An Approach to the Taxonomy of Gram-positive Anaerobic Cocci
1. Isolation and Identification

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Abstract:
The aim of this study was to collect and identify fresh isolates of Gram-positive anaerobic cocci (GPAC) from clinical materials as a basis for the study of taxonomy of GPAC.
A total of 116 strains of GPAC, isolated from clinical materials collected from the Department of Microbiology, in St. Lukes Hospital, Guilford and St. Thomas Hospital, London, were investigated. The reference strains were obtained from the American Type Culture Collection (ATCC), Virginia Polytechnic Institute and State University (VPI) and the National Collection of Industrial Bacteria (NCIB). GPAC were identified on the basis of morphology, biochemical examination and the detection of the end products from the fermentation in peptone yeast extract glucose or chopped meat glucose media by means of Gas Liquid Chromatography (GLC). All of the strains studied were obligately anaerobic Gram-positive cocci (GPAC). They occurred in singles, pairs, tetrads, short or long chains. Peptone yeast extract without addition of carbohydrate supported their growth. The strains grew at 37°C. The G.L.C. profiles showed that all strains produced acetic acid and lactic acid.
One hundred and three (89%) out of 116 isolates were considered to be different from each other at the species level. These were *Peptostreptococcus (Pst.) anaerobius* (26.7%), *Peptococcus (Pc.) magnus* (19%), *Streptococcus (Str.) asaccharolyticus* (6.9%), *Peptostreptococcus (Pst.) micros* (3.5%), *Peptococcus (Pc.) asaccharolyticus* (2.6%), *Gaffkya (G.) anaerobia* (1.7%), and *Megosphaera (M.) elsdenni* (0.9%). Thirteen (11.1%) out of 116 strains could not be speciated.

Introduction:
The first report of micro-organisms arranged in packets was in 1842.[1] However, the first isolation of the gram-positive anaerobic cocci (GPAC) was, in 1893, from a case of suppurative bartholinitis.[2] GPAC are a heterogeneous group of organisms defined by their morphological appearance and their inability to grow in the presence of oxygen. GPAC are part of the normal flora of the mouth, upper respiratory and gastrointensinal tracts, female genitourinary system and skin.[3-7]
GPAC are commonly present in human clinical specimens; data from four surveys of anaerobic infections are consistent that they account for about 25 to 30% of all anaerobic isolates. They are not involved in any single specific disease process; rather they may be present in a great variety of infections involving all areas of the human body. These infections may range in severity from mild skin abscesses, which disappear spontaneously after incision and drainage, to more serious and life-threatening infections such as brain abscess, bacteremia, necrotizing pneumonia, and septic abortion. Infection by GPAC usually involves invasion of devitalized tissue by organisms that are part of the normal flora of the affected tissue or of the surrounding area.[8-11]
GPAC have been poorly studied for several reasons; which include an inadequate classification, difficulties with laboratory identification, and the mixed nature of the infections from which they are usually isolated. GPAC have been fair game for amateur taxonomists. The classification has always been very unsatisfactory; but a few chemical tests can be helpful. Hare and co-workers put forward the original scheme to classify the GPAC into nine groups according to fermentation of carbohydrates (glucose, fructose, maltose and sucrose) and organic acids.
All strains of the nine groups are Gram-positive (except group V which is Gram-negative) cocci arranging either in clusters or in chains, with the exception of group VI and VII showing no particular arrangement, under the microscope.

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On the basis of fermentation of carbohydrates and organic acids group I is characterized by its ability to ferment glucose, fructose and maltose. Group III and VI ferment all carbohydrates tested, the former group differs from the latter in the fermentation of pyruvate. Group IV is readily separated from other groups, since fructose is the only sugar utilized. Both group II and IX do not ferment carbohydrates, but the former is different from the latter in the fermentation of pyruvate and citrate (group II produces gas from pyruvate and citrate). The remaining groups VII and VIII are separated from the others on the basis of the fermentation of glucose and fructose only, and the only difference between these two groups is the production of gas from carbohydrates (glucose and fructose) and pyruvate by group VIII. [12-15] (Table 1).

Table 1: Fermentation characteristics of anaerobic Gram-positive cocci.

Group	Morphology	Fermentation of carbohydrate				Fermentation of organic acid		
		Glucose	Fructose	Maltose	Sucrose	Pyruvate	Citrate	Lactate
(Str. Putridus)	Gram-positive chains, cooci small	AG	AG	AG	-	G	-	-
II	Gram-positive, clusters	-	-	-	-	G	G	-
III	Gram-positive, clusters, large cocci	AG	AG	AG	AG	G	-	-
IV	Gram-positive, clusters	-	A	-	-	G+	-	-
V	Gram-positive, clusters	-	-	-	-	G	-	-
(Veillonellase?)								
VI	Gram-positive chains and clusters	A	A	A	A	-	-	-
VII	Gram-positive chains and clusters	A	A	-	-	-	-	-
VIII	Gram-positive clusters	AG	AG	-	-	-	-	-
IX	Gram-positive clusters	-	-	-	-	-	-	-

A = acid; G = Gas; + = moderate amount.

However, this conventional carbohydrate Metabolism has proven unsuccessful in distinguishing between the various anaerobic cocci.[15]

The aim of this study was to collect and identify fresh isolates of GPAC from clinical materials as a basis for the study of taxonomy of GPAC.

Materials and Methods:

The sources of cultures were a total of 116 strains of GPAC isolated from clinical materials in St. Luke's Hospital, Guilford and St. Thomas's Hospital, London. All specimens were routinely examined for anaerobic bacteria. The reference strains, 24, were obtained from the American Type Culture Collection (ATCC), Virginia Polytechnic Institute and State University (VPI) and the National Collection of Industrial Bacteria (NCIB).

The specimens were streaked onto freshly prepared blood agar plates using a swab or a sterile platinum loop. The remainder of the specimen was incubated in chopped meat medium. The inoculated blood agar plates were placed in anaerobic jars. An inoculated blood agar with a culture of *Clostridium (Cl.) tetani* was incubated in the anaerobic jar to act as a control for adequate anaerobiosis.[15] A modified sachet containing platinum catalyst was reactivated each time the jar was used by heating to 160° for 90 minutes.

The anaerobic jars were prepared by the use of the evacuation-replacement method.[16,17] In this method the anaerobic jar was filled with freshened catalyst. A tube of freshly prepared methylene blue indicator was placed in the loaded jar. The jar was sealed and evacuated to an internal pressure of about -440 mmHg by water pump. After three series of partial evacuation the anaerobic jar was filled with a gas mixture containing 90% hydrogen and 10% carbon dioxide and left for 25 minutes. Since the catalysis creates a reduced pressure within the jar due to decrease in the amount of gases, the valve to the mixed gas supply was reopened and the jar was filled with mixed gas

and incubated. The colourless appearances of the methylene blue in the indicator capsule and the presence of swarming growth in the *Cl. tetani* plate, were an indication of good anaerobiosis in the jar.

After 6 days' incubation, the various types of colonies were examined and marked. On the basis of primary screening using the Gram stain, the colonies of GPAC were picked, inoculated into chopped meat media (C.M.) and incubated for 24 hours. The cultures of GPAC were transferred to the microbiological laboratory in Surrey University for further study.

All strains were plated in parallel on three Brain Heart Infusion Agar (BHIA); one of which was incubated aerobically, the second in candle jar, and the third in an anaerobic atmosphere.

All plates in different atmospheres were incubated at 37°C for 6 days. After this period of incubation, the strains were accepted as GPAC if they had grown only anerobically. One pure colony was picked and used for subsequent inoculation of C.M. medium and for Gram stain. The inoculated C.M. medium was incubated in an anaerobic cabinet overnight, stoppered firmly, and the culture was preserved at 4°C until needed.

The identification of the isolates was based on the method described by Holdeman et al, in the Virginia Polytechnic Institute Laboratory Manual of Anaerobes.[18] Accordingly, GPAC were identified on the basis of morphology, biochemical examination and the detection of the end products from the fermentation in peptone yeast extract glucose or chopped meat glucose media by means of Gas Liquid Chromotography (GLC).

Morphology:

The study of morphology of GPAC was achieved by Gram stain reaction of 24 hour C.M. media cultures.

The arrangement of cells was recorded as follows: singles, pairs, tetrads, packets, clusters and chains. Cell size was not measured as this characteristic is variable.

Carbohydrate fermentation:

Carbohydrate fermentation tests included arabinose, cellobiose, fructose, glucose, lactose, maltose, mannose, sorbitol, sucrose, xylose and aesculin and starch hydrolysis. The basal medium was peptone yeast extract containing 0.5 g for both peptone and tripticase; 1.0 g yeast extract; and 0.4 ml salt solution per 100 ml of distilled water. The basal medium was supplemented (per 100 ml) with 0.5 μg vitamin K_1; 0.5 mg of haemin; 0.05 g of cysteine hydrochloride as reducing agent, 0.0001 g of resazurin as an indicator of anaerobiosis; and 0.02 ml of Tween-80. All sugars were added to the basal medium in a final concentration of 1% except for aesculin 0.5%.

Test for catalase production:

0.5 ml of 24 hour chopped meat culture was exposed to air at least 30 minutes in a small tube. 0.5 ml of 3% H_2O_2 was added. If continuous bubbles were observed the reaction was positive for catalase production.

Test for indole production:

Two ml of C.M. medium were aseptically pipetted. One ml of xylene was added. The contents were shaken well in a small test tube and left for two minutes. 0.5 ml of Ehrlich's reagent was slowly added down side of the tube. Pink or fuchsia ring within 15 minutes was positive, yellow ring was negative.

Test for aesculin hydrolysis:

The ability of isolates to hydrolyse aesculin to aesculetin and glucose was tested as follows: two or three drops of 1% ferric ammonium citrate solution were added to culture in aesculin medium, which was incubated for 24 hr. and to non-incubated aesculin medium which served as control test. The immediate brownish-black colour reaction was read as positive, and no colour change as negative.

Test for nitrate reduction:

Nitrate reagents A and B were prepared as recommended by Suffer et al.[19] The ability of the test strain to reduce nitrate to nitrite was estimated by growing the organism in indole-nitrate medium supplemented with haemin and vitamin K_1.

Test for starch hydrolysis:

Two to three drops of Gram's iodine were added to the culture in starch medium. The colour change was observed immediately. No colour change was taken as positive for starch hydrolysis, and blue-blackish colour as negative.

Preparation of the media for biochemical tests:

The pre-reduction of the media was carried out inside the anaerobic cabinet. The weighed solids were dissolved by magnetic stirring hot

plate. The medium containing resazurin was boiled until the colour of the indicator changed from pink to coulourless. The bottle was stoppered and left to cool. The pH was adjusted to 7.0-7.1 using 8 N NaoH and 5 N HCl and placed in the anaerobic cabinet until the gases were exchanged i,e. the resazurin indicator became colourless. Vit K1, haemin solution and cysteine were added and the medium was mixed by inversion and dispensed in 3 ml lots with a Cornwall syringe in Bijou bottles (5 ml). The media were autoclaved at 15 lb/in^2 for 15 minutes and cooled at room temperature.

Storage of the prepared media:
The prepared media were stored in an anaerobic jar and kept at 4°C in the cold room until needed. Any bottles that became pink before use were discarded.

Inoculation and incubation:
All media were inoculated in an anaerobic cabinet, with 5 drops from pure-24 h-culture in chopped meat media, and incubated in anaerobic atmosphere containing 85% nitrogen, 10% carbon dioxide and 5% hydrogen in an anaerobic cabinet, at 37°C for 3 days.
The fermentation of carbohydrates was determined by measuring the acidity directly in the incubated culture by pH meter and long thin combination electrode (ORION combination pH catalogue No. 91.15). The reading was compared with that of non-inoculated but incubated medium. The results were recorded as follows: below pH 5.5 was acid, 5.5 to 6.0 weak acid, and above 6.0 was negative production (with exception of xylose, acid = below 5.7).

Gas-liquid chromatography:
A Perkin-Elmer gas chromatograph, fitted with a flame ionization indicator was used throughout this study. Pyrex glass column of 6ft. length with an internal diameter of 3 mm was packed with 5% free fatty acid phase, acid washed, of 80-100 mesh. The gas chromatography was operated isothermally at 140°C with hydrogen as a carrier gas, the flow rate was 40 ml per min.

Extraction of volatile fatty acids and alcohol:
One ml of incubated peptone yeast extract glucose medium (PYG) was acidified with 0.2 ml of 50% aqueous sulphuric acid in a screw capped bottle. 0.4 g of NaCl and 1 ml of diethyl ether were added. The vial was closed and the contents were mixed with gentle inversion and centrifuged for 5 min. at 4000 rpm. 10 micro liters of the ether layer at the top was injected into the column using Hamilton micro-syringe.

Extraction of non-volatile fatty acids:
One ml of culture in PYG medium was acidified with 0.4 ml of 50% aqueous sulphuric acid in a reacti-vial (5 ml). Two ml of methanol were added and the vial was stoppered and heated in a dry-block heater, at 60°C for 30 min. One ml of water and 0.5 ml of chloroform were added and the vial was stoppered and the contents were mixed with gentle inversion. Ten micro-liters from the chloroform layer at the bottom were injected onto the column. Peaks on the chromatogram were compared with those of standard solution and identified. Standard solutions of volatile and non-volatile fatty acids and alcohols were obtained from Clinical Analysis Products Co. (Capaco).

Results:
The results of the biochemical properties of the 116 fresh isolates and the reference strains of GPAC are summarized in Table 2. The number of isolates are indicated as the fraction positive of the total identified. The weak acid production from carbohydrates was considered as a positive reaction. Thus the isolates were identified on the basis of biochemical reactions and the analysis of the end-products from the fermentation PYG medium by means of G.L.C. as recommended by Holdeman et al (1977).[18]
All of the strains studied shared certain features in common. They were all obligately anaerobic Gram positive cocci. They occurred in singles, pairs, tetrads, short or long chains. Usually the chains were not easily observed. Peptone yeast extract without addition of carbohydrate supported their growth. The strains grew at 37°C, and all biochemical tests were carried out at this temperature. The GLC profiles showed that all strains produced acetic acid and lactic acid.
One hundred and three (89%) out of 116 isolates were considered to be different from each other at the species level (Table 3). These were *Pst. anaerobius* (26.7%), *Pc. magnus* (19%), *Str. asaccharolyticus* (6.9%), *Pst. micros* (3.5%), *Pc. saccharolyticus* (2.6%), *G. anaerobia* (1.7%), and *M. elsdenni* (0.9%).

Thirteen (11.1%) out of 116 strains could not be speciated.

Discussion:

Since most obligate anaerobic Gram-positive cocci are weakly saccharolytic or asacchharolytic, at the present the specific identification of these organisms requires a knowledge of their general morphology by Gram reaction, biochemical tests and the analysis of metabolic products by G.L.C. For these reasons the methods of Virginia Polytechnic Institute (VPI) described by Holdeman et al (1977)[18] were utilized.

The present results showed that of 116 cultures isolated from various clinical specimens, primarily abscesses and infected wounds, 53 (45.7%) were peptococcus species and 46 (39.7%) were peptostreptococcus species. Eleven per cent (13 strains) of the total isolates could not be identified to the species level, because of the difficulty of retaining viable cultures for retesting.

The most frequent isolates of GPAC were the species of *Pst. anaerobius* and *Pc. magnus*, followed by the species of *Str. intermedius*, *Pc. prevotii*, *Pst. productus*, and *Pst. asaccharolyticus* respectively. The species of *Pst. micros*, *Pc. saccharolyticus*, *G. anaerobia* and *M. elsdenii* represented the minority of GPAC. These findings generally agree with those of other workers.[22-25]

However, these contradict the results of Holland and co-workers[26] who reported *Pc. magnus* as the most common isolate (20%) and Porschen and Staton[27] who found *Pc. asaccharolyticus* as the most frequent isolate (29%).

Despite the prevalence of GPAC, most of peptococci and peptostreptcocci are difficult to identify to genus species levels because:

(1) The identification of GPAC is technically difficult. Schemes of identification on the basis of VPI manual include such technical advances as anaerobic cabinets, pre-reduced anaerobically sterilized (PRAS) media, and gas-liquid chromatography.

Many microbiological laboratories have been reluctant to utilize these methods because of the time consumption, the cost and complexity of the equipment.[2]

Because of the unreliability of Gram-stain reaction and the absence of chain formation of certain strains of peptostreptococcus, it is impossible to differentiate them morphologically from peptococcus.[28-29] Therefore, there is doubt about the identify of the species of *Pc. magnus*, which is one of the most common isolates in this study. Strains of *Pst. micros* probably merge with those of *Pc. magnus* due to the chemical similarity. Their separation was achieved by the recognition of the chain formation of the cultures of *Pst. micros*. The only discrimination is that the cells of *Pc. magnus* are larger than those of *Pst. micros*.[18]

However, some strains of *Pc. magnus* have small cells.[38] This is in agreement with the contention of Barnes (1980) that the most unreliable properties in the classification of anaerobes are the cell size and shape because of the extreme pleomorphism encountered among many anaerobes.[30] On the other hand, it was Sneath (1972) who advocated that for the adequate recognition of a species there should be at least three bacteriological tests which separate the majority of strains of two species.[31]

The present data indicate that species of peptococcus possessed a variable result for catalase production which strengthens the findings of Gordon and Jong (1968)[32] and Holdman et al (1977).[18] In contrast Dowell and Hawkins (1977)[17] found that peptococci produce catalase and are distinguishable from peptostreptococci. The species peptococcus (*Pc., magnus*, *Pc. prevotii*, and *Pc. asaccharolyticus*) are common isolates. All these organisms together with the species of *Pst. micros*, some species of *Pst. anaerobius* and the reference strains of *Pc. indolicus* and *Pc. variabilis* are asaccharolytic. Both the species *Pc. asaccharolyticus* and Pc. *indolicus* are characterized by their production of indole. Culture of *Pc. indolicus* differs from *Pc. asaccharolyticus* in their production of coagulase.

The separation of all these anaerobic cocci is difficult unless the metabolic by products are analyzed by G.L.C.[18] However, the metabolism of a given substrate by various organisms to identical end products does not necessarily involve identical enzymatic reaction.[33]

This is consistent with the case of *Pc. magnus* and *Pc. micros* and probably the same for the pairs of *Pc. asaccharolyticus* and *Pc. aerogenes* and *Pc. magnus* and *Pc. variabilis* (*Pc. anaerobius*). Therefore, classification based on putting together organisms with similar end products should be revised according to the nature of these products.

Here, the isolates of *Str. intermedius* were strict anaerobic, and separated from GPAC on the basis of the production of lactic acid as the

major product from glucose (if so, homofermentation – streptococcus; if not, heterofermentation – peptococcus or peptostreptococcus).[18]

The taxonomic status of the species of streptococcus (*Str. constellatus*, *Str. intermedius*, *Str. morbillorum*) that are aerotolerant to strictly anaerobic is debatable. The species of *Str. intermedius* was first classified as a member of the genus streptococcus and transferred to the genus peptococcus.[34-35]

All of these organisms except *Str. constellatus* are excluded from the new family peptococcaceae, since the main product was the lactic acid. They are also excluded from the new family streptococcaceae because they are not facultative anaerobes.[36-37]

However, Rogosa (1974)[37] suggested that because their major fermentation product is dextrorotatory lactic acid, they should be included in the genus streptococcus, a view also held by Holdman et al (1977).[18] Therefore, although these organisms are excluded from the 8th edition of Bergey's manual of determinative bacteriology, they are included in this study as streptococci because these organisms appear in the current literature as members of the genus streptococcus, and they are frequently isolated from clinical material together with GPAC. Thus taxonomic problems in the GPAC exist because (a) many different classification schemes have been developed, (b) schemes without using G.L.C. do not agree well with the VPI system such as that proposed by Dowell and Hawkin (1974),[17] and (c) many synonyms are still in use. Such problems will be discussed in detail in the section on the utilization of amino acids by GPAC.

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Table 2: Biochemical characteristics of the fresh isolates and the reference strains of AGPC

Characteristic	*G. anaerobic*		*M. elsdenii*		*Pc. asaccherolyticus*						*Pc. magnus*			
	2 strains		1 strain ATCC 25940		8 strains	ATCC 29743	ATCC 14963	*Pc. aerogenes* NCIB 10074	*Pc. glycinophilus* ATCC 23195	*Pc. indolicus* ATCC 29427	22 strains	ATCC 29427	*Pc. anaerobius* ATCC 14955	*Pc. anaerobius* ATCC 14956
Acid from PY + the following carbohydrate:														
Arabinose	0/2	-	1/1	-	2/8	-	-	-	-	-	7/22	-	-	-
Cellobiose	0/2	-	0/1	-	0/8	-	-	-	-	-	0/22	-	-	-
Fructose	2/2	+	1/1	+	0/8	-	-	-	-	-	0/22	-	-	-
Glucose	2/2	+	1/1	+	0/8	-	-	-	-	-	0/22	-	-	-
Lactose	0/2	-	0.1	-	0/8	-	-	-	-	-	0/22	-	-	-
Maltose	2/2	+	0/1	-	0/8	-	-	-	-	-	0/22	-	-	-
Mannose	2/2	+	1/1	-	0/8	-	-	-	-	-	0/22	-	-	-
Sorbitol	1/2	-	0/1	-	0/8	-	-	-	-	-	0/22	-	-	-
Sucrose	2/2	+	0/1	-	0/8	-	-	-	-	-	0/22	-	-	-
Xylose	0/2	-	0/1	-	1/8	-	-	-	-	-	0/22	-	-	-
Aesculin hydrolysis	0/2	+	0/1	-	0/8	-	-	-	-	-	0/22	-	-	-
Strach hydrolysis	0/2	-	0/1	-	0/8	-	-	-	-	-	0/22	-	-	-
Indole production	0/2	-	0/1	-	8/8	+	+	+	-	+	0/22	-	-	-
Nitrate reduction	0/2	-	0/1	-	0/8	-	-	-	-	-	0/22	-	-	-
Catalase production	0/2	-	0/1	-	0/8	-	-	-	-	-	13/22	-	-	-
Coagulase production	0/2	-	0/1	-	0/8	-	-	-	-	+	0/22	-	-	-
Detection of the following end-products from PYG:														
Ethanol	0/2	-	0/1	-	0/8	-	-	-	-	-	0/22	-	-	-
Acetic acid	2/2	+	0/1	+	7/8	+	+	+	+	+	22/22	+	+	+
Propionic acid	0/2	+	1/1	+	0/8	-	+	-	-	+	5/22	-	-	-
Isobutyric acid	0/2	-	1/1	+	3/8	-	-	-	-	-	0/22	-	-	-
Butyric acid	2/2	+	1/1	+	8/8	+	+	+	-	+	0/22	-	-	-
Isovaleric acid	0/2	-	1/1	+	0/8	-	-	-	-	-	0/22	-	-	-
Valeric acid	0/2	-	1/1	+	0/8	-	-	-	-	-	0/22	-	-	-
Isocaproic acid	0/2	-	1/1	-	1/8	-	-	-	-	-	0/22	-	-	-
Caproic acid	0/2	-	1/1	+	0/8	-	-	-	-	-	0/22	-	-	-
Pyruvic acid	0/2	-	0/1	-	0/8	+	-	-	-	-	0/22	-	-	-
Lactic acid [b]	0/2	-	0/1	-	0/8	-	-	-	-	-	0/22	-	-	-
Succinic acid	1/2	+	1/1	+	5/8	+	+	+	-	+	22/22	-	-	-

Continued (Table 2: Biochemical characteristics of the fresh isolates and the reference strains of AGPC)

Characteristic		*Pc. prevotii*		*Pc. saccharolyticus*		*Pst. anaerobius*		*Pst. micros*			*Pst. productus*	
	Pc. niger ATCC 27731	11 srtrains	ATCC 14952	3 strains	ATCC 14953	31 strains	ATCC 27337	4 strains		*Pst. parvulus* VPI 0546	9 strains	ATCC 27340
Acid from PY + the following carbohydrate:												
Arabinose	-	0/11	-	0/3	-	0/31	-	0/4	-	-	6/9	+
Cellobiose	-	0/11	-	0/3	-	0/31	-	0/4	-	+	8/9	+
Fructose	-	1/11	-	3/3	+	7/31	+	0/4	-	+	9/9	+
Glucose	-	0/11	-	3/3	+	15/31	+	0/4	-	+	9/9	+
Lactose	-	0/11	-	0/3	-	0/31	-	0/4	-	+	9/9	+
Maltose	-	0/11	-	1/3	-	4/31	-	0/4	-	+	7/9	+
Mannose	-	0/11	-	3/3	+	0/31	-	0/4	-	+	9/9	+
Sorbitol	-	0/11	-	0/3	-	0/31	-	2/4	-	-	5/9	+
Sucrose	-	0/11	-	0/3	-	0/31	-	0/4	-	-	9/9	+
Xylose	-	3/11	-	0/3	-	1/31	-	0/4	-	-	9/9	+
Aesculin hydrolysis	-	0/11	-	0/3	-	0/31	-	0/4	-	+	3/9	+
Strach hydrolysis	-	0/11	-	0/3	-	0/31	-	0/4	-	-	0/9	-
Indole production	-	0/11	-	0/3	-	0/31	-	0/4	-	-	0/9	-
Nitrate reduction	-	0/11	-	0/3	+	2/31	-	0/4	-	-	0/9	-
Catalase production	-	0/11	-	2/3	+	1/31	-	0/4	-	-	0/9	-
Coagulase production	+	0/11	-	0/3	-	0/31	-	0/4	-	-	0/9	-
Detection of the following end-products from PYG:												
Ethanol	-	0/11	-	1/3	+	12/31	+	0/4	-	-	0/9	-
Acetic acid	+	11/11	+	3/3	+	31/31	+	4/4	+	+	9/9	+
Propionic acid	+	6/11	+	0/3	-	15/31	-	0/4	-	-	4/6	+
Isobutyric acid	+	3/11	-	2/3	-	22/31	+	0/4	-	-	0/9	-
Butyric acid	+	11/11	+	0/3	-	17/31	+	0/4	-	-	0/9	-
Isovaleric acid	+	0/11	-	0/3	-	27/31	+	0/4	-	-	0/9	-
Valeric acid	-	0/11	-	0/3	-	6/31	-	0/4	-	-	0/9	-
Isocaproic acid	-	0/11	-	1/3	-	21/31	+	0/4	-	-	0/9	-
Caproic acid	+	0/11	-	0/3	-	0/31	+	0/4	-	-	0/9	-
Pyruvic acid	-	4/11	-	3/3	-	5/31	-	0/4	-	+	2/9	-
Lactic acid [b]	-	0/11	-	0/3	-	0/31	-	0/4	-	-	0/9	-
Succinic acid	+	11/11	+	1/3	+	30/31	-	4/4	+	+	5/9	+

Continued (Table 2: Biochemical characteristics of the fresh isolates and the reference strains of AGPC)

Characteristic			*St. intermedius*				Unidentified AGPC	
	S. Ventriculi VPI 0218	*Str. constellatus* ATCC 27823	12 strains	ATCC 27335	*Str. morbillorum* ATCC 27527	*Str. morbillorum* ATCC 27824	13 strains	Strains No.:
Acid from PY + the following carbohydrate:								
Arabinose	-	-	9/12	-	-	-	1/13	106
Cellobiose	-	+	12/12	+	-	-	3/13	4, 11 93
Fructose	+	+	12/12	+	-	-	8/13	4, 6, 11, 14, 38, 56, 82, 93
Glucose	+	+	12/12	+	+	+	6/13	4, 11, 38 61, 82, 93
Lactose	+	-	12/12	+	-	-	1/13	4
Maltose	+	+	12/12	+	+	-	5/13	4, 11, 38, 82, 93
Mannose	+	+	12/12	+	+	+	6/13	4, 6, 11, 14 , 38, 93
Sorbitol	-	-	0/12	-	-	-	1/13	82
Sucrose	+	+	12/12	+	+	+	2/13	11, 93
Xylose	-	-	4/12	-	-	-	0/13	
Aesculin hydrolysis	+	-	11/12	+	-	-	3/13	14, 33, 101
Strach hydrolysis	-	-	3/12	-	-	-	0/13	
Indole production	-	-	0/12	-	-	-	3/13	24, 56, 101
Nitrate reduction	+	-	0/12	-	-	-	1/13	106
Catalase production	-	-	0/12	-	-	-	1/13	61
Coagulase production	-	-	0/12	-	-	-	0/13	
Detection of the following end-products from PYG:								
Ethanol	+	-	0/12	-	-	-	4/13	11, 33, 56, 61
Acetic acid	+	+	7/12	+	+	+	12/13	4, 6, 11, 14, 24, 33, 38, 56, 61, 82, 101, 106
Propionic acid	-	-	0/12	-	-	-	4/13	11, 56, 61, 101
Isobutyric acid	-	-	0/12	-	-	-	2/13	24, 101
Butyric acid	+	-	0/12	-	-	-	5/13	4, 14, 24, 56, 101
Isovaleric acid	-	-	0/12	-	-	-	4/13	4, 11, 14, 24
Valeric acid	-	-	0/12	-	-	-	3/13	11, 14, 24
Isocaproic acid	-	-	0/12	-	-	-	5/13	4, 11, 24, 61, 106
Caproic acid	-	-	0/12	-	-	-	1/13	11
Pyruvic acid	-	-	0/12	-	+	-	4/13	4, 33, 38, 61
Lactic acid [b]	-	+	12/12	+	+	+	1/13	93
Succinic acid	+	+	10/12	+	+	+	13/13	4, 6, 11, 14, 24, 33, 38 56, 61, 82, 101, 106

a = Number of strains positive/ number of strains tested. b = Lactic acid is considered positive if it's amount had exceeded that of the standard solution (more than 1 meg/100ml).
c = Formic acid was not detected. d = All of the four strain of *Pst. micros* formed chains.

Table 3: Summary of identified isolates and designation

Species	Strain numbers	No. of strains	% of total collection
Gaffkya anaerobia	62, 81	2	1.7
Megasphaere elsdenii	15	1	0.9
Peptococcus asaccharoiyticus	9, 25, 55, 60, 89, 97, 103, 111	8	6.9
Peptococcus magnus	3, 5, 29, 31, 36, 49, 51, 52, 67, 71, 72, 79, 86, 91, 94, 100, 104, 109, 113, 115	22	19.0
Peptococcus saccharolyiticus	68, 76, 83	3	206
Peptococcus prevotii	12, 13, 16, 28, 30, 37, 43, 58, 77, 85, 98	11	9.5
Peptococcus app.	6, 14, 24, 33, 38, 56, 61, 82, 101	9	7.8
Peptostreptococcus anaerobius	1, 8, 10, 18, 19, 21, 22, 26, 27, 32, 34, 35, 40, 41, 44, 45, 47, 48, 54, 57, 63, 66, 70, 75, 80, 87, 96, 102, 107, 108, 114	31	26.7
Peptostreptococcus micros	59, 69, 84, 99	4	3.5
Peptostreptococcus productus	2, 7, 20, 42, 50, 74, 88, 92, 110	9	7.8
Peptostreptococcus spp.	10, 106	2	1.7
Streptococcus intermedius	17, 23, 39, 46, 65, 73, 78, 90, 105, 112, 116	12	10.3
Streptococcus spp.	93	1	0.9
Unidentified anaerobic gram-positive cocci	4, 11	2	1.7
Total		116	

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