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Numerical taxonomy of an 'atypical' population of Gram-positive cocci isolated from freshly dressed lamb carcasses

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Abstract

One hundred Gram-positive, catalase-positive strains were isolated from freshly dressed lamb carcasses. They were randomly selected from a non-selective medium and tested for 75 characters. Only nine cultures could be identified by conventional methods. A numerical taxonomic study was conducted on the whole population and 25 reference strains. At the 80% similarity level (S_{sm}), ten clusters were formed. Five of them were entirely composed of reference strains. Phena V and VIII contained seven isolates and two reference strains of *Micrococcus*. Phena VI (six unidentified isolates), VII (nine staphylococci) and IX (69 unidentified isolates) were more related to *M. kristinae* than to the remaining reference strains. Properties with possible implications in meat spoilage were: strong lipolytic activity (76%), anaerobic growth (85%), tolerance to 15% (w/v) NaCl (95%) and ability to grow at 15°C (95%) and 4°C (26%).

Keywords: Micrococcaceae; Lamb; Carcasses

1. Introduction

A high percentage of fresh carcasses is contaminated with staphylococci (ICMSF, 1980; Bergdoll, 1989). Nevertheless, it is widely accepted that their presence is of little direct significance for two reasons: (i) the organisms are poor competitors and do not grow well in the presence of psychrotrophic bacteria; and (ii) raw meats

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do not provide a good medium for their growth (Mossel and Van Netten, 1990; Bergdoll, 1989; Varnam and Evans, 1991).

In a previous work, Prieto et al. (1993) observed that staphylococci grew on the surface of lamb carcasses during aerobic storage at low temperatures. The incidence amongst isolates obtained a few hours (6–8) after slaughtering was 17% and increased as spoilage progressed (39.9% at the spoilage time). The *S. saprophyticus* species complex (mainly *S. xylosus*) proved to be dominant and it seemed as if it would be favoured by primary actions due to *Pseudomonas* and *Brochothrix* (Prieto et al., 1993).

Although the microbiology of carcass meats depends on the time and conditions of storage, it is clear that bacteria present at the moment of slaughter will influence the evolution of the microbial associations. Therefore, it was considered of interest to establish if the species of Gram-positive, catalase-positive cocci found on sheep carcasses at the completion of the slaughtering process were those associated with spoilage by Prieto et al. (1993). Using conventional taxonomic schemes (Kloos and Lambe, 1991), the identity of 100 isolates was uncertain because most of them failed the description of the four genera of Micrococcaceae.

In this paper we present a numerical taxonomic study of this 'atypical' population of Micrococcaceae.

2. Materials and methods

2.1. Sampling

Three commercial abattoirs located at three different towns were visited twice and thirty lamb carcasses were examined. On each visit, five individual carcasses were removed immediately after final washing, hung from a detention rail adjacent to the main line and sampled. Swabs from three areas of 50 cm^2 (neck, leg and flank) were collected and pooled together according to ICMSF (1986).

2.2. Field strains

Counts were determined on Plate Count agar (ICMSF, 1986). Colonies were randomly picked and screened for catalase production and Gram-positive reaction. After purification, 100 isolates (obtained from different carcasses) were selected. Of them, 91 could not be identified on the basis of routine tests.

2.3. Reference strains

The following reference strains were included in the numerical analysis: *Staphylococcus epidermidis* ATCC 29663, *S. capitis* ATCC 27840, *S. warneri* ATCC 27836, *S. haemolyticus* ATCC 29970, *S. saccharolyticus* ATCC 14953, *S. caprae* CCM 3573 and *S. hominis* ATCC 27884 (*S. epidermidis* group). *S. saprophyticus* ATCC 14953, *S. cohnii* subsp. *cohnii* ATCC 29974 and *S. xylosus* ATCC 29971 (*S. saprophyticus*

group). *S. gallinarum* CCM 3572, *S. kloosii* DSM 20676, *S. equorum* DSM 20674 and *S. arlettae* DSM 20672. *S. simulans* ATCC 27848 and *S. carnosus* DSM 20501 (*S. simulans* group), *S. sciuri* ATCC 29062 and *S. lentus* ATCC 29070 (*S. sciuri* group). *Micrococcus luteus* ATCC 4698, *M. lylae* ATCC 27566, *M. varians* ATCC 15306, *M. kristinae* ATCC 27570, *M. sedentarius* ATCC 14392, *M. halobius* ATCC 21727, and *Stomatococcus mucilaginosus* ATCC 25296.

Reference strains used as biological cultures in tests were those recommended by Lanyi (1987).

2.4. Phenotypic characterization

In all, 75 characters were determined. the following tests were performed using conventional methods: cell morphology and arrangement (Kloos et al., 1974), colony morphology (size, consistency, profile, edge and lustre) and pigmentation (Kloos and Lambe, 1991); type of growth in broth (Harrigan and McCance, 1976; Mead and Dodd, 1990), motility (hanging drop technique and motility medium) (Cowan, 1974; Harrigan and McCance, 1976), lysostaphin and lysozyme susceptibility (Schleifer and Kloos, 1975); growth on furazolidone agar (Rheinbaben and Hadlok, 1981), anaerobic growth in semisolid thioglycollate medium (Evans and Kloos, 1972); ability to produce acid anaerobically and aerobically from glucose and mannitol (Subcommittee on Taxonomy of Staphylococci and Micrococci, 1965); growth temperature range (4, 15, 30, 35 and 37°C) (Baird-Parker, 1979); nitrate reduction (Kloos et al., 1974); aesculin hydrolysis (Schleifer et al., 1981); oxidase and benzidine tests (Faller and Schleifer, 1981); ability to grow on inorganic nitrogen agar (Kloos et al., 1974); acid from glycerol-erythromycin medium (Schleifer and Kloos, 1975), salt tolerance (7.5%, 10% and 15%, w/v), free coagulase and thermonuclease production (Baird-Parker, 1979), clumping factor reaction (Devriese and Hajek, 1980), novobiocin resistance (Baird-Parker, 1979), acid production from carbohydrates (D-xylose, L-arabinose, D-cellobiose, raffinose, salicin, sucrose, maltose, D-mannitol, D-mannose, D-trehalose, α-lactose, D-galactose, β-D-fructose, D-melizitose, D-ribose and xylitol), egg yolk reaction and hydrolysis of Tween (80, 60, 40 and 20) (Gutiérrez et al., 1982); tributyrin hydrolysis (Harrigan and McCance, 1976), proteolytic activity on casein and gelatin (Cowan, 1974); arginine dihydrolase and urease activities (Krasuski, 1981); production of nuclease, β-galactosidase and acetoin (Kloos and Lambe, 1991); methyl red and Voges Proskauer reactions, haemolysis of sheep blood; hyaluronidase production (Smith and Willett, 1968) and utilization of citrate (Kloos et al., 1974).

2.5. Numerical analysis

The characters were coded as negative (0), positive (1) or doubtful (2). The simple matching coefficient (S_{sm}, Sokal and Michener, 1958) and the Jaccard coefficient (S_j, Sneath, 1957; Austin and Priest, 1986) were used and clustering achieved by unweighted pair groups average linkage (UPGMA, Sneath and Sokal, 1973). The analysis was performed in a Compaq Deskpro 386S personal computer

(Compaq Computer Corp., TX, USA). The software used was SPSS PC + (SPSS Inc., Ch., USA) for clustering and Basic computer programs (Prieto, 1990) for similarity coefficients. The correlation coefficient between the similarity matrix and the levels on the dendrogram derived from that matrix (cophenetic correlation) was calculated (Sneath, 1978a). Character frequency tables were generated by computer and used to determine the most discriminatory characters (Sneath, 1978b).

All tests were repeated on six strains. The average probability of error (P) was calculated according to Sneath and Johnson (1972).

3. Results

The average probability of error (P) was 2.2, which would not produce important distortion of the taxonomic structure. The cophenetic correlation values were 0.913 (S_{sm}) and 0.841 (S_j).

At the 80% similarity level (% S_{sm}), ten clusters were formed and designated groups I to X, respectively (Fig. 1). Five of them (Phena I, II, III, IV and X) contained reference strains only and the remaining five (Phena V, VI, VII, VIII and IX) contained 91 isolates and two reference strains. There were 11 unclustered strains, of which two were reference strains (*Stomatococcus mucilaginosus* and *Micrococcus varians*) and nine field strains.

The reference strains of all the *Staphylococcus* species fell into clusters I (*S. saprophyticus* group), II (*S. epidermidis* and *S. simulans* groups), III (*S. gallinarum, S. arlettae, S. equorum* and *S. kloosii*) and IV (*S. sciuri* group) while three reference strains of *Micrococcus* formed cluster X (*M. lylae, M. luteus* and *M. sedentarius*). Sixty-nine of our isolates were recovered in cluster IX. Cluster V contained two isolates and the type strain of *M. halobius*. Cluster VI consisted of six unidentified field strains. The nine isolates in cluster VII had been identified as staphylococci and cluster VIII comprised 6 strains including the reference strain of *M. kristinae.*

At the 44% similarity level (S_j), strains were divided into seven clusters. With the S_j coefficient, clusters of reference strains were more diffuse and all but one of the field strains grouped together in a cluster.

4. Discussion

In practice, it has often been considered that members of the genus *Staphylococcus* can be clearly separated from other Gram-positive, catalase-positive cocci on the basis of simple tests. Phenotypic characters can also be very useful in the classification and identification of the species of Micrococcaceae (Schleifer, 1986; Schleifer and Kroppenstedt, 1990; Kloos, 1990; Gilmour and Harvey, 1990; Kloos and Lambe, 1991). Furthermore, numerical taxonomy has been used successfully to examine strains of micrococci and staphylococci isolated from different habitats (Mortensen and Benzton, 1976; Feltham, 1979).

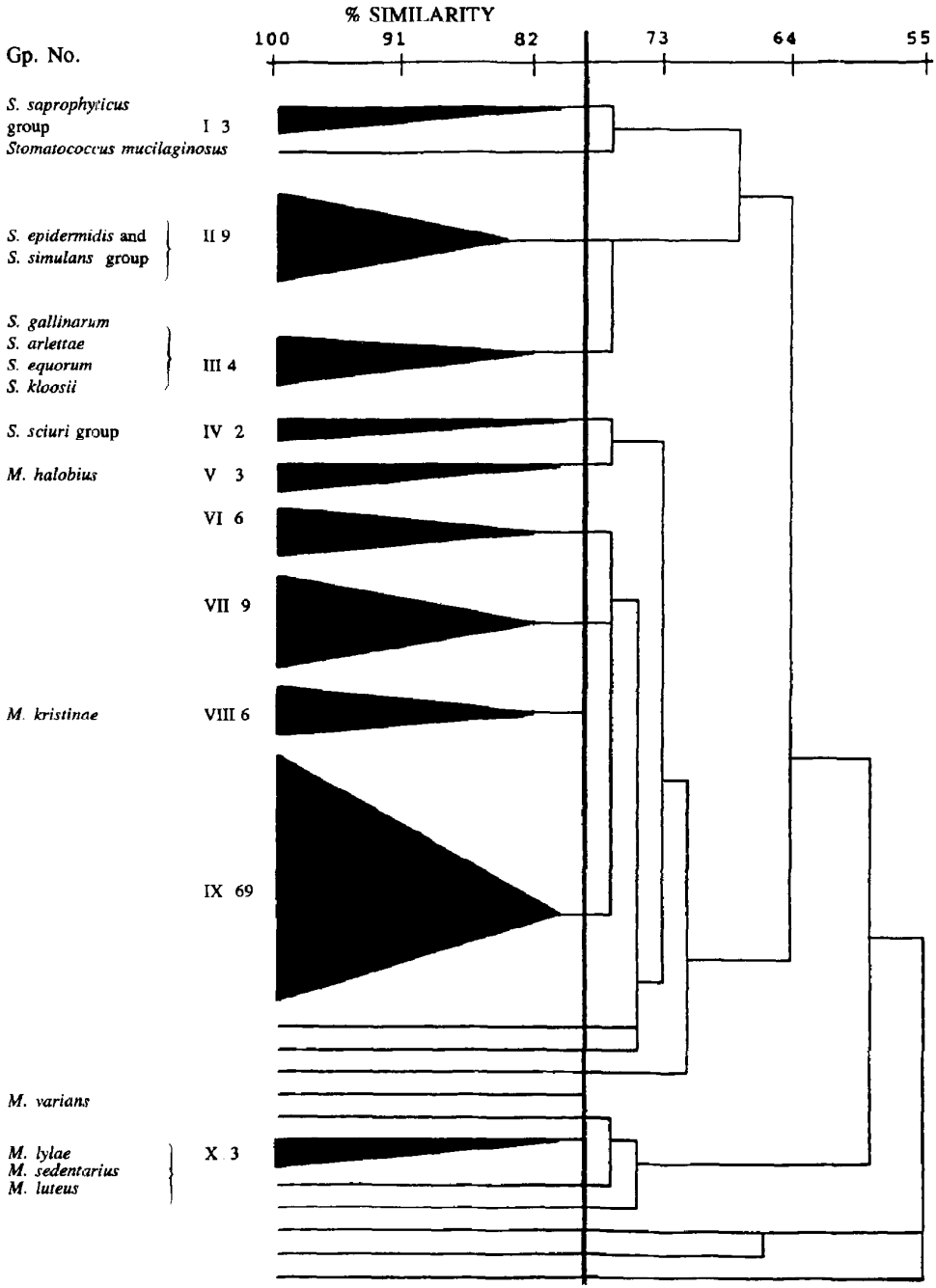

Fig. 1. Simplified dendrogram showing the phena formed at the 80% similarity level and unweighted group average linkage clustering. Gp, group; No., number of strains per group.

In previous studies on the characterization of Micrococcaceae obtained from raw milk and dairy products, red meat and food handlers (García et al., 1980, 1986, 1988; Gutiérrez et al., 1982; Menes et al., 1984) we observed that the majority of the isolates could be identified to the species level by classical approaches. The only exception was a group of strains found in cheeses made from pasteurized ewes' milk. It is interesting that the clustering of those strains by numerical analysis correlated well with their DNA base composition (mol % G + C) and that coagulase-positive and coagulase-negative, novobiocin-resistant isolates formed satisfactory clusters (García et al., 1990).

The application of numerical taxonomy methods to the atypical population of Gram-positive, catalase-positive cocci examined in this study was not successful in helping to discriminate the genera and/or species of Micrococcaceae to which they could belong. thus, the isolates in phenon VII (nine strains fitting the description of novobiocin-resistant, coagulase-negative staphylococci) joined phenon IV (*S. sciuri* group) at the 73% similarity level (S_{sm}) but they were more closely related (82.36%s) to the *M. kristinae* cluster (phenon VIII). Phena VI (six isolates) and IX (69 isolates) also showed closer resemblance to *M. kristinae* than to the remaining reference strains (77.9% and 79.86%, respectively). The relationship among the above clusters (Fig. 1) does not allow us to conclude that the majority of the Gram-positive, catalase-positive cocci isolated from freshly dressed lamb carcasses were *M. kristinae*. The main reasons are the lack of genetic and chemotaxonomic data and certain 'key' properties of the Phena. According to several authors (Sneath, 1978a; Austin and Price, 1986), 'species' can be defined at the 80% similarity level using S_{sm}-UPGM generated dendrograms and genera at about 60% but this is a rough guide which needs to be substantiated by chemotaxonomic evidence. On the other hand, significant properties of certain clusters (i.e. phenon IX) were characteristic of *Staphylococcus*. It would be tempting to speculate that most of our isolates were 'new' Micrococcaceae. However, the accurate identification would require additional tests with a higher power of resolution (i.e. nucleic acid analyses, cell wall composition, etc.). These were not performed because a basic taxonomy study was beyond the initial aim of this work which only attempted to contribute to the clarification of the role of Micrococcaceae as spoilage bacteria on lamb carcasses.

Characteristics with potential significance in meat spoilage (Table 1) were the strong lipolytic activity of the 69 isolates belonging to phenon IX and the ability to hydrolyse casein and gelatin showed by isolates in phena VI, VII and VIII. In all, 96 cultures had extracellular enzyme activity though the 'lipolytic' isolates were non proteolytic and vice versa. Other properties with possible implications in spoilage processes under certain storage conditions were anaerobic growth (85 cultures), salt tolerance (95 cultures grew in 15% (w/v) NaCl) and the ability to grow at 15 and even 4°C.

Aerobic spoilage of meat and other proteinaceous foods are usually attributed to Gram-negative motile and non-motile bacteria. However, recent advances in taxonomy have shown the significance of previously unrecognized species and genera of bacteria (*Pseudomonas lundensis, Carnobacterium* and *Leuconostoc*)

Table 1
Characteristics with potential significance in spoilage of 100 field strains of Micrococcaceae isolated from freshly dressed lamb carcasses

	Clusters					
	V	VI	VII	VIII	IX	U [a]
No. of isolates [b]	2	6	9	5	69	9
Growth at 4°C	0 [c]	0	100	0	25	0
15°C	100	100	100	100	100	44
Salt tolerance						
7.5% (w/v)	100	100	100	100	100	100
10% (w/v)	100	100	100	100	96	100
15% (w/v)	100	100	89	100	94	100
Egg yolk reaction	0	0	0	0	100	78
Hydrolysis of Tween						
20	0	0	0	0	100	78
40	0	0	0	0	50	0
60	0	0	0	0	0	0
80	0	0	0	0	7	0
Tributyrin hydrolysis	0	0	0	0	100	78
Proteolytic activity						
gelatin	0	100	100	80	0	0
casein	0	83	100	100	15	0
Anaerobic growth	100	100	89	100	82	89

[a] unclustered field strains; [b] number of field strains recovered in each cluster; [c] percentage of positive isolates.

(Dainty and Mackey, 1992). Although it is arguable whether Micrococcaceae are important contributors to the spoilage of lamb carcasses, we have observed the predominance of staphylococci and other unidentified strains of Micrococcaceae at the end of storage life (Prieto, 1990; Prieto et al., 1993). An additional consideration is the fact that the mechanisms that allow certain microorganisms to become dominant among meat microflora are not fully understood. For example, we detected that storage in air of lamb carcasses at 4–5°C tends to favour the growth of *Brochothrix thermosphacta*, which become dominant after 1 week (Prieto, 1990; Prieto et al., 1993). A factor which may have influenced our data on types and proportions of organisms associated with lamb spoilage is the method of selection of the strains tested (random selection from a non selective medium).

The most important *Staphylococcus* species associated with sheep are those of the *S. sciuri* group and some others of the *S. saprophyticus* group (Devriese, 1990). the primary habitat of many *Micrococcus* species is mammalian skin while *Stomatococcus* and *Planococcus* are mainly found in humans and in marine environments, respectively. Where did our isolates originate? They could be inhabitants of the skin, mucous membranes or other regions of the sheep body but also they could have been acquired during processing. The persistance of 'endemic' staphylococci in poultry processing plants, despite regular cleaning and disinfection, and the difficulty to remove staphylococcal contamination of carcasses by washing have been reported by Notermans et al. (1982) and by Mead and Dodd (1990). A

Table 2
Major features distinguishing the Phena formed at the 80% similarity level and unweighted group average linkage clustering

	Separation index [a]	Clusters									
		I (3) [b]	II (9)	III (4)	IV (2)	V (3)	VI (6)	VII (9)	VIII (6)	IX (69)	X (3)
Colony appearance (lustre)	24	+ [c]	+	+	+	−	−	−	−	−	−
Lysostaphin sensitivity	20	+	+	+	+	−	−	+	V	−	−
Aesculin hydrolysis	18	+	−	−	V	+	+	+	+	+	−
Growth on furazolidone agar	16	−	−	−	−	+	−	−	−	−	+
Colony appearance (profile)	15	−	V	−	−	+	V	+	V	+	−
Growth at 45°C	14	−	+	+	−	−	−	−	−	−	V

[a] Sneath (1978); [b] number of strains recovered in each phenon; [c] +, property present in more than 85% of the strains; −, properties present in less than 15% of the strains; V, percentage of positive strains between 15% and 85%.

distinctive character of the majority of our isolates (ca. 80%–100% in phenon IX-) was the formation of flocculent turbidity and clumps in broth culture. Mead and Dodd (1990) indicate that mucoid growth would provide a means of surface attachment.

Although the high similarity of strains in phenon IX suggests a common source, they were obtained from 25 animals belonging to different flocks and slaughtered in separated abattoirs.

Table 2 shows the major features distinguishing clusters obtained with the S_{sm} coefficient. Two of them are differential characteristics of staphylococci and micrococci (growth on furazolidone agar and lysostaphin sensitivity). Aesculin hydrolysis and growth at 45°C are used for the identification of species of *Micrococcus* and *Staphylococcus*, respectively. In this study, the high separation index of these properties was mainly due to the clustering of the reference strains since phena containing the majority of our isolates (i.e., VI and IX) showed mixed patterns. Finally, the colony appearance is considered by Kloos (1990) as a very useful character in staphylococci identification.

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