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ORIGINAL ARTICLE

Similarities of *Geobacillus* bacteria based on their profiles of antimicrobial susceptibility in milk samples

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Abstract The genus *Geobacillus* is composed of thermophilic bacteria that exhibit diverse biotechnological potentialities. Specifically, *Geobacillus stearothermophilus* is included as a test bacterium in commercial microbiological inhibition methods, although it exhibits limited sensitivity to aminoglycosides, macrolides, and quinolones. Therefore, this article evaluates the antibiotic susceptibility profiles of five test bacteria (*G. stearothermophilus* subsp. *calidolactis* C953, *Geobacillus thermocatenulatus* LMG 19007, *Geobacillus thermoleovorans* LMG 9823, *Geobacillus kaustophilus* DSM 7263 and *Geobacillus vulcani* 13174). For that purpose, the minimum inhibitory concentrations (MICs) of 21 antibiotics were determined in milk samples for five test bacteria using the radial diffusion microbiological inhibition method. Subsequently, the similarities between bacteria and antibiotics were analyzed using cluster analysis. The dendrogram of this multivariate analysis shows an association between a group formed by *G. thermocatenulatus* and *G. stearothermophilus* and another by *G. thermoleovorans*, *G. kaustophilus* and *G. vulcani*. Finally, future microbiological methods could be developed in microtiter plates using *G. thermocatenulatus* as test bacterium, as it exhibits similar sensitivities to *G. stearothermophilus*. Conversely, *G. vulcani*, *G. thermoleovorans* and *G. kaustophilus* show higher MICs than *G. thermocatenulatus*.

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PALABRAS CLAVE
Geobacillus;
Susceptibilidad antimicrobiana;
Residuos de antibióticos;
Concentraciones mínimas inhibitorias;
Análisis conglomerados

Similitudes de las bacterias de *Geobacillus* según sus perfiles de susceptibilidad antimicrobiana en muestras de leche

Resumen El género *Geobacillus* está compuesto por bacterias termófilas que poseen diversas potencialidades biotecnológicas. Puntualmente, *G. stearothermophilus* se incluye como bacteria utilizadas para testeo en pruebas comerciales de inhibición microbiana para detectar residuos de antibióticos en alimentos, aunque presenta una limitada sensibilidad frente a aminoglucósidos, macrólidos y quinolonas. En este trabajo se evaluaron los perfiles de sensibilidad antibiótica de cinco bacterias utilizadas para testeo: *G. stearothermophilus* subsp. *calidolactis* C953, *G. thermocatenulatus* LMG 19007, *G. thermoleovorans* LMG 9823, *G. kaustophilus* DSM 7263 y *G. vulcani* 13174. Para ello, se determinaron las concentraciones inhibitorias mínimas (CIM) de 21 antibióticos en muestras de leche frente a las bacterias mencionadas mediante el método de inhibición microbiológica de difusión radial. Posteriormente, se analizaron las similitudes entre estas bacterias y entre los diferentes antibióticos evaluados mediante el análisis de clusters. El dendrograma de este análisis multivariante detectó un grupo formado por *G. thermocatenulatus* y *G. stearothermophilus* y otro constituido por *G. thermoleovorans*, *G. kaustophilus* y *G. vulcani*. En el futuro se podrían desarrollar métodos microbiológicos en placas de microtitulación utilizando *G. thermocatenulatus* como bacteria-test, puesto que este microorganismo posee sensibilidades similares a *G. stearothermophilus*. Por el contrario, *G. vulcani*, *G. thermoleovorans* y *G. kaustophilus* presentan CIM superiores a *G. thermocatenulatus*.

Introduction

The genus *Geobacillus* is characterized by including Gram positive, aerobic or facultative anaerobic rod-shaped bacteria, with the ability to form endospores[32] and grow at a wide range of temperatures (35–80 °C). This group includes *Geobacillus stearothermophilus*, *Geobacillus thermocatenulatus*, *Geobacillus thermoleovorans*, *Geobacillus kaustophilus*, and *Geobacillus vulcani*, among others.

Thermophilic bacteria of this genus are found in a variety of harsh environments such as high-temperature oil fields, marine vents, corroded pipes in extremely deep wells, African and Russian hot springs and the Marianas Trench, but can also be found in manure hay, garden soils, and the Sahara Desert[4,31]. This unexpected or inconsistent distribution of *Geobacillus* spp. has been discussed by Zeigler[32], who correlated this inconsistency with its worldwide spread.

These bacteria are used in various industrial applications[19], such as enzyme production[3,7,10,11,16,25], bioethanol[6,17], papermaking[27,28], bioremediation[15,18,23,30], and animal feed production[9].

In addition, *G. stearothermophilus* is specifically used as a test bacterium in commercial microbiological methods, such as Delvotest®, Eclipse® and Charm BY®, for the detection of antibiotic residues in food[14]. The advantages of *G. stearothermophilus* over other test bacteria include its low response times (2.5 h), high incubation temperature (selective growth of this test bacterium), low cost and easiness to use in laboratories, high sensitivity to a wide group of antibiotics (beta-lactams, tetracyclines and sulfonamides), dichotomous responses (positive *vs.* negative) and possibility to be stored at 4 °C for long periods of time[14]. However, the use of other strains of the genus *Geobacillus* (*G. vulcani*, *G. thermocatenulatus*, *G. thermoleovorans* and *G. kaustophilus*) for the development of methods to detect antibiotic residues in fluid matrices such as milk and meat extract has not yet been reported.

Before developing new bioassays, the susceptibility profiles of thermophilic bacteria to different antibiotics should be investigated. This can be achieved by using different antimicrobial susceptibility testing methods such as the agar dilution method, the broth macrodilution method, the disc diffusion/Kirby–Bauer method, Etest, the agar diffusion method in Petri dishes, and the microdilution method, which allow to estimate the minimum inhibitory concentration (MIC) of each antibiotic[2,24].

Based on the above, the aim of this study was to analyze the antibiotic susceptibility profiles of thermophilic bacteria of the genus *Geobacillus* and identify similarities among the different test bacteria, with the purpose of recommending strains that can be used in future microbiological techniques for the detection of antibiotic residues in fluid matrices.

Materials and methods

Microorganisms

The following commercially acquired strains were used for this study: *G. stearothermophilus* subsp. *calidolactis* C953 (Merck®, Ref. 1.11499, KGAA, Darmstadt, Germany), *G. thermocatenulatus* LMG 19007 (Leibniz Institute DSMZ, Braunschweig, Germany), *G. thermoleovorans* LMG 9823 (Leibniz Institute DSMZ, Braunschweig, Germany), *G. kaustophilus* DSM 7263 (Leibniz Institute DSMZ,

Braunschweig, Germany) and *G. vulcani* 13174 (Leibniz Institute DSMZ, Braunschweig, Germany).

Milk samples

For the preparation of antibiotic solutions, milk samples were used from individual cows (Las Colonias, Santa Fe, Argentina) that were in the middle of the lactation period, untreated and not medicated before or during sampling. In addition, milk samples with low somatic cell count (SCC < 300,000 cells/ml) and low total bacterial count (TBC < 400,000 cells/ml) were selected to avoid potential interference in the inhibition of microbiological methods.

Antibiotic-fortified milk solutions

Twenty-one antibiotics (10 beta-lactams, 4 macrolides, 3 quinolones, 3 tetracyclines, and neomycin) were used. The solutions of each antibiotic were prepared by successive dilutions of a stock solution (1000 mg/l) using antibiotic-free milk samples that tested negative to the Delvotest® ''SP'' method, which is considered the reference method[2,8]. For the preparation of the successive dilutions, volumes of stock solutions of each antibiotic containing less than 1% of the solution in milk were used[13] in order not to alter the composition of the milk samples. In the study of antibiotic susceptibility for each test bacterium, five concentrations were prepared in ascending order of concentration (C_1, C_2, C_3, C_4 and C_5), with each concentration being two-fold higher than the previous one (Table 1), using negative control milk samples.

Preparation of the agar diffusion method

Mueller Hinton agar (38 g/l, Biokar®, Ref. 10272, France) fortified with glucose (10 g/l, Sigma Aldrich®, Ref. G8270, USA) at pH 7 ± 0.1 was used. After sterilizing the culture medium in an autoclave (121 °C − 15 min), the agar was inoculated with a spore suspension of each test bacterium evaluated to achieve a concentration of 1×10^5 spores/ml in the culture medium. Then, a volume of 14 ml of culture medium was added to each Petri dish (90 mm in diameter) to obtain a thickness of 2.2 mm. For each test bacteria (5) and antibiotic (21), 12 microbiological bioassays were prepared in Petri dishes ($5 \times 21 \times 12 = 1260$ bioassays in total). Subsequently, the plates were cooled on a flat level surface to obtain a layer of uniform thickness[2].

Next, once the culture medium solidified, seven cylindrical perforations (8 mm in diameter) were made in each plate. Six of the perforations were distributed at a 60° angle, while the remaining perforation was made in the center of the plate where the antibiotic-free sample (AFS) was dispensed[2].

For each test bacterium, 12 bioassays were prepared using milk samples fortified with the five concentrations (C_1, C_2, C_3, C_4 and C_5) of the antibiotics detailed in Table 1. As shown in Figure 1, the intermediate concentration (C_3) was considered a control sample and was analyzed alternately in triplicate in each of the 12 plates (36 replicates in total). This concentration (C_3) was used for the correction of the inhibitory halos due to possible variations in the preparation of the bioassays[2]. Each of the four remaining concentrations (C_1, C_2, C_4 and C_5) was plated in triplicate on three plates (9 replicates). In each well of the Petri dishes, a volume of 70 μl of antimicrobial-fortified milk sample (Table 1) was dispensed.

Subsequently, the plates were incubated at 64 °C for 6 h until the formation of clear, different and measurable inhibitory halos at the five concentrations of the antibiotics tested. Lastly, the diameters of the inhibitory halos (including the 8-mm well) were measured in duplicate, using a Vernier caliper with a sensitivity of ±0.1 mm. Minimal increases of 2 mm in the diameter of the inhibition halos (8 mm + 2 mm = 10 mm) were recorded as significant inhibitions.

Statistical analysis

Calculation of the minimum inhibitory concentration (MIC)

The relationship between the inhibition halos and the concentrations of each antibiotic and test bacterium was established by using the regression model proposed by Bonev et al.[5]:

$$Y^2 = 4 * D * t * \mathrm{Ln}\, C - 4 * D * t * \mathrm{Ln\,MIC} \quad (1)$$

where Y represents the diameter of the inhibition zone; D is the diffusion coefficient of the antibiotic; t is the incubation time (constant = 6 h); Ln C is the logarithmic transformation of the antibiotic concentration; and Ln MIC is the logarithmic transformation of the MIC at the end of the halo. This Eq. (1) can be solved using the following linear regression model[26]:

$$Y^2 = \beta_1 * \mathrm{Ln}\, C - \beta_0 \quad (2)$$

where Y^2 represents the response variable and Ln C is the predictor variable. The MIC of each antibiotic and test bacterium was calculated using Eq. (3) and the coefficients β_0 and β_1 estimated by the linear regression model, as described below:

$$\beta_0 = 4 * D * t * \mathrm{Ln\,MIC}; \quad \beta_1 = 4 * D * t$$

$$\mathrm{Ln\,MIC} = \frac{\beta_0}{\beta_1} \qquad \mathrm{MIC} = e^{(\beta_0/\beta_1)} \quad (3)$$

Determination of similarities by using cluster analysis

To visualize associations between test bacteria and antibiotics, we used cluster analysis. Initially, the association of antibiotics was studied by using the hierarchical cluster analysis[26] with the Ward algorithm and the Euclidean distance of the MICs. Then, the similarities between the different strains of *Geobacillus* were analyzed by the hierarchical cluster analysis using the Ward algorithm and the Euclidean distance of the MIC values.

Results and discussion

Minimum inhibitory concentrations

Table 2 shows the parameters β_0 and β_1 and the regression coefficient (R) calculated by means of the linear regression

Table 1 Antibiotic concentrations (μg/l) used for each test bacterium in the microbiological inhibition method.

Antibiotics	*G. stearothermophilus*	*G. thermoleovorans*	*G. vulcani*	*G. kaustophilus*	*G. thermocatenulatus*
Beta-lactams					
Amoxycillin	4, 8, 16, 32, 64	8, 16, 32, 64, 128	12, 24, 48, 96, 192	8, 16, 32, 64, 128	4, 8, 16, 32, 64
Ampicillin	4, 8, 16, 32, 64	16, 32, 64, 128, 256	8, 16, 32, 64, 128	8, 16, 32, 64, 128	4, 8, 16, 32, 64
Cloxacillin	100, 200, 400, 800, 1600	200, 400, 800, 1600, 3200	100, 200, 400, 800, 1600	400, 800, 1600, 3200, 6400	15, 30, 60, 120, 240
Oxacillin	15, 30, 60, 120, 240	100, 200, 400, 800, 1600	50, 100, 200, 400, 800	100, 200, 400, 800, 1600	15, 30, 60, 120, 240
Penicillin ''G''	2, 4, 8, 16, 32	8, 16, 32, 64, 128	12, 24, 48, 96, 192	8, 16, 32, 64, 128	4, 8, 16, 32, 64
Cephalexin	100, 200, 400, 800, 1600	200, 400, 800, 1600, 3200	200, 400, 800, 1600, 3200	200, 400, 800, 1600, 3200	100, 200, 400, 800, 1600
Cefadroxil	100, 200, 400, 800, 1600	100, 200, 400, 800, 1600	100, 200, 400, 800, 1600	1000, 2000, 400, 800, 16000	100, 200, 400, 800, 1600
Cefoperazone	100, 200, 400, 800, 1600	100, 200, 400, 800, 1600	100, 200, 400, 800, 1600	100, 200, 400, 800, 1600	100, 200, 400, 800, 1600
Ceftiofur	200, 400, 800, 1600, 3200	100, 200, 400, 800, 1600	100, 200, 400, 800, 1600	200, 400, 800, 1600, 3200	100, 200, 400, 800, 1600
Cefuroxime	100, 200, 400, 800, 1600	100, 200, 400, 800, 1600	100, 200, 400, 800, 1600	200, 400, 800, 1600, 3200	100, 200, 400, 800, 1600
Tetracyclines					
Chlortetracycline	300, 600, 1200, 2400, 4800	800, 1600, 3200, 6400, 12800	400, 800, 1600, 3200, 6400	1000, 2000, 400, 800, 16000	400, 800, 1600, 3200, 6400
Oxytetracycline	200, 400, 800, 1600, 3200	100, 200, 400, 800, 1600	200, 400, 800, 1600, 3200	1000, 2000, 400, 800, 16000	200, 400, 800, 1600, 3200
Tetracycline	200, 400, 800, 1600, 3200	400, 800, 1600, 3200, 6400	200, 400, 800, 1600, 3200	1000, 2000, 400, 800, 16000	200, 400, 800, 1600, 3200
Quinolones					
Ciprofloxacin	5000, 10000, 20000, 40000, 80000	2000, 400, 800, 16000, 32000	3000, 6000, 12000, 24000, 48000	5000, 10000, 20000, 40000, 80000	2000, 400, 800, 16000, 32000
Enrofloxacin	5000, 10000, 20000, 40000, 80000	2000, 400, 800, 16000, 32000	3000, 6000, 12000, 24000, 48000	5000, 10000, 20000, 40000, 80000	2000, 400, 800, 16000, 32000
Marbofloxacin	5000, 10000, 20000, 40000, 80000	2000, 400, 800, 16000, 32000	3000, 6000, 12000, 24000, 48000	5000, 10000, 20000, 40000, 80000	2000, 400, 800, 16000, 32000
Macrolides and neomycin					
Erythromycin	300, 600, 1200, 2400, 4800	100, 200, 400, 800, 1600	1000, 2000, 400, 800, 16000	300, 600, 1200, 2400, 4800	800, 1600, 3200, 6400, 12800
Lincomycin	200, 400, 800, 1600, 3200	150, 300, 600, 1200, 2400	1000, 2000, 400, 800, 16000	1000, 2000, 400, 800, 16000	600, 1200, 2400, 4800, 9600
Neomycin	1500, 3000, 6000, 9000, 18000	750, 1500, 3000, 6000, 12000	1500, 3000, 6000, 9000, 18000	250, 500, 1000, 2000, 4000	8000, 16000, 32000, 64000, 128000
Tilmicosin	800, 1600, 3200, 6400, 12800	50, 100, 200, 400, 800	400, 800, 1600, 3200, 6400	20, 40, 80, 160, 320	400, 800, 1600, 3200, 6400
Tylosin	100, 200, 400, 800, 1600	50, 100, 200, 400, 800	100, 200, 400, 800, 1600	20, 40, 80, 160, 320	100, 200, 400, 800, 1600

Table 2 Parameters estimated by the model for different bacteria of the genus *Geobacillus*.

Antibiotics	*G. stearothermophilus*			*G. thermoleovorans*			*G. vulcani*			*G. kaustophilus*			*G. thermocatenulatus*		
	β_0	β_1	*R*	β_0	β_1	*R*	β_0	β_1	*R*	β_0	β_1	*R*	β_0	β_1	*R*
Beta-lactams															
Amoxycillin	−42.2	199.3	0.968	−223.6	294.8	0.931	−337.7	427.8	0.996	−201.9	276.9	0.945	−146.1	322.1	0.935
Ampicillin	−32	189.9	0.971	−247.7	285.4	0.933	−228.8	343.9	0.984	−116.7	260.8	0.985	−127.1	291.4	0.958
Cloxacillin	−645.4	409.7	0.998	−1220.8	596.0	0.995	−599.1	350.9	0.987	−843.4	402.4	0.966	−237.5	250.4	0.912
Oxacillin	−199.5	243.2	0.977	−1027.7	539.6	0.964	−702.3	446.7	0.981	−751.2	418.1	0.987	−263.9	358.1	0.997
Penicillin ''G''	−4.5	219.2	0.924	−355.9	419	0.946	−387.2	484.2	0.997	−249.1	322.0	0.975	−230.1	419.2	0.946
Cephalexin	−327	195.0	0.922	−794.7	437.4	0.969	−781.2	445.3	0.992	−909.5	425.4	0.972	−760.5	414.5	0.967
Cefadroxil	−505.1	290.9	0.972	−682.3	431.8	0.975	−672.8	428.1	0.998	−617.8	380.3	0.990	−834.6	470.7	0.975
Cefoperazone	−558.4	340.4	0.982	−640.2	350.6	0.964	−441.4	280.0	0.985	−405.5	240.1	0.971	−638.1	415.6	0.974
Ceftiofur	−528.5	310.8	0.993	−779.9	402.2	0.912	−572.1	663.3	0.995	−1012.4	485.0	0.997	−794.9	438.6	0.997
Cefuroxime	−539.4	333.5	0.986	−768.5	463.0	0.979	−481.4	366.0	0.988	−852.7	460.6	0.980	−538.1	337.9	0.937
Tetracyclines															
Chlortetracycline	−307.6	174.3	0.994	−27.9	80.3	0.959	−445.1	217.2	0.963	−625.2	262.0	0.988	−622.5	305.1	0.906
Oxytetracycline	−140.4	115.7	0.982	−572.2	324.9	0.947	−614.6	293.8	0.975	−438.8	217.1	0.911	−455.5	245.5	0.958
Tetracycline	−182.7	134.3	0.984	−620.3	271.8	0.947	−720.5	326.1	0.922	−481.5	245.9	0.986	−589.3	297	0.953
Quinolones															
Ciprofloxacin	−929.3	324.9	0.995	−1126.0	385.2	0.983	−963.9	337.1	0.986	−1257.6	371.5	0.992	−1322.7	452.2	0.969
Enrofloxacin	−835.4	287.7	0.991	−709.9	231.9	0.912	−966.7	315.2	0.991	−1119.5	319.6	0.980	−1060.4	354.7	0.957
Marbofloxacin	−995.7	318.1	0.996	−650.4	216.1	0.919	−1131.9	363.4	0.991	−1246.7	359.7	0.990	−975.7	351.0	0.982
Macrolides and neomycin															
Erythromycin	−428.3	211.0	0.994	−317.2	211.6	0.989	−159.4	109.7	0.986	−350.2	225.4	0.973	−521.5	233.9	0.977
Neomycin	−369.3	161.6	0.993	−215.2	153.7	0.960	−223.3	111.3	0.985	−300.3	177.0	0.953	−527.0	170.3	0.986
Lincomycin	−243.6	207.5	0.962	−376.8	234.6	0.974	−644.3	262.1	0.997	−326.9	195.1	0.965	−610.0	270.5	0.989
Tilmicosin	−398.6	193.1	0.997	−278.4	226.7	0.988	−493.5	228.4	0.974	−97.6	137.6	0.975	−364.1	179.8	0.987
Tylosin	−262.5	183.7	0.985	−366.9	267.8	0.977	−314.5	213.7	0.988	−87.2	174.6	0.968	−271.7	177.0	0.948

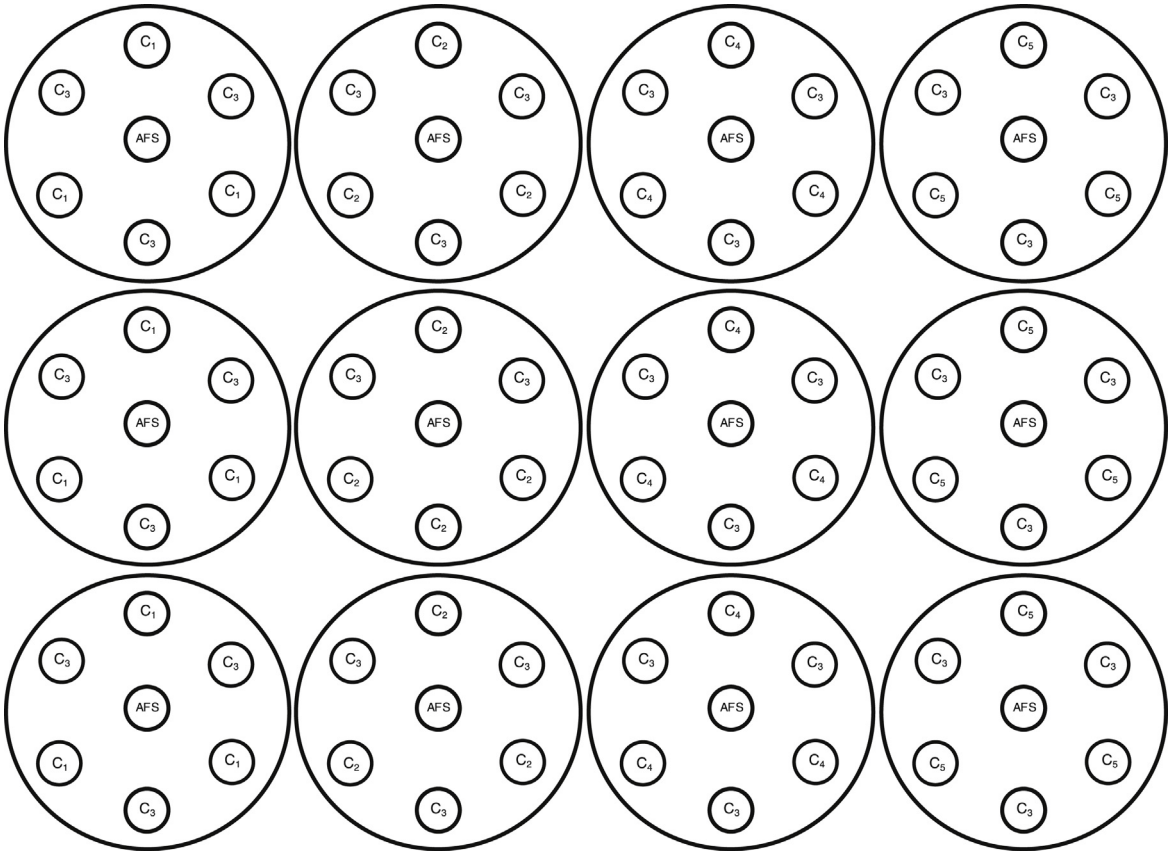

Figure 1 Distribution of the different concentrations of antibiotics in milk samples used in the microbiological inhibition method in Petri dishes. C_1: concentration 1; C_2: concentration 2; C_3: concentration 3 (control sample); C_4: concentration 4; C_5: concentration 5; AFS: antibiotic-free sample.

model for the 21 antibiotics and five test bacteria of the genus *Geobacillus* here evaluated. Regression coefficients were high, with values between 0.906 (chlortetracycline, *G. thermocatenulatus*) and 0.998 (cloxacillin, *G. stearothermophilus*), evidencing the linear proportionality between the squares of the inhibitory halos (Y^2) and the logarithmic transformations of the antibiotic concentrations (Ln C), according to Bonev et al.[5] Next, the MICs were calculated using Eq. (3) for each antibiotic and test bacterium (Table 3).

With respect to penicillins, *G. stearothermophilus* and *G. thermocatenulatus* showed great sensitivity toward these molecules, since their MIC values were low. In contrast, *G. thermoleovorans*, *G. kaustophilus* and *G. vulcani* exhibited MICs of penicillins between 1.5 (3 μg/l of ampicillin, *G. kaustophilus*) and 12.8 times higher (90 μg/l of oxacillin, *G. thermoleovorans*) than those obtained by *G. stearothermophilus* (Table 3).

With respect to cephalosporins, the five test bacteria exhibited similar susceptibilities for the five cephalosporins studied, since their MIC values were between 37 μg/l of ceftiofur for *G. vulcani* and 151 μg/l of cephalexin for *G. kaustophilus*. In addition, the MICs of cephalosporins were higher than those observed for penicillins for all the test bacteria studied.

With regard to tetracyclines, *G. stearothermophilus* exhibited a high level of susceptibility to all three tetracyclines, in comparison to the other test bacteria, with the exception of *G. thermoleovorans* (which showed MIC values of 9 μg/l for chlortetracycline and 70 μg/l for oxytetracycline) and *G. thermocatenulatus* (which showed a MIC value for 87 μg/l of oxytetracycline). On the other hand, *G. vulcani* exhibited lower susceptibility to tetracyclines than *G. stearothermophilus* (Table 3).

It should be noted that none of the five test bacteria analyzed showed susceptibility toward the three fluoroquinolones, since they had to be present at concentrations between 659 μg/l (marbofloxacin, *G. thermocatenulatus*) and 3430 μg/l (enrofloxacin, *G. kaustophilus*) to produce noticeable inhibitory halos (Table 3).

Table 3 shows that the five test bacteria exhibited adequate sensitivities for tylosin residues, but lower for tilmicosin, erythromycin–neomycin (with the exception of *G. stearothermophilus* and *G. thermocatenulatus*) and lincomycin (with the exception of *G. vulcani* and *G. thermocatenulatus*).

In the literature that we reviewed, we found reports only mentioning the detection limits of *G. stearothermophilus* in Petri dishes. Nouws et al.[22], for example, reported similar detection limits in cow milk samples for the five penicillins (amoxycillin, ampicillin, cloxacillin, oxacillin, and penicillin) and for cephalexin, cefoperazone, ceftiofur, cefuroxime and tylosin. Similarly, in a previous study, we obtained detection limits similar to those reported in Table 3 for amoxycillin, ampicillin, penicillin, cephalexin, cefoper-

Table 3 Minimum inhibitory concentrations (μg/l) for test bacteria of the genus *Geobacillus* and maximum residue limits (μg/l) in milk.

Antibiotics	MRL	*G. stearothermophilus*	*G. thermoleovorans*	*G. vulcani*	*G. kaustophilus*	*G. thermocatenulatus*
Beta-lactams						
Amoxycillin	4	2	7	6	6	3
Ampicillin	4	2	9	5	3	3
Cloxacillin	30	38	114	54	148	12
Oxacillin	30	7	90	40	66	6
Penicillin ''G''	4	1	8	6	7	4
Cephalexin	100	65	76	59	151	77
Cefadroxil	–	61	43	37	46	39
Cefoperazone	50	47	76	40	55	70
Ceftiofur	100	52	70	37	124	76
Cefuroxime	–	44	50	22	78	52
Tetracyclines						
Chlortetracycline	100	60	9	149	260	178
Oxytetracycline	100	19	70	135	112	87
Tetracycline	100	26	248	205	101	117
Quinolones						
Ciprofloxacin	100	749	903	782	2667	970
Enrofloxacin	100	849	1593	1217	3430	1167
Marbofloxacin	75	1378	1408	1357	3034	659
Macrolides and neomycin						
Erythromycin	40	110	33	33	43	194
Lincomycin	150	20	46	291	41	190
Neomycin	1500	203	36	114	66	1360
Tilmicosin	50	118	18	164	6	114
Tylosin	50	29	26	32	4	44

azone and tylosin in sheep milk samples, although we did not observe inhibitions when the milk contained quinolone residues[1].

Determination of similarities by the hierarchical cluster analysis

Figure 2 shows the associations of the 21 antibiotics determined by cluster analysis using the MICs of the evaluated five test bacteria. The figure shows the formation of two large clusters (Cluster ''A'' and Cluster ''B''), which reveal two different types of antimicrobial susceptibilities for these test bacteria.

Cluster ''A'' (Euclidean distance less than 4) includes antibiotics with lower MICs (beta-lactams, tetracyclines, macrolides and lincomycin), as described above. The first associations occur for the group of penicillins, cephalosporins and tylosin, which have low MICs, followed by tetracyclines and other antimicrobials (macrolides). Finally, neomycin joins Cluster ''A'' with a greater Euclidean distance.

Cluster ''B'' (Euclidean distance greater than 4) consists of fluoroquinolones, which have high MICs and finally join Cluster ''A'' at a high Euclidean distance (greater than 20). Thus, the low susceptibility of thermophilic bacteria to these antimicrobials is highlighted.

This association of antibiotics for the five *Geobacillus* indicates a high sensitivity of these thermophilic bacteria toward penicillins and cephalosporins followed by a medium sensitivity for tetracyclines and a low sensitivity for quinolones. In addition, sensitivity studies with *G. stearothermophilus* indicate a similar behavior when these antibiotics are analyzed in cow[22] and ewe[1] milk.

To establish a cluster of test bacteria based on susceptibility associations, fluoroquinolones were removed from the analysis because they exhibited high MIC values. Figure 3 shows a cluster made up of the MICs of 18 antibiotics (Table 3) that exhibited inhibitory effects toward the bacteria evaluated. This figure shows two groups: Group A (*G. stearothermophilus* and *G. thermocatenulatus*), with a Euclidean distance similar to 1.0, and Group B (*G. thermoleovorans*, *G. kaustophilus* and *G. vulcani*), with a Euclidean distance greater than 2.0. These associations can be attributed to the high susceptibilities of *G. stearothermophilus* and *G. thermocatenulatus* (Group A) toward penicillins, cephalosporins and other antimicrobials (erythromycin, tilmicosin and tylosin), and the similar susceptibilities of *G. thermoleovorans*, *G. kaustophilus* and *G. vulcani* (Group B) to other antimicrobials (aminoglycosides and macrolides), with the exception of *G. vulcani* (higher MICs of lincomycin and tilmicosin), which is later integrated into Group B.

Regarding the associations of different strains of the genus *Geobacillus*, it should be noted that previous studies have reported similar associations when analyzing gene sequences encoding 16S rRNA[6,19–21]. Nazina et al.[21], for

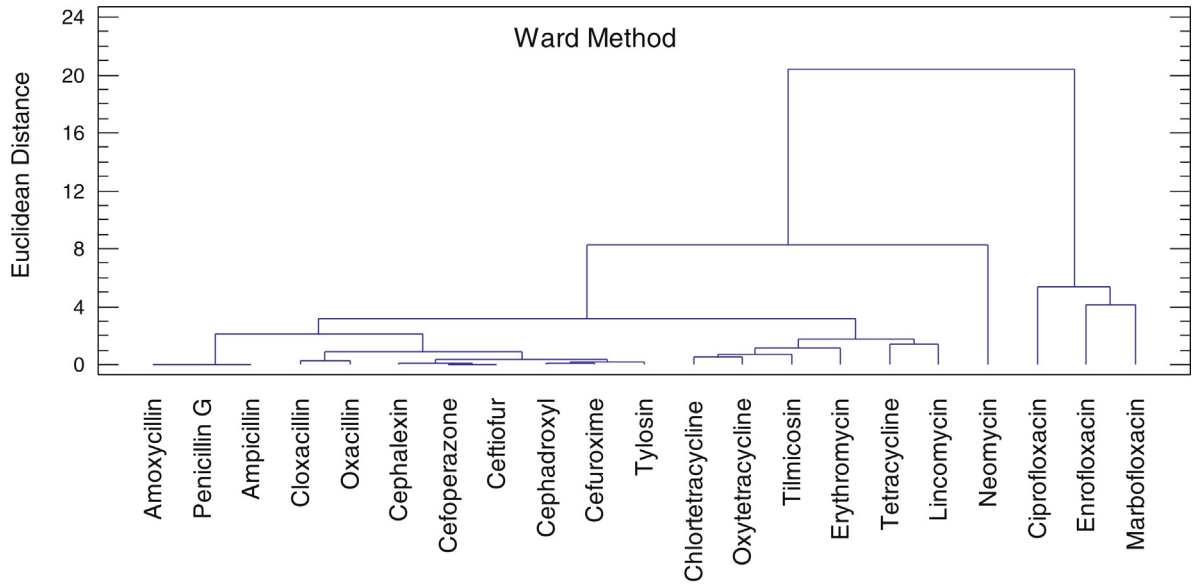

Figure 2 Results of the cluster analysis applied to the minimum inhibitory concentrations (MICs) obtained for each test bacterium.

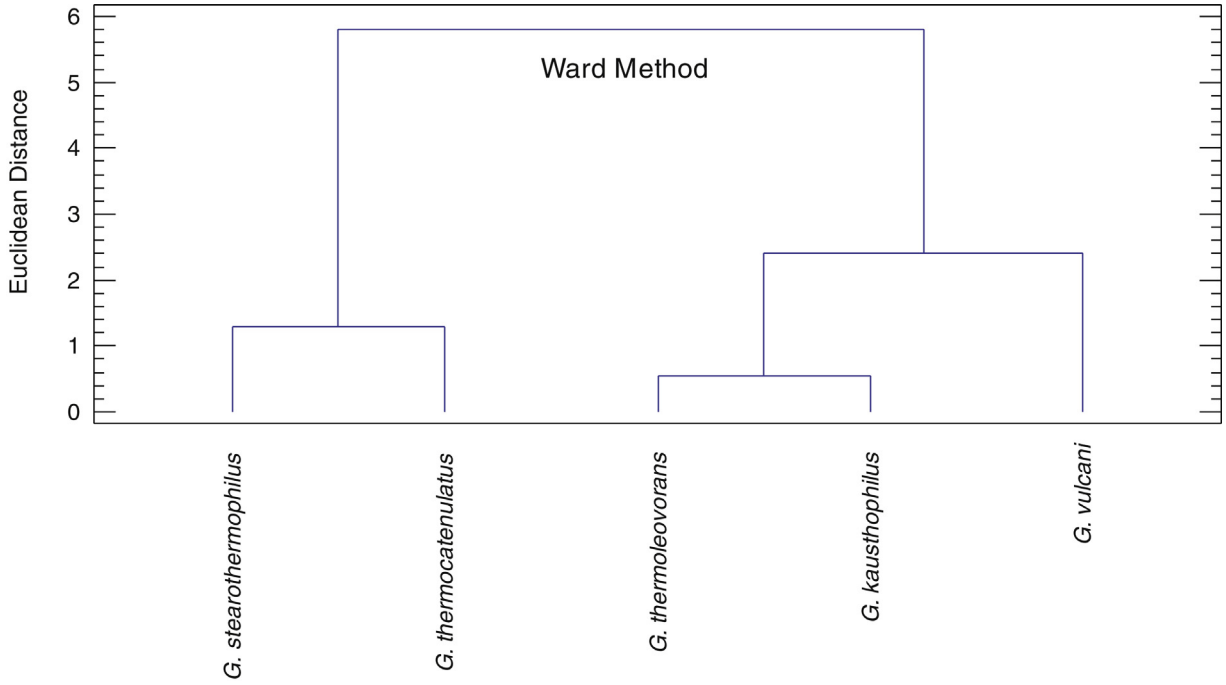

Figure 3 Dendrogram of thermophilic bacteria obtained by cluster analysis with minimum inhibitory concentrations.

example, analyzed the 16S rDNA genes present in 26 strains of *Geobacillus* and constructed a phylogenetic tree using the neighbor-joining method and the bootstrap analysis contained in the Treecon software package[29]. The results showed the presence of a cluster composed of *G. thermoleovorans*, *G. kaustophilus* and *G. vulcani*, and another cluster composed of *G. stearothermophilus* and *G. thermocatenulatus*, in accordance with those determined in this work when the MICs were used as an associative variable. Similarly, Najar and Thakur[19] obtained a phylogenetic tree using the associative neighbor-joining method for the 16S rDNA gene sequences corresponding to 19 strains of *Geobacillus*. The results indicated a cluster composed of *G. thermoleovorans*, *G. lituanicus*, *G. kaustophilus* and *G. vulcani*, into which a cluster containing *G. stearothermophilus* and *G. thermocatenulatus* was subsequently incorporated. This cluster of thermophilic bacteria is similar to the dendrogram in Figure 3, which uses the MIC values as a property of similarity. Brumm et al.[6] also performed a phylogenetic analysis of the nucleotide sequences (16S rDNA) present in 24 strains of *Geobacillus* by applying neighbor-joining and BioNJ algorithms and highlighted a grouping similar to

the cluster shown in Figure 3. The formation of a group composed mainly of *G. thermoleovorans*, *G. vulcani* and *G. kaustophilus*, followed by another group composed of *G. thermocatenulatus* and *G. stearothermophilus*, would reflect the evolutionary history of these thermophilic bacteria.

Due to the similarities between bacterial susceptibilities and bacterial ribosomal DNA (16S rDNA) of different strains of the genus *Geobacillus*, the MICs calculated in this work could be analyzed together with the information of the complete genome of these bacteria through the use of appropriate bioinformatic tools[12]. Thus, associations of susceptibilities with bacterial genes could be revealed.

Conclusions

In conclusion, it can be established that the cluster analysis dendrogram shows two different groups of thermophilic bacteria. The first one is formed by *G. thermocatenulatus* and *G. stearothermophilus*, while the second is made up of *G. thermoleovorans*, *G. kaustophilus* and *G. vulcani*.

Since the minimum inhibitory concentrations of *G. thermocatenulatus* and *G. stearothermophilus* are similar and lower than those observed for other *Geobacillus*, future bioassays in microtiter plates with *G. thermocatenulatus* spores should be optimized. The development of new bioassays using this test bacterium should improve some of the properties of current commercial methods, such as response time, specificity, detection limits and robustness, among others.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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