8:2 **Direct probe mass spectrometry and the classification of lactic acid bacteria from vacuum packed meats**

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INTRODUCTION

Examination of the microbial flora on stored vacuum packed meats usually reveals a predominance of Gram positive, catalase negative rods or cocci referred to collectively as lactic acid bacteria. To understand the microbial ecology and spoilage of vacuum packed meats it is necessary to study the properties of these bacteria, including factors affecting growth, substrates used for growth and end-products. It is essential to examine representative strains of all the common types. To facilitate this it is necessary to establish what groups exist amongst these bacteria.

Shaw and Harding (1984) classified lactic acid bacteria from vacuum packed beef, pork, lamb and bacon by numerical taxonomic methods, using morphological, physiological and biochemical properties. Three groups (clusters I, II and III) were revealed which contained nearly all the strains examined. Cluster I contained homofermentative lactobacilli which were non-aciduric, cluster II contained aciduric streptobacteria and cluster III consisted of Leuconostoc strains. Strains in cluster II were provisionally identified with Lactobacillus sake but the other two groups did not match any named species. That study provided a useful preliminary classification, but it was concluded that chemotaxonomic and genetic data were required to determine whether further groups existed within the clusters.

Recent investigations have demonstrated the potential of direct probe mass spectrometry (DPMS) for the discrimination of different groups of microorganisms (Gutteridge and Puckey, 1982). This paper describes the application of DPMS to the classification of lactic acid bacteria from vacuum packed meats. Most strains examined were taken from the earlier numerical taxonomic study (Shaw and Harding 1984) to allow direct comparison of groupings indicated by the two methods.

MATERIALS AND METHODS

Forty isolates of lactic acid bacteria from vacuum packed beef, pork, lamb and bacon were examined (Table 1).

Mass spectrometry All 40 strains were analysed in triplicate and the resulting 120 samples were pyrolysed in a random order. Each sample consisted of c. 50 µg of whole cells taken from a plate culture after incubation for 48h at 25°C. These cells were transferred to a clean quartz tube which was inverted into a stainless steel probe. Analyses were

Table 1. Strains of lactic acid bacteria used in the study

Strain No.	No. of strains	Shaw & Harding (1984) clusters
LV6,LV13,LV14,LV17,LV31,LV32, LV50,LV56,LV60,LV61,LV64,LV74	12	I (non-aciduric streptobacteria)
LV2,LV5,LV26,LV27,LV34,LV36, LV38,LV40,LV45,LV46,LV47,LV58, LV72,LV77,LV79,LV82,LV88,LV89, LV94,LV97	20	II (aciduric streptobacteria)
LV7,LV28,LV29,LV42,LV51,LV53	6	III (leuconostocs)
LV101,LV102	2	New isolates

performed on a Finnigan 4000 quadrupole mass spectrometer operated as described previously (Gutteridge and Puckey 1982). After insertion into the instrument the probe was temperature-programmed at 60min^{-1} from ambient to 300°C. Spectra were recorded over the mass range m/e 33-400 using a 2 second scan cycle. As the probe was heated the total ionization produced from the sample in the mass spectrometer was recorded as a function of time in an ion current profile. This pulse of ions subsided when the program was halted at 300°C. A single spectrum was produced for each sample by averaging the spectra recorded from the beginning to the amplitude of the pulse and subtracting on averaged ten-scan background taken from before the pulse to remove any underlying contamination.

Data analysis Before analysis each spectrum was normalized to its total ion count to remove varitions due to sample quantity. Each normalized spectrum was then reduced to a subset of ions using the concept of characteristicity developed by Eshuis *et al.* (1977). This reduction removed redundant data and revealed those ions which best discriminated between strains.

Relationships between spectra were examined by non-linear mapping and two cluster analysis techniques.

Non-linear mapping. Average intensity values of each of the 40 most characteristic ions were obtained for each strain by calculating the mean of the three replicate normalized intensity values. These average intensity values were used to determine similarities between the spectra of all strains using proportional similarity coefficients (Kistemaker *et al.* 1975). The best two dimensional representation of the matrix of similarities was then obtained by non-linear mapping (Kruskal 1964; Eshuis *et al.* 1977).

Cluster analysis Calculation of similarities in the cluster analyses used intensity values of the 40 most characteristic ions normalized to their total ions count in each spectrum.

The similarity value (SP_{ij}) based on quantitative pythagorean distances was calculated between the averaged spectra of all pairs of strains using the following formula.

$$SP_{ij} = \frac{1}{40} \sum_{k=1}^{40} (1 - (x_{ik} - x_{jk})^2/R_k^2)$$

where x_{ik}, x_{jk} are the replicate means of the kth ions of strains i and j respectively. R_k is the maximum observed difference in the kth ion between any two of the 40 strains. Using the matrix of similarities strains were clustered by unweighted pair-group average linkage analysis (Sokal and Michener 1958).

A function that takes replicate variation into account and also allows for any correlation between the ions is the Mahalanobis distançe (Mahalanobis 1936). Mahalanobis distances D_{ij} were calculated between the averaged spectra of all pairs of strains and converted to similarities by the following formula.

$$SM_{ij} = 1 - D_{ij} / D_{max}$$

where D is the maximum distance between two samples in the set. Unweighted pair group analysis was again used to cluster strains.

RESULTS

Non-linear mapping A non-linear map derived using the 40 most characteristic ions (not shown) revealed a clearly distinct group (A) of 12 strains (LV6, 13, 14, 17, 31, 32, 50, 56, 60, 61, 64, 74), but the remaining strains could not be grouped with any confidence.

When the characteristicity values of the ions were examined it was observed that the first ten had much higher values than the remainder, and a non-linear map was therefore constructed using only these ions in the calculation of similarities. Four groups (A_1, A_2, B and C) were evident (Figure 1). Groups A_1 and A_2 together contained all 12 strains observed as group A in the non-linear map based on the 40 most characteristic ions.

In a further analysis the 12 distinct group A strains were excluded as they heavily influenced the selection of ions used in the first two analyses, so diminishing the discrimination of other groups. This produced a new set of ions of which the 40 most characteristic were used to produce the non-linear map shown in Figure 2. The 28 strains are now clearly separated into three groups (B, C and D).

Non-linear mapping therefore indicated the presence of five groups amongst the 40 strains: groups A_1 and A_2 as in Figure 1, and groups B, C and D as in Figure 2.

Cluster analysis Average linkage cluster analysis on the matrix of quantitative pythagorean similarities revealed four clusters (A, B, C and D) of strains which corresponded exactly to groups A, B, C and D indicated by non-linear mapping.

Average linkage cluster analysis on the matrix of similarities derived from Mahalanobis distances indicated the presence of four clusters (A_1, A_2, D and E). Clusters A_1 and A_2 corresponded exactly to groups A_1 and A_2 detected by non-linear mapping and Cluster E contained all group C strains less strain LV45 which was unclustered. Cluster D was identical to group D in the non-linear map of the reduced set of 28 strains.

The groupings indicated by the three data analysis techniques are summarized for comparison in Figure 3.

DISCUSSION AND CONCLUSIONS

Direct probe mass spectrometry differentiated the 40 strains into a number of groups. Evidence of group composition revealed by the three data analysis techniques varied slightly (Figure 3), but this was only at the level of subdivision and was not conflicting. Taken together the three techniques of analysis indicated the presence of five groups (A_1, A_2, B, C and D) composed as shown for non-linear mapping in Figure 3.

The classification of strains produced by DPMS both complements and extends that obtained previously (Shaw and Harding 1984) using traditional morphological, physiological and biochemical tests (Figure 3). Groups A_1 and A_2 collectively contained all 12 representative of cluster I (unidentified non-aciduric streptobacteria). Shaw and Harding (1984) observed the same partioning but did not sub-divide this group in their classification scheme in the absence of supporting evidence. Dainty *et al.* (1984) have since shown that members of the two sub-clusters differ in their cellular fatty acid composition and distinction by DPMS provides further evidence of differences in chemical composition. It therefore seems certain that two types of non-aciduric streptobacteria occur on vacuum packed meats.

Groups B and C contained all 21 representatives of cluster II (aciduric streptobacteria provisionally identified with L. sake). Division of those strains by DPMS indicates that two types may be present. However, these were not evident as sub-clusters in the numerical taxonomic study.

The only major discrepancy between classification by DPMS and traditional methods occurred with the Leuconostoc strains. Five strains formed group D but strains LV7 and LV101 grouped with the aciduric streptobacteria. Further studies on more strains from this genus are required to determine whether LV7 and LV101 form the basis of another group of leuconostocs.

It is concluded that the majority of lactic acid bacteria on vacuum packed meats belong to one of four types represented by DPMS groups A_1, A_2, C and D. Group B may represent a fifth group but its distinction from group C requires verification by other techniques. This classification provides the basis for strain selection for use in pure culture studies.

REFERENCES

Dainty, R.H., Hibbard, C.M. & Edwards, R.A. (1984). System. Appl. Microbiol. 5, (in press).
Eshuis, W., Kistemaker, P.G. & Meuzelaar, H.L.C. (1977). In Analytical Pyrolysis. ed. Jones, C.E.R. & Cramers, C.A. pp. 151-166. Amsterdam: Elsevier.
Gutteridge, C.S. & Puckey, D.J. (1982) J.Gen. Microbiol. 128, 721-730.
Kistemaker, P.G., Meuzelaar, H.L.C. & Posthumus, M.A. (1975). In New Approaches t the Identification of Micro-organisms. ed. Heden, C.G. & Illeni, T. pp. 179-181. London: John Wiley & Sons.
Kruskal, J.B. (1964). Psychometrika 29, 1-42.
Mahalanobis, P.C. (1936). Proc. National Institute of Sciences of India 2, 49-56.
Shaw, B.G. & Harding, C.D. (1984). J.Appl.Bact. 56, 25-40.
Sokal, R.R. & Michener, C.D. (1958). Kansas University Science Bulletin 38, 1409-1438.

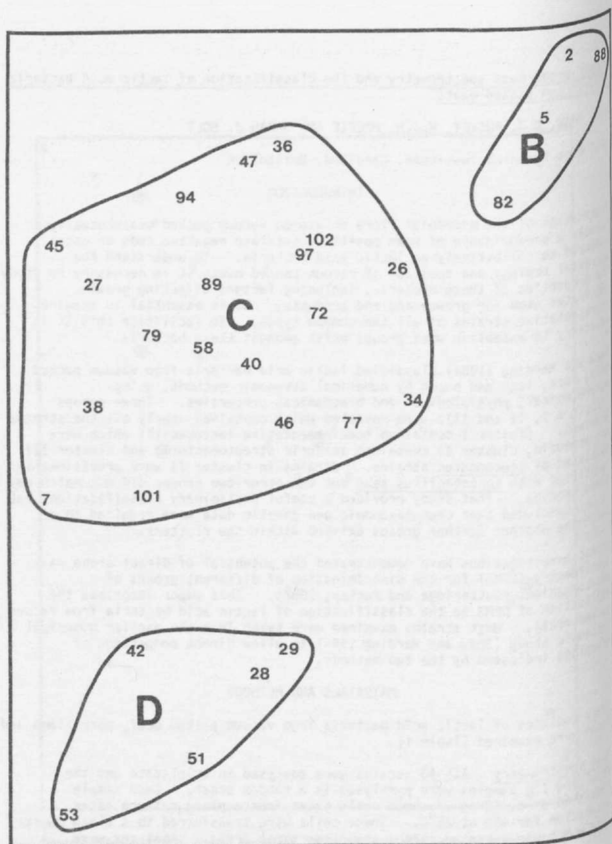

Figure 2. Non-linear map of a restricted set of 28 strains based on similarities calculated using values of the 40 most characteristic ions.

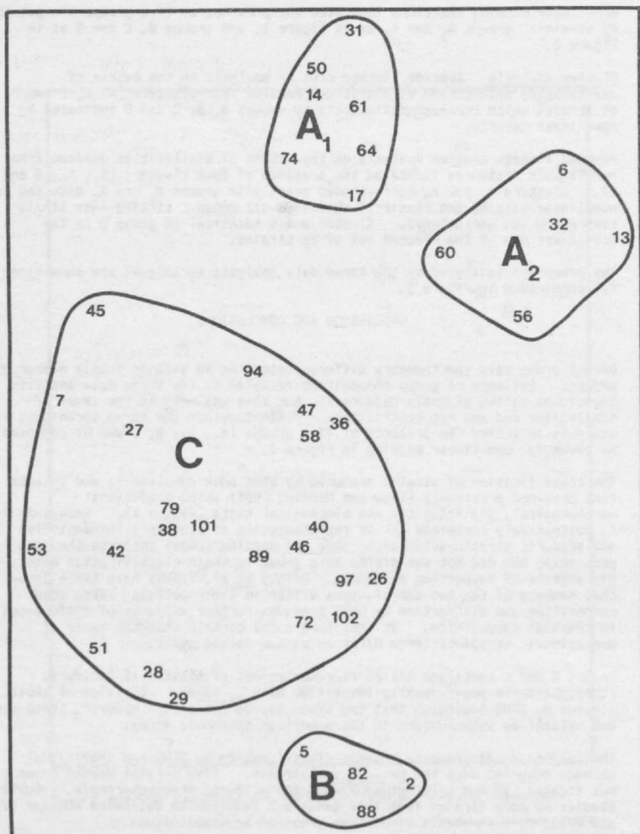

Figure 1. Non-linear map of all 40 strains based on similarities calculated using the 10 most characteristic ions.

Strains	Classification of Shaw & Harding (1984)	Non-linear mapping	Cluster analysis using pythagorean similarities	Cluster analysis from Mahalanobis distances
LV14, 17, 31, 50, 61, 64, 74	Non-aciduric streptobacteria (Cluster I)	A_1	A	A_1
6, 13, 32, 56, 60		A_2		A_2
2, 5, 82, 88	Aciduric streptobacteria (Cluster II)	B	B	E
26, 27, 34, 36, 38, 40, 46, 47, 58, 72, 77, 79, 89, 94, 97, 102		C	C	
7, 101	Leuconostocs (Cluster III)			
28, 29, 42, 51, 53		D	D	D
45	Cluster II	C	C	UNGROUPED

Figure 3. Summary of groupings indicated by non-linear mapping and cluster analysis of DPMS data.