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Characterization of Fatty Acids from Gram Positive Rods Possibly Related to the Genus Microbacterium

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Abstract

Aim: The principle aim of this study is to illustrates fatty acid profile of isolated bacteria from milk and milk product to illustrate the taxonomic position of these isolate to the type species of the genus *Microbacterium* with respect to their role in dairy product spoilage.

Method: Twenty strains of coryneform and related bacteria were obtained from public and private culture collection. All strains were grown on nutrient agar (Difco) after being sterilized by autoclaving at 121°C for15 mines. Cultures were incubated for 48h then were harvested by centrifugation at 8000 rpm for 20 min in MSE high speed M-18 centrifuge, acid methanolysis was followed. Where 100 mg dried cells were mixed with 5 cm^3 methanol, 5 cm^3 toluene and 0.2 cm^3 concentrated Sulphuric acid in 20 cm^3 tubes sealed with PTFE-lined serum caps. Upper hexane layer was collected. Liquid chromatography (GLC) using a Pye unicam chromatogram equipped by flame ionization detector was used.

Results: Two groups of fatty acid were obtained. One group contains a high proportion of straight-chain and unsaturated fatty acid and those composed mainly of branched fatty acids. The former group includes *Microbacterium flavum* and strain number 19 and 20 while the remaining strains contain mainly branched chain fatty acids. The other group of showed fatty acid profile similar to *Microbacterium lacticum* and those contain predominantly C15, C16, and C17 branched chain fatty acid.

Keywords: *Microbacterium, Fatty acid*

Introduction

The classification of all biological entitles, be they animals, plants or bacteria, is subject to change as new information become available. However, bacterial classification are more unstable than those of plants and animals because of the paucity of easily observed morphological details., the virtual absence of a fossil record and the, as yet poor information which is basic to present day bacterial knowledge of generic exchange between bacterial information which is basic for to present day bacterial morphological classifications. The first bacterial calcification scheme separated bacterial groups mainly on morphological criteria together with some biochemical and physiological properties was described by Bergey., *et al*. 1934 [1]. Despite such development, her was still outstanding difficulties encountered in bacterial classification. Although difficulties are encountered with most bacterial groups, the unsatisfactory state of the taxonomy of the coryneform bacteria has been particularly widely acknowledged [1-6]. Thus, in the 8th edition of Bergy's Manual the genus *Microbacterium* was considered as member of the family *Corynebacteriaceae* [6,7]. Recently study considered *Microbacterium* is a genus of bacteria in the family *Microbacteriaceae*. As of 2015 it consists of 96 species [8]. The genus *Microbacerium* created by Orla-Jensen [9] for a group of small gram positive heat resistant rods which are capable of produce lactic acid from sugar. The genus originally consisted of number of specie, *M. mesentaricum, M. lacticum, M. flavum and M. liquefaciens*. Later *M. thermosphactum* was added for strains which are not heat resistant [9]. However, in the 8th edition of Bergey's Manual they were placed [11] under the heading Coryneform group, of bacteria and the genera *Brevibacterium* and *Microbacterium*, hitherto regarded as member of the family *Corynebacterineae* [11] and were

included as genera incertae sedis. The few intervening years between the compilation of the 8th edition of Bergey's Manual have however, seen the application and extension of chemotaxonomic method where modern techniques for classifying bacteria according to their components such as cell wall [12-14] and by their lipid composition [15-19].

The first attempt to correlate lipid with taxonomic classification was made by Abel., *et al.* [20] who showed that qualitative fatty acid analysis could be used to differentiate various organisms, since then a considerable number of studies had been extended and confirmed the usefulness of lipid analysis to microbial taxonomist.

In the past, there were two studies on the fatty acid components of microbacteria. In Shibukawa., *et al*. [21] study, an organism called *Microbacterium ammoniaphilum* was shown to contain C14, C16, C18 straight-chained, stratified fatty acids and C16 (stearic acid) being the major component. They also reported the presence of a trace C18 di-unsaturated fatty acid. In the other study, Shaw and stead [22] isolated two straight-chain fatty acid (C14, C16) together with C15, C17, C19 branched fatty acid from *Microbacterium thermosphactum* however, they did not determine whether the branched fatty acid was iso or anteiso bearing in mind the doubt concerned the taxonomy of *Microbacterium thermosphactum* at that time [23]. Fatty acid of coryneform bacteria fall into two type the straight-chained, saturated and mono-unsaturated fatty acids and type iso- and anteioso-branched fatty acids (Table 2). The proportion of each of these types is quite different in different species [17] and the significant of theses analyses to microbacterial taxonomy remain unclear however significant of fatty acid in bacterial taxonomy was under investigation [23].

The bacteria fatty acid profile has been extensively studied for taxonomic classification purposes, since bacteria, in general, contain particular and rare fatty acids, compared with animal and plant tissues. As for any real-world sample type, the development of rapid and reliable methods for (i) sample identification (in this case, bacterium type), and (ii) constituent identification in this instance, the fatty acid profile) is desirable [24]. Recent study on *Brucella ovis* lipids profile showed the significant of lipid in identification purpose [25].

This study is concerned with an examination of the relationship between strains isolated from dairy products and their relatedness to the genus *Microbacterium*.

Method

Twenty strains of coryneform and related bacteria were obtained from public and private culture collection. The full strain list is shown in Table1. A number of strains representing different taxa defined by Bousfield [26] and Jones [27] where representative of coryneform and related bacteria were included as markers. The unidentified strains were collected from the National Institute for research in Dairying, Sheffield, Reading and the Food research institute, Norwich and were thermoduric isolates from milk, milk products and egg. All strains were originally obtained in freeze dried culture. All strains were grown on nutrient agar (Difco) after being sterilized by autoclaving at 121°C for15 mines. Cultures were incubated for 48h then were harvested by centrifugation at 8000 rpm for 20 min in MSE high speed M-18 centrifuge then washed in distilled water and re-harvested two times.

Extraction of fatty acid from whole organism was followed according to Minikin., *et al.* [28], acid methanolysis was followed. 100 mg dried cells were mixed with 5 cm^3 methanol, 5 cm^3 toluene and 0.2 cm^3 concentrated Sulphuric acid in 20 cm^3 tubes sealed with PTFE-lined serum caps. Methanolysis was carried out for 16 - 18h at 50^0C Gas. The mixture was then subsequently cooled and 2 cm3 hexane was added and the mixture was shaken vigorously. Upper hexane layer was collected. Liquid chromatography (GLC) using a Pye unicam chromatogram equipped by flame ionization detector was used where A 6 meter stainless steel Colum packed with 10% Silar 100C on 100 - 120 mesh Gas Chrom Q (Phase separation LTD). The column temperature used was 140^0C. Nitrogen at flow rate of 40 cm min^{-1} was used as a carrier gas. The identification of individual ester was determined by comparison of their retention times of their standard. Those standards were loaded onto the column as mixture of straight, mono-unsaturated, anteiso-branched and iso-branched methyl esters of fatty acids.

Results

Acid methanolysis method from extracted fatty acids on 100 mg freeze dried samples of strain gave sufficient material for analysis. The analysis of the methyl ester fatty acids produced using the Pye Unicam GVC system gave excellent resolving power. The identity of individual ester was established by comparison with the retention time with standard mixture of straight chained mono-unsaturated fatty acid ester. The running of these standards confirmed the resolving ability of the GLC system Figure 1. Assignment of carbon number for fatty acids which standards were not available (e.g. branched chained) was done using Miwa's., *et al.* [29] equivalent chain length technique. The equivalent chain length of these unknown gave part values (e.g. 14.8) and probably represent branched chain fatty acids (Table 1). In the case of those fatty acid of equivalent chain length 14.8 these probably 15 carbon atom branched fatty acid. The column used here did not allow any distinction between iso- and antiso-branched fatty acids. Surprisingly only five group of fatty acids were considered. Saturated straight chain (14.0) (16.0) and (18.0), group two branched chained (b-15), group three branched (b16), group four branched (17) and group five unsaturated 18.1.

Equivalent chain length (Miwa., *et al.* 1960)			**14.0**	**14.8**	**15.8**	**16.0**	**16.8**	**18.0**	**18.1**
Probable identity			14.0						
Lab no	Strain Designation	Source							
1	*Brevi acterium imperial*	NC189888	=	27.1	27.7	8.7	36.3		
2	*Corynebacterium insidiosum*	NCPPB2414	-	53.5	18.0	2.9	25.5	0.4	
3	*Corynebacterium sepedonicum*	NCPP378	-	28.7	15.6	2.3	53.4		
4	*Corynebacterium mediolanum*	NCI87206	-	36.6	55.9	1.4	6.1		
5	*Corynebacterium imichiganes*	NCPPB1573		44.1	24.3	5.2	26.4		
6	*Corynebacterium nebraskenes*	NCPPB2578	-	36.0	8.6	1.4	6.1		
7	*Microbacerium flavum*	NCI88707	0.3	-	-	38.2			61.5
8	*Microbacterium lacticun*	NCI88540	-	40.3	17.6	2.0	40.0	0.2	
9	*Microbacterium liquefaciens*	NCIB11511	0.1	36.7	15.5	3.0	44.8		
10	Un indentified isolate		1.2	44.8	32.1	2.4	19.4		
12	Un indentified isolate		2.0	34.5	34.8	7.8	19.3		1.6
13	Un indentified isolate		0.3	32.2	27.8	2.4	37.3		
14	Un indentified isolate		0.3	36.9	22.6	1.5	38.0	0.2	
15	Un indentified isolate		0.6	37.0	23.7	1.9	36.8		
16	Un indentified isolate		0.7	12.9	30.5	2.4	33.5		
17	Un indentified isolate		-	39.3	4.5	-	56.2		
18	Un indentified isolate			4.4	87.3	8.3			
19	Un indentified isolate		0.8			27.7			71.5
20	Un indentified isolate		0.3			38.5			61.2

Table 1: *Major fatty acid composition of strains.*

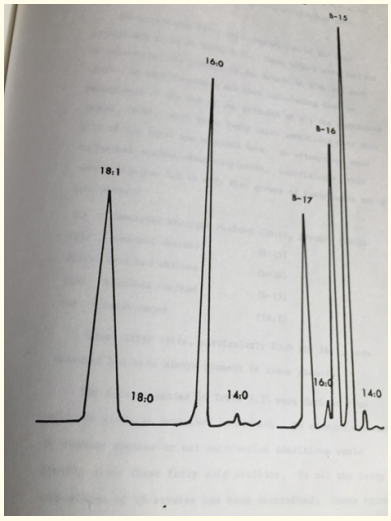

Figure 1: *Trace of GLC elution of standard mixture of fatty acid.*

The data presented in Table1 were derived from cultures grown as described in the method where fatty acids composition of strains has been determined. These strains were divided into two major groups, theses containing a high proportion of straight-chain and unsaturated fatty acid and those composed mainly of branched fatty acids. The former group includes *Microbacterium flavum* and strain number 19 and 20 while the remaining strains contain mainly branched chain fatty acids. These results is consistent with other study [30]. In Collins., *et al.* [30] study they were however, able to resolve iso- and anteiso branched chain fatty acid which is characteristic feature of coryneform fatty acid profile. The major classes of lipids are shown in Table 2. In the qualitative data study presented by Mininikin., *et al*. [28] it was reported that the principal fatty acid of *Corynebacterium insidiosum* were C15 and C16 branched fatty acids together with straight chained C16 and C18, data consistent with that reported here. Likewise, there is general agreement between Minnikin., *et al.* [28] data and data reported here for *Corynebacterium michiganense* and *Corynebacterium sepedonicum.*

Straight chain	$CH_3(CH_2)COOH$ (	steric acid
Unsaturated	$CH_3(Ch_2)_n$ CH-CH$(Ch_2)_m$ COOH	Oleic acid
Iso-acid	CH3-^{CH3}CH$(CH2)_n$COOH	isoplamitic n = 12
Cyclpropane fatty acid	$CH_3(CH_2)_n$ CH----------$_{CH2}$CH-CH-$(CH2)_n$ COOH	

Table 2: *The Major classes of lipids in coryneform taxonomy.*

n=m=7

All strains of *Microbaticum lacticum* used in this study had a similar fatty acid profile containing predominantly C15, C16, and C17 branched chain fatty acid. The three groups of fatty acid being present in the range C15 (33.3-43.0%), C16 (22,8-21.5% and C17(38.5 - 46.6%). Despite some variation in the amount of each observed in different strains, in all cases these branched chain fatty acids made up at the least of 94.9% of the total fatty acid content of strain s of this species. Similar pattern was observed with the strain of *Microbacterium liquefaciens* where branched chain fatty acid make up more than 89% of the total fatty acids. In contrast, the fatty acids of *Microbacterium flavum* were mainly of straight chained and unsaturated fatty acid. No branched chain fatty acids were detected in this strain. All the unidentified isolate used in this study except strain number 19 and 20 showed a preponderance of branched fatty acids. In these strains C15, C16, C17 branched chained fatty acids predominated except with strain number 18 which C17 branched chain fatty acid were not detected. No branched chain fatty acids were detected in strains number 19 and 20. The fatty acids of these two strains is rather similar to that observed with *Microbacterium flavum*.

Discussion

The principle aim of this study was to compare pattern of fatty acid of bacteria isolated from milk and milk product to that of the type species of the genus *Microbacterium* so that the taxonomic position of theses isolates could be improved and more information can be obtained about these organisms that cause dairy product spoilage. Both qualitative and quantitative analysis of fatty acid composition has proven to be useful in assisting to establish the taxonomic relatedness of *Microbacterium* which have been supported by other study done on coryneform bacteria and hence of the genus *Microbacterium* [28] since the taxonomic position of the genus *Microbacterium* was in debate [27]. In this study, the fatty acid composition of eleven known and nine unknown isolates have been determined. The analysis used was sufficient to separate fatty acids of different chain length, degree of unsaturated and ability to distinguish between branched and unbanked fatty acid. The method of analysis was sufficiently separated fatty acid of different chain length and degree of unsaturated and to distinguish between branched and un-branched fatty acids. The results reported in Table 1 have been restricted to components which constitute more than 0.1% of the total acids. The identification of such minor components is difficult and probably adds little to this work. Perhaps the most thorough complication of the fatty acid composition Minikin., *et al*. [28] in which the major fatty acid was reported in his study where he indicated that fatty acid composition cannot be used as confirmation of similarity of organisms but can be used to confirm difference between organisms.

In this study, the major fatty acids in all strain of *Microbacterium lacticum* examined were branched C15 and C17 fatty acid which makes up at least 78% of total fatty acid. The unnamed strain however, shows slightly more C14 straight chained saturated fatty acid than did strain of *M. lacticum* which indicates some resemblance. The results obtained in this study provide evidence that *M. lacticum* is sufficiently distinct from the other named strain used. The chemical evidence here is consistent with this conclusion. Nevertheless, there are a number of issues which require additional study. However, more study still require which might include Polar lipids, isoprenoid quinones and mycolic acids.

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