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Characterization of *Carnobacterium* species by pyrolysis mass spectrometry

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L.N. MANCHESTER, A. TOOLE AND R. GOODACRE. 1995. Forty-eight strains of *Carnobacterium* were examined by pyrolysis mass spectrometry (PyMS). The effects of culture age and reproducibility over a 4 week period were also examined. The results were analysed by multivariate statistical techniques and compared with those from a previous numerical taxonomic study based on morphological, physiological and biochemical characteristics and with studies which used DNA-DNA and 16S rRNA sequence homologies. Taxonomic correlations were observed between the PyMS data and the previous studies. Culture age was observed to have little effect on the mass spectra obtained and the reproducibility study indicated that there was very little variation over the 4 week period. It was concluded that PyMS provides a reliable method for studying carnobacterial classification and provides a rapid way for clarifying and refining subgeneric relationships within the genus *Carnobacterium*. Further work may also show that it offers a potentially very rapid and accurate method for the identification of *Carnobacterium*.

INTRODUCTION

The genus *Carnobacterium* was proposed by Collins *et al.* (1987) to describe the atypical lactobacilli poultry isolates of Thornley and Sharpe (1959), *Lactobacillus divergens* (Holzapfel and Gerber 1983) and *Lact. carnis* (Shaw and Harding 1985). Four species of *Carnobacterium* were described: *Carnobacterium divergens* and *C. piscicola*, which are typically isolated from vacuum-packaged meats such as pork, beef and lamb (Hitchener *et al.* 1982; Shaw and Harding 1984), and *C. gallinarum* and *C. mobile*, which were isolated from poultry (Thornley and Sharpe 1959). *Carnobacterium piscicola* strains have also been recognized as fish pathogens (Hiu *et al.* 1984; Baya *et al.* 1991; Starliper *et al.* 1992). More recently, two new species, *C. alterfunditum* and *C. funditum*, have been proposed for isolates which were not associated with meat, poultry or fish (Franzmann *et al.* 1991). The representatives of these two species were isolated from Antarctic lake water.

Feresu and Jones (1988) performed a numerical taxonomic study of the atypical lactobacilli described by Thornley and Sharpe (1959), the majority of which are now being recognized as *C. divergens*, *C. piscicola*, *C. gallinarum* and *C. mobile*. Work by Collins *et al.* (1989) and Wallbanks *et al.* (1990), using 16S rRNA sequence analysis, showed that the four species had high levels of sequence relatedness (*ca* 96–98%) and formed a group which is phylogenetically distinct from other lactic acid bacteria. *Vagococcus fluvialis* and the enterococci were observed to be the most closely related genera.

The availability of constantly improving rapid, automated systematic techniques has enabled the examination of the relationships between strains, and has also highlighted properties which may be useful in the identification of unknown isolates. One such technique, pyrolysis mass spectrometry (PyMS), has been widely applied to the characterization of microbial systems over a number of years (for reviews see Irwin 1982; Meuzelaar *et al.* 1982; Gutteridge 1987; Berkeley *et al.* 1990) and, because of its high discriminatory ability (Goodacre and Berkeley 1990), presents a powerful fingerprinting technique which is applicable to any (micro) biological material.

Pyrolysis mass spectrometry has been successfully applied to the characterization of a wide range of bacterial species and groups, including actinomycetes (Sanglier *et al.* 1992), *Bacillus* (Shute *et al.* 1984), *Escherichia* (Goodacre *et al.* 1991a; Goodacre and Kell 1993), *Legionella* (Kajioka and Tang 1984), mycobacteria (Wieten *et al.* 1981; Freeman *et al.* 1993), propionibacteria (Goodacre *et al.* 1994), rhizobia (Goodacre *et al.* 1991b; Kay *et al.* 1994), salmonellas (Freeman *et al.* 1990) and streptococci (Magee *et al.* 1991), highlighting the usefulness of the technique in the detection of small differences between microbial samples.

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Pyrolysis is the thermal degradation of complex material in an inert atmosphere or a vacuum. It causes molecules to cleave at their weakest points to produce smaller, volatile fragments called pyrolysate (Irwin 1982). A mass spectrometer can then be used to separate the components of the pyrolysate on the basis of their mass-to-charge ratio (m/z) to produce a pyrolysis mass spectrum, which can then be used as a 'chemical profile' or fingerprint of the complex material analysed.

In the present study, 48 strains representing *C. alterfunditum*, *C. divergens*, *C. gallinarum*, *C. mobile* and *C. piscicola* were analysed by PyMS to evaluate the classification of the strains with other biochemical characteristics and to determine the potential of PyMS and multivariate statistical analysis in the systematics of the genus *Carnobacterium*. Five strains were chosen to study the effect of culture age on the classification produced. In addition, the five type strains were chosen to examine the reproducibility, over a period of 4 weeks, of the groupings produced.

MATERIALS AND METHODS

Strains

The strains used and their sources are shown in Table 1.

Media and growth conditions

All strains were grown on (g l^{-1}): K_2HPO_4, 0·14; KCl, 0·34; $MgCl_2 . 2H_2O$, 6·00; $MgSO_4 . 7H_2O$, 1·00; NH_4Cl, 0·25; $CaCl_2 . 2H_2O$, 0·05; NaCl, 20·00; yeast extract (Oxoid), 3·00; bacteriological peptone (Lab M), 5·00; glucose, 5·00; agar, 15·00; pH 7·0–7·4 (Franzmann, personal communication). Glucose was autoclaved separately and added after sterilization. Cultures were incubated at 30°C for 48 h except for *C. alterfunditum* which was grown at 20°C for 48 h. Five strains were examined in duplicate to assess the reproducibility of the system.

Effect of culture age

The effect of culture age on the pyrolysis mass spectra was studied by growing six strains (Table 1) at 30°C for 24, 48 and 72 h.

Short-term reproducibility

The short-term reproducibility of the method was examined with the five type strains of the species studied (Table 1). The strains were examined on four separate occasions, over a period of 5 weeks, on weeks 1, 2, 4 and 5. The strains were grown, as described previously, at 30°C for 48 h on a single batch of media.

Sample preparation for pyrolysis mass spectrometry

Clean iron-nickel foils (Horizon Instruments Ltd, Heathfield, E. Sussex, UK) were inserted, with clean forceps, into clean pyrolysis tubes (Horizon Instruments), so that 6 mm was protruding from the mouth of the tube. After incubation, disposable plastic loops were used to remove some organisms from the top of one or more well-isolated colonies, avoiding the plate surface. These organisms were spread on 5 mm of a protruding foil to give a thin uniform surface coating. The samples were oven-dried at 50°C for 30 min, then the foils were pushed into the tube using a stainless steel depth gauge so that they were 10 mm from the mouth of the tube. Finally, viton 'O'-rings (Horizon Instruments) were placed on the tubes. All bacterial samples were replicated three times.

Pyrolysis mass spectrometry

The pyrolysis mass spectrometer used in this study was the Horizon Instruments PyMS-200X, as initially described by Aries *et al.* (1986). The sample tube carrying the foil was heated, prior to pyrolysis, at 100°C for 5 s. Curie-point pyrolysis was at 530°C for 3 s, with a temperature rise time of 0·5 s. This pyrolysis temperature was chosen because it has been shown (Windig *et al.* 1980; Goodacre 1992) to give a balance between fragmentation from polysaccharides (carbohydrates) and protein fractions. The pyrolysate then entered a gold-plated expansion chamber heated to 150°C, whence it diffused down a molecular beam tube to the ionization chamber of the mass spectrometer. To minimize secondary fragmentation of the pyrolysate the ionization method used was low voltage electron impact ionization (25 eV). Non-ionized molecules were deposited on a cold trap, cooled by liquid nitrogen. The ionized fragments were focussed by the electrostatic lens of a set of source electrodes, accelerated and directed into a quadrupole mass filter. The ions were separated by the quadrupole, on the basis of their mass-to-charge ratio, and detected and amplified with an electron multiplier. The mass spectrometer scans the ionized pyrolysate 160 times at 0·2 s intervals following pyrolysis. Data were collected over the m/z range 51–200, in one-tenth of a mass-unit intervals. These were then integrated to give unit mass. Given that the charge of the fragment was unity, the mass-to-charge ratio can be accepted as a measure of the mass of pyrolysate fragments. The IBM-compatible PC used to control the PyMS-200X was also programmed (software was provided by the manufacturers) to record spectral information on ion count for the individual masses scanned and the total ion count for each sample analysed. Prior to any analysis the mass spectrometer was calibrated using the chemical standard perfluorokerosene (Aldrich), such that m/z 181 was one-tenth of m/z 69.

Table 1 Designation and cluster assignment of *Carnobacterium* strains

Cluster	Strain number	Name received as	Reference
Cluster group A			
1	NCFB 2762*†	*C. piscicola*	
	MT2, MT3, MT29 MT31‡, MT59	*C. piscicola*	Collins *et al.* (1987)
	ACPC7C12§¶, PC5C6§¶	*C. piscicola*	Toole, unpublished
	PN5I9, PC4C7, PC4C12	*C. piscicola*	Toole, unpublished
	2§¶, 501, 725	*C. piscicola*	Montel *et al.* (1991)
	DX, GN	*C. piscicola*	Lewus *et al.* (1991)
2	508	*C. divergens*	Montel *et al.* (1991)
	545	*C. piscicola*	Montel *et al.* (1991)
3	MT47, MT50	*C. divergens*	Collins *et al.* (1987)
4	MT22, MT46, MT56	*C. divergens*	Collins *et al.* (1987)
5	NCFB 2766*†	*C. gallinarum*	
	MT44	*C. gallinarum*	Collins *et al.* (1987)
6	15, 41	*C. piscicola*	Montel *et al.* (1991)
7	543	*C. piscicola*	Montel *et al.* (1991)
	506§¶	*C. divergens*	Montel *et al.* (1991)
8	DSM 5972*	*C. alterfunditum*	Franzmann *et al.* (1991)
	NCFB 2308	*C. mobile*	
	572	*C. piscicola*	Montel *et al.* (1991)
	694‡	*C. divergens*	Montel *et al.* (1991)
SMC	MT34	*C. mobile*	Collins *et al.* (1987)
Cluster group B			
9	NCFB 2763†	*C. divergens*	
	185‡, 327‡§¶	*C. divergens*	Montel *et al.* (1991)
	MT4	*C. divergens*	Collins *et al.* (1987)
	ANP7C33	*C. divergens*	Toole, unpublished
SMC	MT23	*C. divergens*	Collins *et al.* (1987)
10	PN4I15	*C. divergens*	Toole, unpublished
	554	*C. divergens*	Montel *et al.* (1991)
	17	*C. piscicola*	Montel *et al.* (1991)
11	MT49, MT51, MT53	*C. divergens*	Collins *et al.* (1987)
	544	*C. divergens*	Montel *et al.* (1991)
Cluster group C			
12	NCFB 2765*†‡§¶	*C. mobile*	

* Type strain: NCFB, National Collection of Food Bacteria; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.
† Strains used in short-term reproducibility study.
‡ Duplicate strains.
§¶ Strains used to study effect of incubation period—§ growth for 24 h; ¶ growth for 72 h.
SMC, Single member cluster.

Data analysis

The data from PyMS may be displayed as quantitative pyrolysis mass spectra (Fig. 1). The abscissa represents the m/z ratio whilst the ordinate contains information on the ion count for any particular m/z value ranging from 51 to 200. Data were normalized as a percentage of total ion count to remove the influence of sample size *per se*.

The normalized data were then processed with the GENSTAT package (Nelder 1979) which runs under Microsoft DOS 5.0 on an IBM-compatible PC. This method has been previously described by MacFie and Gutteridge (1982) and Gutteridge *et al.* (1985). In essence, the first stage was the reduction of the data by principal components analysis (Causton 1987; Flury and Riedwyl 1988; Everitt 1993), which is a well-known technique for reducing the dimensionality of multivariate data whilst preserving most of the variance. Data were reduced by keeping only those principal components whose eigenvalues accounted for more than 0·1% of the total variance. Canonical variates analysis (CVA) then separated the samples into

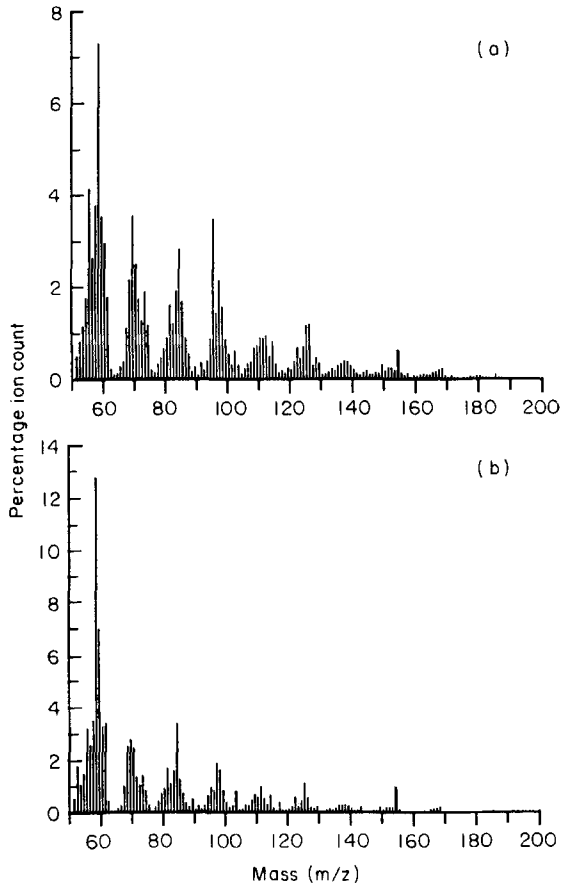

Fig. 1 Normalized pyrolysis mass spectra of (a) *Carnobacterium divergens* and (b) *C. alterfunditum*

groups on the basis of the retained principal components and some *a priori* knowledge of the appropriate number of groupings (MacFie *et al.* 1978; Windig *et al.* 1983). The next stage was the construction of a percentage similarity matrix by transforming the Mahalanobis' distance between *a priori* groups in CVA with the Gower similarity coefficient S_G (Gower 1971). Finally, hierarchical cluster analysis was employed to produce a dendrogram, using average linkage clustering (Gutteridge *et al.* 1985).

RESULTS

Typical PyMS spectra for two of the *Carnobacterium* species studied are shown in Fig. 1. There was little qualitative difference between the spectra from the species type strains but, on close inspection, quantitative differences between the spectra may be observed. Such spectra readily illustrate the need to employ multivariate statistical techniques in the analysis of PyMS data.

In the CVA plot (Fig. 2), in which the first two canonical variates accounted for 87·3% of the total variation in the data, the *C. piscicola* strains were recovered into a distinct group with six outlier strains. This distribution is reflected in the dendrogram (Fig. 3) where 16 of the 22 strains of this species were recovered into cluster 1. The remaining six *C. piscicola* strains were recovered into five other clusters; clusters 2, 6, 7, 8 and 10.

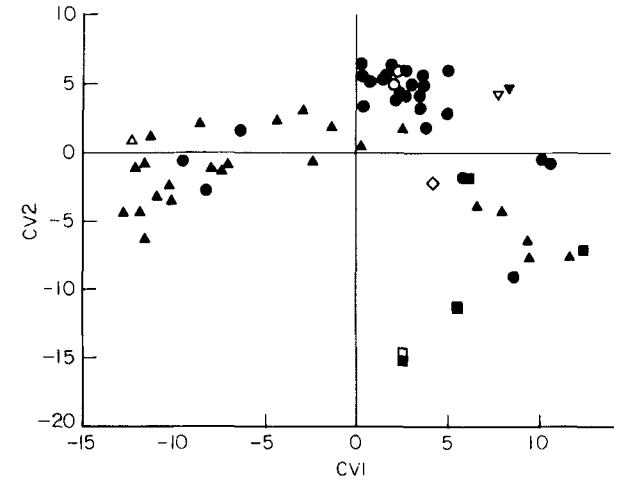

Fig. 2 Ordination plot based on pyrolysis mass spectrometry data analysed by GENSTAT showing the relationship between *Carnobacterium* spp. The first canonical variate accounted for 75·7% of the total variance, and the second accounted for 11·6% of the total variance (87·3% total). ○, *Carnobacterium piscicola* (type strain); ●, *C. piscicola*; □, *C. mobile* (type strain); ■, *C. mobile*; △, *C. divergens* (type strain); ▲, *C. divergens*; ▽, *C. gallinarum* (type strain); ▼, *C. gallinarum*; ◇, *C. alterfunditum* (type strain)

The *C. divergens* strains showed a greater degree of heterogeneity than those of *C. piscicola* in the CVA plot (Fig. 2), with at least two groups of strains being recognized. However, the majority of the strains were still readily separated from the four other species. This distribution is shown in the dendrogram (Fig. 3) where the strains are recovered into two cluster groups, in cluster group A (clusters 2, 3, 4, 5 and 8) and in cluster group B (clusters 9, 10 and 11). Twelve of the total 15 strains recovered in cluster group B were supplied as *C. divergens* strains, including *C. divergens* NCDO 2763, the type strain.

The *C. mobile* strains did not form a tight group in the CVA plot (Fig. 2) and this was reflected in the dendrogram (Fig. 3) where the strains were recovered into three separate clusters. The three examples of the *C. mobile* type strain (NCFB 2765) were recovered into cluster 12 which shows a distinct dissimilarity to the other strains. MT34 was recovered as a single member cluster and *C. mobile* NCFB 2308 was recovered in cluster 8.

The two examples of *C. gallinarum*, the type strain NCFB 2766 and strain MT44, were recovered together in cluster 5 (cluster group A). *Carnobacterium alterfunditum* was recovered into cluster 8.

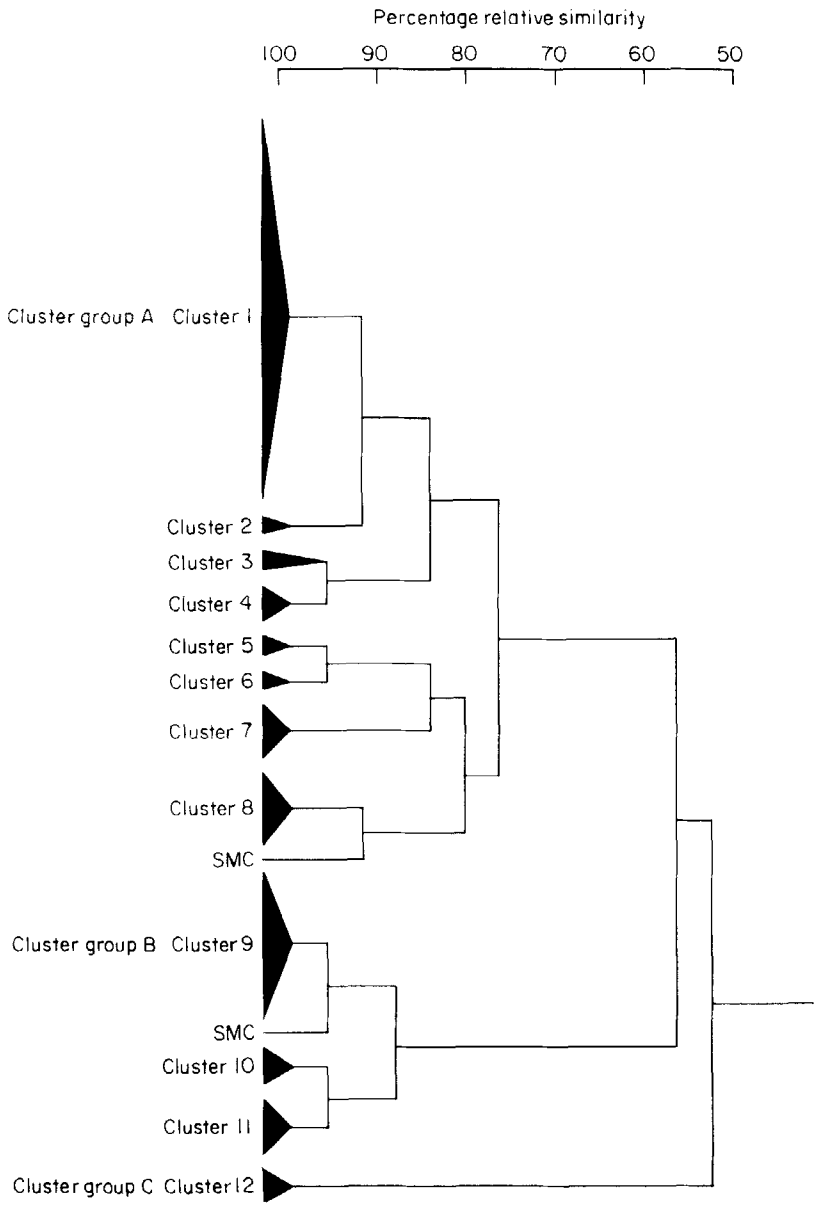

Fig. 3 Dendrogram representing the relationships between *Carnobacterium* spp. based on pyrolysis mass spectrometry data analysed by GENSTAT

In the dendrogram (Fig. 3) all of the duplicated strains, except for strain 2765, showed a similarity of 98·5% or greater. Strain 2765 duplicates showed a similarity of 96·1%. All of the five duplicated strains, except for *C. divergens* 185, exhibited the greatest similarity to their corresponding duplicate. One of the duplicates of strain *C. divergens* 185 showed an identical similarity to MT4 (99·9%) as did to its corresponding duplicate strain.

The effect of incubation time on the spectra obtained was studied with five strains (Table 1). The strains had been grown on the same media batch for either 24, 48 or 72 h and were always recovered into the same cluster. Percentage similarities based on S_G showed that the length of incubation had little effect on the mass spectra obtained, implying that there was very little phenotypic change. The percentage similarities between strains varied from 98·9% for strain PC5C6 grown for 24 and 48 h to 99·9% for PC5C6 grown for 24 and 72 h. These similarities are greater than the similarities between duplicate strains.

The canonical variate plot of the long-term reproducibility study of the type strains is shown in Fig. 4. Strains were analysed on weeks 2, 4 and 5, the strains from week 1 were omitted because of limitations with the computer package, GENSTAT, which was programmed by Horizon Instruments. The first two canonical variates account for 89·9% of the total variation of the data. The three samples of the five species were recovered into five groups. The three samples of *C. divergens* NCFB 2763 were

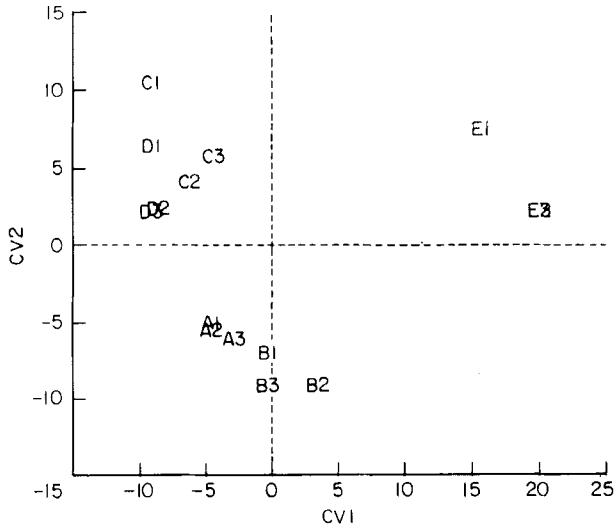

Fig. 4 Ordination plot based on pyrolysis mass spectrometry data analysed by GENSTAT showing the relationship between the carnobacterial type strains analysed over a period of 4 weeks. The first canonical variate accounted for 82·8% of the total variance and the second accounted for 7·1% of the total variance (89·9% total). A, *Carnobacterium gallinarum*; B, *C. piscicola*; C, *C. mobile*; D, *C. alterfunditum*; E, *C. divergens*. Strains analysed on: 1, week 2; 2, week 4; 3, week 5

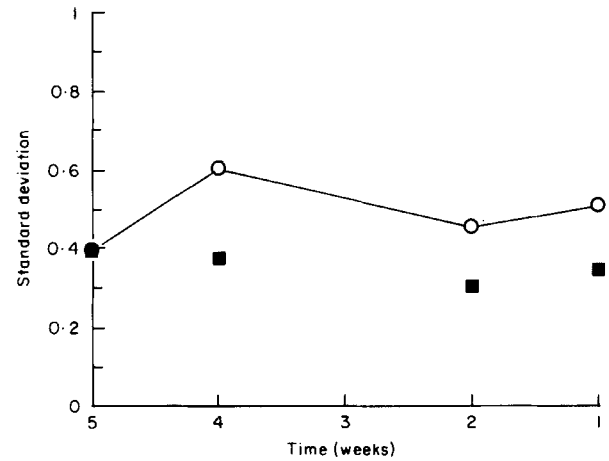

Fig. 5 Graph showing the same day standard deviation (■) and week to week standard deviation (○), compared with week 5. These values are calculated on the top 20 *m/z* values of the mass spectra of the carnobacterial type strains

recovered into a single group distinct from the other four type strains. *Carnobacterium gallinarum* NCFB 2766 and *C. piscicola* NCFB 2762 were recovered into the same area off the plot but were still recognizable as separate groups, as were *C. alterfunditum* DSM 5972 and *C. mobile* NCFB 2765.

Figure 5 presents two measures of variation in the reproducibility study. Firstly, it shows a measure of the standard variation seen between the three replicate spectra which were obtained for each strain in a single day (same day standard deviation). Secondly, it shows the average standard deviation observed when the standard deviations are compared with the spectra obtained from the most recent analysis on week 5 (week-to-week standard deviation). In each case, the standard deviation was based on the 20 most intense masses.

The same day standard deviation of the spectra for the five type strains showed little variation over the time course of each part of the short-term reproducibility study. Not surprisingly, the week-to-week variation showed a greater variation than the same day deviation; this may be because of phenotype changes in the strains or, alternatively, due to instrument drift. It is noteworthy, however, that the variation between spectra did not get any worse after the first week (i.e. week 4 compared with week 5) and that the variation was due to slight phenotypic variability. It is, therefore, likely that the spectra produced by PyMS are stable over the 4 week period and would give robust classification; this is mirrored by these strains clustering together in the CVA plot (Fig. 4).

DISCUSSION

Confidence can be placed in this PyMS study given the good reproducibility of the mass spectra of the duplicated strains. It is encouraging that the majority of the *C. piscicola* strains were recovered into a single cluster, and also that the majority of the *C. divergens* strains were recovered into a single cluster group. Additionally, the short-term reproducibility studies showed both sustainable separation of the five type strain species examined, and that the spectra did not change significantly over the study period.

Numerical taxonomic studies by Feresu and Jones (1988) showed that the strains which constitute the four species *C. divergens*, *C. gallinarum*, *C. mobile* and *C. piscicola* can be separated on the basis of traditional bacteriological techniques. Their work indicated that *C. gallinarum* showed greater similarity to *C. piscicola* than to the other species. Additionally, these two species were more closely related to *C. divergens* than to *C. mobile*. Work by Wallbanks *et al.* (1990), using 16S rRNA sequencing techniques, also indicated that *C. piscicola*, *C. gallinarum* and *C. divergens* were more closely related to each other than they were to *C. mobile*. This taxonomic relationship was also observed in the dendrogram (Fig. 3) where *C. mobile* NCFB 2765 was recovered as a distinct cluster (cluster 14).

Montel *et al.* (1991) suggested that sub-species of *C. piscicola* may exist, on the basis of DNA-DNA homology data. *Carnobacterium piscicola* strains 15, 213, 501, 543, 545 and 572 showed between 73 and 92% DNA-DNA homology with *C. piscicola* NCDO 2762, whilst *C. piscicola*

strains 2, 17 and 41 showed only 40–45% DNA-DNA homology. In this PyMS study the *C. piscicola* strains were not all recovered in a single cluster (Fig. 3) and this may reflect the presence of sub-species of *C. piscicola*. Collins *et al.* (1987) did not observe any heterogeneity for their *C. piscicola* strains and indeed PyMS recovered all of their strains which were included in this study into a single cluster, cluster 1.

The high degree of heterogeneity shown by *C. divergens*, with several centres of variation observable, is not in agreement with previous DNA-DNA homology studies (Collins *et al.* 1987; Montel *et al* 1991). The discrepancies between the PyMS data and those obtained from the other taxonomic studies of *C. divergens* are unexpected and as stated by Manchester *et al.* (1990), highlight a problem in contemporary bacterial systematics: given two or more partially compatible classifications, what criteria can be used to decide which, if any, of the taxonomic structures is most fitting? Clearly, further work is needed to resolve these apparent contradictions.

Carnobacterium mobile, *C. gallinarum* and *C. alterfunditum* are species of which there are few available examples and it is therefore more difficult to draw conclusions based on the few strains included in this study. The recovery of the *C. mobile* type strain in a position which mirrors that seen in the numerical taxonomic study of Feresu and Jones (1988) indicates that, with more examples of this species, PyMS may provide a valid method for separating, and therefore identifying, unknown isolates of the genus *Carnobacterium*. Strains MT34, 2308 and 2765 were selected to represent the spread of variation seen in *C. mobile*, based on the work of Feresu and Jones (1988), and this may account for their recovery into separate clusters. The recovery of the *C. gallinarum* strains in cluster group A (Fig. 3) with the majority of the *C. piscicola* strains was encouraging because Feresu and Jones (1988) showed this species to be more closely related to *C. piscicola* than to *C. divergens*. This phenomenon is further supported by the work of Wallbanks *et al.* (1990) in which *C. gallinarum* and *C. piscicola* were found to show 98·7% 16S rRNA homology whilst *C. gallinarum* and *C. divergens* exhibited only 97% similarity. The close relationship of these two species was also shown during the development of oligonucleotide probes for rapid detection and identification of *Carnobacterium* species in meat by Brooks *et al.* (1992). They found that it was not possible to design an oligonucleotide probe to distinguish between these species.

The observed congruences between the PyMS, numerical taxonomic, DNA homology and 16S rRNA data were very encouraging. This, and the reproducibility of the PyMS technique, indicates that such a system warrants further investigation for its use in carnobacterial systematics. Future work will be to investigate this as a potentially very rapid and accurate method for the identification of *Carnobacterium*. This investigation demonstrates the potential use of PyMS for examining the subspecific taxonomic structure of the *Carnobacterium* genus and provides a rapid method for clarifying and refining subgeneric relationships within the genus *Carnobacterium*.

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