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Effects of Inoculum Size and Incubation Time on Broth Microdilution Susceptibility Testing of Lactic Acid Bacteria▽

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Inoculum size and incubation time were varied during broth microdilution testing of the susceptibilities of 35 strains of lactic acid bacteria to six antibiotics. An increase in either parameter resulted in elevated MICs for all species. An inoculum of 3×10^5 CFU/ml is recommended to assess the antibiotic susceptibilities of these bacteria by using broth microdilution.

Lactic acid bacteria (LAB) are commonly used as food-processing aids and probiotics. Due to their genetic flexibility and widespread occurrence in the food chain and in the intestinal tract, LAB can act as potential reservoirs of antibiotic resistance genes that may be transferred to other bacteria, including human pathogens (4, 21). Thus, the presence of antibiotic resistance genes should be assessed before LAB are used in food applications. For this reason, standardized and reliable testing procedures are needed to define rational microbiological breakpoints for distinguishing between strains with and without acquired resistance genes (22).

There is currently no standard method for antibiotic susceptibility testing of LAB, although several broth microdilution methods have been used (5–7, 9, 16, 19, 20). At present, the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) recommends broth microdilution for bacteria that grow aerobically (3) and for anaerobic bacteria belonging to the *Bacteroides fragilis* group (18). The inoculum size and incubation time are important parameters to evaluate during the development of broth microdilution methods (22) and have been extensively studied for several bacterial species (1, 8, 13, 14, 17) but not for nonenterococcal LAB. Other factors that may affect the susceptibility results are the incubation temperature and the composition of the atmosphere and the growth medium (12, 22). The poor growth of many LAB on established antibiotic susceptibility testing media such as Mueller-Hinton and Iso-Sensitest media has led to the recent development of LAB susceptibility test medium (LSM) (15). The present study was performed to evaluate the effects of the inoculum size and incubation time on antibiotic MICs for LAB using broth microdilution and LSM.

Twenty-nine LAB reference strains were tested: 27 *Lactobacillus* species, encompassing different phylogenetic groups and fermentation pathways, and 1 strain each of *Lactococcus lactis* subsp. *lactis* and *Streptococcus thermophilus*, both species widely used by the food industry (Table 1). Six clinical *Lactobacillus* isolates, recovered from cerebrospinal fluid, blood, dental caries, breast milk, intestines, and feces, respectively, were also tested. *Enterococcus faecalis* LMG 8222 was included as a quality control. Strains were obtained from the BCCM/LMG Bacteria Collection, Ghent University, Ghent, Belgium ($n = 32$), the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany ($n = 3$), and the American Type Culture Collection, Middlesex, United Kingdom ($n = 1$). All strains were grown anaerobically (AnaeroGen; Oxoid). The incubation temperatures and growth media used for each species are shown in Table 1.

Broth microdilution was performed using ACE-ART VetMIC panels (National Veterinary Institute, Uppsala, Sweden). The 96-well microtiter plates contained six antibiotics, air dried in cabinets at approximately 30°C, in serial twofold dilution steps to determine MICs in the following ranges: oxytetracycline, 0.5 to 128 μg/ml; clindamycin, 0.12 to 8 μg/ml; streptomycin, 2 to 256 μg/ml; erythromycin, 0.12 to 16 μg/ml; gentamicin, 0.5 to 32 μg/ml; ampicillin, 0.12 to 8 μg/ml. Colonies from overnight cultures (20 to 24 h) were suspended in the corresponding growth medium (Table 1) to obtain four final densities ranging from 3×10^4 to 3×10^7 CFU/ml for each strain. Bacterial density was measured spectrophotometrically at 600 nm and verified by viable cell counts. Each well was filled with 100 μl of inoculum. The panels were covered with plastic lids and incubated anaerobically for 24 and 48 h. The MIC was defined, according to the manufacturer's recommendations, as the lowest antibiotic concentration for which there was no visible bacterial growth, i.e., the first well without a pellet.

The MICs of the quality control strain determined after 24 h at 3×10^5 CFU/ml were within the range reported by Klare et al. (15) for all antibiotics tested except erythromycin, which displayed a twofold increase in the MIC (data not shown). Interassay reproducibility was evaluated by assessing the susceptibilities of five reference strains (*Lactobacillus acidophilus* LMG 9433^T, *L. amylovorus* LMG 9496^T, *L. plantarum* LMG 6907^T, *L. rhamnosus* LMG 6400^T, and *Lactobacillus sakei* subsp. *sakei* LMG 9468^T) to six antibiotics on five separate occasions. MICs determined at 3×10^5 CFU/ml after both 24 and 48 h incubation were within the accuracy limit of MIC standard tests (plus or minus one twofold dilution step) (2) for all strain-antibiotic combinations tested (data not shown).

The MICs for all strains increased with inoculum size. Increasing the density from 3×10^4 to 3×10^5 CFU/ml resulted

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TABLE 1. LAB strains and growth conditions used for antibiotic susceptibility testing

LAB strain (origin of clinical isolates)	Fermentation pathway[a]	Phylogenetic group[b]	Growth medium	Growth temp (°C)
Lactobacillus acidophilus LMG 9433^T	A	*L. delbrueckii*	LSM[c]	37
Lactobacillus amylovorus LMG 9496^T	A	*L. delbrueckii*	LSM	37
Lactobacillus delbrueckii subsp. *bulgaricus* LMG 6901^T	A	*L. delbrueckii*	LSM	37
Lactobacillus gasseri LMG 9203^T	A	*L. delbrueckii*	LSM	37
L. gasseri LMG 13047 (human feces)	A	*L. delbrueckii*	LSM	37
Lactobacillus helveticus LMG 6413^T	A	*L. delbrueckii*	LSM	37
Lactobacillus johnsonii LMG 9436^T	A	*L. delbrueckii*	LSM	37
L. johnsonii LMG 18175 (human intestine)	A	*L. delbrueckii*	LSM	37
Lactobacillus farciminis LMG 9200^T	A	*L. plantarum*	LSM	37
Lactobacillus salivarius subsp. *salivarius* DSM 20555^T	A	*L. salivarius*	LSM	37
Lactobacillus casei LMG 6904^T	B	*L. casei*	LSM	37
Lactobacillus rhamnosus LMG 6400^T	B	*L. casei*	LSM	37
L. rhamnosus LMG 10768 (blood)	B	*L. casei*	LSM	37
Lactobacillus paracasei subsp. *paracasei* LMG 13087^T	B	*L. casei*	LSM	37
L. paracasei subsp. *paracasei* LMG 10774 (cerebrospinal fluid)	B	*L. casei*	LSM	37
Lactobacillus alimentarius LMG 9187^T	B	*L. plantarum*	LSM	37
Lactobacillus pentosus LMG 10755^T	B	*L. plantarum*	LSM	28
Lactobacillus plantarum LMG 6907^T	B	*L. plantarum*	LSM	28
L. plantarum LMG 9206 (dental caries)	B	*L. plantarum*	LSM	28
Lactobacillus curvatus LMG 9198^T	B	*L. sakei*	LSM	28
Lactobacillus sakei subsp. *sakei* LMG 9468^T	B	*L. sakei*	LSM	28
Lactobacillus agilis LMG 9186^T	B	*L. salivarius*	LSM	37
Lactobacillus bifermentans LMG 9845^T	B		LSM	37
Lactobacillus buchneri DSM 20057^T	C	*L. buchneri*	LSM	37
Lactobacillus fructivorans LMG 9201^T	C	*L. buchneri*	LSM	37
Lactobacillus hilgardii LMG 6895^T	C	*L. buchneri*	LSM	37
Lactobacillus collinoides LMG 9194^T	C	*L. plantarum*	LSM	28
Lactobacillus fermentum LMG 6902^T	C	*L. reuteri*	LSM	37
Lactobacillus panis LMG 21658^T	C	*L. reuteri*	LSM	37
Lactobacillus reuteri DSM 20016^T	C	*L. reuteri*	LSM	37
L. reuteri ATCC 55730 (human breast milk)	C	*L. reuteri*	LSM	37
Lactobacillus suebicus LMG 11408^T	C	*L. reuteri*	LSM	37
Lactobacillus brevis LMG 6906^T	C		LSM	37
Lactococcus lactis subsp. *lactis* LMG 6890^T			IST[d]	32
Streptococcus thermophilus LMG 6896^T			IST + 1% lactose	42
Enterococcus faecalis LMG 8222			LSM	37

[a] As summarized by Hammes and Vogel (11). Group A, obligately homofermentative lactobacilli; group B, facultatively heterofermentative lactobacilli; group C, obligately heterofermentative lactobacilli.
[b] As defined by Hammes and Hertel (10).
[c] Iso-Sensitest broth (90%; Oxoid) plus MRS broth (10%; Oxoid) adjusted to pH 6.7 (15).
[d] Iso-Sensitest broth (Oxoid).

in identical MICs after 48 h of incubation for 144 (69%) of the 210 strain-antibiotic combinations, a twofold increase in MICs for 63 (30%) of the combinations, and a fourfold increase for the remaining 3 (1%) combinations (Fig. 1a). A similar stepwise increase in MICs was observed when the inoculum was further increased to 3×10^6 and 3×10^7 CFU/ml, respectively. The shift in the MIC due to increased inoculum size was independent of incubation time (data not shown).

All strains displayed similar increases in MICs when the incubation time was extended to 48 h. With an inoculum of 3×10^5 CFU/ml, 102 (49%) of the 210 strain-antibiotic combinations were unaffected by prolonged incubation, whereas MICs increased twofold for 96 (46%) of the combinations (Fig. 1b). For the remaining combinations (6%), fourfold MIC increases between 24 and 48 h of incubation were seen mainly with oxytetracycline and clindamycin, but these increases were all in the lower part of the MIC range (maximum, 2 to 8 μg/ml). The MIC increase with time was independent of inoculum size (data not shown). For the clinical isolates, the elevation in MICs due to increased inoculum size or incubation time was in accordance with the results for the LAB reference strains (data not shown).

The use of the two highest inoculum densities (3×10^6 and 3×10^7 CFU/ml) was generally associated with trailing growth, which prevented clear-cut end point readings. In addition, an inoculum of 3×10^4 CFU/ml resulted in poor growth for *L. casei*, *L. collinoides*, *L. brevis*, *L. hilgardii*, and *L. buchneri* after 24 h of incubation, making it difficult to determine MICs accurately. Thus, an inoculum size of 3×10^5 CFU/ml is recommended for broth microdilution susceptibility testing of LAB using LSM. This is in approximate agreement with the standardized inoculum size for aerobic bacteria (3) but is about three times lower than the density recommended for anaerobic bacteria (18). Sufficient growth for MIC determination was obtained at 3×10^5 CFU/ml after both 24 and 48 h of incubation for all strains tested. However, end points were more easily read after 48 h of incubation. In addition, LAB groups that need extended incubation for sufficient growth on LSM, such as most *Bifidobacterium* species (15), can be tested simultaneously when 48 h is used as the standard incubation time.

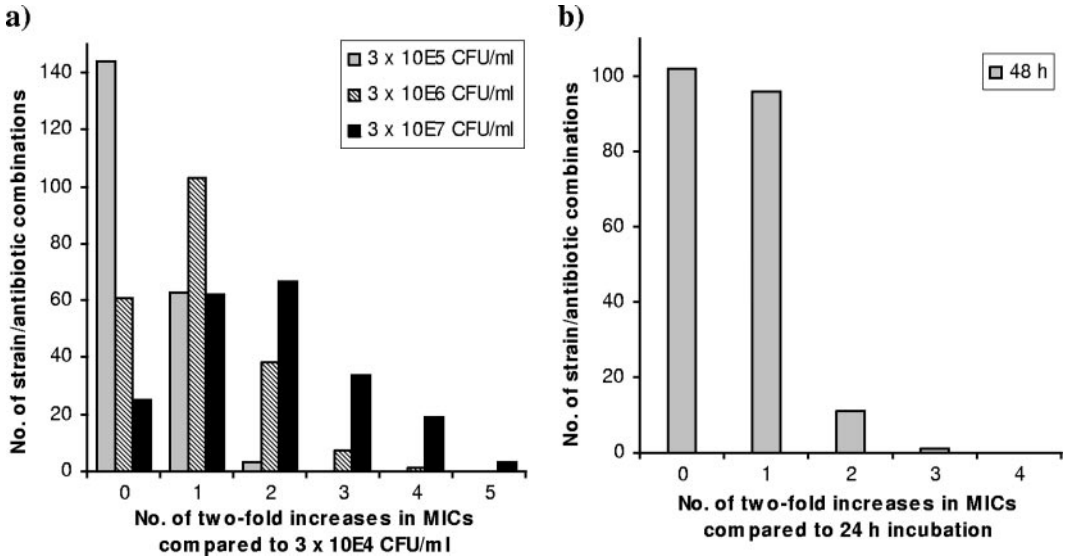

FIG. 1. Effects of increased inoculum size (a) and prolonged incubation time (b) on the MICs of six antibiotics for 35 LAB strains. (a) MICs obtained for the 210 strain-antibiotic combinations with different inoculum sizes, compared to MICs obtained with an inoculum size of 3×10^4 CFU/ml, after 48 h of incubation; (b) MICs obtained with an inoculum size of 3×10^5 CFU/ml after 48 h compared to 24 h of incubation. MICs above the test range are given as the concentration closest to the range, and MICs below the range are given as the lowest concentration.

In conclusion, an increased inoculum size and an extended incubation time resulted in elevated antibiotic MICs for all LAB species, underlining the importance of controlled and standardized conditions for susceptibility testing of LAB. Hopefully, the results from this study will contribute to the development of a standard method for determining the antibiotic susceptibilities of LAB and to subsequent delineation of microbiological breakpoints for individual LAB species.

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