UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

RECLASSIFICATION OF CLOSTRIDIUM CLUSTER XVIII AND DESCRIPTION OF THREE NOVEL TAXA WITHIN THE GRAM-POSITIVE ANAEROBIC COCCI

A THESIS

SUBMITTED TO THE GRADUATE FACULTY

In partial fulfillment of the requirements for the

Degree of

MASTER OF SCIENCE

By

LIZ SAAVEDRA PEREZ
Norman, Oklahoma
2018

RECLASSIFICATION OF CLOSTRIDIUM CLUSTER XVIII AND DESCRIPTION OF THREE NOVEL TAXA WITHIN THE GRAM-POSITIVE ANAEROBIC COCCI

A THESIS APPROVED FOR THE DEPARTMENT OF MICROBIOLOGY AND PLANT BIOLOGY

BY

____
Dr. Paul A. Lawson, Chair

____
Dr. Ralph S. Tanner

____
Dr. Scott D. Russell

©Copyright by LIZ SAAVEDRA PEREZ 2018
All Rights Reserved.

"The heart of the discerning acquires knowledge, for the ears of the wise seek it out."

Proverbs 18:15, NIV

Acknowledgments

First and foremost I want to thank my advisor Paul A. Lawson. It has been an honor to be his Masters student. He has taught me the importance of taxonomy and systematics in the everchanging face of microbiology and has helped me grow into a scientist. I appreciate all of his time, ideas, enthusiasm, and encouragement to make my Masters' experience productive and stimulating. I would also like to acknowledge and thank my committee members for their guidance during my studies at The University of Oklahoma: Dr. Scott D. Russell and Dr. Ralph S. Tanner. Each one has played a role in this thesis and in my development as a student.

Lastly, I would like to thank my family, friends and fellow students who have helped me through this journey. My parents, who have raised me with a love of science and supported me in all of my personal and academic decisions. My brother, who has always walked with me through times of frustration, changes and happiness. Nisha B. Patel, who has represented a teacher and best friend no matter the time, the situation, or the crazy idea.

This thesis could not have been done without any of your support and guidance.

Table of Contents

Acknowledgements iv

Table of Contents v

List of Tables viii

List of Figures ix

Abstract x

1. Literature Review: Firmicutes and The Human Gastrointestinal Microbiome 1

    References 5

2. Reclassification of *Clostridium cocleatum, Clostridium ramosum, Clostridium spiroforme* and *Clostridium saccharogumia* to a novel genus, *Thomasclavelia* gen. nov. 8

    Abstract 8

    Introduction 10

    Materials and Methods 13

        Strain isolation 13

        Culture conditions and cultivation 13

        Morphological, physiological and biochemical characterization 14

        16S rRNA gene sequence and phylogenetic analysis 14

        Chemotaxonomic characterization 15

    Results 17

        Description of Coprobacillaceae fam.. nov. 26

        Description of Thomasclavelia gen. nov. 27

        Description of Thomasclavelia cocleata comb. nov. 27

Description of Thomasclavelia ramosa comb. nov. 28

Description of Thomasclavelia saccharogumia comb. nov. 29

Description of Thomasclavelia spiroformis comb. nov. 29

Discussion 31

References 33

3. Isolation and characterization of novel species belonging to the genera *Parvimonas, Ruoffiella* gen. nov. and *Liuella* gen. nov. of the family *Peptoniphilacea*. 39

Abstract 39

Introduction 41

Materials and Methods 46

Strain isolation 46

Culture conditions and cultivation 46

Morphological, physiological and biochemical characterization 46

Imaging 47

16S rRNA gene sequence and phylogenetic analysis 47

Chemotaxonomic characterization 48

Results 50

Description of Liuella gen. nov. 61

Description of Liuella johnsonii sp.. nov. 61

Description of Ruoffiella gen. nov. 62

Description of Ruoffiella abscessiensis sp. nov. 62

Description of Parvimonas asscharolyticus sp. nov. 63

Discussion 63

References 66

List of Tables

Table 2.1: Bacto™ Brain Heart Infusion medium components. Formula per liter of deionized water 16

Table 2.2: Metabolic fingerprint. BiOLOG's anaerobic identification test panel results from AN MicroPlate (GEN II for Anaerobic Bacteria) 19

Table 2.3: Fatty acid profiles of *Clostridium ramosum, Clostridium cocleatum, Clostridium spiroforme* and *Clostridium saccharogumia* 23

Table 3.1. Hardy Diagnostics Peptone Yeast Glucose (PYG) medium components. Formula per liter of deionized water 49

Table 3.2. Biochemical characteristics of novel GPAC species 53

Table 3.3. Fatty acid profiles of novel GPAC species 57

Table 3.4. Chemotaxonomic characteristics of novel GPAC species 58

List of Figures

Figure 2.1: Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences indicating the position of *Thomasclavelia cocleatum, Thomasclavelia ramosum, Thomasclavelia spiroforme and Thomasclavelia saccharogumia* 21

Figure 2.2: Whole-cell sugar analysis of species within clostridial rRNA cluster XVIII: *Clostridium ramosum* (CCUG 24038^T), *Clostridium cocleatum* (CCUG 46310^T), *Clostridium spiroforme* (CCUG 46510^T) and *Clostridium saccharogumia* (CCUG 51486^T) 24

Figure 2.3: Diagnostic diamino acids analysis of species within clostridial rRNA cluster XVIII: *Clostridium ramosum* (CCUG 24038^T), *Clostridium cocleatum* (CCUG 46310^T), *Clostridium spiroforme* (CCUG 46510^T) and *Clostridium saccharogumia* (CCUG 51486^T) 25

Figure 3.1: Scanning electron microscopy of novel GPAC isolates 51

Figure 3.2: Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences indicating the position of novel clinical species 55

Figure 3.3: Whole-cell sugar analysis of GPAC species. 59

Figure 3.4: Diagnostic diamino Acids analysis of GPAC species 60

Abstract

Current taxonomic classification approaches employ a polyphasic approach to characterize novel species. Most study morphological and biochemical characteristics along with chemotaxonomic characteristics to aid in the general assignment of species to the correct taxa. Numerous clinically isolated species benefit from such studies. One of them is the microbial community in the human gastrointestinal tract (GIT). Specifically, one of its dominant phyla, the phylum *Firmicutes*.

Over the years, polyphasic studies have tried to obtain characteristics to correctly classify them taxonomically and to understand their relationship in the GIT, and so far 16S rRNA gene sequencing has caused significant taxonomic changes. Two important groups, the *Clostridia* and the Gram-positive anaerobic cocci (GPAC), will be discussed though out this thesis. The current taxonomic changes each one of them is currently undergoing are addressed and further changes proposed.

Literature Review: Firmicutes and The Human Gastrointestinal Microbiome.

Abstract

The microbial community of the human gastrointestinal tract (GIT) is dominated by *Bacteroidetes* and *Firmicutes*. Specifically, the phylum *Firmicutes* accommodates species who can have beneficial relations with their host, or some commonly isolated pathogens. Over the years, studies have tried to obtain characteristics using polyphasic approaches to correctly classify them taxonomically and to understand their relationship in the GIT. Specifically, to understand their involvement in plant fiber digestion, carbohydrate digestion, gut microbiome development, immune system modulation, protection against colonization by pathogens, etc.

16S rRNA gene sequencing has caused significant taxonomic changes of the families and genera within the phyla *Firmicutes*. One of these genera is the genus *Clostridium*. Historically, molecular methods emphasized the genus diversity of taxa with a very broad range of morphological and physiological characteristics. These taxonomic changes will be discussed throughout this thesis and for the purpose of this investigation, specific members of two important groups, the *Clostridia* and the Gram-positive anaerobic cocci (GPAC), will be examined.

Firmicutes and The Human Gastrointestinal Microbiome.

The human gastrointestinal tract (GIT) is colonized by a complex community of microbes, which can have major impacts on the host health and disease processes. Representatives of the phyla *Bacteroidetes, Firmicutes, Proteobacteria* and *Actinobacteria* normally dominate this ecosystem with *Verrucomicrobia, Fusobacteria* and *Cyanobacteria* present in lower numbers. 16S rRNA analyses have revealed that more than 90% of bacterial species found within the gut are *Bacteroidetes* and *Firmicutes* [1,2,3,4,]. Functionally, members of these phyla are involved in plant fiber digestion, carbohydrate digestion, gut microbiome development, immune system modulation, and protection against colonization by pathogens [5,6]. Previous case studies reported chronic infections by some commonly isolated pathogens belonging to the *Firmicutes* phylum because of their virulence factors antibiotic resistance. For the purpose of this thesis investigation, specific members of two important groups belonging to the phyla *Firmicutes* will be examined. The first group is found within the genus *Clostridium* located in the family *Clostridiaceae*. The second group is the Gram-positive anaerobic cocci (GPAC) located in the family *Peptophiliaceae*. These families belong to the order *Clostridiales*, phylum *Firmicutes* [7].

In 1968 Murray separated the *Procatyotae* kingdom into three divisions. Each division was based on characteristic cell wall structures which were thought to make them distinct from each other. The *Gracilicutes* diviso nov. was composed of Gram-negative cell wall bacteria, the *Firmacutes* diviso nov. of all Gram-positive cell wall bacteria and the *Mollicutes* diviso nov., of organisms lacking a true cell wall. Since the initial

proposal, the divisions have been a topic of interest for many microbiologists who have challenged them over the years [8]. In Gibbons and Murray's 1978 description, the *Firmacutes* division was made up of aerobic, anaerobic or facultative anaerobic Gram-positive bacteria known to reproduce using binary fission. Their cell shape encompassed a broad range: spheres, rods and branched, or nonbranched filaments. Some produce endospores, and, if motile, they employ flagella for movement; a large portion are capable of both [9]. The broad description, the differences in mol% G+C and the inclusion of the *Mollicutes* division using an unfounded name led Garrity and Holt in 2001 to propose a novel phylum of Gram-positive bacteria with low DNA mol% G+C, the *Firmicutes*. The novel phylum replaced the older name "*Firmacutes*" and further divided it into three classes based on 16S rRNA gene sequence data: the *Clostridia, Bacilli* and *Mollicutes*, although the latter was somewhat controversial [10].

The phylogenetic relationships of the *Clostridia, Bacilli* and *Mollicutes* classes were initially based solely upon 16S rRNA gene sequence and phosphoglycerate kinase (PgK) amino acid sequences reinforced the 16S data [11,12]. Both chronometers agreed on the monophyletic origin the *Mollicutes* have with the *Firmicutes*. Although they shared a common ancestor, the *Mollicutes* were separated because they are phenotypically and morphologically different. An early edition of *Bergey's Manual of Systematic Bacteriology* placed class *Mollicutes* within phylum *Firmicutes*, whereas in the 2nd edition, they were moved to a separate phylum *Tenericutes*. The change was motivated by "their unique phenotypic properties, in particular, the lack of rigid cell walls and the general low support by alternative markers". In the more recent Bergey's publication *Taxonomic Outline of Bacteria and Archaea* (2007), *Mollicutes* were retained within the

phylum *Tenericutes* to accommodate small wall-less prokaryotes with small (usually 0.5–1.5 Mb) genomes and low (typically 25–30 mol%) G + C DNA [13,14, 15].

Although *Firmicutes* represent many different taxa, *Clostridium* and close relatives make up a significant proportion of organisms found in the GIT. [16]. Upon the application of molecular methods, and in particular 16S rRNA gene sequencing, the genus *Clostridium* was found to contain an extraordinary diversity of taxa with a very broad range of morphological and physiological characteristics and will be discussed further in Chapter 2. The Gram-positive anaerobic cocci (GPAC) have also undergone some significant taxonomic changes due to 16S rRNA sequencing and will be discussed in Chapter 3.

References

1. **Hold, G.L., Pryde, S.E., Russell, V.J., Furrie, E. and Flint, H.J**. (2002). Assessment of microbial diversity in human colonic samples by 16S rDNA sequence analysis. FEMS Microbiol. Ecol., **39**(1), pp.33-39.
2. **Wang, X., Heazlewood, S.P., Krause, D.O. and Florin, T.H.J. (2003).** Molecular characterization of the microbial species that colonize human ileal and colonic mucosa by using 16S rDNA sequence analysis. J. Appl. Microbiol., **95**(3), pp.508-520.
3. **Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T. and Mende, D.R. (2010).** A human gut microbial gene catalogue established by metagenomic sequencing. Nature, **464**(7285), p.59-65.
4. **Guinane, C.M., Tadrous, A., Fouhy, F., Ryan, C.A., Dempsey, E.M., Murphy, B., Andrews, E., Cotter, P.D., Stanton, C. and Ross, R.P. (2013).** Microbial composition of human appendices from patients following appendectomy. MBio, **4**(1), pp.e00366-12.
5. **Russell, W.R., Hoyles, L., Flint, H.J. and Dumas, M.E. (2013).** Colonic bacterial metabolites and human health. Curr. Opin. Microbiol., **16**(3), pp.246-254.
6. **Riva, A., Borgo, F., Lassandro, C., Verduci, E., Morace, G., Borghi, E. and Berry, D. (2016).** Pediatric obesity is associated with an altered gut microbiota and discordant shifts in *Firmicutes* populations. Dig. Liver Dis., **48**, pp.e268.

7. **Vos, P., Garrity, G., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.H. and Whitman, W.B. eds. (2011).** Bergey's manual of systematic bacteriology: Volume 3: The *Firmicutes* (vol. 3). Springer Science & Business Media.
8. **Edward, D. G. ff. and Freundt, E. A. (1967).** Proposal for *Mollicutes* as the name of the class established for the order *Mycoplasmatales*. Int. J. Syst. Bacteriol, **17**, pp.267-268.
9. **Gibbons, N. E., and R. G. E. Murray. (1978).** Proposals concerning the higher taxa of bacteria. Int. J. Syst. Bacteriol. **28**, pp.1-6.
10. **Garrity, G.M. and Holt, J.G. (2001).** The road map to the manual. In *Bergey's Manual® of Systematic Bacteriology* (pp. 119-166). Springer New York.
11. **Wolf, M., Müller, T., Dandekar, T. and Pollack, J.D. (2004).** Phylogeny of *Firmicutes* with special reference to *Mycoplasma* (*Mollicutes*) as inferred from phosphoglycerate kinase amino acid sequence data. Int. J. Syst. Evol. Microbiol., **54**(3), pp.871-875.
12. **Pollack, J.D., Li, Q. and Pearl, D.K. (2005).** Taxonomic utility of a phylogenetic analysis of phosphoglycerate kinase proteins of Archaea, Bacteria, and Eukaryota: insights by Bayesian analyses. Mol. Phylogenet. Evol., **35**(2), pp.420-430.
13. **Garrity, G.M., Lilburn, T.G., Cole, J.R., Harrison, S.H., Euzeby, J. and Tindall, B.J. (2007).** The taxonomic outline of bacteria and archaea. TOBA release, **7**(10.1601).
14. **Ludwig, W. and Schleifer, K.H. (2005).** Molecular phylogeny of bacteria based on comparative sequence analysis of conserved genes. *In* Sapp (editor), Microbial

phylogeny and evolution, concepts and controversies. Oxford University Press, New York, pp. 70–98.

15. **Murray, R.G.E. (1984).** The higher taxa, or, a place for everything...? *In* Krieg and Holt (editors), Bergey's Manual of Systematic Bacteriology (**vol. 1**). Williams and Wilkins, Baltimore, pp. 31–34.
16. **Cato, E. P. & Stackebrandt, E. (1989).** Taxonomy and Phylogeny. *In* Clostridia, pp. 1–26. Boston, MA: Springer US. Edited by N. P. Minton & D. J. Clarke.

Reclassification of *Clostridium cocleatum, Clostridium ramosum, Clostridium spiroforme* and *Clostridium saccharogumia* to a novel genus, *Thomasclavelia* gen. nov.

Abstract

Historically, the genus *Clostridium* was a repository for organisms that were Gram-positive staining, anaerobic and spore-formers. Consequently, the genus embraced a huge phylogenetic and physiological diversity that was more clearly revealed by the application of molecular methods. Although this diversity had been reported by several studies it, was not until Collins and co-workers (1994) that the large range of taxa was finally revealed by identifying 19 16S rRNA clusters, with cluster I representing the true *Clostridium*. However, it was not until 2016 that Lawson and Rainey formally proposed to restrict the genus to include only the type species of the genus, *Clostridium butyricum*, and its nearest relatives in rRNA cluster I as *Clostridium sensu stricto*. Consequently, species located outside this group are regarded as misclassified *Clostridium.* Efforts are underway to identify these organisms to continue the taxonomic revision of this important genus. One such group identified by comparative 16S rRNA gene sequencing, biochemical and physiological characteristics are a group of species located in clostridial rRNA cluster XVIII, *Clostridium cocleatum, Clostridium ramosum, Clostridium spiroforme* and *Clostridium saccharogumia*. These organisms are far removed from rRNA cluster I, *Clostridium sensu stricto*. In this study, all isolates were Gram-stain positive, non-motile, obligate anaerobes. The dominant long-chain fatty acids were $C_{16:0}$ (*Clostridium cocleatum,* 27.9%; *Clostridium ramosum,* 23.8%; *Clostridium*

spiroforme, 23.1%; *Clostridium saccharogumia,* 15.9%) and $C_{18:1\ w9c}$ (*Clostridium cocleatum,* 29.6%; *Clostridium ramosum,* 15.1%; *Clostridium spiroforme,* 15.4%; *Clostridium saccharogumia,* 15.4%), while glucose and ribose were the carbohydrates present within the cells and in the cell walls. The characteristic cell wall murein was *meso*-2,6-diaminopimelic acid (*meso*-DAP). Further biochemical profiles derived from BiOLOG were also consistent with closely related species. Based upon phenotypic and phylogenetic findings the creation of a novel genus, "*Thomasclavelia*", accommodating *Clostridium cocleatum, Clostridium ramosum, Clostridium spiroforme* and *Clostridium saccharogumia* as "*Thomasclavelia cocleatum* gen. nov. comb. nov.", "*Thomasclavelia ramosum* comb. nov.", "*Thomasclavelia saccharogumia* comb. nov." and "*Thomasclavelia spiroforme* comb. nov." is proposed. The type species of the genus is *Thomasclavelia cocleatum* DSM 1551^T (Kaneuchi, Miyazato, Shinto, and Mitsuoka 1979). Furthermore, the genera *Coprobacillus*, *Catenibacterium*, *Eggerthia*, *Kandleria*, *Longibaculum*, *Sharpea* and *Thomasclavelia* share a phylogenetic ancestry. Based on phylogenetic, biochemical and chemotaxonomic information the designation of *Coprobacillaceae* fam. nov., a sister family to *Erysipelotrichaceae*, was proposed.

Introduction

Prazmowski first described the genus *Clostridium* in 1880. Since then, it has become a depository for non-sulfate-reducing, spore-forming, obligate anaerobes with Gram-positive type cell walls [1]. Over time the morphological, physiological and chemotaxonomic features of the genus expanded resulting in the discovery and description of over 200 species (http://www.bacterio.net/clostridium.html, 15th of June, 2018). This range of characteristics is observed in the different phenotypes which include psychrophiles, thermophiles and acidophiles along with organisms that synthesize cytochromes and quinones and whose G+C content of chromosomal DNA ranges from approximately 21 to 54% [2]. Still, the application of molecular methods in addition to older cultivation methods, have emphasized the need for revision. 16S rRNA gene sequencing revealed a broad diversity range between species and over the past 43 years researchers have worked to identify species that should be within the *Clostridium* genus, while properly reclassifying those which should not.

The genetic relationships of *Clostridium* were first studied by Johnson and Francis in 1975. They utilized rRNA homologies to demonstrate that 56 clostridial species had low homologies between them. These homology patterns were employed to establish RNA groups within the genus [3]. Six years later, Tanner et al. (1981) and Woese (1987) continued this work with oligonucleotide cataloging in which short 5 to 7 nucleotide long fragments were used to provide some preliminary insight into the phylogenetic relationships of different species within the genus *Clostridium* [4,5]. Their work oficially questioned the validity of the taxonomic distinctions in the genus *Clostridium*.

In 1994, Collins and colleagues used 16S rRNA gene sequencing to more precisely demonstrate the phylogenetic diversity present within the genus [6]. Nineteen distinct phylogenetic clusters were defined and cluster I was reserved for the 'true' members of the genus. Additionally, Collins and colleagues proposed 5 new genera and 11 novel species combinations [7]. Gupta and Gao reinforced these relationships in 2009 by demonstrating that cluster I species contained unique protein signatures specific to this cluster [8]. Their results supported the separation of cluster I organisms as true clostridia. Subsequently, in 2016, Lawson and Rainey (2016) formally proposed the restriction of the *Clostridium* genus to include only *Clostridium butyricum* and related species within cluster I while recognizing *Clostridium senus stricto* as the true clostridial species [9,10]. In their proposal member of cluster I held 16S rRNA gene sequence similarities and conserved three unique indels within three highly conserved proteins: a 1 aa deletion in ATP synthase β subunit, a 4 aa insert in DNA gyrase A and a 1aa insert in the S2 ribosomal protein.

The genus remains in a state of taxonomic transition with many organisms still retaining the designation Clostridium and thus requiring further taxonomic restructuring. One such group in need of reclassification identified using phylogenetic studies based on the 16S rRNA gene. Four species from cluster XVIII, *Clostridium saccharogumia, Clostridium ramosum, Clostridium cocleatum* and *Clostridium spiroforme*, were investigated due to their significance in clinical microbiology. Particularly, species in cluster XVIII have specific roles in clinical microbiology, including gut communities. *Clostridium saccharogumia* impacts the gut microbiome as it aids in the digestion of lignans found in Western diets [11]. *Clostridium cocleatum* is a known mucin utilizer

when colonizing the digestive tract of mice [12]. *Clostridium ramosum* is known to enhance diet-induced obesity in mice and in 2014 was proposed to have a similar effect in a simplified human intestinal (SIHUMI) microbiota [13]. *Clostridium spiroforme* has been associated with spontaneous antibiotic-associated diarrhea and colitis [14].

In this study a polyphasic approach including phylogenetic, phenotypic and chemotaxonomic methods were applied to augment 16S rRNA gene studies. The analysis demonstrated the shared phylogenetic ancestry between the genera *Coprobacillus*, *Catenibacterium*, *Eggerthia*, *Kandleria*, *Longibaculum*, *Sharpea* and *Thomasclavelia* that warrants a new family designation for which the name *Coprobacillaceae* fam. nov. is proposed.

Materials and Methods

Strain isolation

The strains in this study were isolated in multiple locations around the world and deposited at the Culture Collection at the University of Göteborg (CCUG), Sweden. *Clostridium cocleatum* strain CCUG 46310^T was initially isolated from a mouse cecum in Japan in 2002. *Clostridium spiroforme* strain CCUG 46510^T was initially isolated from human feces in 1993 in the United States. *Clostridium saccharogumia* strain CCUG 51486^T was initially isolated from human feces in Germany in 2005. *Clostridium ramosum* strain CCUG 24038^T was deposited in the Prévot collection in 1927 without an indicated source. Strains CCUG 46310^T and CCUG 46510^T were isolated and purified in chocolate agar while strains CCUG 51486^T and CCUG 24038^T were isolated and purified in Brain Heart Infusion agar amended with 5% defibrinated sheep blood and chopped meat medium, respectively. All strains were grown under anaerobic conditions at 37°C.

Culture conditions and cultivation

Clostridium ramosum (CCUG 24038^T), *Clostridium saccharogumia* (CCUG 51486^T), *Clostridium cocleatum* (CCUG 46310^T) and *Clostridium spiroforme* (CCUG 46510^T) strains were provided as lyophilized cultures. The strains were initially grown in BD Bacto^{TM} Brain Heart Infusion (Sparks, MD, USA) broth amended with vitamin K and rumen fluid at 37°C with 10 psi anaerobic headspace (N_2:H_2:CO_2, 70:20:10). After seven days, 100 μL samples were transferred onto BD Bacto^{TM} Brain Heart Infusion (Sparks, MD, USA) agar amended with 5% defibrinated sheep's blood (pH 7.4) and incubated under the same conditions (Table 2.1).

Morphological, physiological and biochemical characterization

Colonies used for analyses were grown anaerobically for seven days at 37ºC on BD Bacto™ Brain Heart Infusion agar with 5% defibrinated sheep's blood, unless stated otherwise. Gram-stains and wet mounts were performed in accordance with previously described methods to analyze cell wall characteristics and motility (15,16). Cells were examined with an Olympus CX41 microscope using phase contrast at 1000X magnification.

Biochemical characterization was performed using BiOLOG AN MicroPlate GEN II (Hayward, CA, USA) following the manufacturer's instructions. The plates were incubated anaerobically, read after 24 hours and reviewed after 48 hours; all tests were performed in duplicate. In addition, the API Rapid ID 32A system (bioMérieux, France) was used to determine additional biochemical and enzymatic reactions following the manufacturer's instructions. All tests were performed in duplicate.

16S rRNA gene sequence and phylogenetic analysis

DNA isolation, 16S rRNA gene sequencing and phylogenetic analysis were used to examine and establish phylogenetic relationships with nearest neighbors. The complete 16S rRNA gene sequence was obtained from DNA extracted using a PowerSoil® DNA Isolation Kit (MoBio Laboratories, Inc.). Primers corresponding to positions 8-28 (universal pA forward primer) and 1522-1542 (universal pH reverse primer) of the *Escherichia coli* numbering were used to amplify the 16S rRNA gene [17].

Amplicons were purified using ExoSAP-IT (USB Corporation) and sequenced with primers specific to the conserved positions of the rRNA gene; 522-539 (pD, reverse primer), 339–358 (anti γ, forward primer), 1090-1109 (3*, forward primer) and 1510-1492 (1492R, reverse primer) [17]. 16S rRNA gene sequencing was carried out at the University of Oklahoma's Biology Core Molecular Laboratory. Approximately 1,440 bases of the 16S rRNA gene were determined for all strains. Nearest phylogenetic relatives were identified using the EzTaxon server (http://eztaxon-e.ezbiocloud.net/) [18]. The 16S rRNA gene sequences of all strains and the type strains of their nearest relatives were aligned in MEGA using Muscle according to the maximum-likelihood method and the stability of the groupings was estimated by bootstrap analysis (1000 replications) [19-21].

Chemotaxonomic characterization

Chemotaxonomic analyses were used to examine characteristics of the cell membrane components. Whole cell sugars and diagnostic diamino acids features were analyzed as previously described [22,23,38]. Fatty acid methyl esters were extracted from lyophilized cell material and analyzed by gas chromatography in accordance to the Sherlock Microbial Identification System protocol (MIDI Labs Inc., Newark, DE) version 6.1 [24,25]. Fatty acid concentrations were expressed as percentages in the QBA1 peak naming databases, and a value of 10% as the threshold for major and minor fatty acids.

Table 2.1: BD Bacto™ Brain Heart Infusion medium components. Formula per liter of deionized water.

Formulae BD Bacto™ Brain Heart Infusion		
Calf brains, Infusion from 200 g	7.7	g
Beef Heart, Infusion from 250 g	9.8	g
Proteose Peptone	10.0	g
Dextrose	2.0	g
Sodium Chloride	5.0	g
Disodium Phosphate	2.5	g
Agar	15.0	g

Final pH 7.4 +/- 0.2 at 25 ^oC.

Autoclave at 121 ^oC.

Results

Morphological, phenotypic, and biochemical characterization studies

The morphological, phenotypic, and biochemical results obtained in this study were concordant with previously established data [26].

All strains recovered from solid media required seven days of growth under strictly anaerobic conditions. *Clostridium ramosum* (CCUG 24038^{T}) and *Clostridium saccharogumia* (CCUG 51486^{T}) produced round colonies, with slight convex elevation, white pigmentation and smooth, entire margin. *Clostridium ramosum* (CCUG 24038^{T}) cells were straight rods, 0.5-0.8 x 2-10 μm in size and occurring singly, in pairs or short chains arranged as "V". *Clostridium saccharogumia* (CCUG 51486^{T}) cells were helically coiled rods, 0.8 x 2 μm in size and occurring as single cells. *Clostridium cocleatum* (CCUG 46310^{T}) colonies were round, with slight convex elevation, light yellow pigmentation, and a smooth, entire margin. Cells were semicircular rods, 0.4 x 5 μm in size and occurring singly. *Clostridium spiroforme* (CCUG 46510^{T}) produced small, round colonies with convex elevation, off-white pigmentation, and a smooth, entire margin. The cells were tightly coiled rods, 0.4-0.5 x 3.0-8.0 μm in size occurring as long chains. Under microscopic examination all cells contained a thick peptidoglycan layer and a thin cytoplasmic membrane as evident by a Gram-positive staining result. Motility was not observed.

BiOLOG AN MicroPlate II (Hayward, CA, USA) analysis at 24 hours provided evidence that all strains utilized D-fructose, but L-fucose and palatinose were used only by three of the four strains (*Clostridium ramosum*; CCUG 24038^{T}, *Clostridium cocleatum*; CCUG 46310^{T} and *Clostridium saccharogumia*; CCUG 51486^{T}) (Table 2.2).

The results correspond with previously published data for *Clostridium cocleatum, Clostridium ramosum, Clostridium spiroforme* and *Clostridium saccharogumia* with minimal differences [17]. N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, arbutin, dextrin, gentibiose, α-D-glucose, glucose-6-phosphate, lactulose, matotriose, 3-methy-D-glucose, and turanose were utilized by *Clostridium ramosum*; CCUG 24038^{T} and *Clostridium saccharogumia*; CCUG 51486^{T}. D-galacturonic acid, D-glucosaminic acid, and L-rhamnose were utilized by *Clostridium cocleatum*; CCUG 46310^{T} and *Clostridium saccharogumia*; CCUG 51486^{T}. Glucose-1-phosphate was utilized by *Clostridium cocleatum*; CCUG 46310^{T} and α-methyl-D-galactoside, β-methyl-D-galactoside, β-methyl-D-glucoside, turanose, pyruvic acid, pyruvic acid methyl ester, L-methionine, L-phenylalanine, L-valine, inosine and uridine were utilized by *Clostridium ramosum*; CCUG 24038^{T}.

API RapidID 32An test system (bioMérieux, France) analysis at 24 hours provided evidence that all strains produced acid from β-galactosidase. CCUG 51486^{T}, CCUG 24038^{T} and CCUG 46310^{T} produced acid from β-glucosidase, but only CCUG 51486^{T} and CCUG 24038^{T} produced acid from mannose. CCUG 51486^{T} and CCUG 46310^{T} produced acid from α-galactosidase. Additionally CCUG 46310^{T} produced acid from alkaline phosphatase and CCUG 24038^{T} produced acid from β-galactosidase-6-phosphate.

Table 2.2: Metabolic fingerprint as derived from BiOLOG AN MicroPlate (GEN II for anaerobic bacteria).

Test	1	**2**	**3**	**4**	**5**	6	7	8	9	10
α-galactosidase	-	+	-	+	-	-	-	+	-	+
β-galactosidase	+	+	+	+	+	-	-	+	+	+
β -galactosidase-6-phosphate	+	-	-	-	+	+	w	-	-	-
α -glucosidase	+	-	-	-	-	-	+	+	-	+
β -glucosidase	+	w	-	+	+	+	+	+	-	+
β -glucuronidase	-	-	-	-	-	-	-	-	+	-
Mannose	+	-	-	+	+	+	-	+	-	+
Raffinose	-	-	-	-	+	-	-	-	-	-
Alkaline phosphatase	-	+	-	-	-	-	-	-	-	-
Arginine arylamidase	-	-	-	-	+	-	+	+	-	+
Leucine arylamidase	-	-	-	-	-	-	w	w	-	w
Pyroglutamic acid arylamidase	-	-	-	-	+	+	+	-	+	-
Glycine arylamidase	-	-	-	-	+	-	w	+	-	w
Histindine arylamidase	-	-	-	-	+	-	-	+	+	w

Clostridium ramosum CCUG 24038^T this study; 2., *Clostridium cocleatum* CCUG 46310^T this study; 3., *Clostridium spiroforme* CCUG 46384^T this study; 4., *Clostridium saccharogumia* CCUG 51486^T this study; 5., *Catenibacterium mitsuokai* JCM 10609^T [27]; 6., *Coprobacillus cateniformis* JCM 10604^T; [28]; 7., *Eggerthia catenaformis* OT 569^T[29]; 8., *Kandleria vitulina* DSM 20405^T; [29]; *9., Longibaculum muris* DSM 29481^T[30]; 10., *Sharpea azabuensis* JCM 14210^T[31]. +, positive; -, negative; w, weak positive.

Profiles were established using cultures grown in Brain Heart Infusion Agar + 5% sheep blood at 37°C and a N_2:H_2:CO_2 gas mix for 24 hours. Results for strains within *Clostridium* cluster XVIII are emphasized in bold.

Phylogenetic studies

A phylogenetic tree constructed by the maximum-likelihood method based on 16S rRNA sequencing depicting the phylogenetic affinity of these organisms (*Clostridium saccharogumia, Clostridium ramosum, Clostridium cocleatum* and *Clostridium spiroforme)* and close relatives are shown in Figure 2.1. The results confirmed that *Clostridium saccharogumia, Clostridium ramosum, Clostridium cocleatum* and *Clostridium spiroforme* belong to the clostridial rRNA cluster XVIII as previously reported with *Coprobacillus cateneformis* being somewhat more distantly related (91.4 %) [9,10]. These values support the proposal that these species represent a novel genus based on the accepted value of 94.0% now routinely used to demarcate this rank [32].

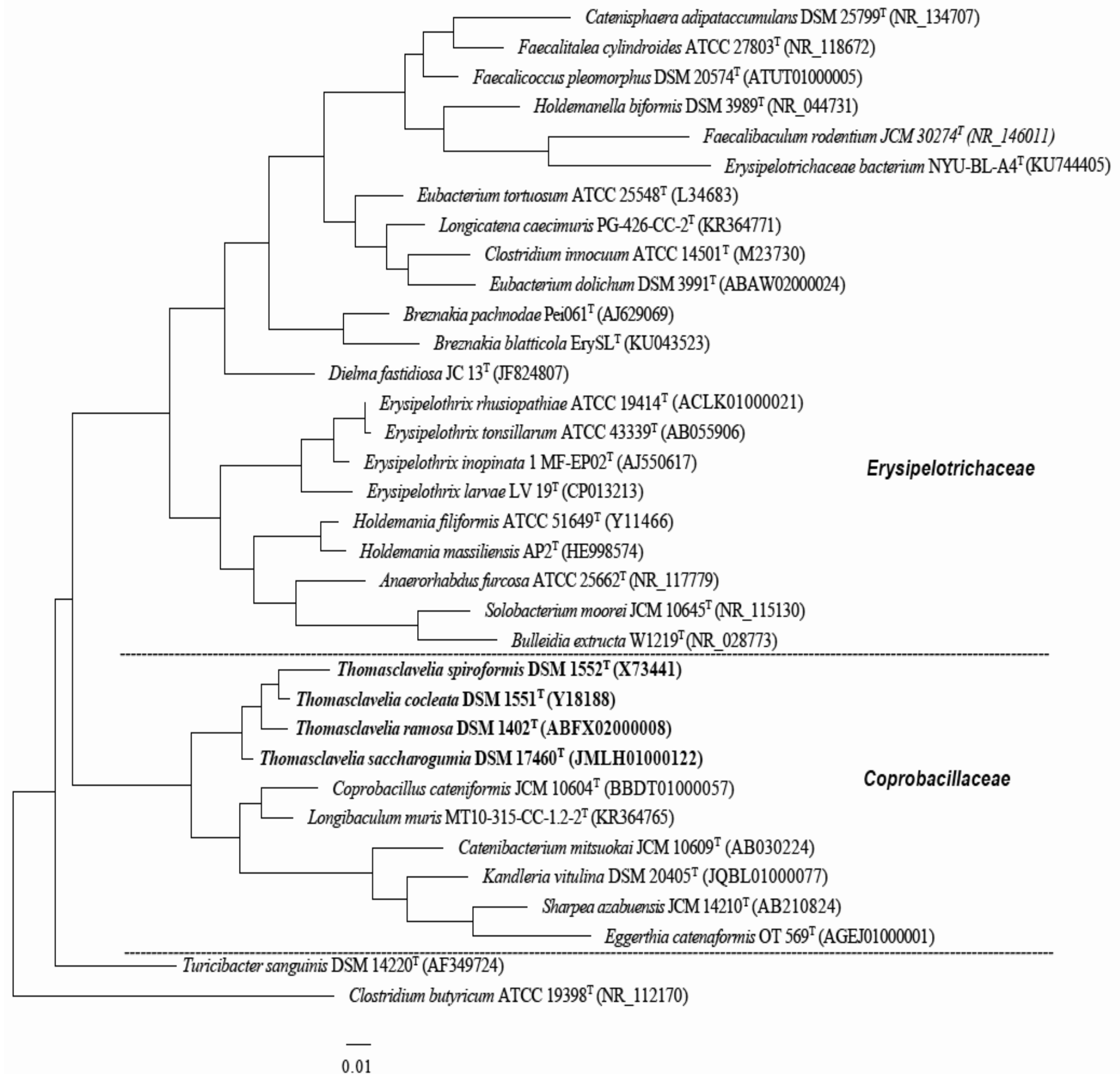

Figure 2.1: Unrooted tree showing the phylogenetic inter-relationships of members of the genus ***Thomasclavelia* gen. nov.** with its nearest relatives. Phylogenetic analyses were performed on 1300 nucleotides using the maximum-likelihood algorithm. Bar = 1% sequence divergence. Cluster XVIII species with new names are emphasized in bold.

Chemotaxonomic studies

The dominant fatty acids of all the species were $C_{16:0}$ (17.0%), and $C_{18:1\ w9c}$ (13%) (Table 2.3). Additionally, *Clostridium ramosum* (CCUG 24038^{T}) contained $C_{18:1}$ w7c (14.8%) and $C_{16:1\ w9c}$ (12.1%), *Clostridium saccharogumia* (CCUG 51486^{T}) contained $C_{16:0\ aldehyde}$ (29.2%) and $C_{18:1}$ w7c (13.1%), *Clostridium cocleatum* (CCUG 46310^{T}) contained $C_{14:0}$ (10.2) and $C_{18:0}$ (11.1%), and *Clostridium spiroforme* (CCUG 46510^{T}) contained $C_{15:0\ iso}$ (10.3%). Whole-cell sugar analysis revealed the sugars present within the cells of each isolate were glucose and ribose. In addition rhamnose was also present in *Clostridium ramosum* (CCUG 24028^{T}) and *Clostridium saccharogumia* (CCUG 51486^{T}) (Figure 2.2). *Meso*-2,6-diaminopimelic acid (*meso*-DAP) was present as the diagnostic diamino acid in the third position of the stem peptide chain along with the universal L-alanine and D-glutamic acid. Lastly the interpeptide bridge appeared to contain L- lysine (Figure 2.3).

Table 2.3: Fatty acid profiles of *Clostridium ramosum* (CCUG 24038^T)*, Clostridium cocleatum* (CCUG 46310^T)*, Clostridium spiroforme* (CCUG 46510^T) and *Clostridium saccharogumia* (CCUG 51486^T).

Fatty acid[a]	**1**	**2**	**3**	**4**
$C_{14:0}$	6.3	**10.2**	9.3	5.3
$C_{15:0}$		1.0		
$C_{15:0\ iso}$			**10.3**	
$C_{16:0\ aldehyde}$	9.1	1.1	1.7	**29.2**
$C_{16:1\ w9c}$	**12.1**			3.6
$C_{16:1\ w7c}$	6.2	3.3		3.6
$C_{16:0}$	**23.8**	**27.9**	**23.1**	**15.9**
$C_{17:0}$		1.3		
$C_{17:1\ w8c}$	1.9			6.4
$C_{18:2\ w6,9c}$	3.2	5.1	2.5	
$C_{18:1\ w9c}$	**15.1**	**29.6**	**15.4**	**15.4**
$C_{18:1\ w7c}$	**14.8**	2.8		**13.1**
$C_{18:0}$	6.1	**11.1**	6.4	3.8

Clostridium ramosum (CCUG 24038^T) this study; 1.*, Clostridium cocleatum* (CCUG 46310^T) this study; 2.*, Clostridium spiroforme* (CCUG 46510^T) this study; 3. and *Clostridium saccharogumia* (CCUG 51486^T) this study; 4.

Profiles were established using cultures grown in Brain Heart Infusion Agar + 5% sheep's blood at 37°C and a N_2, H_2, CO_2 gas mix. [a]Major fatty acids were analyzed in the QBA1 library under 10% standard percentage abundance and bolded for emphasis.

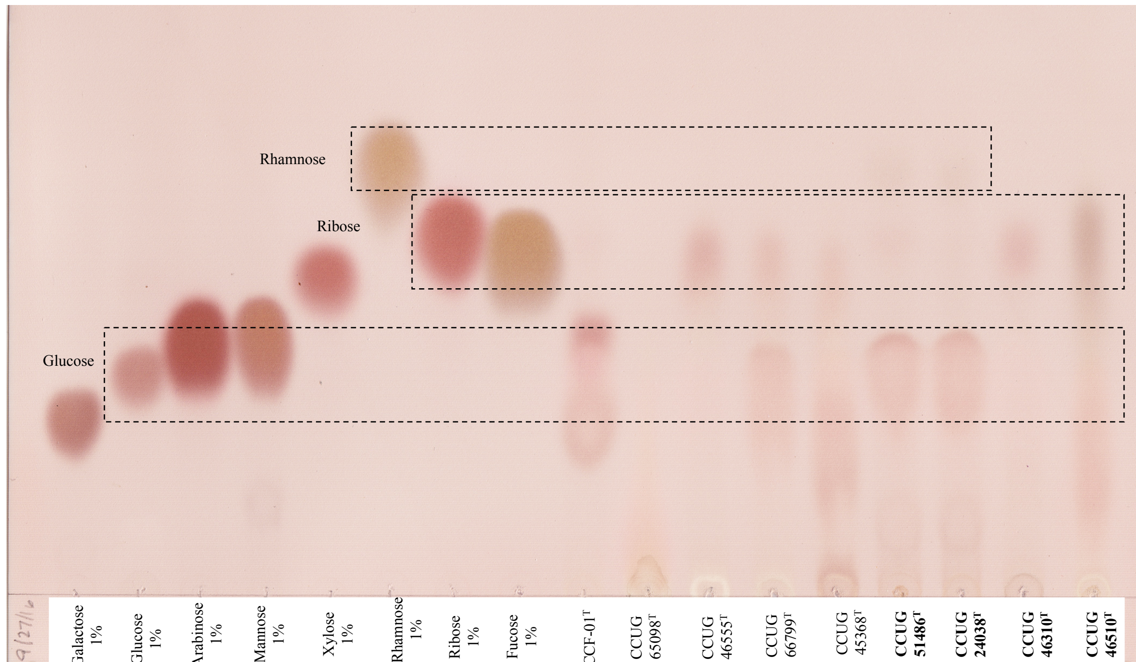

Figure 2.2: Whole-cell sugar analysis of species within clostridial rRNA cluster XVIII: *Clostridium ramosum* (CCUG 24038^T)*, Clostridium cocleatum* (CCUG 46310^T)*, Clostridium spiroforme* (CCUG 46510^T) and *Clostridium saccharogumia* (CCUG 51486^T).

Profiles were established using lyophilized cell material from cultures grown in Brain Heart Infusion Agar + 5% sheep's blood at 37°C and a N_2, H_2, CO_2 gas mix.

Lane 1 to 8: sugar standards.

Lane 9 and 13: Controls (*Lawsonella clevelandensis*, CCF-01^T)

Lanes 14 to 17: CCUG 51486^T, CCUG 24038^T, CCUG 46310^T, CCUG 46510^T

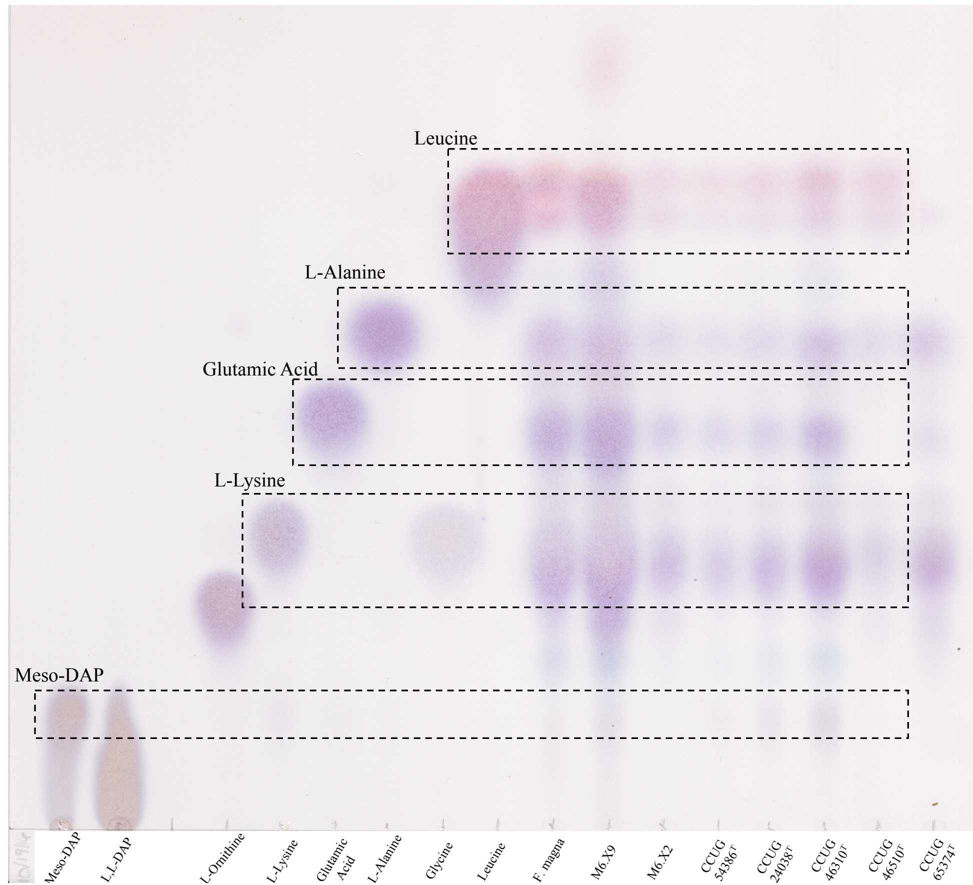

Figure 2.3: Diagnostic diamino acids analysis of species within clostridial rRNA cluster XVIII: *Clostridium ramosum* (CCUG 24038^T)*, Clostridium cocleatum* (CCUG 46310^T)*, Clostridium spiroforme* (CCUG 46510^T) and *Clostridium saccharogumia* (CCUG 51486^T).

Profiles were established using lyophilized cell material from cultures grown in Brain Heart Infusion Agar + 5% sheep's blood at 37°C and a N_2, H_2, CO_2 gas mix.

Lane 1 to 8: amino acid standards.

Lane 9 to 11, 16: Controls

Lanes 12 to 15: CCUG 51486^T, CCUG 24038^T, CCUG 46310^T, CCUG 46510^T

Based on the data, we propose the reclassification of *Clostridium* cluster XVIII species into a novel genus, *Thomasclavelia* gen. nov., and the creation of a novel family, *Coprobacillaceae* fam. nov.. Their new descriptions are as follows:

Description of *Coprobacillaceae* fam. nov.

***Coprobacillaceae* fam. nov.** (Co.pro.ba.cil.la.ce'ae. N.L. masc. n. *Coprobacillus*, type genus of the family, -*aceae* ending to denote a family, N.L. pl.
fem.n. *Coprobacillaceae* the *Coprobacillus*
family).

Type genus: *Coprobacillus* Kageyama and Benno 2000.

The description of the family is based on that given by Verbarg *et al*. [15]. The family falls within the order *Erysipelotrichales* and its delineation is primarily based on 16S rRNA gene sequence phylogeny which is supported by morphological, biochemical and chemotaxonomic characteristics. The family includes the genera *Catenibacterium*, *Coprobacillus*, *Eggerthia*, *Kandleria*, *Longibaculum, Sharpea and Thomasclavelia*. Strictly anaerobic, spores may be present. Peptidoglycan contains *meso*-DAP as the diagnostic diamino acid, but one species has been reported to contain L-lysine. The predominate fatty acid is $C_{16:0}$. Phylogenetically a sister clade of *Erysipelotrichaceae*, phylum *Firmicutes*. Isolated from human and horse feces, human infections, rumen of cattle. The DNA G + C content is between 26-39 mol%. The type genus is *Coprobacillus*.

Description of *Thomasclavelia* gen. nov.

Thomasclavelia gen. nov. (Tho.mas.cla.vel'i.a. N.L. fem. n. *Thomasclavelia*, in honor of the contemporary Germany microbiologist Thomas Clavel in recognition of his many contributions to gastrointestinal microbiology).

Cells are Gram-positive staining, non-motile and exhibit various degrees of coiling although some strains are seen as straight rods. All species are anaerobic and nonhemolytic. Spores are round and located both centrally and terminally. Cell wall murein is based on *meso*-DAP as the diagnostic diamino acid, and glucose, ribose and xylose are the major whole cell sugars. Isolated from animal and human sources. The G + C content of the DNA is 27-32 mol %. The type species of the genus is *Thomasclavelia cocleatum* and belongs to the family *Coprobacillaceae*.

Description of *Thomasclavelia cocleata* comb. nov.

Thomasclavelia cocleata (co.cle.a'ta. L. n. *coclea* a snail shell or whirlpool; referring to the cell morphology of the type strain).

Basonym: *Clostridium cocleatum* Kaneuchi et al. 1979.

The description of *Thomasclavelia cocleata* is identical to that proposed for *Clostridium cocleatum* [16] with the additional details. Using the BiOLOG AN Microplate (GEN II for anaerobic bacteria), positive reactions are given with D-fructose, L-fucose, D-galacturonic acid, D-glucosaminic acid, glucose-1-phosphate, glucose-6-phosphate, palatinose and L-rhamnose. Using the API RapidID 32An test system, acid is produced with alkaline phosphatase, α-glactosidase, β–galactosidase, and β-glucosidase (weak). The major fatty acids are $C_{14:0}$, $C_{16:0}$, $C_{16:0\ aldehyde}$, $C_{16:1\omega9c}$ and $C_{18:1\omega9c}$. Cell wall murein

is based on *meso*-DAP as the diagnostic diamino acid. The type strain is CCUG 46310^T (= ATCC 29902^T = DSM 1551^T = NCTC 11210^T) [29].

Description of *Thomasclavelia ramosa* comb. nov.

Thomasclavelia ramosa (ra.mo'sa. L. fem. adj. *ramosa* much-branched).

Basonym: *Clostridium ramosum* Veillon and Zuber 1898; Holdeman, Cato and Moore 1971.

The description of *Thomasclavelia ramosa* is identical to that proposed for *Clostridium ramosum* Holdeman, Cato and Moore 1971) with the additional details. Using the BiOLOG AN Microplate (GEN II for anaerobic bacteria), positive reactions are obtained with N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, amygdalin, arbutin, D-cellobiose, dextrin, D-fructose, L-fucose, D-galactose, gentibiose, α-D-glucose, glucose-6-phosphate, α-D-lactose, lactulose, maltose, matotriose, D-mannitol, D-melibiose, 3-methyl-D-glucose, α-methyl-D-galactoside, β-methyl-D-galactoside, β-methyl-D-glucoside, palatinose, D-raffinose, salicin, sucrose, D-trehalose, turanose, pyruvic acid, pyruvic acid methyl ester, L-methionine, L-phenylalanine, L-valine, inosine, uridine. Using the API RapidID 32An test system, acid is produced with β-galactosidase, β-glactosidase-6-phosphate, α-glucosidase, β-glucosidase and mannose. The type strain is CCUG 24038^T (= ATCC 25582^T = DSM 1551^T = NCTC 11210^T).

Description of *Thomasclavelia saccharogumia* comb. nov.

Thomasclavelia saccharogumia (sac. cha. ro. gu' mi.a. Gr. neut. n. *saccharon* sugar, L. fem. n. *gumia* eater, N. L. fem. n. saccharogumia sugar eater

Basonym: *Clostridium saccharogumia* Clavel et al. 2007.

The description of *Thomasclavelia saccharogumia* is identical to that proposed for *Clostridium saccharogumia* Clavel et al. 2007. The type strain is SDG-Mt85-3Db^T (=CCUG 51486^T = DSM 17460^T). Using the BiOLOG AN Microplate (GEN II for anaerobic bacteria), positive reactions are obtained with N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, amygdalin, arbutin, D-cellobiose, dextrin, D-fructose, L-fucose, D-galacturonic acid, gentibiose, D-glucosaminic acid, α-D-glucose, glucose-6-phosphate, α-D-lactose, lactulose, maltose, matotriose, D-mannose, 3-methy-D-glucose, β-methyl-D-glucoside, palatinose, D-raffinose, D-rhamnose, salicin, sucrose, D-trehalose, turanose, pyruvic acid methyl ester. Using the API RapidID 32An test system, acid is produced with α-galactosidase, β-galactosidase, β-glucosidase, and mannose. The type strain is SDG-Mt85-3Db^T (CCUG 51486^T = DSM 17460^T).

Description of *Thomasclavelia spiroformis* comb. nov.

Thomasclavelia spiroformis (spi.ro.for'mis. Gr. n. *spira* a coil; L. n. *forma* shape; N. L. fem. adj. spiroformis in the shape of a coil).

Basonym: *Clostridium spiroforme* Kaneuchi et al. 1979.

The description of *Thomasclavelia spiroformis* is identical to that proposed for *Clostridium spiroforme* Kaneuchi et al. 1979. Using the BiOLOG AN Microplate (GEN

II for anaerobic bacteria), positive reactions are obtained with amygdalin, D-cellobiose, D-fructose, D-mannose and salicin. Using the API RapidID 32An test system, acid is produced with β-galactosidase. The type strain is CCUG 46510^T (= ATCC 29900^T = DSM 1552^T = NCTC 11211^T) [29].

Discussion

The human intestinal tract harbors an enormous diversity of microorganisms which play a decisive role in the health and physiology of the host [33-35]. One of the genera found to be a major component of this diversity is *Clostridium*, but throughout the years the taxonomy of this large group has been debated. Presently, only species found in cluster I are recognized as true members of the genus *Clostridium*; those located outside this cluster are regarded as misclassified clostridia [1-10, 34]. With the objective of improving the taxonomic structure of the genus *Clostridium*, a polyphasic approach was employed in this study, and the results confirmed the need for reclassification of *Clostridium cocleatum, Clostridium saccharogumia, Clostridium ramosum* and *Clostridium spiroforme*.

Based on the results presented, *Clostridium cocleatum*, *Clostridium ramosum*, *Clostridium spiroforme* and *Clostridium saccharogumia*, possess consistent characteristics that strongly support their inclusion into a novel genus. 16S rRNA gene sequencing shows relatedness between the species greater than 96.0% and places them into cluster XVIII, demonstrating that they are well removed from *Clostridium sensu stricto*. This rRNA cluster is adjacent to cluster XVI containing the organism *Erysipelotrix* and related organisms and was the nuclei of the family *Erysipelotrichaceae* established to accommodate the genera *Erysipelothrix*, *Holdemania*, *Bulleidia*, *Solobacterium* and some misclassified members of other genera, *Clostridium innocuum*, *Streptococcus pleomorphus* and *Eubacterium biforme* [6] . Chemotaxonomic analysis utilizing fatty acids, sugars and peptidoglycan composition of each species, as well as the presence or absence of a wide variety of enzymes confirmed the close association

between these species. Therefore, it is proposed that a novel genus should be created for which the name *Thomasclavelia* gen. nov. is proposed to include *Clostridium cocleatum*, *Clostridium saccharogumia*, *Clostridium ramosum* and *Clostridium spiroforme*. Characteristics that serve to distinguish members of *Thomasclavelia* are given in Figures 2.1, 2.2 and 2.3, and Tables 2.1, 2.2 and 2.3.

Furthermore, the genera *Coprobacillus*, *Catenibacterium*, *Eggerthia*, *Kandleria*, *Longibaculum*, *Sharpea* and now *Thomasclavelia* share a phylogenetic ancestry within the *Firmicutes* and form a robust group. Based on phylogenetic, biochemical and chemotaxonomic information the designation of *Coprobacillaceae* fam. nov., a sister family to *Erysipelotrichaceae* was proposed. It is important to note this family is based upon that of the same name as proposed by Verbarg et al., [37] but due to technical reasons, improper proposal to create a genus with a family name, validation was not allowed in IJSEM.

References

1. **Prazmowski, A. (1880).** Untersuchung u˙ber die Entwickelungsgeschichte und Fermentwirking einiger Bacterien-Arten. Inaugural Dissertation. Hugo Voigt, Leipzig, Germany.
2. "Genus *Clostridium*." List of Prokaryotic Names with Standing in Nomenclature. LPSN (1997). http://www.bacterio.net/clostridium.html. 15th of June 2018.
3. **Johnson, J.L. and Francis, B.S., 1975.** Taxonomy of the clostridia: ribosomal ribonucleic acid homologies among the species. Microbiology, **88**(2), pp.229-244.
4. **Tanner, R.S., Stackebrandt, E., Fox, G.E. and Woese, C.R. (1981).** A phylogenetic analysis of *Acetobacterium woodii, Clostridium barkeri, Clostridium butyricum, Clostridium lituseburense, Eubacterium limosum,* and *Eubacterium tenue*. Current Microbiology, **5**(1), pp.35-38.
5. **Woese, C.R. (1987).** Bacterial evolution. Microbiol. Rev., **51**(2), p.221.
6. **Collins, M.D., Lawson, P.A., Willems, A., Cordoba, J.J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. and Farrow, J.A.E. (1994).** The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. Int. J. Syst. Evol. Microbiol., **44**(4), pp.812-826.
7. **Stackebrandt E. (2009).** *Erysipelotrichaceae*. In: de Vos P, Garrity GM, Jones D, et al. (eds). Bergey's Manual of Systematic Bacteriology. Springer US, pp. 1299.

8. **Gupta, R.S. and Gao, B. (2009).** Phylogenomic analyses of clostridia and identification of novel protein signatures that are specific to the genus *Clostridium sensu stricto* (cluster I). Int. J. Syst. Evol. Microbiol., **59**(2), pp.285-294.

9. **Lawson P.A., Rainey F.A. (2016).** Proposal to restrict the genus *Clostridium* Prazmowski to *Clostridium butyricum* and related species. Int. J. Syst. Evol. Microbiol., **66**(2), pp.1009–1016.

10. **Hutson RA, Thompson DE, Collins MD. (1993).** Genetic interrelationships of saccharolytic *Clostridium botulinum* types B, E and F clostridia as revealed by small-subunit sequences. FEMS Microbiol. Lett.,**108**(1), pp.103–110.

11. **Clavel, T., Borrmann, D., Braune, A., Dor´e, J. and Blaut, M. (2006a).** Occurrence and activity of human intestinal bacteria involved in the conversion of dietary lignans. Anaerobe, **12**, pp. 140–147.

12. **Boureau, H., Decre, D., Carlier, J.P., Guichet, C. and Bourlioux, P. (1993).** Identification of a *Clostridium cocleatum* strain involved in an anti-*Clostridium difficile* barrier effect and determination of its mucin-degrading enzymes. Res. Microbiol., **144**(5), pp.405-410.

13. **Woting, A., Pfeiffer, N., Loh, G., Klaus, S. and Blaut, M. (2014).** *Clostridium ramosum* promotes high-fat diet-induced obesity in gnotobiotic mouse models. MBio, **5**(5), pp.e01530-14.

14. **Borriello, S.P. and Carman, R.J. (1983).** Association of iota-like toxin and *Clostridium spiroforme* with both spontaneous and antibiotic-associated diarrhea and colitis in rabbits. J. Clin. Microbiol., **17**(3), pp.414-418.

15. **Gram, C. (1884).** Ueber die isolirte Farbung der Schizomyceten in Schnitt-und Trockenpraparaten. Fortschritte der Medicin, **2**, pp.185-189.

16. **Iino, T. and Enomoto, M. (1971).** Chapter IV Motility. In Methods in Microbiology (**Vol. 5**), pp. 145-163. Academic Press.

17. **Edwards, U., Rogall, T., Blöcker, H., Emde, M. and Böttger, E.C. (1989).** Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res., **17**(19), pp.7843-7853.

18. **Yoon, S.H., Ha, S.M., Kwon, S., Lim, J., Kim, Y., Seo, H. and Chun, J. (2017).** Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. Int. J. Syst. Evol. Microbiol., **67**(5), pp.1613-1617.

19. **Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011).** MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol., **28**(10), pp.2731–2739.

20. **Edgar, R.C. (2004a).** MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics, **5**(1), pp.113.

21. **Edgar, R.C. (2004b).** MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res., **32**(5), pp. 1792–1797.

22. **Rainey, F. and Oren, A. (2011).** Taxonomy of prokaryotes (vol. 38). Academic Press, London, UK, pp. 7, 101-182.

23. **Schumann P. (2011).** Peptidoglycan Structure. In: Oren A (ed) Methods in Microbiology (Vol. 38). Elsevier, London, UK, pp. 101–129.

24. **Kämpfer, P. and Kroppenstedt, R.M. (1996).** Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. Can. J. Microbiol., **42**(10), pp.989-1005.

25. **Sasser, M. (1990).** Identification of bacteria by gas chromatography of cellular fatty acids. Technical Note 101, revised February 2001. MIDI Labs Inc., Newark, DE, pp. 1-6

26. **Vos, P., Garrity, G., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A., Schleifer, K.H. and Whitman, W.B. eds. (2011).** Bergey's manual of systematic bacteriology: The *Firmicutes* (**vol. 3**). Springer Science & Business Media.

27. **Kageyama, A. and Benno, Y. (2000).** *Catenibacterium mitsuokai* gen. nov., sp. nov., a Gram-positive anaerobic bacterium isolated from human faeces. Int. J. Syst. Evol. Microbiol., **50**(4), pp.1595-1599.

28. **Kageyama, A. and Benno, Y. (2000).** *Coprobacillus catenaformis* gen. nov., sp. nov., a new genus and species isolated from human feces. J. Microbiol. Immunol. Infect., **44**(1), pp.23-28.

29. **Salvetti, E., Felis, G.E., Dellaglio, F., Castioni, A., Torriani, S. and Lawson, P.A. (2011).** Reclassification of *Lactobacillus catenaformis* Eggerth 1935; Moore and Holdeman 1970 and *Lactobacillus vitulinus* Sharpe et al. 1973 as *Eggerthia catenaformis* gen. nov., comb. nov. and *Kandleria vitulina* gen. nov., comb. nov., respectively. Int. J. Syst. Evol. Microbiol., **61**(10), pp.2520-2524.

30. **Lagkouvardos, I., Pukall, R., Abt, B., Foesel, B.U., Meier-Kolthoff, J.P., Kumar, N., Bresciani, A., Martínez, I., Just, S., Ziegler, C. and Brugiroux, S. (2016).** The Mouse Intestinal Bacterial Collection (miBC) provides host-specific insight into cultured diversity and functional potential of the gut microbiota. Nat. Microbiol., **1**(10), p.16131.

31. **Morita, H., Shiratori, C., Murakami, M., Takami, H., Toh, H., Kato, Y., Nakajima, F., Takagi, M., Akita, H., Masaoka, T. and Hattori, M. (2008).** *Sharpea azabuensis* gen. nov., sp. nov., a Gram-positive, strictly anaerobic bacterium isolated from the faeces of thoroughbred horses. Int. J. Syst. Evol. Microbiol., **58**(12), pp.2682-2686.

32. **Yarza, P., Yilmaz, P., Pruesse, E., Glöckner, F.O., Ludwig, W., Schleifer, K.H., Whitman, W.B., Euzéby, J., Amann, R. and Rosselló-Móra, R. (2014).** Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. Nat. Rev. Microbiol., **12**(9), p.635.

33. **Dethlefsen, L., Eckburg, P.B., Bik, E.M. and Relman, D.A. (2006).** Assembly of the human intestinal microbiota. Trends Ecol. Evol., **21**(9), pp.517-523.

34. **Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S.R., Nelson, K.E. and Relman, D.A. (2005).** Diversity of the human intestinal microbial flora. Science, **308**(5728), pp.1635-1638.

35. **Macfarlane, G. T., Gibson, G. R., Drasar, B. S. & Cummings, J. H. (1995).** Metabolic significance of the gut microflora. Gastrointestinal and oesophageal pathology, 2nd Edn., pp.249-274.

36. **Kaneuchi C, Miyazato T, Shinjo T, Mitsuoka T. (1979).** Taxonomic study of

helically coiled, sporeforming anaerobes isolated from the intestines of humans and other animals: *Clostridium cocleatum* sp. nov. and *Clostridium spiroforme* sp. nov. Int. J. Syst.c Baceriol., **29**(1), pp.1–12.

37. **Verbarg, S., Göker, M., Scheuner, C., Schumann, P. and Stackebrandt, E. (2014).** The Families *Erysipelotrichaceae* emend., *Coprobacillaceae* fam. nov., and *Turicibacteraceae* fam. nov. *In* The prokaryotes. Springer, Berlin, Heidelberg, pp. 79-105.

38. **Fox, G.C.A., Stackebrandt, E., Hespell, R.B., Gibson, J., Maniloff, J., Dyer, T.A., Wolfe, R.S., Balch, W.E., Tanner, R.S., Magrum, L.J. and Zablen, L.B. (1980).** The phylogeny of prokaryotes. Science, **209**(4455), pp.457-463.

Isolation and characterization of novel species belonging to the genera *Parvimonas, Ruoffiella* gen. nov. and *Liuella* gen. nov. of the family *Peptoniphilacea*.

Abstract

The Gram-positive anaerobic cocci (GPAC) are part of the commensal flora of humans and animals. They account for approximately 30% of all isolated anaerobic bacteria from clinical specimens. Historically identification of these anaerobes has been limited in the diagnostic laboratory, mainly due to requirements for prolonged incubation times, slow developing genotypic identification and common isolation from polymicrobial infections in which known pathogens may take precedence. However, more recent developments in molecular methods have resulted in considerable changes to the taxonomy of GPAC enabling the reclassification of many existing taxa in addition to the description of novel species originating from clinical material. The present study employed a polyphasic investigation that included phylogenetic, phenotypic and chemotaxonomic methods to characterize three novel organisms belonging to the GPAC group. The consistent chemotaxonomic features shared by the three species included L-ornithine as the diagnostic diamino acid, glucose as the common sugar present in the cell wall, and an inability to utilize carbohydrates. In addition to low 16S rRNA similarity values, the biochemical profiles of *Ruoffiella abscessiensis* sp. nov., CCUG 66799^T, and *Liuella johnsonii* sp. nov., CCUG 65098^T**,** differed from those of their nearest relatives, but that of *Parvimonas asscharolyticus* sp. nov., CCUG 667360^T, showed concordance with other species of the genus *Parvimonas*. 16S rRNA gene sequencing shows sequence similarity between *Parvimonas asscharolyticus* sp. nov., CCUG 667360^T, and

Parvimonas micra was less than 95.3%, lower than the 98.8% threshold value used to delineate novel species. The additional two organisms examined demonstrated sequence similarities below the 94% value routinely used for delineation of novel genera. Therefore, based on phylogenetic, phenotypic and chemotaxonomic results, two novel genera, *Ruoffiella* gen. nov. (CCUG 66799^{T}) and *Liuella* gen. nov. (CCUG 65098^{T}) are proposed. The type species of the genera are *Ruoffiella abscessiensis* sp. nov. and *Liuella johnsonii* sp. nov. Furthermore, a novel species belonging to the genus *Parvimonas* is proposed, *Parvimonas asaccharolyticus* sp. nov. (CCUG 67360^{T}).

Introduction

An important group known as the Gram-Positive Anaerobic Cocci (GPAC) bacteria is often found as part of the commensal microbiota in the respiratory and gastrointestinal tracts, the female genitourinary system and skin. Consisting of obligately anaerobic non-spore-forming cocci, species in this group have been reported to make up as much as 30% of pathogenic isolates recovered from clinical material, but their etiology remains unclear. However, antibiotic resistance and some other virulence factors have been identified [1-4]. Typically, commensal bacteria don't breach the protective immunological barriers, but under certain conditions (wounds, immune-compromised systems, abscesses, etc.) infections caused by these opportunistic pathogens have the potential to cause severe damage. Body locations of most GPACs are located in the oral flora (*Parvimonas micra*) and the skin flora (*Finegoldia magna* and *Peptinophilus asaccharolyticus*) [5]. However, transfer or carry-over mechanisms to sites of infections are not well understood. The gap in our knowledge is due mainly to the long periods of growth required for GPACs recovery during which carry-over/transfer is hypothesized to take place in addition to difficulties isolating species from polymicrobial infections. In severe cases, isolates of *Finegoldia magna* can often be associated not only with skin infections, but also with soft tissue, bone and joint infections. Therefore it is almost impossible to precisely identify the original location of the infection.

The GPACs are part of the order *Clostridiales* and have undergone extensive taxonomic changes including reclassification and the formation of a new family *Peptoniphileace*. The changes embrace a number of genera (*Anaerococcus, Anaerosphaera, Helcoccus, Finegoldia, Gallicola, Parvimonas* and *Murdochiella*) that

were previously classified under Family XI *Incertae sedis* denoting their unknown precise taxonomic affiliations [6].

The background of this group is long and complex. In 1936 Kluyver and van Niel described two genera, *Peptostreptococcus* and *Peptococcus*, separated solely by morphological characteristics. Species belonging to *Peptococcus* were arranged in clumps and placed in the *Micrococcaceae* family while those in *Peptostreptococcus* were in chains and placed in the genus *Streptococcus* [7]. In 1948, Prévot further divided them into eight genera when more microscopic morphological characteristics were observed. In 1971 he reclassified half of them, *Peptococcus, Peptostreptococcus, Sarcina* and *Ruminococcus*, into the family *Peptococcaceae* [8, 9]. The new family was composed of strictly anaerobic Gram-positive cocci or coccobacillus. Peptococci and peptostreptococci were differentiated from ruminococci and sarcinae due nutritional requirements derived from protein as the sole energy source rather than carbohydrates [10]. Rogosa's study led Holdeman and Moore to transfer all microaerophilic species to the genus *Streptococcus* based on their ability to withstand and grow under low oxygen levels in addition to oxidase and catalase negative reactions, and the transfer was later supported by Wexler's cell wall structure analysis and nucleic acid analysis as described by Huss et al. [11-13].

The increasing application of DNA hybridization techniques in the 1980's began to emphasize discrepancies in older classification systems [14]. Ezaki et al. (1983) shed light on the nucleic acid and biochemical properties that again revealed discrepancies with the original classification. The G+C content differences they obtained allowed them to reclassify four *Peptococcus* species into the *Peptostreptococcus* genus leaving

Peptococcus niger as the sole species of the *Peptococcus* genus while also disposing of the original cellular arrangement distinction between peptococci and peptostreptococci [15]. Still, these observations were not without controversy as in 1984 Huss et al. challenged their nucleic acid analysis. The study by Huss et al. detected no DNA homology between certain strains as well as different G+C content values at the genus level [16]. The discrepancies were not initially accepted, but additional studies over time, 1984, confirmed the results of Huss et al. case for a more in-depth restructuring of the GPAC [17, 18].

More recently, 16S rRNA gene sequence data have confirmed the distant relationship of *Peptococcaceae*'s type species, *Peptococcus niger*, to GPAC species while emphasizing the relatedness between the type species of the genera *Peptostreptococcus* and *Clostridium* [19, 20, 21, 22]. In 1994 Collins et al. compared over 100 reference strains and created nineteen 16S rRNA gene clusters within the *Clostridium* genus. Cluster XIII contains eleven species from the *Peptostreptococcus* genus in addition to *Helococcus kunzii*; cluster XI contains one GPAC species, *Peptostreptococcus anaerobius, Eubacterium tenue* and eighteen *Clostridium* species [17,23]. The location of various *Peptostreptococcus* species in different clusters, along with varying biochemical results led Huss et al. to propose further revisions of the genus.

Murdoch and Shah (1999) reanalyzed 16s rRNA gene sequences and showed that *Peptostreptococcus anaerobius*, the type species of the genus *Peptrostreptococcus*, was phylogenetically distinct from other species in the genus and therefore represented a subgroup within clostridia. Furthermore *Peptostreptococcus anaerobius* contained higher mol% G+C reinforcing its separateness. *Peptostreptococcus micros* and

Peptostreptococcus magnus were closely related to each other, but distinct from the type species and were reclassified into novel genera, *Micromonas* gen. nov. and *Finegoldia* gen. nov., respectively [22]. Murdoch and Shah's only error was nomenclature because of the usage of a homonym when naming the *Micromonas* genus. Tindall and Euzeby believed Manton and Parke's 1960 algal genus name *Micromonas* took precedence and in 2016 proposed replacing *Micromonas* with a new genus name, *Parvimonas* [23].

Ezaki continued working on the reclassification of GPAC and in 2011 further phylogenetical analysis using 16S rDNA sequences allowed reclassification of the remaining members to *Peptoniphilus* gen. nov., *Anaerococcus* gen. nov. and *Gallicola* gen. nov. [24]. The application of molecular methods continued to demonstrate the diversity of the GPAC within the polymicrobial biofilm communities found in various wound types [25, 26]. Such diversity has gathered importance due to the increase of clinical manifestations in our growing geriatric and/or immunocompromised populations. Cases with abscesses or necrotic tissue following the introduction of implants suggest that a wound's development is caused initially by aerobic bacteria. Aerobic bacteria promote anaerobic growth through induction of tissue hypoxia, reduction of redox potential and finally through the production of essential nutrients (amino acids, peptides and proteins). The ability of GPAC to use the amino acids, peptides and proteins exacerbate the condition of the wounds and in some cases lead to chronic infections containing polymicrobial communities resistant to a wide range of antibiotics. Additionally, metabolites produced during the infection cycle may impair normal wound-healing processes (inflammation, remodeling of extracellular matrix, etc.) [27].

In this study, three Gram-positive, obligately anaerobic non-spore-forming cocci originating from bodily abscesses were characterized using a polyphasic approach. Phylogenetic and phenotypic analyses demonstrated the strains represented one novel species of *Parvimonas* and two novel organisms not phylogenetically related to any currently described taxa, therefore representing novel GPAC genera for which the names *Liuella* gen. nov. and *Ruoffiella* gen. nov. are proposed.

Materials and Methods

Strain isolation

The strains in this study were isolated in multiple locations around the world and deposited at the Culture Collection at the University of Göteborg (CCUG), Sweden. Strain CCUG 67360^T was initially isolated in 2005 from a clinical blood sample at the Sahlgrenska University Hospital's department of microbiology. Strain CCUG 65098^T was initially isolated from human tissue (ankle) at the General Diagnostic Department in Göteborg, Sweden. Strain CCUG 66799^T was initially isolated from human feces. Strains CCUG 65098^T and CCUG 66799^T were deposited in the Culture Collection at the University of Göteborg (CCUG) without specified isolation dates. All strains were isolated and purified in chocolate agar under anaerobic conditions at 37°C.

Culture conditions and cultivation

Following initial isolation, the strains were received at the Lawson laboratory as lyophilized cultures and grown in Hardy Diagnostics Peptone Yeast Glucose (PYG) broth (Santa Maria, CA, USA) at 37°C for 48 hours under anaerobic headspace (N_2:H_2:CO_2, 70:20:15; 10 psi). At 48 hours, 100 µL samples were transferred onto Hardy Diagnostics Peptone Yeast Glucose (PYG) agar (Santa Maria, CA, USA) amended with 5% defibrinated sheep blood (pH 7.3) and incubated under the same conditions (Table 3.1).

Morphological, physiological and biochemical characterization

Gram-stain and catalase production were determined for all provided species as previously described [28, 29]. Biochemical characterizations of each strain were carried

out at the Culture Collection at the University of Göteborg (CCUG) in Sweden via API (Analytical Profile Index) Rapid ID32A (bioMérieux, France).

Imaging

An Olympus CX41 using phase contrast at 1000X magnification and Zeiss NEON 40 EsB high resolution scanning electron microscope were used to determine cell morphology in accordance to Huang et al., 2000 [30]. Cells for scanning electron microscopy were fixed with equal volumes of 2.5% glutaraldehyde in 0.1 M cacodylate buffer and attached to polylysine coated glass coverslips usinh 0.1% polylysine. Dehydration was carried out through a 30-100% ethanol series (30,50,70,90, 100x3) followed by critical point drying, attachment to SEM stubs and sputter coated using iridium.

16S rRNA gene sequence and phylogenetic analysis

Chromosomal DNA was extracted and purified using the PowerSoil® DNA Isolation Kit (MoBio Laboratories, Inc.). The 16S rRNA gene fragments were amplified via PCR using universal primers corresponding to positions 8-27 (8F, forward primer) and 1521-1542 (pH*, reverse primer) of the *Escherichia coli* numbering system [29].
Unincorporated primers and dNTPs were removed from resulting amplicons with ExoSAP-IT (USB Corporation). Purified amplicons were sequenced at the University of Oklahoma's Biology Core Molecular Laboratory with primers directed towards conserved regions of the rRNA gene: 522-539 (pD), 339–358 (anti γ), 1090-1109 (3*) and 1510-1492 (1492R) [29]. Approximately 1,460 bases of the 16S rRNA gene were

determined for all strains. Nearest relatives were identified by a calculated pairwise sequence similarities alignment algorithm in the EzBiocloud server (http:// eztaxon-e.ezbiocloud.net/) [30]. The sequences of the nearest relatives were retrieved and aligned using Muscle. A phylogenetic tree was re-constructed in MEGA according to the maximum-likelihood method and TREEVIEW (31,32). The stability of the groupings was established via bootstrap analysis employing 1000 replicons.

Chemotaxonomic characterization

Chemotaxonomic analyses were used to examined characteristics cell membrane components. Whole cell sugars and diagnostic diamino acids were analyzed as previously specified (33,34). Cellular fatty acid methyl ester (FAME) products were extracted using the Sherlock Microbial Identification System (MIDI Labs Inc., Newark, DE) version 6.1, analyzed with an Agilent Technologies 6890N gas chromatograph (GC). The GC was coupled with a phenyl methyl silicone fused silica capillary column (HP-2 25m x 0.2 mm x 0.33 μm film thickness), hydrogen as the carrier gas and a flame ionization detector (35,36). Fatty acids' concentrations were expressed as percentages in the QBA1 peak naming database, and 10% cut off value was used as the threshold for major and minor fatty acids.

Table 3.1. Hardy Diagnostics Peptone Yeast Glucose (PYG) medium components. Formula per liter of deionized water.

Formulae **Hardy Diagnostics Peptone Yeast Glucose (PYG)**		
Pancreatic Digest of Casein	20.0	g
Yeast Extract	10.0	g
Glucose	10.0	g
L-Cysteine	0.5	g
Sodium Bicarbonate	0.4	g
Sodium Chloride	0.08	g
Monopotassium Phosphate	0.04	g
Dipotassium Phosphate	0.04	g
Calcium Chloride, Anhydrous	0.008	g
Magnesium Sulfate, Anhydrous	0.008	g
Hemin Solution, 0.1%	5.0	ml
Vitamin K Solution, 1%	0.1	ml
Agar	15.0	g

Final pH 7.2 +/- 0.2 at 25 ^{o}C.

Autoclave at 121 ^{o}C.

Results

Morphological and phenotypic studies

All organisms required 48 hours of growth under strictly anaerobic conditions and were Gram-positive, non-motile, non-spore forming cocci. CCUG 65098^T and CCUG 66799^T produced small, round, off white colonies while CCUG 67360^T produced small, round, light yellow colonies with spreading edges. Individual cells had the following diameters. CCUG 67360^T cells were 0.50 μm in diameter. CCUG 66799^T cells were 0.70 μm in diameter. CCUG 65098^T cells were 0.50 μm in diameter. All cell walls were uneven, but only extracellular material secreted by CCUG 66799^T or CCUG 65098^T cells connected individual cells to each other after seven days of incubation.

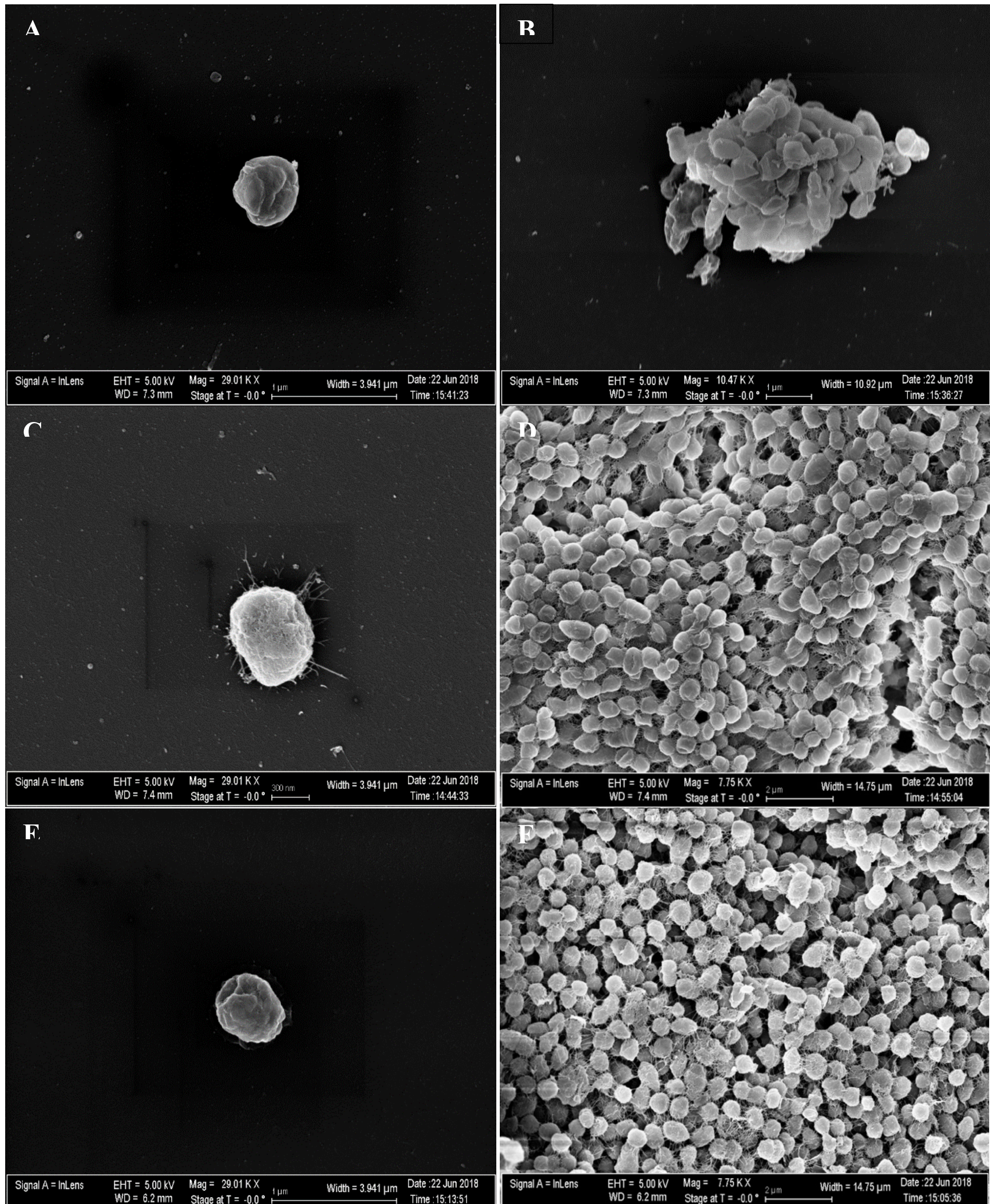

Figure 3.1: Scanning electron microscopy of novel GPAC isolates. At lower magnification, extracellular material was observed connecting *Ruoffiella* or *Liuella* cells (D,F), but was not seen in the cells of *Parvimonas* (B). A, B: *Parvimonas* sp. nov., CCUG 67360^{T}; C,D: *Ruoffiella sp. nov.*, CCUG 66799^{T}; E,F: *Liuella sp. nov.*, CCUG 65098^{T}.

Biochemical studies

Biochemical characteristics are listed in Table 3.2. Using the Rapid ID32A kit, all isolates were positive for arginine arylamidase and leucine arylamidase. Additionally CCUG 65098^T and CCUG 67360^T were positive for histidine arylamidase. CCUG 67360^T was also positive for phenyl arylamidase, proline arylamidase, tyrosine arylamidase and serine arylamidase. CCUG 66799^T was weakly positive for indole. All remaining tests were negative.

Table 3.2. Biochemical characteristics of novel GPAC species.

Biochemical Characterization	**1**	**2**	**3**
Rapid ID32AN			
Urease	-	-	-
Arginine dihydrate	-	-	-
α-galctosidase	-	-	-
β-galactosidase	-	-	-
β-gal-6-phospate	-	-	-
α-glucosidase	-	-	-
β-glucosidase	-	-	-
α-arabinosidase	-	-	-
β-glucuronidase	-	-	-
N-acetyl-β-glucosamine	-	-	-
Mannose	-	-	-
Raffinose	-	-	-
Nitrate	-	-	-
Indole	-	W+	-
Alkaline phosphatase	-	-	-
Arginine arylamidase	S+	S+	S+
Proline Arylamidase	-	-	S+
Leucyl gly arylamidase	-	-	-
Phenyl arylamidase	-	-	S+
Leucine arylamidase	S+	S+	S+
Pyroglu acid arylamidase	-	-	-
Tyrosine arylamidase	-	-	S+
Alanine arylamidase	-	-	-
Glycine arylamidase	-	-	-
Glutamic acid decarboxylase	-	-	-
α-fucosidase	-	-	-
Histidine arylamidase	S+	-	S+
Glycine arylamidase	-	-	S+
Serine arylamidase	-	-	S+

W+ weakly positive

S+ strongly positive

- Negative

CCUG65098^T in this study; 1., CCUG66799^T in this study; 2. and CCUG67360^T in this study; 3.

Phylogenetic studies

Maximum-likelihood tree analysis demonstrated the organisms belonged to the order *Clostridiales*. A pairwise comparison revealed CCUG 66799^T had approximately 89.5% sequence similarity to the type strain of its closest valid species, *Finegoldia magna*, CCUG 17636^T. CCUG 65098^T had approximately 90.71% sequence similarity to the type strain of its closest validly named species, *Peptoniphilus ivorii*, DSM 10022^T, while CCUG 67360^T had approximately 95.30% sequence similarity to the type strain of its closest validly named species, *Parvimonas micra*, ATCC 33270^T. A phylogenetic tree, constructed by maximum-likelihood method, depicting the phylogenetic affinity of all species and their close relatives, is shown in Figure 3.2.

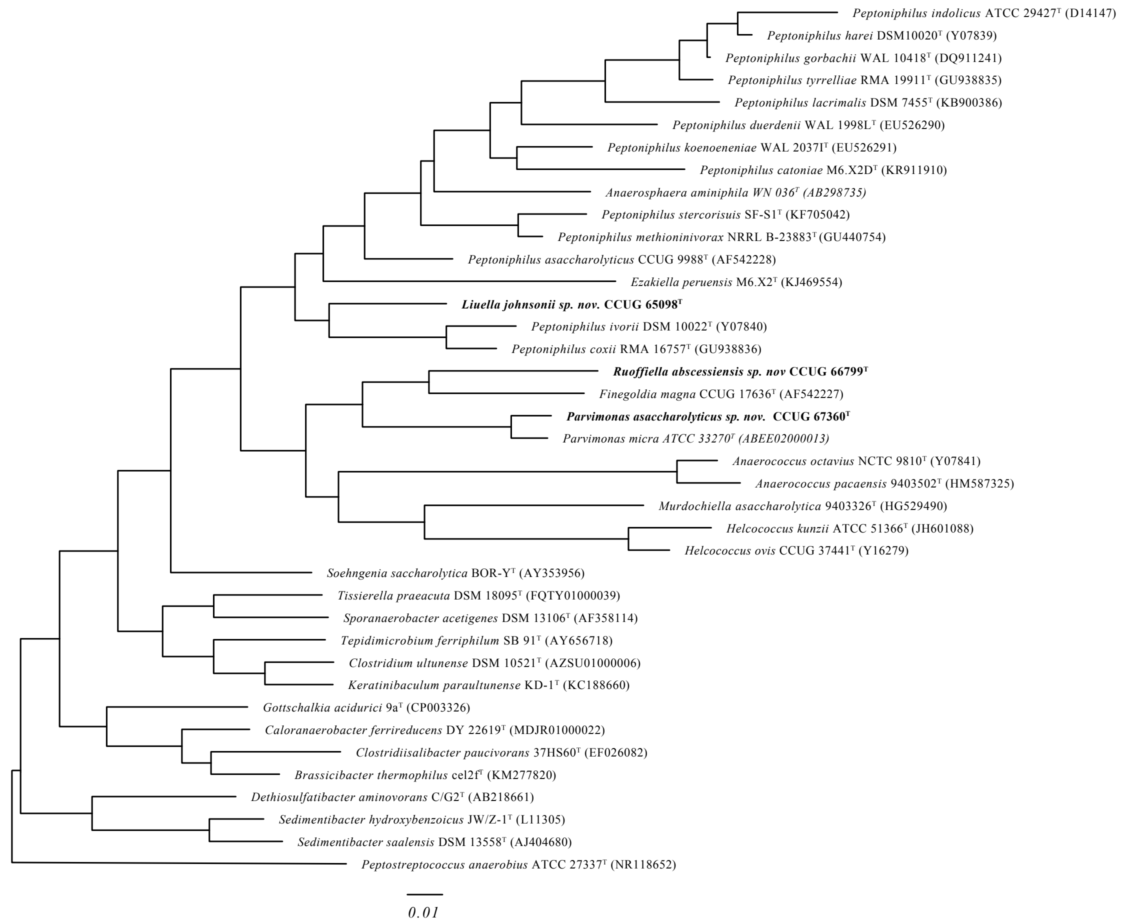

Figure 3.2: Unrooted tree showing the phylogenetic inter-relationships of a novel member of the genus *Parvimonas and the novel genera Liuella gen. nov. and Ruofiella gen.nov.* with their nearest relatives. Phylogenetic analyses were performed on 1300 nucleotides using the maximum-likelihood algorithm. Bootstrap values expressed as a percentage of 1000 replications, are given at branching points. Bar = 1% sequence divergence. Three novel clinical species *Liuella johnsonii* sp. nov, *Ruoffiella abscessiensis* sp. nov. and *Parvimonas asaccharolyticus* sp. nov. were identified.

Chemotaxonomic studies

The dominant fatty acids shared by all species examined were $C_{16:0}$ and $C_{18:1\ w6c}/C_{18:1\ w7c}$ (both >13.0%). In addition, CCUG 65098^T contained $C_{13:0\ anteiso}$ (11.0%), CCUG 67360^T contained $C_{17:0\ cyclo}$/ Unknown17.975/$C_{18:0}$ (10.3%) and CCUG 66799^T contained $C_{10:0}$ (28.4%) and $C_{15:1\ w8c}$ (13.2%) as major fatty acids (Table 3.3). Whole-cell sugar analysis using TLC plates revealed glucose to be the common sugar present within the cells and in the cell walls of all species. Xylose was also present in CCUG 67360^T and CCUG 66799^T. The N-acetylmuramic acid's crosslinked peptide chains found in the peptidoglycan contained relativeness between all species. L-ornithine was present as the diagnostic diamino acid in the third position of the stem peptide chain with L-alanine and glutamic acid which correspond with a type A4β peptidoglycan structure (34). Differences were observed between species. CCUG 65098^T contained L-lysine in the stem peptide chain while CCUG67360^T and CCUG66799^T contained glycine. HPLC analysis of fermentative end products revealed that CCUG65098^T produced isobutyrate and butyrate under anaerobic conditions, while CCUG67360^T and CCUG 66799^T produced acetic acid (Table 3.4, Figure 3.3, Figure 3.4).

Table 3.3. Fatty acid profiles of novel GPAC species.

[a]Fatty acid	1	2	3
	QBA1		
$C_{9:0}$	-	-	-
$C_{10:0}$	-	-	**28.4**
$C_{12:0}$	4.2	2.3	4.8
$C_{13:0\ anteiso}$	**11.0**	8.1	4.2
$C_{14:1\ w5c}$	-	-	0.7
$C_{14:0}$	9.1	4.1	2.0
$C_{14:0\ 2OH}$	5.8	-	2.2
$C_{15:1\ w8c}$	3.8	-	**13.2**
$C_{16:0}$	**13.3**	**25.2**	**13.2**
$C_{16:0\ 2OH}$	-	-	0.5
$C_{17:0\ anteiso}$	-	-	0.5
$C_{17:1\ w8c}$	-	-	3.0
$C_{17:1\ w6c}$	-	-	1.0
$C_{17:0}$	-	1.6	1.4
$C_{17:0\ 2OH}$	-	-	0.6
$C_{18:2\ w6,9c}$	-	-	-
$C_{18:1\ w9c}$	-	-	-
$C_{18:0}$	-	-	-
Summed Feature 3	-	-	4.2
Summed Feature 4	-	2.2	1.5
Summed Feature 5	-	8.2	9.0
Summed Feature 8	**43.4**	**38.0**	**16.7**
Summed Feature 10	9.4	**10.3**	2.8
Summed Feature 13	-	-	0.6

Profiles were established using cultures grown in Hardy Diagnostics Peptone Yeast Glucose agar + 5% sheep's blood at 37°C and a N_2:H_2:CO_2 gas mix. [a]Major fatty acids were analyzed in the QBA1 library under 10% standard percentage abundance(emphasized in bold). Summed feature 3: $C_{16:1\ w7c}$/$C_{16:1\ w6c}$. Summed Feature 4: $C_{15:0}$/Unknown15.040. Summed Feature 5: $C_{18:0\ ante}$/ $C_{18:2\ w6,9c}$. Summed Feature 8: $C_{18:1\ w6c}$/$C_{18:1\ w7c}$. Summed Feature 10: $C_{17:0\ cyclo}$/ Unknown17.975/$C_{18:0}$. Summed Feature 13: Unknown 16.612/$C_{17:0\ iso}$.CCUG65098[T] in this study; 1., CCUG67360[T] in this study; 2. and CCUG66799[T] in this study; 3.

Table 3.4. Chemotaxonomic characteristics of novel GPAC species.

Characteristics	1	2	3
Diagnostic Diamino Acids			
Meso-Diamino acid	-	-	-
L,L-Diamino acid	-	-	-
L-Ornithine	+	+	+
L-Lysine	+	-	-
Glutamine	+	-	-
Glycine	+	+	+
L-Alanine	+	+	+
Leucine	+	-	-
Whole Cell Sugars			
Galactose	-	ND	-
Glucose	+	ND	+
Arabinose	-	ND	-
Manose	-	ND	-
Xylose	-	ND	+
Rhamnose	-	ND	-
Ribose	-	ND	-
Fucose	-	ND	-
End Products			
Pyruvate	-	-	-
Lactate	-	-	-
Formate	-	-	-
L-Aspartic Acid	-	-	-
Isobutyrate	+	-	-
Butyrate	+	-	-
Acetic Acid	-	+	+

Profiles were established using cultures grown in Hardy Diagnostics Peptone Yeast Glucose agar + 5% sheep's blood at 37°C and a N_2:H_2:CO_2 gas mix.
- Negative
+ Positive
ND Not Determined
CCUG65098^{T} in this study; 1., CCUG67360^{T} in this study; 2. and CCUG66799^{T} in this study; 3.

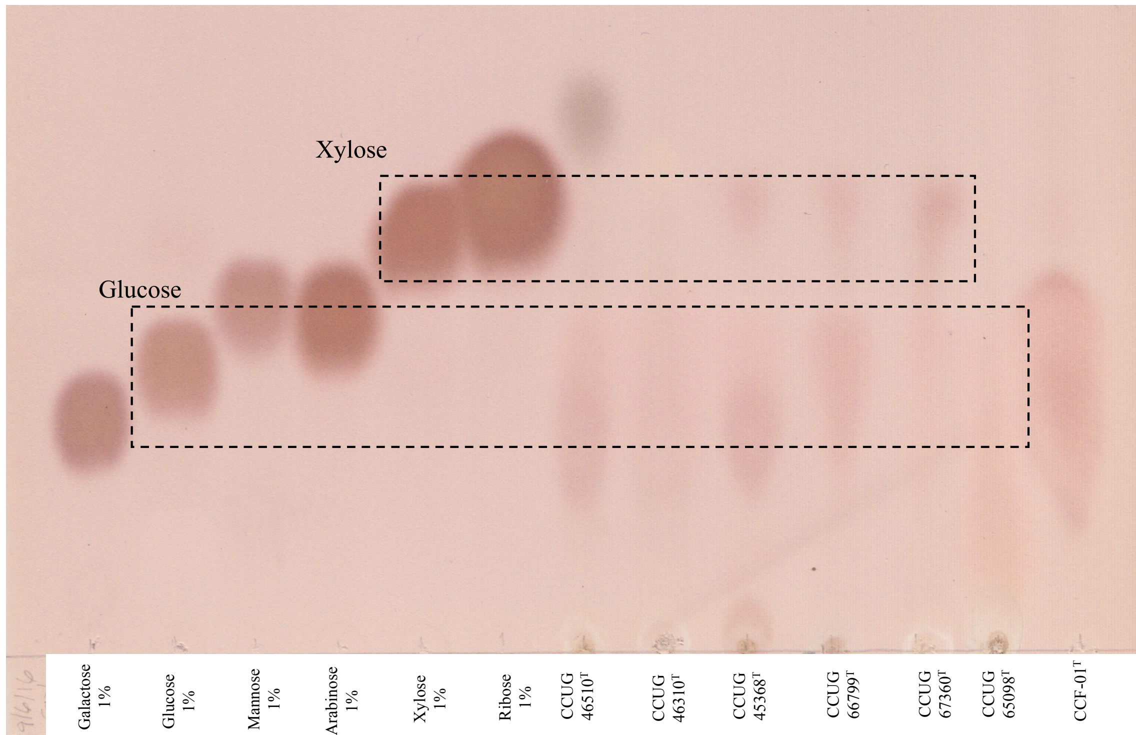

Figure 3.3: Whole-cell sugar analysis of GPAC species.

Profiles were established using cultures grown in Hardy Diagnostics Peptone Yeast Glucose agar + 5% sheep's blood at 37°C and a N_2:H_2:CO_2 gas mix.

Lane 1 to 6: sugar standards.

Lane 7-9 and 13: Controls

Lanes 10 to 12: **CCUG 66799^T**, **CCUG 67360^T**, **CCUG 65098^T**

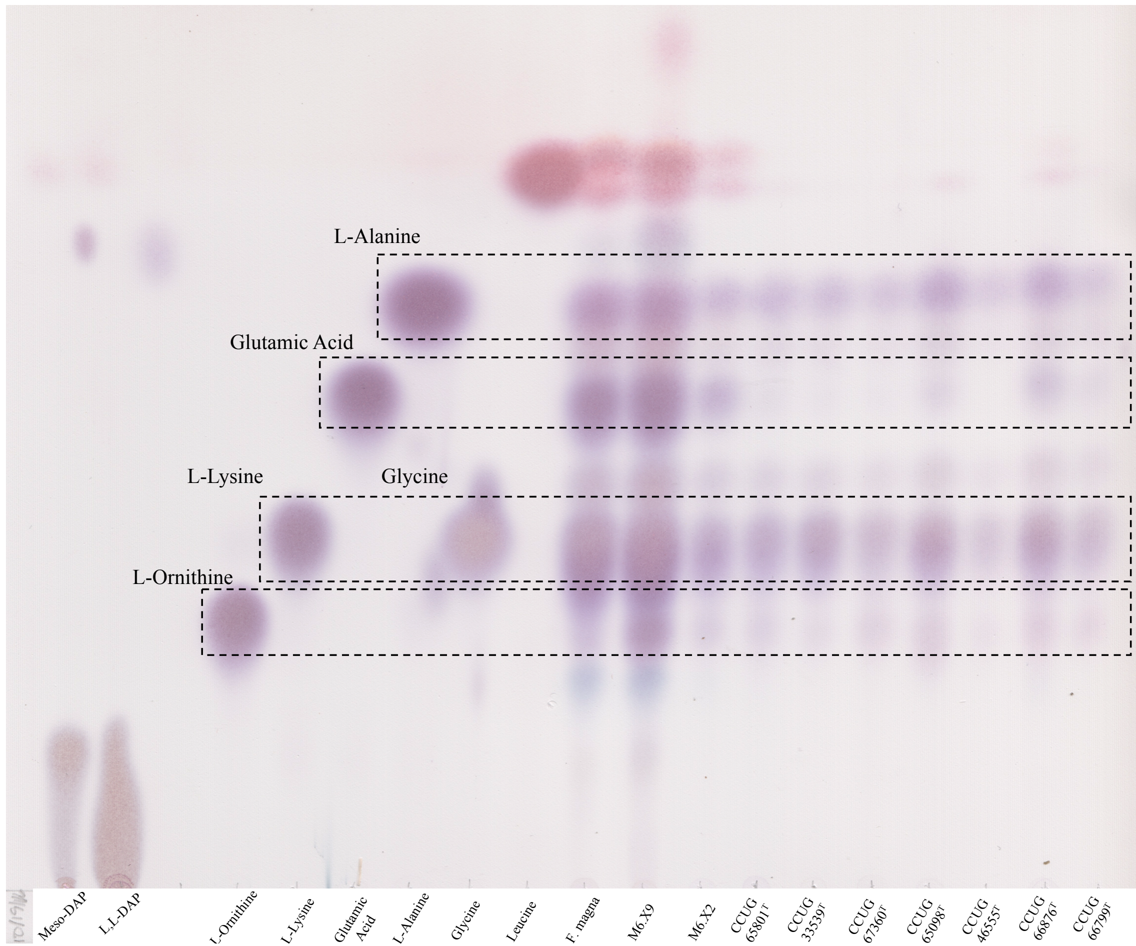

Figure 3.4: Diagnostic diamino Acids analysis of GPAC species.

Profiles were established using cultures grown in Hardy Diagnostics Peptone Yeast Glucose agar + 5% sheep's blood at 37°C and a N_2:H_2:CO_2 gas mix.

Lane 1 to 8: amino acid standards.

Lane 9 to 11: Controls

Lanes 12 to 18: CCUG 65801^{T}, CCUG 33539^{T}, **CCUG 67360^{T}**, **CCUG 65098^{T},** CCUG 46555^{T}, CCUG 66876^{T}, CCUG 66799^{T}

Description of *Liuella* gen. nov.

N.L. fem. dim. n. *liuella*, in honor of Chengxu Liu, a microbiologist from the United States for his contributions to microbial taxonomy.

Cells are Gram-stain- positive cocci. The predominant fatty acids are $C_{13:0\ anteiso}$, $C_{16:0}$ and summed features8 ($C_{18:1\ w6c}$/$C_{18:1\ w7c}$). The end products from PYG are isobutyrate and butyrate. Positive for: arginine arylamidase, leucine arylamidase and histidine arylamidase. The type species is *Liuella johnsonii*.

Description of *Liuella johnsonii* sp. nov.

N.L. fem. dim. n. *johnsonii*, in honor of Crystal N. Johnson, a microbiologist from the United States for her contributions to the taxonomy of Gram-positive anaerobic cocci.

Cells are Gram-positive cocci 0.5 μm in diameter. After 7 days of incubation, colonies are small, round, and off white. Urease, nitrate and indole are negative. They are sacchrolytic. Using the API Rapid ID32 AN kit, cells were positive for arginine arylamidase, leucine arylamidase and histidine arylamidase. Cells were negative for arginine dihydrate, α-galctosidase, β-galactosidase, β-gal-6-phospate, α-glucosidase, β-glucosidase, α-arabinosidase, β-glucuronidase, N-acetyl-β-glucosamine, alkaline phosphatase, leucyl gly arylamidase, pyroglutamic acid arylamidase, alanine arylamidase, proline arylamidase, leucyl gly arylamidase, glycine arylamidase, phenyl arylamidase, tyrosine arylamidase, glutamic acid decarboxylase and α-fucosidase. The isolate is from a human clinical specimens; abscess. The type strain is *Liuella johnsonii* (CCUG 65098^{T}).

Description of *Ruoffiella* gen. nov.

N.L. fem. dim. n. ruoffiella, in honor of Kathryn L. Ruoff, a microbiologist from the United States for her contributions to the taxonomy of Gram-positive anaerobic cocci.

Cells are Gram-positive staining cocci, carbohydrates are not normally utilized. Predominant fatty acids are $C_{10:0}$, $C_{15:1\ w8c}$, $C_{16:0}$ and a summed feature 8 ($C_{18:1\ w6c}$/$C_{18:1\ w7c}$) and major end product from PYG is acetic acid. Cells are positive for enzymes: arginine arylamidase, and leucine arylamidase. The type species is *Ruoffiella abscessiensis.*

Ruoffiella abscessiensis sp. nov.

ab.sce.ssien'sis, from abscesses from where the organism was first isolated.

Cells are Gram-positive staining cocci 0.7 μm in diameter. After 7 days of incubation, colonies are small, round and off white. Indole is positive, but urease and nitrate are negative. They are asacchrolytic. Using the API Rapid ID32 AN kit, cells were positive for arginine arylamidase and leucine arylamidase. Cells were negative for arginine dihydrate, α-galctosidase, β-galactosidase, β-gal-6-phospate, α-glucosidase, β-glucosidase, α-arabinosidase, β-glucuronidase, N-acetyl-β-glucosamine, alkaline phosphatase, leucyl gly arylamidase, pyroglutamic acid arylamidase, alanine arylamidase, proline arylamidase, leucyl gly arylamidase, glycine arylamidase, phenyl arylamidase, serine arylamidase, tyrosine arylamidase, glutamic acid decarboxylase and α-fucosidase. The isolate is from a human clinical specimen; abscess. The type strain is *Ruoffiella abscessiensis* sp. nov (CCUG 66799^T).

Description of *Parvimonas asscharolyticus* sp. nov.

a.sac.cha.ro.ly'ti.cus. Gr. N. *sakchar* sugar; N.L. fem. adj. *lytica* (from Gr. fem. adj. *lutike*) able to loosen, able to dissolve; N.L. fem. adj. *asacharolytica* not digesting sugar.

Cells are Gram-positive cocci 0.5 μm in diameter. After 7 days of incubation, colonies are small, round and light yellow with spreading edges. Urease, nitrate and indole are negative. They are asacchrolytic. Using the API Rapid ID32 AN kit, cells were positive for arginine arylamidase, proline arylamidase, leucine arylamidase phenyl arylamidase, histidine arylamidase, glycine arylamidase and serine arylamidase. Cells were negative for arginine dihydrate, α-galctosidase, β-galactosidase, β-gal-6-phospate, α-glucosidase, β-glucosidase, α-arabinosidase, β-glucuronidase, N-acetyl-β-glucosamine, alkaline phosphatase, leucyl gly arylamidase, pyroglutamic acid arylamidase, alanine arylamidase, glycine arylamidase, glutamic acid decarboxylase and α-fucosidase. The isolate is from a human clinical specimens; abscess. The type strain is *Parvimonas asaccharolyticus* sp. nov. (CCUG 67360^T).

Discussion

The commensal microbiota of humans contain a group known as the Gram-positive anaerobic cocci (GPAC) that account for 30 % of clinically isolated anaerobes Over the years, significant taxonomic reclassification with the objective of correct and comprehensive identification has addressed the differences between GPAC species and their nearest relatives [1-4]. In doing so, more effective antibiotic treatments have been and will be ensured along with potential data that understands GPAC pathogens' transfer or carry-over mechanisms from colonization sites to infection sites.

Polyphasic studies are essential when classifying or reclassifying species within the GPAC because they provide a wide range of characteristics connecting phenotypic and genotypic data[33]. This study has focused on an approach to mimic effective taxonomic reclassification standards and contains an equivalent objective; proper taxonomic classification and characterization of three novel GPAC species using a polyphasic approach. Based on the results presented, *Ruoffiella* gen. nov. sp. nov, and *Liuella* gen. nov. sp. nov, possess consistent characteristics that strongly support their inclusion into the novel genera. These characteristics are the ~ 90% sequence similarity of each novel genera's type species to their closest valid specie, cell wall characteristics (fatty acid and whole-cell sugar composition) and end product profiles. *Ruoffiella abscessiensis* sp. nov. and *Liuella asaccharolyticus* sp. nov. sequence similarities are below the 94% value routinely used for delineation of novel genera [37]. *Parvimonas asscharolyticus* sp. nov. possesses characteristics that are consistent for its inclusion in the genus *Parvimonas*. 16S rRNA gene sequencing shows sequence similarity between *Parvimonas assachrolyticus* sp. and other species of the genus is less than 95.3% which

is less than the threshold value (98.8%) used to delineate novel speciesFurthermore, the results of the phylogenetic analysis clearly indicate that *Peptoniphilus ivorii* and *Peptoniphilus coxii* should now be regarded as misclassified members of the genus *Peptoniphilus* as they form a cluster with *Liuella johnsonii* sp. nov. while separating themselves from other members of their current genus.

Chemotaxonomic analysis utilizing the fatty acid composition, end products and peptidoglycan structure of each organism, as well as the presence or absence of a wide variety of enzymes, confirmed their unique characteristics. Therefore, the creation of two novel genera, *Ruoffiella* gen. nov. and *Liuella* gen. nov. along with the description of *Parvimonas asscharolyticus* sp. nov. are proposed.

Further, ongoing work, will address the identification of known GPAC virulence factors from these species' annotated genomes. These factors include, but are not limited to, capsule formation, adherence, ability to form hydrogen sulphide from gluthathione, etc (38-41). This work will also be completed with the objective of publication and validation of two novel genera and a novel *Parvimonas* specie in the International Journal of Systematic and Evolutionary Microbiology.

References

1. **Ezaki, T., H. Oyaizu, and E. Yabuuchi. (1992).** The anaerobic Gram-positive cocci, p. 1879–1892. In A. Balows, H. G. Tru¨per, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes, 2nd ed. Springer-Verlag KG, Berlin, Germany.

2. **Brook, I. (1995).** Anaerobic cocci, p. 2204–2206. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), The principles and practice of infectious diseases, 4th ed. Churchill Livingstone, Inc., New York, N.Y.

3. **Holdeman Moore, L. V., Johnson, J. L. and Moore, W. E. C. (1986).** Genus Peptostreptococcus, p. 1083–1092. In P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore, Md.

4. **Murphy, E.C. and Frick, I.M. (2013).** Gram-positive anaerobic cocci–commensals and opportunistic pathogens. FEMS Microbiol. Rev., **37**(4), pp.520-553.

5. **Song Y. and Finegold S. (2015).** *Peptostreptococcus, Finegoldia, Anaerococcus, Peptoniphilus, Veillonella*, and Other Anaerobic Cocci, p 909-919. *In* Jorgensen J, Pfaller M, Carroll K, Funke G, Landry M, Richter S, Warnock D (ed), Manual of Clinical Microbiology, 11th Edn. ASM Press, Washington, DC. **doi: 10.1128/9781555817381.ch51**

6. **Kluyver, A. J., and van Niel, C. B. (1936).** Prospects for a natural system of classification of bacteria. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. II, **94**, pp.369–403.

7. **Prevot, A. R. (1948).** Manuel de classification et de determination des bacteries anaerobies, 2nd ed. Masson, Paris, France.
8. **Rogosa, M. (1971).** *Peptococcaceae,* a new family to include the gram-positive, anaerobic cocci of the genera *Peptococcus, Peptostreptococcus*, and *Ruminococcus*. Int. J. Syst. Bacteriol., **21**(3), pp.234–237.
9. **Rogosa, M. (1974).** Family III. *Peptococcaceae*, p. 517–527. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore, Md.
10. **Holdeman, L. V., and Moore, W. E. C. (1974).** New genus, *Coprococcus*, twelve new species, and emended descriptions of four previously described species of bacteria from human feces. Int. J. Syst. Bacteriol., **24**(2), pp.260–277.
11. **Wexler, H. M. (1993).** Susceptibility testing of anaerobic bacteria—the state of the art. Clin. Infect. Dis. 16(Suppl. 4):S328–S333.
12. **Huss, V. A. R., Festl, H. and Schleifer, K. H. (1984).** Nucleic acid hybridisation studies and deoxyribonucleic acid base compositions of anaerobic Gram-positive cocci. Int. J. Syst. Bacteriol., **34**(2), pp.95–101.
13. **Ezaki, T., Oyaizu, H. and Yabuuchi, E. (1992).** The anaerobic Gram-positive cocci, p. 1879–1892. *In* A. Balows, H. G. Tru¨per, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes, 2nd Edn. Springer-Verlag KG, Berlin, Germany.
14. **Ezaki, T., Yamamoto, N., Ninomiya, K., Suzuki, S. and Yabuuchi, E. (1983).** Transfer of *Peptococcus indolicus, Peptococcus asaccharolyticus, Peptococcus prevotii* and *Peptococcus magnus* to the genus *Peptostreptococcus* and proposal

of *Peptostreptococcus tetradius* sp. nov. Int. J. Syst. Bacteriol., **33**(4), pp. 683–698.

15. **Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J. J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. and Farrow, J. A. E. (1994).** The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. Int. J. Syst. Bacteriol., **44**(4), pp. 812–826.
16. **Ezaki, T., Li, N., Hashimoto, Y., Miura, H. and Yamamoto, H. (1994).** 16S ribosomal DNA sequences of anaerobic cocci and proposal of *Ruminococcus hansenii* comb. nov. and *Ruminococcus productus* comb. nov. Int. J. Syst. Bacteriol., **44**(1), pp.130–136.
17. **Lawson, P. A., Llop-Perez, P., Hutson, R. A., Hippe, H. and Collins, M. D. (1993).** Towards a phylogeny of the clostridia based on 16S rRNA sequences. FEMS Microbiol. Lett., **113**(1), pp. 87–92.
18. **Paster, B. J., Russell, J. B., Yang, C. M. J., Chow, J. M., Woese, C. R. and Tanner, R.S. (1993).** Phylogeny of the ammonia-producing ruminal bacteria *Peptostreptococcus anaerobius, Clostridium sticklandii,* and *Clostridium aminophilum* sp. nov. Int. J. Syst. Bacteriol., **43**(1),pp. 107–110.
19. **Willems, A., and Collins, M. D. (1994).** Phylogenetic placement of *Sarcina ventriculi* and *Sarcina maxima* within Group I *Clostridium*, a possible problem for future revision of the genus *Clostridium*. Request for an opinion. Int. J. Syst. Bacteriol., **44**(3),pp. 591–593.
20. **Murdoch, D.A. and Shah, H.N. (1999).** Reclassification of *Peptostreptococcus magnus* (Prevot 1933) Holdeman and Moore 1972 as *Finegoldia magna* comb.

nov. and *Peptostreptococcus micros* (Prevot 1933) Smith 1957 as *Micromonas micros* comb. nov. Anaerobe, **5**(5), pp.555–559.

21. **Tindall, B.J. and Euzéby, J.P. (2006).** Proposal of *Parvimonas* gen. nov. and *Quatrionicoccus* gen. nov. as replacements for the illegitimate, prokaryotic, generic names *Micromonas* Murdoch and Shah 2000 and *Quadricoccus* Maszenan et al. 2002, respectively. Int. J. Syst. Evol. Microbiol., **56**(11), pp. 2711-2713.
22. **Ezaki, T., Kawamura, Y., Li, N., Li, Z.Y., Zhao, L. and Shu, S.E. (2001).** Proposal of the genera *Anaerococcus* gen. nov., *Peptoniphilus* gen. nov. and *Gallicola* gen. nov. for members of the genus *Peptostreptococcus*. Int. J. Syst. Evol. Microbiol., **51**(4), pp.1521-1528.
23. **Murdoch, D.A., Mitchelmore, I.J. and Tabaqchali, S. (1988).** *Peptostreptococcus micros* in polymicrobial abscesses. The Lancet, **331**(8585), p.594-602.
24. **Han, A., Zenilman, J.M., Melendez, J.H., Shirtliff, M.E., Agostinho, A., James, G., Stewart, P.S., Mongodin, E.F., Rao, D., Rickard, A.H. and Lazarus, G.S. (2011).** The importance of a multifaceted approach to characterizing the microbial flora of chronic wounds. Wound Repair Regen., **19**(5), pp.532-541.
25. **Bjarnsholt, T., Kirketerp-Møller, K., Jensen, P.Ø., Madsen, K.G., Phipps, R., Krogfelt, K., Høiby, N. and Givskov, M. (2008).** Why chronic wounds will not heal: a novel hypothesis. Wound Repair Regen., **16**(1), pp.2-10.

26. **Huang, C.Y., Garcia, J.L., Patel, B.K., Cayol, J.L., Baresi, L. and Mah, R.A. (2000).** *Salinivibrio costicola* subsp. *vallismortis* subsp. nov., a halotolerant facultative anaerobe from Death Valley, and emended description of *Salinivibrio costicola*. Int. J. Syst. Evol. Microbiol., **50**(2), pp.615-622.
27. **Hutson R.A., Thompson D.E. and Collins, M.D. (1993).** Genetic interrelationships of saccharolytic *Clostridium botulinum* types B, E and F clostridia as revealed by small-subunit sequences. FEMS Microbiol. Lett., **108**(1), pp.103–110.
28. **Gram, C. (1884).** Ueber die isolirte Farbung der Schizomyceten in Schnitt-und Trockenpraparaten. Fortschritte der Medicin, **2**, pp.185-189.
29. **Iino, T. and Enomoto, M. (1971).** Chapter IV Motility. *In* Methods in Microbiology (**vol. 5**), pp. 145-163. Academic Press.
30. **Clarke, H. and Cowan, S.T. (1952).** Biochemical methods for bacteriology. J. Gen. Microbiol. **6**, pp.187–197.
31. **Yoon, S.H., Ha, S.M., Kwon, S., Lim, J., Kim, Y., Seo, H. and Chun, J. (2017).** Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. Int. J. Syst. Evol. Microbiol., **67**(5), pp. 1613-1617.
32. **Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011).** MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol., **28**(10), 2731–2739.

33. **Rainey, F. and Oren, A. (2011).** Taxonomy of prokaryotes (vol. 38). Academic Press, London, UK, pp. 7, 101-182.

34. **Schumann P. (2011).** Peptidoglycan Structure. In: Oren A (ed) Methods in Microbiology (Vol. 38). Elsevier, London, UK, pp. 101–129.

35. **Kämpfer, P. and Kroppenstedt, R.M. (1996).** Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. Can. J. Microbiol., **42**(10), pp.989-1005.

36. **Sasser, M. (1990).** Identification of bacteria by gas chromatography of cellular fatty acids. Technical Note 101, revised February 2001. MIDI Labs Inc., Newark, DE, pp. 1-6

37. **Yarza, P., Yilmaz, P., Pruesse, E., Glöckner, F.O., Ludwig, W., Schleifer, K.-H., Whitman, B., Euzeby, J., Amann, R. and Rosselló-Móra, R. (2014).** Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. Nat. Rev. Microbiol., **12**(9), pp. 635–645.

38. **Brook, I. and Walker, R.I. (1985).** The role of encapsulation in the pathogenesis of anaerobic gram-positive cocci. Can. J. Microbiol., **31**(2), pp.176-180.

39. **Brook, I. (1987).** Bacteraemia and seeding of capsulate *Bacteroides* spp. and anaerobic cocci. J. Med. Microbiol., **23**(1), pp.61-67.

40. **Brook, I. (1988).** Enhancement of growth of aerobic, anaerobic, and facultative bacteria in mixed infections with anaerobic and facultative gram-positive cocci. J. Surg. Res., **45**(2), pp.222-227.

41. **Carlsson, J., Larsen, J.T. and Edlund, M.B. (1993).** *Peptostreptococcus micros* has a uniquely high capacity to form hydrogen sulfide from glutathione. Oral Microbiol. Immunol., **8**(1), pp.42-45.