulations, all including Iso-Sensitest broth as a basic component, were used as test media. The overall agreement of median MIC ranges ± 1 $\log_2$ dilution determined by the VetMIC and Etest methods with the median MICs determined by the reference method was very good for tetracycline, ampicillin, and streptomycin (92.3 to 100%) but low for erythromycin (19.5 to 30.7%) and clindamycin (50.0 to 80.8%). There was a consensus among the participating laboratories that VetMIC was preferred over Etest because of its lower cost, better growth support, and more uniform criteria for MIC end point reading. With the range for acceptable intralaboratory reproducibility being defined as the median MIC ± 1 $\log_2$ dilution, VetMIC results (with 69.2% of all data sets in the acceptable range) were shown to display greater reproducibility than Etest results (with 58.8% of all data sets in the acceptable range). Also at the interlaboratory level, the proportion of MIC values obtained with VetMIC that belonged to the complete agreement category (60.0%) was higher than the proportion of such values obtained with Etest (47.0%), which indicates a higher degree of interlaboratory reproducibility for the former method. Apart from some agent-specific effects, the majority of VetMIC and Etest replicate data sets were situated within a 1- to 2-$\log_2$ dilution range, suggesting that the two methods can be considered to be equivalent for recognizing resistance phenotypes. This multicenter study has further validated the standard use of LSM and related mixed-medium formulations with commercially available systems and formed the basis for the ongoing development of the ISO 10932/IDF 223 standard for susceptibility testing of NELAB and bifidobacteria.

Because of their distinctive fermentative, functional, and potentially health-promoting properties, bifidobacteria and nonenterococcal lactic acid bacteria (NELAB) such as lactobacilli, lactococci, and *Streptococcus thermophilus* are intensively used in the food industry as starter cultures, adjunct cultures, and probiotics (29). Although the majority of NELAB and *Bifidobacterium* species are food-grade organisms, the large-scale application and deliberate introduction of such cultures into the food chain has opened the debate over whether or not criteria that document their safety for human and animal use should be defined (35). Despite the overall low pathogenic potential of these organisms, several studies have indicated that especially NELAB can act as reservoirs of potentially transferable antimicrobial resistance genes (1, 10, 15, 31). In the field of probiotics, the absence of acquired resistance traits has been recommended as a safety criterion in the selection of new commercial culture probiotic strains for human use (12, 27, 28, 33).

Due mainly to the limited clinical relevance of NELAB and bifidobacteria, the development and optimization of methods for antimicrobial susceptibility testing of these organisms have long been underappreciated. Moreover, the fact that many of these organisms have specific nutritional and atmospheric re-

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quirements for growth does not allow uniform use of standardized susceptibility test media such as Mueller-Hinton broth and Iso-Sensitest (IST) broth. There are indications that de Man, Rogosa, and Sharpe (MRS) medium, which is commonly used as a growth medium for most of these organisms, may exhibit antagonistic effects with supplemental antimicrobials in susceptibility testing (13). To address the apparent limitations of using single media, a mixed formulation of IST broth (90%, vol/vol) and MRS broth (10%, vol/vol) referred to as lactic acid bacterium susceptibility test medium (LSM) was developed recently (16). This new formulation has proven to support the growth of a wide taxonomic range of lactobacilli and *Bifidobacterium* spp. (when supplemented with 0.03% cysteine) and minimizes potential antagonism between medium components and tested antimicrobials. So far, LSM and related mixed-medium formulations, all containing IST broth as the basic component, have been successfully used to determine MICs for members of the genera *Bifidobacterium*, *Lactobacillus*, *Lactococcus*, *Pediococcus*, and *Streptococcus* by microdilution and Etest methods (2, 5, 7, 8, 17, 18, 20–24, 32).

In the near future, it is expected that the increased use of LSM as the standard medium for susceptibility testing of NELAB and bifidobacteria will produce a large amount of MIC data, enabling the definition of epidemiological cutoffs (ECOFFS). As proposed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST [http://www.eucast.org/]), ECOFFS provide an objective basis to differentiate wild-type organisms, which lack acquired and mutational resistance mechanisms, from non-wild-type members of the same species that contain one or more mechanisms conferring antimicrobial resistance. For this purpose, evaluation of the internal and external quality assurance procedures for reference methods and commercial systems for susceptibility testing is absolutely crucial for the correct interpretation of these ECOFFS. Several harmonization studies of susceptibility testing of clinical (14, 30), veterinary (26, 34), and aquatic (11, 25) organisms have provided important insights into the reproducibility of results from standardized methods at the intra- and/or interlaboratory level. Although such studies would also be highly valuable to all with an interest in evidence-based biosafety assessments of NELAB and bifidobacteria for human and animal use, the performances of susceptibility test methods using LSM within and across laboratories have to our knowledge not been evaluated.

Within the framework of an international research project, the European Union Assessment and Critical Evaluation of Antibiotic Resistance Transferability in Food Chain (EU-ACE-ART), a small-scale harmonization study involving nine laboratories in eight European countries was conducted. The study set out to examine the performances of two commercial susceptibility test methods widely used throughout the EU-ACE-ART project, i.e., the VetMIC system (broth microdilution) and Etest (agar diffusion), at the intra- and interlaboratory levels. For this purpose, MICs of six antimicrobials for the type strains of 25 NELAB and *Bifidobacterium* species and MICs of vancomycin for a selection of relevant taxa were determined using LSM and related mixed-medium formulations as test media.

TABLE 1. Growth conditions for antimicrobial susceptibility testing of NELAB and *Bifidobacterium* type strains[a]

Strain	Medium	Temp (°C)
B. adolescentis LMG 10502^T	LSM-cysteine	37
B. animalis subsp. *animalis* LMG 10508^T	LSM-cysteine	37
B. bifidum LMG 11041^T	LSM-cysteine	37
B. breve LMG 13208^T	LSM-cysteine	37
B. longum subsp. *longum* LMG 13197^T	LSM-cysteine	37
B. pseudolongum subsp. *pseudolongum* LMG 11571^T	LSM-cysteine	37
B. thermophilum LMG 21813^T	LSM-cysteine	37
E. faecalis LMG 8222	IST broth	37
Lb. acidophilus LMG 9433^T	LSM	37
Lb. amylovorus LMG 9496^T	LSM	37
Lb. brevis LMG 6906^T	LSM	28
Lb. casei LMG 6904^T	LSM	28
Lb. delbrueckii subsp. *bulgaricus* LMG 6901^T	LSM	37
Lb. fermentum LMG 6902^T	LSM	37
Lb. gasseri LMG 9203^T	LSM	37
Lb. helveticus LMG 6413^T	LSM	37
Lb. johnsonii LMG 9436^T	LSM	37
Lb. paracasei subsp. *paracasei* LMG 13087^T	LSM	28
Lb. pentosus LMG 10755^T	LSM	28
Lb. plantarum LMG 6907^T	LSM	28
Lb. reuteri LMG 9213^T	LSM	37
Lb. rhamnosus LMG 6400^T	LSM	37
Lb. sakei subsp. *carnosus* LMG 17302^T	LSM	28
Lb. sakei subsp. *sakei* LMG 9468^T	LSM	28
Lc. lactis subsp. *lactis* LMG 6890^T	IST broth	32
S. thermophilus LMG 6896^T	IST-M17-lactose	42

[a] All strains were grown under anaerobic conditions. LMG strains were obtained from the BCCM/LMG Bacteria Collection, Ghent University, Ghent, Belgium.

MATERIALS AND METHODS

Study design and culture conditions. Susceptibility testing by VetMIC and Etest systems was performed according to standard operating procedures (SOPs) that were approved by all nine participants in the harmonization study: Laboratory of Microbiology, Ghent University, Ghent, Belgium; Istituto di Microbiologia, Universita Cattolica del Sacro Cuore—Piacenza, Piacenza, Italy; Chr. Hansen A/S, Hoersholm, Denmark; Instituto de Productos Lácteos de Asturias, Villaviciosa, Asturias, Spain; VTT Biotechnology, Espoo, Finland; Nofima Mat AS (previously Matforsk AS), As, Norway; University of Kuopio, Kuopio, Finland; Department of Food Science and Technology, University of Natural Resources and Applied Life Sciences, Vienna, Austria; and Institution of Microbiology, Research and Development, National Food Administration, Uppsala, Sweden. In addition to taking part in the interlaboratory harmonization study, one participant also determined the MICs for all strain-antimicrobial combinations (except those with vancomycin) by a noncommercial reference method based on the use of fresh broth microdilution panels.

The test panel comprised the type strains of 18 NELAB and 7 *Bifidobacterium* species and *Enterococcus faecalis* ATCC 29212 (LMG 8222) obtained from the BCCM/LMG Bacteria Collection, Ghent University, Ghent, Belgium (http://bccm.belspo.be/about/lmg.php) (Table 1). The strains were distributed to the respective participating laboratories as lyophilized cultures. Upon arrival in the labs, cultures were recovered on MRS medium and maintained on cryobeads during the entire study. Each strain was tested in three different laboratories using VetMIC and Etest and in a single laboratory using a broth microdilution reference method. Per strain-antimicrobial combination tested with VetMIC and Etest, each participating laboratory had to conduct a series of five independent consecutive replications starting from new subcultures. For combinations tested with the broth microdilution reference method, four consecutive replications were conducted.

Prior to the actual susceptibility assay, strains were precultured overnight (16 to 24 h, except for bifidobacteria, which usually required 40 to 48 h) on the agar version of the test medium under the recommended incubation conditions (Table 1). For lactobacilli, the LSM formulation (i.e., 90% IST broth [code no.

CM0471; Oxoid], 10% MRS broth [code no. CM0359; Oxoid]) was used, whereas for bifidobacteria, LSM broth was supplemented with 0.3 g/liter l-cysteine–HCl (yielding a formulation hereinafter referred to as LSM-cysteine). The pHs of LSM and LSM-cysteine were adjusted to 6.7 after autoclaving of the preparations with sterile HCl solution (16). For *S. thermophilus*, IST broth (90%) was supplemented with M17 broth (10% [code no. CM0817; Oxoid]) and 1% filter-sterilized lactose (yielding a formulation hereinafter referred to as IST-M17-lactose) (32). Pure IST broth was used for *Lactococcus lactis* subsp. *lactis*. In Etest assays, agar versions (Oxoid) of the respective broth media were used, except for MRS broth, which was supplemented with 15 g/liter bacteriological agar no. 1 (code no. LP0011; Oxoid).

VetMIC assay. After overnight incubation, agar cultures were checked for purity. For inoculum preparation, individual colonies were suspended in a sterile glass or plastic culture tube containing 2 to 5 ml sterile saline (i.e., 0.85% NaCl solution) until a density corresponding to a McFarland (McF) standard of 1 or a spectrophotometric equivalent (3×10^8 CFU/ml) was obtained. The inoculated saline suspension was diluted 1:1,000 (for inoculation of VetMIC plates) or 1:500 (for inoculation of vancomycin microdilution plates) in the appropriate test medium (Table 1) to obtain a final concentration of 3×10^5 or 6×10^5 CFU/ml, respectively, as recommended previously (6).

VetMIC (National Veterinary Institute, Uppsala, Sweden [http://www.sva.se/en/Target-navigation/Services–Products/VetMIC/]) is a commercially available microtiter-based system comprising dried antimicrobials in serial 2-fold dilutions that can be stored for 2 years at room temperature. For the ACE-ART project, a tailor-made VetMIC plate was designed, enabling susceptibility testing of two strains per plate for the following six agents: tetracycline (0.5 to 128 μg/ml), erythromycin (0.12 to 16 μg/ml), streptomycin (2 to 256 μg/ml), gentamicin (0.5 to 32 μg/ml), clindamycin (0.12 to 8 μg/ml), and ampicillin (0.12 to 8 μg/ml). Susceptibility to vancomycin was tested in a separate broth microdilution assay for bifidobacteria and for members of the *Lactobacillus acidophilus* group, i.e., *Lb. acidophilus*, *Lb. amylovorus*, *Lb. johnsonii*, and *Lb. gasseri*.

One hundred microliters of the 3×10^5 CFU/ml inoculum was added to each well (yielding 3×10^4 CFU/well) in columns 1 to 6 (strain 1) or 7 to 12 (strain 2) of individual VetMIC plates within 30 min after the preparation of the standardized inoculum. According to the VetMIC manufacturer, no additional homogenization step was required because the antimicrobial compound in each well dissolves easily in the test medium and diffuses to achieve equilibrium throughout the well. Microplates were incubated under the species-specific conditions listed in Table 1. When anaerobic jars were used, plates were piled with a lid between every two plates to generate a homogeneous environment throughout the jar. Depending on whether the tests were performed at 28 or 37°C, *Lb. plantarum* ATCC 14917^T (LMG 6907^T) or *E. faecalis* ATCC 29212, respectively, was included as a control strain during each susceptibility assay. As positive and negative controls, a standardized inoculum and uninoculated test medium, respectively, were added to wells without an antimicrobial compound.

For vancomycin microdilution assays, a stock solution of 1,280 μg/ml vancomycin (product no. V2002; Sigma) in water was prepared, and samples of this solution were diluted in LSM broth (for members of the *Lb. acidophilus* group) or LSM-cysteine (for bifidobacteria) to obtain intermediate concentrations over the range from 0.25 to 256 μg/ml. Subsequently, 50-μl aliquots of the final solutions were dispensed into microdilution plates, which were then sealed in plastic bags and immediately frozen at or below −20°C until needed. Upon usage, plates were thawed under anaerobic conditions. Each well containing 50 μl of a serial vancomycin dilution was inoculated with 50 μl of the bacterial inoculum (6×10^5 CFU/ml of LSM broth [for members of the *Lb. acidophilus* group] or LSM-cysteine [for bifidobacteria]), resulting in a final bacterial concentration of 3×10^5 CFU/ml (3×10^4 CFU/well) and in a final antibiotic concentration of 0.12 to 128 μg/ml. The last row in each plate was used as a sterility control and was inoculated with pure LSM broth or LSM-cysteine. The same control strains used for VetMIC plates were used.

After 48 h of incubation, growth in VetMIC and vancomycin microdilution assay systems was evaluated visually by comparing the pellet at the bottom of a well with the positive and negative controls. Any series of wells in which discontinuity in growth was observed were discarded. Irrespective of the bactericidal or bacteriostatic mechanism of the tested agent, the MIC was defined as the lowest antimicrobial concentration for which at least 80% visual reduction in growth was reported.

Etest. Strain inocula with densities corresponding to a McF standard of 1 or a spectrophotometric equivalent (3×10^8 CFU/ml) were prepared as described above. A sterile cotton swab was dipped into the standardized inoculum and used to inoculate an agar plate of the appropriate test medium (Table 1). Inoculated plates were allowed to dry for approximately 15 min before application of the Etest (AB bioMérieux, Solna, Sweden) and incubated under the recommended conditions (Table 1). Etest strips with preformed antimicrobial gradients in the test range from 0.016 to 256 μg/ml were applied for tetracycline (article no. 5100 2258; AB Biodisk), erythromycin (article no. 5100 1058; AB Biodisk), streptomycin (article no. 5100 2188; AB Biodisk), gentamicin (article no. 5100 1258; AB Biodisk), clindamycin (article no. 5100 0958; AB Biodisk), ampicillin (article no. 5100 0158; AB Biodisk), and (for members of the *Lb. acidophilus* group and *Bifidobacterium* spp.) vancomycin (article no. 5100 2558; AB Biodisk).

After 48 h of incubation, the MIC was defined as the value corresponding to the first point on the Etest strip where growth did not occur along the inhibition ellipse. For bacteriostatic agents (e.g., tetracycline, erythromycin, and clindamycin), the MIC was read at the point where growth was inhibited by 80% (i.e., the first point of significant inhibition as judged by the naked eye).

Broth microdilution reference assay. Cultures at a McF standard of 1 were prepared as described for the VetMIC assay. The resulting saline culture suspension was diluted 1:500 in the appropriate test medium (Table 1) to obtain a final concentration of 6×10^5 CFU/ml.

The MICs of the following antimicrobial agents were determined: tetracycline (range, 0.5 to 128 μg/ml [product no. T3383; Sigma]), erythromycin (range, 0.12 to 16 μg/ml [product no. E5389; Sigma]), streptomycin (range, 2 to 256 μg/ml [product no. S6501; Sigma]), gentamicin (range, 0.5 to 32 μg/ml [product no. G1264; Sigma]), clindamycin (range, 0.12 to 8 μg/ml [product no. C5269; Sigma]), and ampicillin (range, 0.12 to 8 μg/ml [product no. A0166; Sigma]). For each agent, two freshly prepared sterile stock solutions were diluted in the appropriate test medium to obtain 2-fold dilution series, each encompassing a range of four concentrations. Each well containing 50 μl of a serial antibiotic dilution was inoculated with 50 μl of the bacterial inoculum (6×10^5 CFU/ml of the appropriate broth), resulting in a final bacterial concentration of 3×10^5 CFU/ml (3×10^4 CFU/well). The last row in each plate was used as a sterility control and was inoculated with pure broth. The control strains were the same used for the other assays. After 48 h of incubation, MICs were determined as described for the VetMIC assay.

Data processing and statistical analysis. For each participating laboratory, MIC data and remarks on end point reading were filed in a standard table format and sent to a central laboratory, where they were collected in a central database.

For evaluation of intralaboratory reproducibility, MIC data generated with VetMIC and Etest were first converted to $\log_2$ values. In the case of Etest readings, MIC data that ranked in between 2-fold dilutions were rounded up to the next 2-fold dilution before conversion to the $\log_2$ scale. For each series of five replicate MIC measurements for a specific strain-antimicrobial combination, the median of the $\log_2$ series and the $\log_2$ value of the median were determined. Finally, the mathematical differences between each of the five $\log_2$ values in the replicate series and the $\log_2$ value of the median were summed. Analysis of these summed $\log_2$ distances (SLD) across all strain-antimicrobial combinations allowed classification of the variations in MIC data from the individual participating laboratories into different SLD categories. Intralaboratory reproducibility was considered acceptable for data sets classified into categories with an SLD of 0 or 1, corresponding to the range encompassing the median MIC ± 1 dilution step.

To assess the interlaboratory variation among results from each group of three laboratories that tested the same strain-antimicrobial combinations, the nonparametric Kruskal-Wallis one-way analysis of variance was used because no normalized distribution of MIC data could be assumed (19). Based on the defined threshold level, the degree of variance per strain-antimicrobial combination was expressed as one of three categories: (i) total agreement (i.e., results from all three laboratories were under the threshold), (ii) partial agreement (i.e., results from two of the three laboratories were under the threshold), and (iii) total disagreement (i.e., results from all three laboratories were above the threshold). In addition, the chi-square test was used to check if the two methods performed in similar ways. The confidence interval corresponded to P values of <0.05.

RESULTS

Protocol and study design agreement. In the initial phase of the study, each of the nine participating laboratories evaluated the test conditions described in the draft SOPs using *Lb. plantarum* ATCC 14917^T or *E. faecalis* ATCC 29212 for susceptibility assays at 28 or 37°C, respectively. During this test phase, several laboratories reported that accurate reading of Etest end points was not always possible due to different shapes of inhibition ellipses. Especially for Etest reading of clindamycin

TABLE 2. Percentages of agreement between results from commercial VetMIC and Etest assays and results from the broth microdilution reference method

Antibiotic	% Agreement[a] between results from broth microdilution and:	
	VetMIC	Etest
Erythromycin	19.5	30.7
Tetracycline	96.2	96.2
Streptomycin	92.3	96.2
Ampicillin	92.3	100
Gentamicin	73.1	69.2
Clindamycin	50.0	80.8
Total	70.5	78.8

[a] Values are percentages of agreement between the median MICs determined by broth microdilution (the reference method) in laboratory 8 and the interlaboratory range of the median MICs (± 1 $\log_2$ dilution) determined by the VetMIC and Etest methods.

MICs, both bulb-like and narrowing ellipses were observed. In order to ensure that all ellipse types were read at 80% inhibition, it was agreed that bulb-like ellipses would be read at the intersection where the head of the bulb ended in order to exclude microcolonies. If the ellipse was narrowing, clindamycin MICs were read at the actual intersection and distinct individual colonies within the lower part of the narrowing ellipse were included if they were situated very close to the strip.

Once the SOPs were finalized, *Lb. plantarum* ATCC 14917^T and *E. faecalis* ATCC 29212 were included as control strains in all assays in the actual harmonization trial. A crossover scheme was developed to ensure that each laboratory tested seven to nine type strains and that each strain was tested in three different laboratories using VetMIC and Etest methods. Laboratory 5 used only VetMIC, and this arrangement was partially compensated for by laboratory 2's conducting a higher number of Etest assays than the other laboratories. Per strain-antimicrobial combination, a set of five independently obtained MIC values were collected, resulting in totals of 2,875 and 2,755 MIC values generated by VetMIC and Etest procedures, respectively. In addition, laboratory 8 also determined MICs using the broth microdilution reference method, resulting in 624 MIC values.

Comparison of VetMIC and Etest with the broth microdilution reference method. In order to compare MIC data obtained by commercial VetMIC and Etest assays with those obtained by the broth microdilution reference method, the median value in the series of replicate MIC measurements for each strain-antimicrobial combination analyzed in a given laboratory was determined. Interlaboratory ranges of the median values for VetMIC and Etest MICs were then compared to the median values for broth microdilution MICs determined in laboratory 8 (see Table S1 in the supplemental material), and percentages of agreement were calculated (Table 2). Depending on the antimicrobial tested, marked differences in the percentages of agreement between the median MICs from the two commercial assays and those from the reference method were found. For tetracycline, streptomycin, and ampicillin, 92.3 to 96.2% and 96.2 to 100% of the median MICs determined with the reference method were within the interlaboratory range of the median values for VetMIC and Etest MICs $\pm$ 1 $\log_2$ dilution, indicating very good and excellent agreement, respectively. In contrast, low-level (19.5 to 30.7%) agreement between the results from commercial assays and those from the reference method was observed for erythromycin. In between these two groups of antimicrobials, moderate agreement for gentamicin (69.2 to 73.1%) and clindamycin (50.0 to 80.8%) was found. Except for clindamycin MICs, however, no pronounced differences between VetMIC and Etest in the level of agreement with the reference method were observed.

Intralaboratory performance of susceptibility testing. Each series of five independent replicate determinations per strain-antimicrobial combination submitted by an individual laboratory was classified into an SLD category expressing the intralaboratory variation between replicate MIC results (Table 3). The category with an SLD of 0, in which no variation among the five replicate values was recorded, contained a clearly higher proportion of the VetMIC data sets for strain-antimicrobial combinations (47.8%) than of the Etest data sets (30.9%). This difference was apparent for all labs (except laboratory 5) but was not necessarily observed for all strains in the test panel. For instance, for control strain *E. faecalis* ATCC

TABLE 3. Intralaboratory reproducibility of VetMIC and Etest results expressed as SLD values[a]

Laboratory	No. of combinations tested (no. of values) for:		No. (%) of data sets with SLD of:									
			0		1		2		3		>3	
	VetMIC	Etest	VetMIC	Etest	VetMIC	Etest	VetMIC	Etest	VetMIC	Etest	VetMIC	Etest
1	69 (345)	68 (340)	23	18	9	14	27	23	4	4	6	9
2	60 (300)	79 (395)	29	22	12	29	14	25	4	2	1	1
3	65 (325)	65 (325)	35	20	20	16	6	22	4	4		3
4	94 (470)	94 (470)	56	20	22	30	13	33	3	8		3
5	42 (210)	ND	11	ND	9	ND	13	ND	5	ND	4	ND
6	68 (340)	68 (340)	12	11	13	15	18	14	15	12	10	16
7	75 (375)	75 (375)	38	34	21	24	15	17	1			
8	52 (260)	52 (260)	39	27	9	15	4	10				
9	50 (250)	50 (250)	32	18	8	11	10	13		7		1
Total	575 (2,875)	551 (2,755)	275 (47.8)	170 (30.9)	123 (21.4)	154 (27.9)	120 (20.9)	157 (28.5)	36 (6.3)	37 (6.7)	21 (3.6)	33 (6.0)

[a] Laboratories were assigned numbers in random order. All MIC results were read after 48 h. ND, not determined.

TABLE 4. Interlaboratory reproducibility of VetMIC and Etest results expressed as degrees of variance per antimicrobial agent

Degree of variance	Procedure	% of MICs[b] of:							
		Tetracycline (26)	Erythromycin (26)	Streptomycin (26)	Gentamicin (26)	Clindamycin (26)	Ampicillin (26)	Vancomycin (12)	All agents (168)
Complete agreement	VetMIC	42.3	69.2	53.8	53.8	69.2	73.1	50.0	59.5
	Etest	61.5	38.5	34.6	42.3	38.5	57.7	66.7	47.0
Partial agreement	VetMIC	26.9	19.2	23.1	19.2	15.4	23.1	33.3	22.0
	Etest	23.0	23.1	26.9	26.9	34.6	19.2	16.7	25.0
Complete disagreement	VetMIC	30.8	11.5	23.1	26.9	15.4	3.8	16.7	18.5
	Etest	15.4	38.5	38.5	30.8	26.9	23.1	16.7	28.0
P[a]		0.104	0.198	0.083	0.029	0.182	0.099	0.099	0.034

[a] P values of <0.05 were within the confidence interval.
[b] All MICs were read after 48 h. Numbers in parentheses after names of agents are numbers of strains tested.

29212, the difference in reproducibility of results between methods was evident, with 69.0% of the series of results obtained with VetMIC situated in the category with an SLD of 0, compared to only 36.6% of the series of results obtained with Etest (data not shown). In contrast, no difference for control strain *Lb. plantarum* ATCC 14917^T was recorded, as approximately 37% of the submitted series of results from both methods were in the category with an SLD of 0.

The lower number of Etest results for individual strain-antimicrobial combinations in the category with an SLD of 0 was compensated for by the numbers of Etest data sets assigned to categories with SLD of 1 and 2 (Table 3). These categories contained proportionally more results for strain-antimicrobial combinations assayed with Etest (27.9 and 28.5%, respectively) than for those assayed with VetMIC (21.4 and 20.9%, respectively). In these categories, intralaboratory variation was reflected by the fact that one of the five repeated MIC measurements deviated by 1 $\log_2$ dilution from the median (SLD = 1) or that one MIC value deviated by 2 $\log_2$ dilutions from the median or that two MIC values deviated by 1 $\log_2$ dilution from the median (SLD = 2). The proportions of strain-antimicrobial combinations with the highest range of intralaboratory variation (those with data sets with an SLD of $\geq$3) were relatively comparable for the VetMIC (9.9%) and Etest (12.7%) methods. Laboratory 6 submitted the highest proportional numbers of data sets with SLD of $\geq$3 (25 of 57 for VetMIC and 28 of 70 for Etest). Regardless of the antimicrobial tested, the combinations tested in laboratory 6 that yielded an SLD of $\geq$3 involved mainly *Lactobacillus* strains, as no bifidobacteria were included in the test panel for this laboratory.

Interlaboratory performance of susceptibility testing. The performances of both susceptibility test methods across all participating laboratories were classified into three variance categories representing total agreement, partial agreement, or total disagreement among results from each set of three laboratories that tested the same strain-antimicrobial combinations (Table 4). Globally, a higher proportion of MIC values obtained with VetMIC (60.0%) than of those obtained with Etest (47.0%) belonged to the complete agreement category, which indicates higher interlaboratory reproducibility of results from the VetMIC method. Overall, $\geq$50% of VetMIC values for all antimicrobials except tetracycline (42.3%) belonged to this category. Conversely, $\leq$50% of the Etest MIC values for all antimicrobials except tetracycline (61.5%), ampicillin (57.7%), and vancomycin (66.7%) were in complete agreement among three laboratories. The complete disagreement category contained a higher proportion of Etest MIC values (28.0%) than of VetMIC MIC values (18.5%). For all antimicrobials except tetracycline (15.4%) and vancomycin (16.7%), $\geq$20% of the Etest MIC values were in total disagreement among three laboratories. In contrast, $\leq$20% of the VetMIC values obtained for all agents except tetracycline (30.8%), streptomycin (23.1%), and gentamicin (26.9%) belonged to this category. Notably, cases of complete disagreement tended to occur more frequently among strain-antimicrobial combinations including a strain of bifidobacteria (38 of 98 combinations [38.8%]) than among those including a strain of lactobacilli (39 of 200 combinations [19.5%]) (data not shown).

The interlaboratory reproducibility of results from the two methods was also statistically analyzed using the chi-square test (Table 4). This analysis further substantiated the results from the variance analysis, indicating that VetMIC and Etest essentially performed differently among labs for all tested antimicrobials except gentamicin. Across all antimicrobials, however, the difference between the two methods was not found to be statistically significant ($P = 0.034$).

DISCUSSION

Most NELAB and bifidobacteria used in food and feed production have a long history of safe use (35). In this context, producers usually rely on the GRAS (generally recognized as safe) definition used in the United States and the QPS (qualified presumption of safety) approach of the European Food Safety Authority (http://www.efsa.europa.eu/en.html) for the risk assessment of commercial strains. However, the facts that acquired antimicrobial resistance mechanisms are known to occur in NELAB and bifidobacteria (1, 10, 15, 31), that the antimicrobial resistance pattern is strain specific rather than species specific, that relatively few resistance data are available for these bacterial groups, and that standardization of susceptibility test methods is currently lacking have raised some concern about the practical implementation of GRAS and QPS

approaches. The present study is the first to determine the intra- and interlaboratory performances of two protocols for antimicrobial susceptibility testing of NELAB and bifidobacteria by using appropriate growth-supporting test media with IST broth as a basic component.

The general remarks received from the participating laboratories indicated that the preparation and use of LSM and other mixed-medium formulations were very straightforward and that VetMIC was preferred over Etest for a number of practical reasons. First, it was argued that the VetMIC system, although it offers less flexibility in choice and concentration range of test agents, clearly is less expensive than Etest. Also, better overall growth of the selected NELAB and *Bifidobacterium* strains was observed in the broth version (used for VetMIC) than on the agar version (used for Etest) of a given test medium. For instance, several laboratories reported sufficient growth of *Lb. amylovorus* LMG 9496^T and *Lb. brevis* LMG 6906^T in LSM broth but very weak growth on LSM agar. For either of the two methods, 48 h of incubation was required because several NELAB strains and especially bifidobacteria failed to produce visible growth on LSM after 24 h during initial growth trials (data not shown). Likewise, Egervärn and colleagues (6) observed previously that end points in LSM-based microdilution testing of *Lactobacillus* species were more easily read after 48 h of incubation. Finally, accurate reading of Etest clindamycin MICs, in contrast to VetMIC clindamycin MICs, was not always possible due to different shapes of inhibition ellipses and required the addition of specific guidelines in the SOPs.

Unlike the participants in the present study, other researchers (3, 24) have observed ingrowth of resistant microcolonies in elliptical inhibition zones during Etest analyses of NELAB. Possibly, this may be due to the high rate of spontaneous mutation after prolonged (>24- to 48-h) incubation with subinhibitory antibiotic concentrations. In a recent study of *Lb. acidophilus* group members in which VetMIC was compared to the agar-based disk diffusion and Etest methods, a minor satisfying level of agreement was specifically found for results of susceptibility testing with bacteriostatic agents such as clindamycin (24). In the present study, VetMIC and Etest were compared to broth microdilution as a noncommercial reference method, and MICs of tetracycline, streptomycin, gentamicin, and ampicillin determined by the two assays were in good agreement with those determined by the reference method. In contrast to what could be expected given the reported difficulties with reading of Etest results, a higher percentage of agreement with the reference MICs within a range of ± 1 $\log_2$ dilution was observed for Etest results than for VetMIC results. The low levels of agreement between the MICs of erythromycin determined by the commercial assays and the reference method were surprising and require further attention. It is the lack of congruence observed for clindamycin and erythromycin MICs that largely accounted for the differences in overall agreement with the reference method between VetMIC (70.5%) and Etest (78.8%). On the other hand, it should be kept in mind that the broth microdilution reference method was conducted at only one of the participating laboratories and that, ideally, equivalent numbers of MIC measurements by different methods are needed to compare different test systems.

The apparent differences in the support of test organism growth and the uniformity of end point reading between VetMIC and Etest were also reflected in the mathematical scoring of their performances within and across laboratories. Based on the criterion that only data sets categorized as having SLD of 0 or 1 (i.e., those within the range of the median MIC $\pm$ 1 dilution step) were considered to have acceptable intralaboratory reproducibility, VetMIC (with 69.2% of data sets meeting this standard) appeared to display better reproducibility of results than Etest (with 58.8% of data sets meeting this standard). Clearly, the category with an SLD of 0 had the largest impact on this difference, i.e., 16.9% of all replicate series that exhibited no intralaboratory variation when tested with VetMIC showed a variation of ≥ 1 $\log_2$ steps at the intralaboratory level using the Etest system. Accordingly, there was a shift of the number of Etest series allocated into categories with SLD of 1 and 2, which was 14.1% higher than the corresponding number of VetMIC series. A more global analysis indicates that >87% of all VetMIC (87.3%) and Etest (90.1%) replicate data sets were situated within a 1 to 2 $\log_2$ deviation range from the median, thus demonstrating that results from the two methods correlate well at the intralaboratory level. Previous comparisons between Etest and microdilution methods revealed differences of up to 2 $\log_2$ dilutions but indicated the two methods to be equivalent in recognizing resistance phenotypes (4, 9).

Analysis of interlaboratory performance was based on a study with each strain-antimicrobial combination being tested by three laboratories. Although results from such small-scale studies have to be interpreted with caution, variance analysis per agent indicated that for all antimicrobials except gentamicin, VetMIC appeared to be more suitable for interlaboratory harmonization than Etest. However, when both VetMIC and Etest were compared to a reference method (i.e., broth microdilution), the preference for VetMIC was no longer pronounced. Despite the availability of well-defined SOPs, the fact that experienced as well as nonexperienced users of Etest contributed to the harmonization trial is likely to have influenced the outcome of the study. In most cases, reading of Etest results is straightforward, but in cases of ingrowth and/or diffuse inhibition zone edges, precise reading may require the assistance of an experienced user. Also, simultaneous analyses of intra- and interlaboratory performances of different susceptibility test methods for bacterial groups with complex growth requirements such as NELAB probably requires a larger set of MIC measurements. Preferably, future harmonization studies should include a higher number of participants to more reliably investigate the influence of previous working experience on the test performances.

In this study, we opted to include mainly type strains primarily because of their universal availability in public culture collections worldwide and their well-defined taxonomic status. However, because most of these type strains appeared to be susceptible to the tested antimicrobials, it is not clear to what extent the performance differences observed between VetMIC and Etest for susceptibility testing of NELAB and bifidobacteria may affect the recognition of resistance phenotypes. In this respect, the inclusion of reference strains with phenotypically and/or genotypically documented resistance in future harmonization studies would certainly help to assess the im-

pact of these intra- and interlaboratory differences. Undoubtedly, accurate and reproducible determination of antimicrobial MICs remains crucial in the scientific communication between food/feed industries and regulatory authorities (33). On the other hand, it is unlikely that intermethod deviations of 1 to 2 $\log_2$ dilutions as commonly measured in the present study will lead to failure to differentiate between susceptible wild-type populations and non-wild-type resistant populations, for which the respective MIC ranges are usually ≥ 4 $\log_2$ dilution steps apart. Clearly, this discrimination will also depend on the availability, agreement, and interpretation of ECOFFs based on data for a sufficiently large number of geographically and biologically diverse members of a species.

In conclusion, this multicenter study has further validated the use of LSM and related mixed-medium formulations for susceptibility testing of bifidobacteria and NELAB with commercially available assays. Noteworthily, the general protocol that was followed throughout this harmonization study has been used as a basis to develop the ISO 10932/IDF 223 standard "Milk and Milk Products—Determination of the Minimal Inhibitory Concentration (MIC) of Antibiotics Applicable to Bifidobacteria and Non-Enterococcal Lactic Acid Bacteria (LAB)" (http://www.iso.org/iso/iso_catalogue/catalogue_tc/catalogue_detail.htm?csnumber=46434). Provided that this new standard becomes widely adopted for susceptibility testing of NELAB and bifidobacteria, the definition of ECOFFs is the next step toward a more standardized approach in assessing the biosafety of these organisms in food and feed production. The recent transfer of all approved MIC data from the ACE-ART project to EUCAST for incorporation in an on-line MIC distribution database (G. Kahlmeter, personal communication) is expected to stimulate that process in the near future.

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