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ORIGINAL ARTICLE

Determination of the profile of fatty acids of 4 species of *Shigella* spp by chromatography of gases

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ABSTRACT. S*higella boydii, Shigella flexneri, Shigella dysenteriae* and *Shigella sonnei* were identified using gas chromatography instead of the traditional techniques. Their acid methyl esters profiles were determined using a gas chromatograph Hewlett Packard 5890A and a RSL-150 heliflex capillary column. A total of 192 samples were analyzed both reference strains (ATCC 8700, INDRE B2188, B2194 and B2199) and environmental isolates. 12 fatty acids were included in the profiles from which 3-hydroxytetradecanoic acid (peak 12), trans 9-octadecanoic acid (peak 22), heptadecanoic acid (peak 18) and octadecanoic acid (peak 23), were the most important for the differentiation of the species analyzed.

Key words: Gas chromatography, *Shigella boydii, Shigella flexneri, Shigella dysenteriae,* and *Shigella sonnei.*

RESUMEN. Con el objeto de identificar *Shigella boydii, Shigella flexneri, Shigella dysenteriae* y *Shigella sonnei*, utilizando la técnica de cromatografía de gases en sustitución de las pruebas tradicionales, se compararon los perfiles de ácidos grasos a través de sus ésteres metílicos; que se obtuvieron en un cromatógrafo de gases Hewlett Packard 5890A con columna capilar RSL-150 heliflex. Se realizaron un total de 192 análisis en muestras tanto de cepas de referencia (ATCC 8700, INDRE B2188, B2194 y B2199) como de cepas ambientales, determinándose que su perfil cromatográfico está constituido por 12 *ácidos grasos*, de los cuales el 3-hydroxitetradecanoico (pico 12), el trans 9-octadecanoico (pico 22), el heptadecanoico (pico 18) y el octadecanoico (pico 23) resultaron ser determinantes para la diferenciación entre las especies estudiadas.

Palabras clave: Cromatografía de gases, *Shigella boydii, Shigella flexneri, Shigella dysenteriae, Shigella sonnei.*

INTRODUCTION

Inside of the chromatographic analysis, the determination of the bacterial fatty acids permit to identify in a fast way and exact to the microorganism in research.

Among the different researchers who initiated the utilization of this technique we can mention to Abel *et al.*[1] who in 1963 proposed the classification of 11 groups of the enterobacteriaceae family by means of the chromatographic analysis of lipids.

Subsequently, different authors realized works directed mainly to the enterobacteriaceae and Vibrionaceae families like: Boe y Gjerde (1980),[4] Moss *et al.* (1980),[16] Mayberry (1981),[15] Jantzen *et al.* (1982),[13] Bousfield *et al.* (1983),[6] Wallace *et al.* (1988),[26] Cookson (1989),[8] Urdaci *et al.* (1990),[25] Srinivas *et al.* (1994),[22] Murria *et al.* (1995),[17] Robles *et al.* (1999).[20]

Likewise, another authors continue using the principle of this technique for it application such as in systematic as in aspects of ecophysiology of microorganisms.[5,2,12,24,18]

The principle of this technique is that in the analysis of the total fatty acids of the bacterium, of which the bigger percentage correspond at the membrane and which composition is characteristic for each specie, which permit the differentiation among them. From the enter bacteria which have a broad repercussion in public health are the next species of Shigella: *Shigella disenteriae* causing of the bacillary dysentery which have affected significally places like India, Japan, China, some regions of Asia, America Central and Mexico.[9]

S. dysenteriae is the motive of mortal epidemics between the populations more poor whereas *S. flexneri* and *S. sonnei* are the causing of the endemic form of the illness.[21] *Shigella flexneri* which has been the dysenteric bacillus more commonly observed and which presents illnesses more severe than S. Sonnei.[7] Shigella sonnei which has converted in the responsible specie of shigellosis, more important in the United States[9] and Israel in the study realized by Cohen[7] in the 2001 had the higher incidence near to *S. flexneriy*. *Shigella boydii* is observed in places like India where persist conditions few hygienic.[21]

In this work it was determinate the profiles of fatty acids of *S. dysenteriae, S. flexneri, S. sonnei* and *S. boydii* to make the comparative analysis between these species, with the objective of establish the peaks more relevant for it differentiation.

MATERIAL AND METHODS

It were used 8 pure strains of each specie, of which 4 were typified (one proper of the American Type Culture Collection ATCC 8700, three proper of the National Institute of Epidemiological Reference (INDRE) with the codes

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B2188, B2194, B2199 and four isolated of the environment, obtained from the Laboratory of Microbiology of the National School of Biological Sciences of IPN and the stockage of the Chemical Faculty of UNAM).

It were run 40 chromatograms for each one of the typified and 8 repetitions for each environmental stock.

With the object of determine the profile of the fatty acids of each bacterium, it was standardized the quantity of biomass (20 mg). Each bacterial stock it cultivated in nutritive agar (DIFCO), during 24h. to 37° C.

It was used the same brand and share of medium of crop, with the object to avoid interferences reported for several authors.[10,11,19,20]

Once harvested, it was washed the biomass with a solution of formaldehyde to 0.5% centrifuging it during 10 min. to 15,000 rpm/min, washing it again with NaCl to 0.85% centrifuging and repeating the washed twice more, the biomass obtained it was lyophilized to –50° C with a vacuum of 25 microns.[11]

Once lyophilized the biomass, it was proceeded to esterificated it with methoxyd of sodium according to the technique of Glass.

The fatty acids already esterificates it were extracted in hexane, concentrating the resultant solution with nitrogen gas until a volume approximate of 10 ml.

To obtain the profile of the fatty acids by chromatography of gases it was followed the technique proposed by Häusler using a chromatograph of gases Hewlett Packard (Hp5890A) with detector of ionization of flame, a heliflex hair column (RSL-150, of 30,. With indoor diameter of 0.25 mm and thickness of film of 0.25 µm.

The temperatures of injector and detector it were maintained to 250° C and the column it was programmed from 120 to 250° C with an interval of 4°C/min using nitrogen like carrier gas with a flow of 30 ml/min and a volume of injection of 1 µl.

The identification of the fatty acids through their esters in the four species it was realized by means of the comparation of their times of retention with the patron solution (Bacterial Acid methyl estermix.10 mg/ml methyl caproate, 1ml Supelco catalogue No. 4-7080).

The areas of each peak it normalized it, staying the expressed worth in relatives percentages, take it like 100% the peak correspondent to the hexadecanoic acid C 16:0 which presented the higher area.[11]

To make the comparation of the fatty acids obtained between the four species and determine those peaks which have bigger weight in their differentiation, it was used an analysis discriminate.

This analysis included the calculation of discriminates functions and distances of Mahalanobis with their respective levels of significance.

The analysis it was proceeded using the statistical packet Statistica V 4.3 for Windows.

RESULTS

In the 192 chromatograms obtained of the 4 species such as in the proofs of the stocks of reference as in the environmental, it were determinate a total of 12 different fatty acids (peaks), that coincided it in times of retention like in area with some of the 26, presents in the standard mixture of fatty acids.

The peaks identified were: 3 (dodecanoic acid), 7 (tetradecanoic acid), 10 (pentadecanoic acid), 12 (3-hydroxitetradecanoic acid), 14 (cis-9-hexadecanoic acid), 15 (hexadecanoic acid), 17 (cis-9,10 methylen- hexadecanoic acid), 18 (heptadecanoic acid), 21 (cis-9 octadecanoic acid), 22 (trans-9-octadecanoic acid and cis-11-octadecanoic acid), 23 (octadecanoic acid), and 24 (cis-9,10.methylen-octadecanoic acid), Table 1. From this it can observe that as the stocks of *Shigella* typified as the environmental presented the fatty acids characteristic of the family

Table 1. Comparation of the fatty acids of *S. dysenteriae*, *S. boydii*, *S. flexneri* and *S. sonnei* in relation to the standard.

Number of Peak	Fatty acids of the standard	*S. dysenteriae*	*S. boydii*	*S. flexneri*	*S. sonnei*
1	C 11:0				
2	2-OH C10:0				
3	C 12:0	X	X	X	X
4	C 13:0				
5	2-OH C 12:0				
6	3-OH C 12:0				
7	C 14:0	X	X	X	X
8	I-C 15:0				
9	a- C 15:0				
10	C 15:0	X	X	X	X
11	2-OH C 14:0				
12	3-OH C 14:0	X	X	X	X
13	i- C 16:0				
14	C $16:1^{9}$	X	X	X	X
15	C 16:0	X	X	X	X
16	i- C 17:0				
17	C 17:0	X	X	X	X
18	C 17:O	X	X	X	X
19	2-OH C 16:0				
20	C $18:2^{9,12}$				
21	C $18:1^{9}$	X	X	X	X
22	C $18:1^{9,11}$	X	X	X	X
23	C 18:0	X	X	X	X
24	C 19:0	X	X	X	X

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Enterobacteriaceae that are the cis-9-hexadecanoic acid (peak 14), the hexadecanoic acid (peak 15) being this the most abundant just like in the others members of the family and the heptadecanoic acid (peak 18).[4]

Once identified the fatty acids, the facts it were normalized comparing them against the peak 15 (C 16:0) that presented the bigger area under the curve by the what it was assigned the worth of 100%.

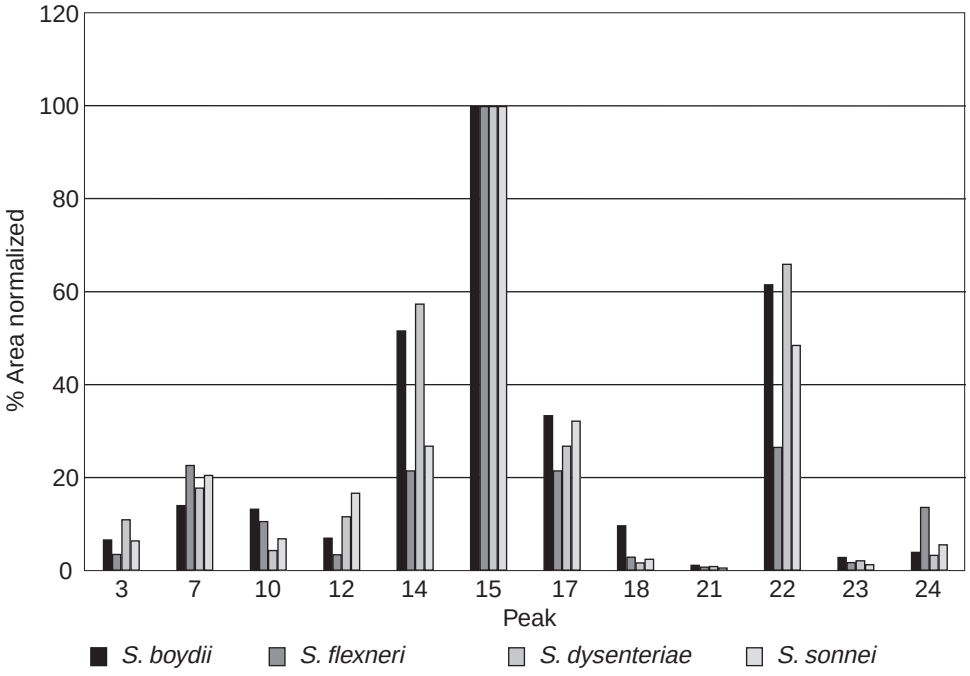

Figure 1. *Comparation of the profile of fatty acids of* S. boydii, S. flexneri, S. dysenteriae *and* S. sonnei.

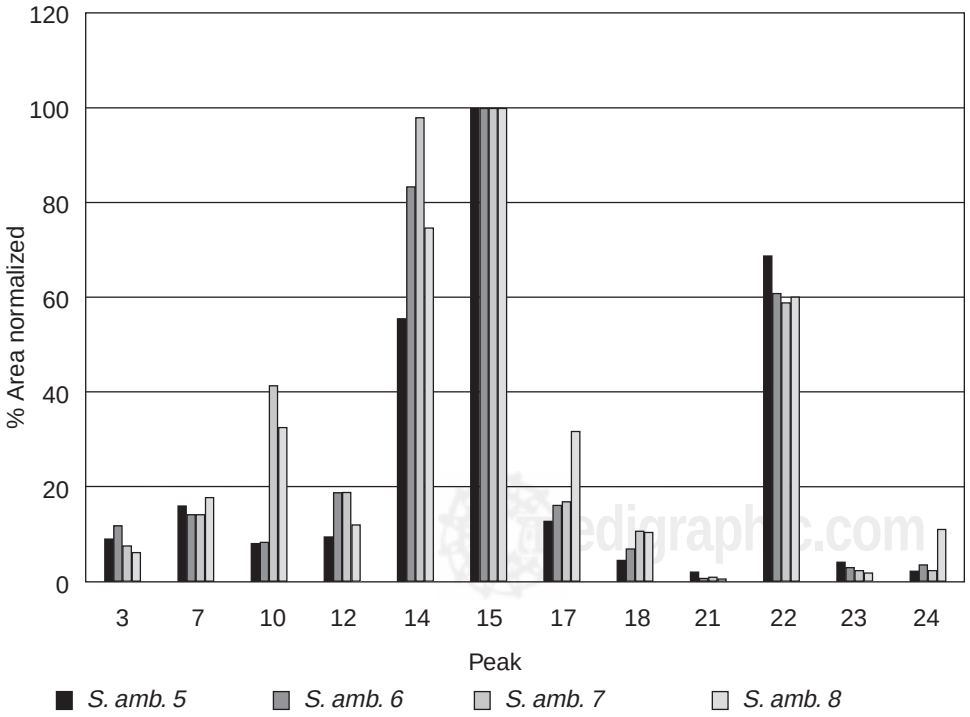

Figure 2. *Comparation of the profile of fatty acids of environmental proofs.*

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Table 2. Distances of Mahalanobis of the 8 stocks of *Shigella* studied it.

Species	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
Group 1	0	75.38	81.16	82.55	53.48	35.34	96.08	61.63
Group 2	75.38	0	49.43	28.14	75.31	78.42	178.62	127.74
Group 3	81.16	49.43	0	17.17	29.15	38.38	226.17	177.29
Group 4	82.55	28.14	17.17	0	45.65	38.26	192.98	149.74
Group 5	53.48	75.31	29.15	45.65	0	28.23	171.70	131.27
Group 6	35.34	78.42	38.38	38.26	28.23	0	132.27	99.43
Group 7	96.08	178.62	226.17	192.98	174.71	132.27	0	28.05
Group 8	61.63	127.74	177.29	149.74	131.23	99.43	28.05	0

With the facts obtained it was calculated for each one of the peaks the mean and the standard deviation, as the areas as the times of retention and it were represent the profile of fatty acids for each specie (Figs. 1 and 2).

S. dysenteriae presented the means of area normalized it higher in the peaks: 22 (66.04%) and 14 (57.45%).

The lowest worth it were presented in the peaks: 12 (11.72%), 3 (11.04%), 10 (4.46%), 24 (3.35%), 23 (2.23%), 18 (1.77%) and 21 (0.99%).

For *S. boydii* the highest peaks were: 22 (61.60%), 14 (51.71%) and 17 (33.48%) and the lowest were: 12 (7.10%), 3 (6.70%), 24 (4.07), 23 (2.92%) and 21 (1.25%).

For *S. flexneri* the highest peak was the 17 (63.51%) and the lowest; the 3 (3.57%), 12 (3.49%), 18 (3.01%), 23 (1.82%) and 21 (0.86%).

For *S. sonnei* the highest worth it presented the peaks 14 (70.63%) and 22 (48.55%) being the lowest the 12 (16.78%), 10 (7%), 3 (6.51%), 24 (5.71%), 18 (2.55%), 23 (1.37%) and 21 (0.66%).

For the environmental proof 2 the highest peaks were the 14 with 83.41% and the 22 with 60.87%. The lowest peaks were the 12 (18.85%), 17 (16.19%), 7 (14.22%), 3 (11.84%), 10 (8.39%), 18 (5.96%), 24 (3.59%), the 23 (3.04%) and the 21 (0.78%).

In the environmental proof 4 the highest peaks were the 14 (74.76%), and the 22 (60.13%) and the lowest, the 12 (12.08%), 24 (11.14%), 18 (10.44%), the 3 (6.23%) and the 23 (1.88%).

To determine if existed it significative differences between the 8 species studied with respect to the areas of the fatty acids, it was proceeded to apply discriminate multivariate analysis, obtaining it the next results:

Function	χ^2	P	%
1	1648.93	0.0	56.47
2	1075.42	0.0	80.05

Both functions resulted statically significatives ($p < 0.05$), the first function explained the 56.47% of the total variation of the means integrated by the peaks 12 and 22, and the second explained the 23.58% of the variation and stayed integrated by the peaks 18 and 23, this mean that sonner peaks were those which more differentiated to the species.

The results of the comparation between the 8 species, using the distances of Mahalanobis shown that exists significatives differences ($p < 0.05$) among them and it were appreciated eight fundamental groups which correspond 4 to the typified stocks and four of the environmental. Inside of the typified it encounter conforming the group 1: *S. boydii*, the group 2: *S. flexneri*, the group 3: *S. dysenteriae* and the group 4: *S. sonnei*.

Inside of the environmental the groups 5,6,7 and 8 respectively (Table 2).

DISCUSSION

The fatty acids plays an important role in determine the physical-chemical properties of the lipids of the cell and of the membrane, so that the microorganisms possess specific profiles of fatty acids per specie.

In the other hand the microbial lipids are indicators of the microbial mass, of the structure of the community and the metabolic status; this it has made by means of the analysis of the phospholipids, which are the structural components of all the biological membranes.[2,18]

With base in the previous is that it has considerate that the analysis of the fatty acids can help in an important way to realize the characterization of bacteria.

With the obtained results in relation to the contained of fatty acids of each specie as typified as environmental it proceeded the correspondents profiles. From the statistical analysis it was obtained two functions statically significatuves ($p < 0.05$), the first function explained the 56.47% of the total variation of the means integrated by the peaks 12

and 22 and the second explained the 23.58% of the variation and stayed integrated by the peaks 18 and 23, this mean that sooner peaks were those which more differentiated at the species.

In accordance to this analysis it observe that in the typified proofs the distances of group 1 with respect to the groups: 2 is of 75.38% at 3 is of 81.16% and at 4 is of 82.55%. These differences it can justify if it bear in mind that the stocks belong to different species inside of the type.

From the three restant groups; the groups 3 and 4 it encounter among them in a distance of 17.17 whereas the groups 2 and 3 is of 49.43% and the groups 2 and 4 is of 28.14%. With this is clear that exist significative differences that permit the establishment of the fundamental groups of *Shigella* type.

In the other hand, in the groups of the environmental proofs 5 to 8 and in accordance to the distance of Mahalanobis it can observe that the group 5 its draw near in a way more marked at 3 with a distance of 29.15, for what it can deduce that correspond at groups *S. dysenteriae*, this it support in the obtained results with the traditional biochemical proofs, confirming that the group 5 corresponds to *S. dysenteriae*. The group 6 it found closer at 1 with a worth of 35.34% indicating with that correspond to *S. boydii*, which also was corroborated with the biochemical proofs. The groups 7 and 8 it find a little separated of the four correspondent groups to the typified stocks. In the comparation of the profiles of the fatty acids of the four typified species it were obtained 4 fatty acids that permit the differentiation between the species of the *Shigella* type.

These acids were: the 3-hydroxitetradecanoic (peak 12), the trans 9-octadecanoic (peak 22), the heptadecanoic (peak 18) and the octadecanoic (peak 23), same that also established it differences between the environmental stocks of *Shigella*.

From the comparation to the profiles of the stocks of reference against the environmental stocks it were found significative differences among them, however the stock of environment group 5 was which presented the characteristics closers to *S. dysenteriae*, those which it was confirmed with the traditional biochemical proofs. Also the three environmental restant stocks coincided with *S. boydii*.

The profiles of the fatty acids obtained for the four typified stocks it could use like patrons of reference in the identification of environmental means by means of the comparation of the chromatograms of the profiles of these with reference´s of.

It can says that this technique in comparation with the traditional techniques offers bigger quickness and exactness by what in the last years it has proposed the employment of the analysis of the methylic esters of the fatty acids for the identification of microorganisms and like tool in the epidemiology of illnesses of plants and animals.[3,14,23,]

This technique is adequate for the identification of species of isolated enter bacteria of the environment, not considering the cerotype since the technique not analyze particularly the antigen O constitute by sugars.

There are not relation between the composition of fatty acids with the antigenic groups of Shigella by the which the identification just was limitated until species.

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