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Cell size of various lactic acid bacteria as determined by scanning electron microscope and image analysis

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Abstract — Cells from 48 pure lactic acid bacterial strains from the ACA-DC collection were studied under the scanning electron microscope (SEM) and the images were photographed and processed with an Image Analysis software. The diameters of several cocci and the lengths and widths of several lactobacilli strains were measured, then the size parameters were statistically analyzed by variance analysis. Significant differences were found between the cell sizes of the cocci strains (*Streptococcus* 1.07 to 1.21 μm, *Enterococcus* 0.87 to 1.01 μm, *Lactococcus* 0.75 to 0.95 μm), but not between lengths and widths of the bacilli strains because too high variations were found in the last measurements. © Inra/Elsevier, Paris.

lactic acid bacteria / bacterial cell / SEM / image analysis / cell size

Résumé — Étude granulométrique des cellules de bactéries lactiques par microscopie électronique à balayage associée à l'analyse d'images. Des cellules de 48 souches pures de bactéries lactiques de la collection ACA-DC ont été étudiées par microscopie électronique à balayage (MEB). Les photographies obtenues ont été scannées puis analysées à l'aide de l'analyseur d'images Sigmascan. Les diamètres des cocci ainsi que les longueurs et largeurs des lactobacilles ont été mesurés. L'analyse des variances montre des différences caractéristiques entre les différents genres de cocci (*Streptococcus* de 1,07 à 1,21 μm, *Enterococcus* de 0,87 à 1,01 μm, *Lactococcus* de 0,75 à 0,95 μm). En revanche, dans les conditions expérimentales utilisées, il n'a pas été possible de montrer de différences significatives entre les genres de lactobacilles. © Inra/Elsevier, Paris.

bactérie lactique / cellule bactérienne / MEB / analyse d'image / taille de cellule

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1. INTRODUCTION

Bacterial cell size is still a useful morphological characteristic used in the classification and identification of bacteria. Morphology is easy to study and analyze, particularly in eucaryotic micro-organisms and the more complex procaryotes. In addition, morphological comparisons are valuable because structural features which depend on the expression of many genes, are usually genetically stable [16]. Thus, morphological similarity is a good indication of phylogenetic relatedness. Although the light microscope (LM) has always been a very important tool, its resolving power of about 0.2 μm decreases its value in viewing smaller micro-organisms and structures. During the last decades the electron microscope has become a powerful tool in the examination of bacterial cells. The transmission electron microscope (TEM) is mainly now used by microbiologists for the study of the intracellular or cell wall structure [12], or for a quick examination of the bacterial cell with negative staining. The scanning electron microscope (SEM) is used for the study of the cell surface and its properties [1, 5, 6, 19] or in the greater field of food science and technology, usually for the structure analysis of food products [8] such as milk products [11, 13], cheese [3] meats, raisins, etc. Others have used the SEM for the observation of the microflora in some food products [2, 17]. The SEM together with the LM, according to Zenseky et al. [20], is becoming increasingly more useful in imaging techniques for microbial study.

Moreover; image analysis, a new and developing technique, is now applicable in almost all sciences dealing with images in great detail. The major scope of the image analysis is to define parameters such as size, number, shape, position and optical density of the objects which are recognized in an image [7]. In collaboration with image processing that improves the image in viewing (e.g., zooming, sharpening, segmentation), it is becoming a powerful tool for scientists because of its great accuracy, repetition and fidelity.

The aim of this work was to determine whether the small differences in cell sizes of the lactic acid bacteria (LAB) we observed with the LM were real and stable and could be used for their identification and classification.

The most commonly employed method for measuring bacteria is by means of an ocular micrometer. Measurements can also be made by using a camera lucid attachment and drawing oculars, or by projecting the real image on a screen and measuring the bacteria. In this work, an effort was made to use image analysis techniques. Since these techniques require well-defined images, SEM visualization was considered the most appropriate because it gives highly magnified, well-focused, high resolution images.

2. MATERIALS AND METHODS

A total of 48 strains of LAB from the ACA-DC collection of the Laboratory of Dairy Technology of the Agricultural University of Athens, all isolated from traditional Greek products, were examined. The number of strains from each species and their collection code is shown on *table I.*

2.1. Growth conditions

All the strains were in the form of pure frozen cultures and were subcultured three times in growth media containing 10% skimmed milk powder supplemented with 0.2% yeast extract (Difco). The growth temperature was 37 °C for all strains except those of the *Lactococcus lactis* subsp *lactis* which were incubated at 30 °C. The inoculum amount was 3% for the *Lactobacillus delbrueckii* subsp *bulgaricus* strains and 2% for all the others. The incubation time was 6 h for the cocci strains and 8 h for the bacilli strains.

2.2. Sample preparation for SEM

After the third subculture, 0.5 mL were taken from the coagulated media and washed with

Table I. Strains studied and their collection codes.
Tableau I. Souches étudiées avec leur code dans la collection.

Strain name	ACA-DC collection codes
Streptococcus thermophilus	2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15
Lactococcus lactis subsp *lactis*	47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58
Enterococcus faecalis	138, 239, 240, 252
Enterococcus faecium	165, 174, 241, 225
Enterococcus durans	242, 223, 226, 228
Lactobacillus delbrueckii subsp. *bulgaricus*	84, 85, 86, 87, 88, 90, 100, 101, 102, 103, 104, 105

0.5 mL of phosphate buffer (0.1 mol·L^{-1}, pH 7.2). After three washes, each was followed by a centrifugation (2 000 rpm, 10 min), the cells were resuspended in 1 mL of the buffer. From that media a small amount (approximately 200 µL) was taken and mounted directly on aluminium SEM stubs without prior fixation, air dried at 40 °C and sputter coated with gold. Preliminary experiments showed that fixation, dehydration and critical point drying offered no advantages to the preservation of the cells. The samples were then observed and photographed with a Cambridge Stereoscan S-150. Because the method relies on the accuracy of measurements, care was taken to always use the microscope at the same viewing angle.

2.3. Scanner settings and image analysis

To scan the received pictures we used the GT 9000 EPSON scanner model under the following settings:

gray levels: 64; sharpness: very sharp; brightness: normal; resolution x, y: 663 dpi; graphic file type: bitmaps for Windows (.bmp).

For the image analysis of the micrographs we used the SigmaScan image software (Jandel Scientific, San Rafael, CA, USA). The analysis was limited in measuring the diameters of the cocci cells and the widths and lengths of the bacilli cells. After calibrating the program according to the scale bar on the micrographs, cells were magnified for better definition of the cell edges; the cells were then measured by moving the pointer from one cell edge to the other. The program was keeping the measurements in memory and calculating some statistical values.

3. RESULTS AND DISCUSSION

Tables II, IV, VI, VII and VIII show the data and statistics obtained with the image analysis software. Each table is for one genus. Before any discussion we divided the means of each genus into classes, as shown on *tables III, V* and *IX* for better comparison. The class intervals in each of these tables were calculated using the model of Sturges where the class interval (C) is given by the type C = R/q where R = Max value – Min value and $q = 1 + 3.32 \log_{10}(n)$ where n is the number of measurements.

Beginning the interpretation of the results for each genus separately, we can see on *tables II* and *III* that all the *Streptococcus thermophilus* strains (*figure 1a*) have diameters apparently bigger than 1.00 µm with the most common class interval between 1.07 and 1.21 µm. Eight out of the 12 strains are found in this class interval. According to [4] cells of the genus *Streptococcus* are smaller than 2 µm in diameter while the *Streptococcus thermophilus* cells have a diameter between 0.7 to 0.9 µm, although these figures differ from our observations regarding cellular sizes. The *Lactococcus lactis* subsp *lactis* strains (*figure 1b*), as shown on *tables IV* and *V*, are smaller than the *Streptococcus thermophilus* ones with a diameter of approximately 0.75 to 0.95 µm. According to Mundt [15], the *Streptococcus lactis* (known now as *Lactococcus lactis*) cells are 0.5 to 1.0 µm in diameter which is similar to

Table II. Statistical results for the cell diameter of *Streptococcus thermophilus* strains.
Tableau II. Résultats statistiques sur le diamètre cellulaire des souches de *Streptococcus thermophilus*.

Strain code	Diameter $\bar{\chi}$ (µm)	Standard deviation (µm)	Standard error (µm)	95% confidence space (µm)	99% confidence space (µm)	n (number of cells measured)	$\sum_{1}^{n} x$ (µm)	Minimum values (µm)	Maximum values (µm)
2	1.21	0.01	0.02	0.04	0.05	30	36.26	1.03	1.45
3	1.18	0.04	0.007	0.02	0.02	32	37.64	1.08	1.26
4	1.12	0.13	0.02	0.05	0.016	34	38.20	0.87	1.39
5	1.06	0.15	0.03	0.06	0.09	24	25.36	0.84	1.49
6	1.17	0.14	0.03	0.05	0.07	30	35.23	0.98	1.44
7	1.32	0.17	0.03	0.06	0.08	30	39.63	1.04	1.65
8	1.19	0.15	0.03	0.06	0.07	29	34.39	0.88	1.49
9	1.17	0.1	0.02	0.03	0.04	40	46.92	0.94	1.34
10	1.13	0.09	0.02	0.03	0.05	23	31.74	0.93	1.33
11	1.14	0.11	0.02	0.04	0.05	33	37.73	0.97	1.39
12	1.02	0.1	0.02	0.04	0.05	29	29.66	0.83	1.23
15	1.23	0.01	0.02	0.04	0.05	30	37.05	1	1.42

Table III. Classification into groups of the *Streptococcus thermophilus* strains according to the mean of their cell diameter.
Tableau III. Classification des souches de *Streptococcus thermophilus* en groupes suivant leur diamètre cellulaire moyen.

Groups (in µm)	Strains
1.00 – 1.07	5, 12
1.07 – 1.14	4, 10, 11
1.14 – 1.21	2, 3, 6, 8, 9
1.21 – 1.28	15
1.28 – 1.35	7

Table IV. Statistical results for the cell diameter of *Lactococcus lactis* subsp. *lactis* strains.
Tableau IV. Résultats statistiques sur le diamètre cellulaire des souches de *Lactococccus lactis* subsp. *lactis*.

Strain code	Diameter $\overline{x}$ (µm)	Standard deviation (µm)	Standard error (µm)	95% confidence space (µm)	99% confidence space (µm)	n (number of cells measured)	$\sum_{1}^{n} x$ (µm)	Minimum values (µm)	Maximum values (µm)
47	0.83	0.06	0.01	0.02	0.03	34	28.29	0.72	0.98
48	0.84	0.06	0.01	0.02	0.03	29	24.4	0.69	0.97
49	0.81	0.14	0.03	0.06	0.08	26	21.06	0.57	1.07
50	0.85	0.1	0.02	0.04	0.06	24	20.42	0.69	1.14
51	0.77	0.06	0.01	0.03	0.04	21	16.12	0.65	0.86
52	0.95	0.16	0.04	0.07	0.1	20	18.95	0.66	1.15
53	0.81	0.08	0.02	0.03	0.04	29	23.53	0.59	0.99
54	0.82	0.08	0.01	0.03	0.04	27	22.26	0.73	1.06
55	0.86	0.07	0.02	0.03	0.04	23	19.72	0.71	0.99
56	0.81	0.06	0.01	0.03	0.04	26	21.08	0.7	0.98
57	0.82	0.08	0.02	0.04	0.05	20	16.47	0.68	0.99
58	1.17	0.07	0.02	0.03	0.04	20	23.37	1.04	1.33

Table V. Classification into groups of the *Lactococcus lactis* subsp. lactis strains according to the mean of their cell diameter.
Tableau V. Classification des souches de *Lactococcus lactis* subsp. *lactis* en groupes suivant leur diamètre cellulaire moyen.

Groups (in µm)	Strains
0.75 – 0.84	47, 48, 49, 51, 53, 54, 56, 57
0.84 – 0.93	50, 55
0.93 – 1.02	52
1.02 – 1.11	–
1.11 – 1.2	58

Table VI. Statistical results for the cell diameter of *Enterococcus faecalis* strains.

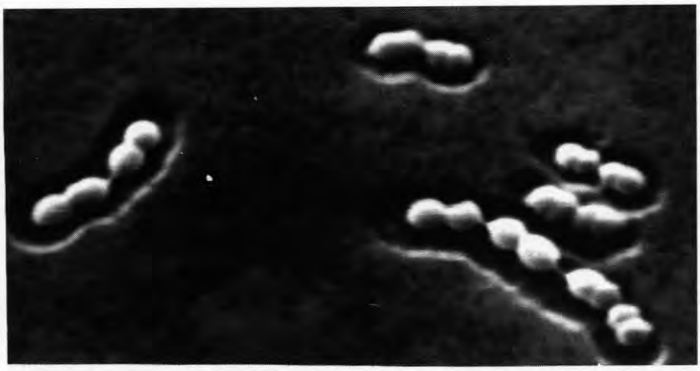
Tableau VI. Résultats statistiques sur le diamètre cellulaire des souches d'*Enterococcus faecalis*.

Strain code	Diameter $\overline{\chi}$ (µm)	Standard deviation (µm)	Standard error (µm)	95% confidence space (µm)	99% confidence space (µm)	*n* (number of cells measured)	$\sum_{1}^{n}$ x (µm)	Minimum values (µm)	Maximum values (µm)
138	0.84	0.09	0.02	0.04	0.06	19	15.94	0.69	0.99
239	0.9	008	0.02	0.04	0.05	22	19.87	0.79	1.07
240	0.88	0.08	0.02	0.04	0.05	21	18.5	0.72	1.10
252	1.14	0.08	0.02	0.03	0.04	24	27.31	0.96	1.28

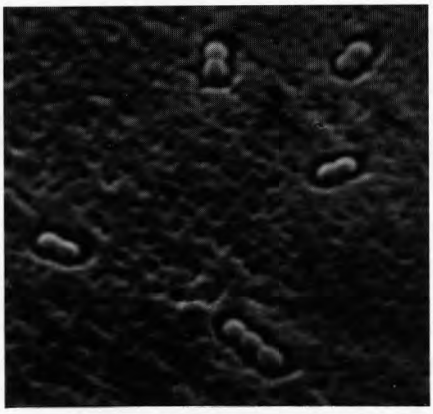
Table VII. Statistical results for the cell diameter of *Enterococcus faecium* strains.

Tableau VII. Résultats statistiques sur le diamètre cellulaire des souches d'*Enterococcus faecium*.

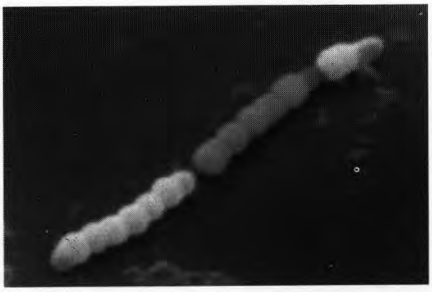
Strain code	Diameter $\overline{\chi}$ (µm)	Standard deviation (µm)	Standard error (µm)	95% confidence space (µm)	99% confidence space (µm)	*n* (number of cells measured)	$\sum_{1}^{n}$ x (µm)	Minimum values (µm)	Maximum values (µm)
165	0.92	0.01	0.02	0.04	0.06	23	21.26	0.74	1.1
174	0.98	0.1	0.02	0.05	0.06	23	22.5	0.78	1.18
241	0.92	0.07	0.01	0.03	0.04	24	22.04	0.81	1.04
225	0.84	0.12	0.02	0.05	0.07	24	20.04	0.62	1.03



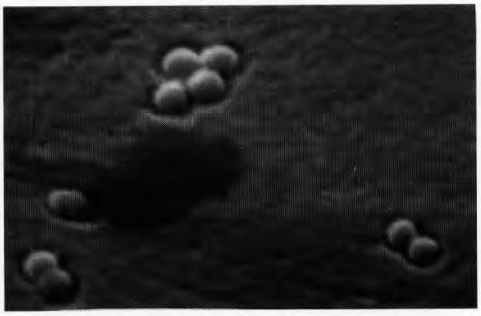
a. *Streptococcus thermophilus* ACA-DC 9 (x4000)



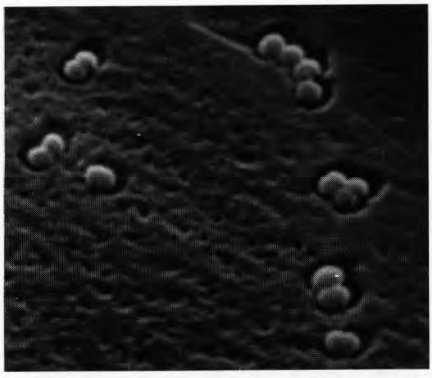
b. *Lactococcus lactis subsp lactis* ACA-DC 53 (x4000)



c. *Enterococcus faecalis* ACA-DC 252 (x4000)



d. *Enterococcus faecium* ACA-DC 174 (x4000)



e. *Enterococcus durans* ACA-DC 226 (x4000)

Figure 1a–e. Pictures of the cocci genera studied as received from SEM.
Figure 1a–e. Photographies des genres de cocci étudiés obtenues par MEB.

Table VIII. Statistical results for the cell diameter of *Enterococcus durans* strains.
Tableau VIII. Résultats statistiques sur le diamètre cellulaire des souches d'*Enterococcus durans*.

Strain code	Diameter $\bar{\chi}$ (μm)	Standard deviation (μm)	Standard error (μm)	95% confidence space (μm)	99% confidence space (μm)	n (number of cells measured)	$\sum_{1}^{n} x$ (μm)	Minimum values (μm)	Maximum values (μm)
242	0.9	0.09	0.02	0.03	0.05	30	26.86	0.75	1.1
223	0.89	0.08	0.02	0.04	0.05	20	17.88	0.79	1.05
226	0.94	0.08	0.01	0.03	0.04	33	31	0.78	1.08
228	0.96	0.08	0.02	0.03	0.05	24	23.05	0.76	1.08

what we found. The strain ACA-DC 58 is bigger in diameter than the other *Lactococcus lactis* subsp *lactis* strains (1.17 μm), a fact that causes some queries. In *tables VI–IX* we can see that most of the *Enterococci* strains (*figure 1 c–e*) – a total of 9 – have diameters ranging between 0.87 and 1.01 μm while the other three have more or less the same diameters. The *Streptococcus faecalis* cells (known now as *Enterococcus faecalis*), as described by Mundt [14], are 0.5 to 1.0 μm in diameter which is more or less similar to what we found.

A look at the statistics of the cocci genera can persuade us that the results are quite acceptable. The standard deviation, which is an indication of great importance for the accuracy of the results, is low or around 10% of the mean value. This percentage might be decreased by increasing the number of measurements; however, 10% is quite acceptable for the statistical method of ANOVA described below.

As far as the twelve *Lactobacillus delbrueckii* subsp *bulgaricus* strains are concerned (*figure 2*), we cannot make any deductions concerning their width and length (data not shown). The width varies between 0.7 and 1.00 μm with more than half of the strains above 0.88 μm. Great differences are found in the length of the cells. This can be partly explained with the fact that, with a few exceptions, young cells are much longer than old or mature ones. Knaysi [10] showed that cells of *B. subtilis* from a 4-h culture measured from five to seven times the length of cells from a 24-h culture. Variations in width are much less pronounced. A decrease in cell length and width appears to be due to a variety of factors. The major causes appear to be changes in the environment with the accumulation of waste products. An increase in the osmotic pressure of the medium will also cause a decrease in cell size and may possibly be the most important factor [18].

It is worth mentioning that it was impossible to measure the length of many cells due to their curved shape. Additionally, the cell edges were, in many cases, difficult to

Table IX. Classification into groups of the *Enterococci* strains according to the mean of their cell diameter.
Tableau IX. Classification des souches d'*Enterococci* en groupes suivant leur diamètre cellulaire moyen.

Groups (in µm)	Strains
0.8 – 0.87	138, 225
0.87 – 0.94	239, 240, 165, 241, 242, 223, 226
0.94 – 1.01	174, 228
1.01 – 1.08	–
1.08 – 1.15	252

Table X. ANOVA table.
Tableau X. Résultats de l'ANOVA.

Source	Sum of squares	d.f.	Mean square	F-ratio
Between groups	SSA = 0.598	2	MSA = 0.299	38.33
Within groups	SSE = 0.260	33	MSE = 0.0078	
Total	SST = 0.858	35		

Figure 2. Picture of a lactobacilli strain as received from SEM.
Figure 2. Photographie d'une souche de lactobacille obtenue par MEB.

distinguish and therefore measurement was impossible. For those with a straight cell and clearly visible, their length varied considerably between 2.8 µm and 5.35 µm. As far as we know the *Lactobacillus delbrueckii* cells are 0.5 to 0.8 µm in width and 2 to 9 µm in length [9], which is quite similar to our results. Moreover, it was not possible to find any relationship between the widths and the lengths of each strain despite the fact that all the culture conditions were the same for all strains. This should be considered for further research.

By comparing the data of the three cocci genera, it becomes evident that there is a significant difference in the size of their cells. The *Streptococcus thermophilus* strains seem to be the biggest, with the Ente-

rococci strains being smaller, whereas those of the *Lactococcus lactis* subsp *lactis* being the smallest. This was also verified by applying the ANOVA (analysis of variance) statistical method to the mean values of the sizes of the different strains of each different genus. Our aim was to find any statistically significant differences of the mean values of the cell diameter of the three cocci genera at the 5% level of significance. The features of the analysis of variance are shown on table *X*. Since $F_{2,\ 33;\ 0.05} = 3.3$ and $F = 38.33 > 3.3 = F_{2,\ 33;\ 0.05}$, we can safely say that there is a significant difference between the diameter of the three cocci genera. This also supports the impression we had when examining these genera with the light microscope or analyzing the results with the image analysis.

The application of this work in numerous different strains from different genera, could give us the ability to create a database containing their cell sizes, which would be a very useful data for the numerical taxonomy of bacteria. Consequently, the cell size in addition to the classic methods can provide us with a more precise identification and classification of an unknown strain, and that is of great importance.

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