Accepted Manuscript

Title: Detection, isolation and characterization of *Fusobacterium gastrosuis* sp. nov. colonizing the stomach of pigs

Author: C. De Witte B. Flahou R. Ducatelle A. Smet E. De Eruyne M. Cnockaert B. Taminiau G. Daube P. Vandamme F. Haesebrouck

PII: S0723-2020(16)30111-4
DOI: http://dx.doi.org/doi:10.1016/j.syapm.2016.10.001
Reference: SYAPM 25804

To appear in:

Received date: 24-8-2016
Revised date: 12-10-2016
Accepted date: 17-10-2016

Please cite this article as: C.De Witte, B.Flahou, R.Ducatelle, A.Smet, E.De Eruyne, M.Cnockaert, B.Taminiau, G.Daube, P.Vandamme, F.Haesebrouck, Detection, isolation and characterization of Fusobacterium gastrosuis sp.nov.colonizing the stomach of pigs, Systematic and Applied Microbiology http://dx.doi.org/10.1016/j.syapm.2016.10.001

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

DETECTION, ISOLATION AND CHARACTERIZATION OF *FUSOBACTERIUM GASTROSUIS* SP. NOV. COLONIZING THE STOMACH OF PIGS

DE WITTE C.[1], FLAHOU B.[1], DUCATELLE R.[1], SMET A.[1], DE BRUYNE E.[1], CNOCKAERT M.[2], TAMINIAU B.[3], DAUBE G.[3], VANDAMME P.[2*], HAESEBROUCK F.[1*]

[1] Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium; [2] Department of Biochemistry and Microbiology, Faculty of Sciences, Ghent University, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium; [3] Department of Food Sciences, FARAH, Université de Liège, Avenue de Cureghem 10, 4000 Liège, Belgium; * shared senior authorship

ACCEPTED MANUSCRIPT

Summary

Nine strains of a novel *Fusobacterium* sp. were isolated from the stomach of 6-8 months old and adult pigs. The isolates were obligately anaerobic, although they endured 2 hours exposure to air. Phylogenetic analysis based on *16S rRNA* and *gyrase B* genes demonstrated that the isolates showed high sequence similarity with *Fusobacterium mortiferum, Fusobacterium ulcerans, Fusobacterium varium, Fusobacterium russii* and *Fusobacterium necrogenes*, but formed a distinct lineage in the genus *Fusobacterium.* Comparative analysis of the genome of the type strain of this novel *Fusobacterium* sp. confirmed that it is different from other recognized *Fusobacterium* spp. DNA-DNA hybridization, fingerprinting and genomic %GC determination further supported the conclusion that the isolates belong to a new, distinct species. The isolates were also distinguishable from these and other *Fusobacterium* spp. by phenotypical characterization. The strains produced indole and exhibited proline arylamidase and glutamic acid decarboxylase activity. They did not hydrolyse esculin, did not exhibit pyroglutamic acid arylamidase, valine arylamidase, α-galactosidase, β-galactosidase, β-galactosidase-6-phosphate or α-glucosidase activity nor produced acid from cellobiose, glucose, lactose, mannitol, mannose, maltose, raffinose, saccharose, salicin or trehalose. The major fatty acids were C16 : 0 and C18 : 1ω9c. The name *Fusobacterium gastrosuis* sp. nov. is proposed for the novel isolates with the type strain CDW1(T) (= DSM 101753(T) = LMG 29236(T)). We also demonstrated that *Clostridium rectum* and *Fusobacterium mortiferum* represent the same species, with nomenclatural priority for the latter.

Key words: *Fusobacterium* – *Fusobacterium gastrosuis* - pig – stomach – *Clostridium rectum*

[1] Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium; [2] Department of Biochemistry and Microbiology, Faculty of Sciences, Ghent University, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium; [3] Department of Food Sciences, FARAH, Université de Liège, Avenue de Cureghem 10, 4000 Liège, Belgium; * shared senior authorship

Introduction

Fusobacteria have been described as anaerobic, non-motile, non-sporulating, fastidious Gram-negative rods that produce butyric acid as major end product of their metabolism [15]. The genus currently consists of 15 recognized species [8,11,17]. Although Fusobacteria are normal constituents of the oropharyngeal, gastrointestinal and genital microbiota, they are the second most frequently isolated anaerobic microbial group from clinical samples of both human and animal origin, especially from cases of pyonecrotic infections [38]. Considering their fastidious nature, this reported detection frequency still may be an underestimation of the true frequency [21]. In human patients *Fusobacterium* spp. have been described to play a role in gingivitis and dental plaque formation [3,7,16], whereas in pigs they are associated with lameness and facial skin necrosis [6,19,46]. In cattle and sheep they are involved in necrotic laryngitis and footrot [19,37]. In horses they may be associated with necrotic oral and lower respiratory tract diseases as well as intra-abdominal abscesses [19].

Ulceration of the non-glandular *pars oesophagea* of the stomach is very common in pigs and can lead to discomfort, pain, decreased daily weight gain and even sudden death. *Helicobacter* (*H.*) *suis*, *Lactobacillus* spp. and *Bacillus* spp. have all been suggested to play a role in the development of gastric ulceration. Nevertheless, the exact etiology of this disease still is a matter of debate and is clearly multifactorial [18,21,24]. Results of a recent metagenomic analysis of the gastric microbiota of 20 pigs of 6-8 months old showed that an unidentified *Fusobacterium* sp. was abundantly present, representing up to 20% of the gastric microbial community. Compared to *H. suis*-negative animals, higher numbers of this *Fusobacterium* sp. were detected in *H. suis*-infected animals (unpublished results).

The main aim of the present study was to isolate and characterize this putative new *Fusobacterium* sp. This is required to enable further research into its possible pathogenic significance and role in the pathogenesis of ulceration of the non-glandular part of the stomach in pigs. Therefore, *Fusobacterium* isolates obtained from porcine stomachs were characterized phenotypically and genotypically. The new *Fusobacterium* sp. showed the highest sequence similarity with *Fusobacterium* (*F.*) *mortiferum, F. russii, F. ulcerans, F. varium, F. necrogenes* and, surprisingly,

ACCEPTED MANUSCRIPT

Clostridium (*C.*) *rectum.* Although clostridia are Gram-positive bacteria capable of producing endospores, in previous studies it was described that *C. rectum* is closely related to *Fusobacterium* spp. [4,10,26]. The second aim of the present study was, therefore, to try and solve this inconsistency in the classification of *C. rectum*.

Material and methods

Isolation from porcine stomachs

Thirty five stomachs of 6-8 months old pigs and 25 stomachs of adult sows were collected over a period of 8 months from different slaughterhouses in Flanders, Belgium. The stomachs were transported immediately to the laboratory and stored at 4°C until further examination within 2 hours. The stomachs were opened along the greater curvature one at the time and rinsed with sterile tap water. Swabs were taken from each stomach region (*pars oesophagea*, cardia, fundus and antrum), streaked on Columbia agar plates® (Oxoid, Basingstoke, United Kingdom) supplemented with 5% defibrinated sheep blood® (E&O laboratories, Bonnybridge, Scotland), 100 mg/L Neomycin®, 5 mg/L Vancomycin® and 1 mg/L Erythromycin® (Sigma-Aldrich, Saint Louis, Missouri), and incubated anaerobically for 3 days at 37°C. Based on previous descriptions of the colony morphology of *Fusobacterium* spp. [5,38,40], colonies of interest were purified on Columbia agar plates® supplemented with 5% defibrinated sheep blood® and incubated anaerobically for 3 days at 37°C. A Gram-staining was performed on purified cultures. When this staining revealed Gram-negative rods, several colonies were suspended in 200 µl of an in-house bacterial preservation medium [21] for storage at -70°C. In order to identify the bacterium at species level, DNA was extracted using PrepMan Ultra Sample Preparation Reagent® (Life Technologies, Carlsbad, California) according to the manufacturer's instructions. The near complete *16S rRNA* gene was amplified with αβ-NOT and ωMB primers [2] and sequenced by GATC Biotech, Supremerun sequencing® (Constance, Germany). The obtained sequences were analyzed with Vector NTI® (Life Technologies, Carlsbad, California). Finally, a comparison was made between the 16S rRNA sequences of the isolates and the previously detected *Fusobacterium* sp. (pig gastric microbiota metagenomic analysis, unpublished results). Colonies were considered as the putative new *Fusobacterium* sp. of interest when at least 99% identity was obtained.

Determination of species with high sequence similarities

A consensus sequence of the *16S rRNA* gene of the 9 isolates was obtained using the BioEdit Sequence Alignment Editor and ClustalIW Multiple Alignment® tools (Ibis Biosciences, California, United States) in order to identify potential nucleotide differences between the isolates. The 16S rRNA sequences of the 9 isolates were blasted using EZ taxon database of EZBioCloud® (ChunLab, Korea) and the species showing the highest sequence similarities were selected for further characterization.

The following type strains of *F. mortiferum, F. russii, F. ulcerans, F. varium, F. necrogenes* and *C. rectum* were obtained from the Culture Collection of University of Göteborg (CCUG) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) bacterial culture collection: CCUG 14475^T, CCUG 45924, CCUG 50053^T, CCUG 4858^T, CCUG 4949^T and DSM 1295^T, respectively. In order to perform the characterization tests, the organisms were grown on Columbia agar plates® supplemented with 5% defibrinated sheep blood® for 3 days at 37°C.

Genotypic characterization

Both *16S rRNA* and *gyrase B* (*gyrB*) genes were selected for phylogenetic analysis. The *16S rRNA* gene of the 9 isolates and the species showing high sequence similarities, were amplified and sequenced as described above. The *gyrB* gene was amplified using UP-1 and UP-2r primers as described previously [44], except that 35 cycles were used with an annealing temperature of 57°C. A consensus sequence of the 9 isolates was also created for the *gyrB* gene, as described above for the *16S rRNA* gene, to determine the sequence similarity. The sequences of both genes were compared with those in the NCBI database using the BLAST search tool. The available *16S rRNA* and *gyrB* gene sequences of the type strains of all recognized *Fusobacterium* spp., were selected for phylogenetic analysis. A multiple alignment was performed using MUSCLE® (EMBL-EBI, Cambridge, United Kingdom) with Gblocks as alignment curation. A phylogenetic tree was created using PhyML® (ATGC, Montpellier, France) with the

ACCEPTED MANUSCRIPT

maximum likelihood method and a bootstrap value of 1000 to estimate the robustness of the topology of the tree. Finally, the 16S rRNA and gyrB trees were visualized using TreeDyn® (GEMI Bioinformatics, Montpellier, France). Maximum parsimony and neighbour-joining algorithm based trees were compared with the maximum likelihood based tree in order to determine the closest phylogenetic neighbours and conserved roots in a reliable way. Finally, gyrB derived amino-acid trees were constructed and compared to the nucleotide based trees.

Isolate CDW1 was chosen as type strain. The genomic DNA G+C content of this strain and *F. mortiferum, F. necrogenes* and *C. rectum* were determined [9] and DNA-DNA hybridizations were performed [14]. Repetitive sequence-based PCR fingerprinting with the $(GTG)_5$ primer [39] was also performed to confirm the non-clonal nature of the 9 isolates.

In order to sequence the genome of isolate CDW1, it was cultivated on Columbia agar plates® supplemented with 5% defibrinated sheep blood® and incubated anaerobically for 3 days at 37°C. Subsequently, genomic DNA was extracted using the Gentra Puregene Yeast/Bact. Kit® (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The genome was sequenced using SMRT® Technology PacBio RS II (GATC Biotech®, Constance, Germany), with an average genome coverage of 100 and a run mode of 240-min movie. Gene finding and automatic annotation were performed using the Rapid Annotation Subsystems Technology (RAST) server [1,34]. The available annotated draft and complete genomes of different *Fusobacterium* spp. were obtained from the NCBI database and selected for further phylogenetic comparison. After analyzing these annotated genome assemblies, pangenomes were created using the rapid large-scale prokaryote pan genome analysis (Roary) tool [35]. Briefly, the annotated proteins from all isolates were used for a BLASTP all-versus-all sequence similarity search. From the BLASTP output, groups of orthologous proteins were predicted using the Orthagogue and MCL software [12]. Orthologous groups with exactly one representative protein from each of the input strains were considered to be part of the *Fusobacterium* core genome. This obtained core genome alignment was then used for phylogenetic tree construction using PhyML® (ATGC, Montpellier, France) with maximum likelihood method and a bootstrap value of 1000. Finally, the tree was visualized using the interactive tree of life (iTOL) tool (http://itol.embl.de/). Using the Genome-to-Genome Distance calculator (GGDC; http://ggdc.dsmz.de), whole-genome distances were determined in order to assess the degree of DNA-DNA hybridization between isolate CDW1 and other *Fusobacterium* spp. In addition, the average nucleotide identity (ANI) values were obtained using the online “average nucleotide identity calculator” tool (enve-omics.ce.gatech.edu/ani/index). In order to assess genomic changes, for example due to recombination, the multiple genome alignment tool (Mauve) was used (The Darling lab, http://darlinglab.org/mauve/mauve.html). This tool identifies conserved regions internally free from genome rearrangements which are referred to as Locally Collinear Blocks (LCBs).

Phenotypic characterization

API 20A, Rapid ID 32A and API ZYM systems® (Biomérieux, Marcy l'Etoile, France) were used to test substrate utilization and enzymes properties of the isolates and the species showing the highest sequence similarities (i.e. *F. mortiferum, F. russii, F. ulcerans, F. varium, C. rectum* and *F. necrogenes*). The instructions of the manufacturer were followed, with the exception that API ZYM was incubated anaerobically. Whole-cell fatty acid methyl esters (FAME) composition of the isolates and their phylogenetic neighbours were determined using an Agilent Technologies 6890N gas chromatograph® (Agilent Technologies, Santa Clara, United States). Extraction and analysis of the FAME were performed according to the recommendations of the Microbial Identification System, Sherlock version 3.10® (MIDI, Delaware, United States). Peaks of the FAME profiles were identified using the TSBA50 identification library version 5.0® (MIDI, Delaware, United States). For the characterization tests, the organisms were grown under identical conditions, i.e. on Columbia agar plates® supplemented with 5% defibrinated sheep blood® for 3 days at 37°C under anaerobic conditions.

GenBank/EMBL/DDBJ Accession numbers

The GenBank/EMBL/DDBJ accession numbers of the *16S rRNA* gene sequences of *Fusobacterium gastrosuis* DSM 101753^T (= LMG 29236^T = CDW1^T), *Fusobacterium mortiferum* CCUG 14475^T, *Fusobacterium necrogenes* CCUG 4949^T, *Clostridium rectum* DSM 1295^T, *Fusobacterium varium* CCUG 4858^T, *Fusobacterium ulcerans* CCUG 50053^T and *Fusobacterium russii* CCUG 45924 are LN906797.1, LT574675.1, LT574677.1, LT574676.1,

ACCEPTED MANUSCRIPT

LT594100.1, LT594101.1 and LT594099.2 respectively. The gyrB sequences were deposited under the accession numbers LN906798.1, LT574675.1, LT574677.1, LT574676.1, LT594103.1, LT594104.1 and LT594102.1, respectively. The GenBank/EMBL/DDBJ accession number of the complete and closed genome of *Fusobacterium gastrosuis* sp. nov. DSM 101753^T (= LMG 29236^T = CDW1^T) is LT607734.1.

Results and discussion

Isolation of 9 strains of the putative new Fusobacterium *sp.*

Nine isolates of the putative new *Fusobacterium* sp. were obtained: 6 from pigs at slaughter age (CDW1-6) and 3 from adult sows (CDW7-9). CDW1-CDW4 and CDW7-CDW9 originated from the non-glandular *pars oesophagea* and CDW 5-6 from the cardia and antrum, respectively [38]. All isolates formed circular, white, slightly elevated colonies of approximately 0.4 cm in diameter surrounded by a very narrow zone of complete hemolysis, often only observed under the colony (supplementary figure S1). The isolates were obligately anaerobic, although they endured 2 hours of exposure to air. Gram-staining revealed 1.5-2 µm long and 0.3-0.5 µm wide Gram-negative rods with rounded ends, non-capsulated, with presence of swelling and globular forms and occasionally long filaments. The species showing high sequence similarity, namely *F. mortiferum, F. russii, F. ulcerans, F. varium, C. rectum and F. necrogenes*, also appeared as Gram-negative rods. In addition, *C. rectum* showed presence of a low number of endospores.

Genotypic characterization reveals a clear distinction between the novel Fusobacterium *sp. and other* Fusobacterium *spp.*

The near complete 16S rRNA consensus sequence was 1364 bp long and showed presence of only 2 wobbles, demonstrating that the isolates showed 99% similarity with each other. Based on the 16S rRNA sequences of the 9 isolates, *F. mortiferum, F. ulcerans, F. varium, F. russii, F. necrogenes* and *C. rectum* were identified as the species with the highest sequence similarities, showing average values of 96%, 96%, 96%, 96%, 95% and 95%, respectively. These low similarities already suggested that the isolated *Fusobacterium* sp. represented a new species.

Apart from the *16S rRNA* gene, the *gyrB* gene was selected for genotypic characterization as housekeeping genes often possess high discriminatory power for phylogenetic analysis [9]. The sequence of this gene was available for the majority of *Fusobacterium* spp. in the NCBI database, in strong contrast with other housekeeping genes that were only available for a few species. Additionally, the *16S rRNA* gene already showed similarity values below 97% with other *Fusobacterium* spp. For those reasons 1 additional housekeeping gene was sequenced in order to confirm the results obtained by 16S rRNA sequencing. The gyrB consensus sequence was 1066 bp long with presence of 21 wobblers. The 9 isolates showed 98-99% similarity with each other and 84%, 75%, 75%, 74%, 74% and 72% similarity with *F. russii, F. mortiferum, C. rectum, F. necrogenes, F. varium* and *F. ulcerans,* respectively.

Phylogenetic trees based on *16S rRNA* and *gyrB* genes demonstrated that the isolates formed a distinct lineage in the genus *Fusobacterium* and this clustering was further supported by bootstrap values of 99-100% (figures 1 and 2). The different methods for construction of the trees resulted in similar clustering and positioning of roots, however, the neighbour-joining method yielded higher bootstrap values than the maximum likelihood. As indicated by the lower similarities, *gyrB* seemed to be more sensitive than *16S rRNA* to differentiate the novel *Fusobacterium* sp. from other *Fusobacterium* spp. In contrast with the conserved *16S rRNA* gene, protein encoding genes, such as *gyrB*, evolve faster and are therefore more useful to discriminate among closely related species [37]. The gyrB derived amino-acid trees (supplementary figure S2) were comparable with the nucleotide based tree, although seemingly less reliable for further (sub)species identification. For example, *F. periodonticum, F. nucleatum* subspecies*, F. canifelinum* and *F. naviforme* clustered together as one group.

Isolate CDW1 was chosen as type strain since this was the first isolated strain and all isolates showed similar genotypic and phenotypic properties. *F. mortiferum* was selected for genomic G+C content determination, since this species showed the highest sequence similarity with the *16S rRNA* sequences of the isolates. Since other studies had shown that *C. rectum* clustered together with *F. necrogenes* [4,10,26], these 2 species were also selected for further genotypic characterization. The genomic G+C contents were 28.5, 29.9, 29.7 and 29.7 mol% for the novel

ACCEPTED MANUSCRIPT

Fusobacterium sp., *F. necrogenes* CCUG 4949^T, *F. mortiferum* CCUG 14475^T and *C. rectum* DSM 1295^T, respectively. The 16S rRNA and gyrB sequences and GC% were identical for *F. mortiferum* CCUG 14475^T and *C. rectum* DSM 1295^T, strongly suggesting that these presented the same species. The repetitive sequence-based PCR fingerprinting with the $(GTG)_5$ primer showed a banding pattern for 7 of the 9 isolates (CDW 1, CDW 3-5, CDW 7-9), *F. mortiferum, F. necrogenes* and *C. rectum*. It was not possible to obtain a banding pattern for strains CDW 2 and 6 using the $(GTG)_5$ primer or other primers. Analysis confirmed that the 7 isolates were different from each other, since no identical pattern was obtained (supplementary figure S3). This was not surprisingly as all isolates were obtained from different pigs that were sampled on different days from 2 distinct slaughterhouses. Although no banding pattern was obtained for strain CDW 2 and 6, they were most likely also non-clonal strains since they were obtained from different stomachs and since the gyrB sequence similarity between the 9 isolates was not 100%. The level of DNA-DNA hybridization between *F. mortiferum* CCUG 14475^T and *C. rectum* DSM 1295^T was 95.5% (reciprocal hybridization values of 95 and 96%) and DNA fingerprinting showed an identical pattern, demonstrating that these are the same species.

To further identify these isolates as a distinct species, whole genome sequencing was performed. The complete and closed genome of the type strain of the new *Fusobacterium* sp. was 1.82 Mb large, with following characteristics: 28.2% GC, 1771 coding sequences, 255 subsystems, 359 hypothetical proteins and 64 RNAs. The phylogenetic tree based on 486 core genes confirmed that the novel *Fusobacterium* sp. formed a distinct lineage in the *Fusobacterium* genus, supported by bootstrap values of 100% (figure 3). DNA-DNA hybridization parameters, assessed by calculating whole-genome distances, varied between 12 and 40%, which is lower than the threshold of 70% for belonging to the same species [41]. In addition, using logistic regression this resulted in a probability of 0.0 to 2.86% that the putative new *Fusobacterium* sp. belonged to a recognized *Fusobacterium* species or subspecies. The ANI values between the new *Fusobacterium* sp. and other *Fusobacterium* spp. varied between 75 and 80%, which is below the generally accepted threshold of 95% for belonging to the same species [23]. Finally, when aligning the genome of the new *Fusobacterium* sp. with other *Fusobacterium* spp., LCBs values varied between 120 and 250. When comparing isolate CDW1 with its closest phylogenetic neighbour, *F. russii*, it was clear that the genomes were sufficiently different, showing a LCB value of 143 (supplementary figure S4). As genome sequences are not yet available for some *Fusobacterium* spp., the results of this genomic analysis should be considered as incomplete. However, they provide further evidence that the isolated *Fusobacterium* sp. belongs to a new species.

Phenotypic characterization confirms the distinction of the novel Fusobacterium *sp.*

Substrate utilization and enzyme properties were identical for all 9 isolates. These isolates and *F. mortiferum, F. russii, F. varium, F. ulcerans, C. rectum* and *F. necrogenes* did not exhibit urease, catalase, nitrate reduction, gelatin hydrolysis, α-fucosidase, β-glucosidase, β-glucuronidase, α-mannosidase, lipase (C14) or trypsin activity. Activity of acid phosphatase, alkaline phosphatase and esterase (C4, although weakly) was detected. They did not produce acid from arabinose, melezitose, rhamnose or sorbitol. Differences in properties between the isolates and *F. mortiferum, F. russii, F. ulcerans, F. varium, C. rectum* and *F. necrogenes* are presented in table 1. The cellular fatty acid profiles are presented in table 2.

Reclassification of Clostridium rectum

Phylogenetic analysis and phenotypical characterization revealed virtually no differences between *C. rectum* DSM 1295^T and *F. mortiferum* CCUG 14475^T. Gram-staining revealed the presence of endospores for *C. rectum* DSM 1295^T, but not for *F. mortiferum* CCUG 14475^T. However, analysis of the *F. mortiferum* (ACDB00000000.2) genome by RAST showed presence of coding sequences for spore maturation protein A and B (spmA, spmB) and spore photoproduct lyase (SPL) [36]. The SPL coding sequence was also detected in the novel *Fusobacterium* sp. Similarly, another study demonstrated the presence of the putative septation protein in *F. nucleatum* [32]. Due to the ability of endospore formation of *C. rectum* and due to the presence of genes associated with spore formation in other *Fusobacterium* spp., the *Fusobacterium* taxon should be emended to take into account the possibility of spore formation, as already stated by Collins and colleagues [10]. *C. rectum* DSM 1295^T might thus be considered as a variant of *F. mortiferum* that not only contains, but also expresses genes associated with spore formation.

ACCEPTED MANUSCRIPT

Although not all available *C. rectum* type strain subcultures were tested in the present study, similar results can be expected since the 16S rRNA sequence of *C. rectum* DSM 1295^T showed 99.9% similarity with ATCC 25751^T (=NCIMB 10651^T, accession number X77850.1) and JCM 1412^T (accession number LC053839.1) and subcultures of the *C. rectum* type strain that are present in other bacteria collections originated from ATCC 25751^T. In addition, the characteristics originally described for *C. rectum* VPI 2488^T [22,30,42] were almost identical to the phenotypic traits of DSM 1295^T obtained in the present study, with the following additions. The API ZYM showed negative reactions for leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, alfa-chymotrypsin, N-acetyl-beta-glucosaminidase, alfa-mannosidase, alfa-fucosidase, beta-glucosidase, beta-glucuronidase and esterase lipase (C8), but positive reactions for alkaline phosphatase, esterase (C4, although weakly) and naphtol-AS-BI-phosphohydrolase. Tests for enzyme activities by use of Rapid ID 32 A showed positive reactions for beta-galactosidase 6 phosphatase, pyroglutamic acid arylamidase, but not for arginine dihydrolase, alfa-arabinosidase, glutamic acid decarboxylase, alfa-fucosidase, arginine arylamidase, proline arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, glutamyl glutamic acid arylamidase, serine arylamidase and urease. The API 20 A showed a negative reaction for catalase activity. Deviations from the original description were that *C. rectum* DSM 1295^T produced acid, although weakly, from maltose, mannose, raffinose and trehalose, but this was most likely due to differences in sensitivity of the applied test procedures. The genotypic and phenotypic similarities with the original *C. rectum* strain strongly indicate that the present results were not caused by a contamination in the lineage maintained at the DSMZ collection and that a subculture of the original *C. rectum* VPI 2488^T, which is no longer available, was used.

These findings were similar to those of Lee and colleagues who demonstrated a close phylogenetic relationship between *C. rectum* ATCC 25751^T and *F. mortiferum* DSM 19809^T [26]. Additionally, the 16S rRNA sequence based tree (Release LTPs123) obtained from the SILVA database also showed clustering of *C. rectum* NCIMB 10651^T within the *Fusobacterium* genus [45].

As already suggested in several other studies [4,10,26,33,43], the classification of *C. rectum* must be revised. Data from our and previous studies show that *F. mortiferum* and *C. rectum* must be considered as heterotypic synonyms. Phylogenetically, these bacteria belong to the genus *Fusobacterium* and therefore the name *F. mortiferum* has nomenclatural priority. An emended species description is presented below.

Description of *Fusobacterium gastrosuis* sp. nov.

Fusobacterium gastrosuis sp. nov. (gas. tro. su'is., Gr. n. gaster gastros, stomach; L. n. sus suis, a pig; L. gen. n. gastrosuis, from the stomach of a pig). After anaerobic incubation on Columbia agar plates® supplemented with 5% defibrinated sheep blood® for 3 days at 37°C, the colonies are circular, white with a translucent border, slightly elevated with a smooth edge and 0.4 cm in diameter with presence of a narrow zone of complete hemolysis. Gram-staining of these colonies shows 1.5-2 µm long and 0.3-0.5 µm wide Gram-negative rods with rounded ends, non-capsulated with presence of swelling and globular forms and occasionally long filaments. The strains produce indole and exhibit proline arylamidase and glutamic acid decarboxylase activity. They do not hydrolyse esculin, do not exhibit pyroglutamic acid arylamidase, valine arylamidase, α-galactosidase, β-galactosidase, β-galactosidase-6-phosphate or α-glucosidase activity nor produce acid from cellobiose, glucose, glycerol, lactose, mannitol, mannose, maltose, raffinose, saccharose, salicin or trehalose. The major fatty acids are C16 : 0 and C18 : 1ω9c. The type strain, CDW1(T) (= DSM 101753(T) = LMG 29236(T)), was isolated from the *pars oesophagea* of a 6-months old pig, in Ghent (Belgium) in 2014. The G+C content of the DNA of the type strain is 28.2 mol%.

Transfer of *Clostridium rectum* [22,30] to *Fusobacterium mortiferum* [20,29]

Phylogenetic analysis and phenotypic characterization indicate that *C. rectum* and *F. mortiferum* should be considered as a single species. The names *C. rectum* [22,30] and *F. mortiferum* [20,29] can be considered as heterotopic synonyms. According to Rule 15 and 17 of the *Bacteriological Code*, we conclude that *C. rectum* should be given the name *F. mortiferum*, with strain ATCC 25557(T) (=350A(T), CCUG 14475(T), DSM 19809(T), VPI 4123A(T)) as type strain.

ACCEPTED MANUSCRIPT

Emended description of *Fusobacterium mortiferum*

Fusobacterium mortiferum (mor.ti'fer.um., L. neut. adj. *mortiferum* death-bringing, death-bearing).

The description is as given for *F. mortiferum* (type strain = CCUG 14475(T), 350A(T), ATCC 25557(T), DSM 19809(T), VPI 4123A(T)) [42] with the following additions. The API ZYM shows negative reactions for leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, alfa-chymotrypsin, N-acetyl-beta-glucosaminidase, alfa-mannosidase, alfa-fucosidase, beta-glucosidase, beta-glucuronidase and esterase lipase (C8), but positive reactions for alkaline phosphatase, esterase (C4, although weakly) and naphtol-AS-BI-phosphohydrolase. Tests for enzyme activities by use of Rapid ID 32 A show positive reactions for beta-galactosidase 6 phosphatase, pyroglutamic acid arylamidase, but not for arginine dihydrolase, alfa-arabinosidase, glutamic acid decarboxylase, alfa-fucosidase, arginine arylamidase, proline arylamidase, leucyl glycine arylamidase, phenylalanine arylamidase, leucine arylamidase, tyrosine arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, glutamyl glutamic acid arylamidase, serine arylamidase and urease. The API 20 A shows a negative reaction for catalase. The major fatty acids (>10% of the total fatty acids) are C14 : 0, C16 : 0 and C18 : 1ω9c. Some strains (= ATCC 25751, NCIB 10651, DSM 1295, JCM 1412) possess the possibility to produce endospores that are oval, subterminal and swell the cell.

ACCEPTED MANUSCRIPT

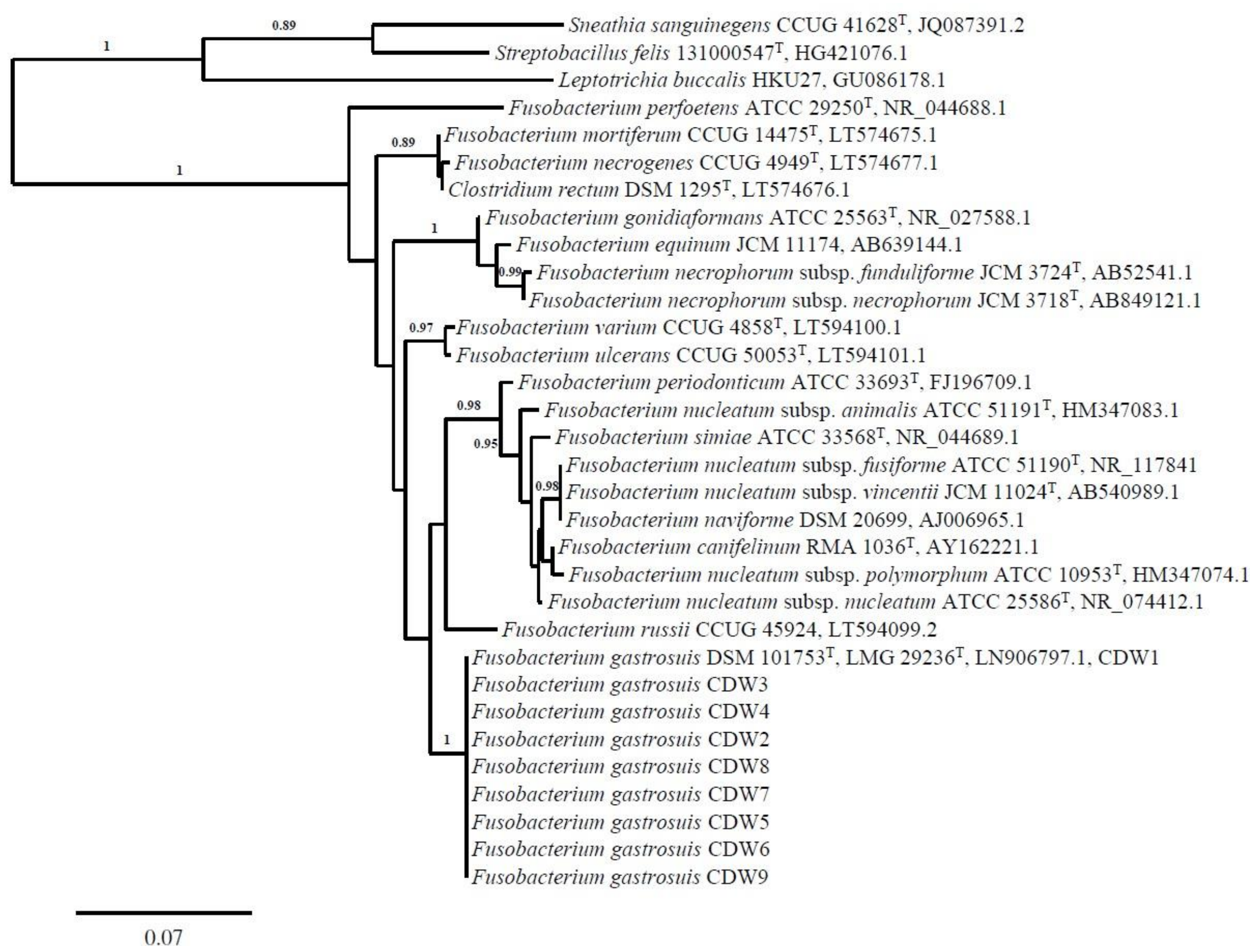

Fig. 1: Phylogenetic tree based on 16S rRNA sequences and maximum likelihood method shows the genetic relationships between *Fusobacterium* spp. and the 9 isolates of *Fusobacterium gastrosuis* sp. nov. (CDW1-9). The scale-bar represents 7% differences in nucleotide sequences; bootstrap values (≥0.7) of 1000 replicates are displayed next to the corresponding branch and GenBank accession numbers are included. *Leptotrichia buccalis* HKU27, *Sneathia sanguinegens* CCUG 41628^T and *Streptobacillus felis* 131000547^T were used as outgroups. All present nodes were coincident in the tree generated with the neighbor algorithm.

ACCEPTED MANUSCRIPT

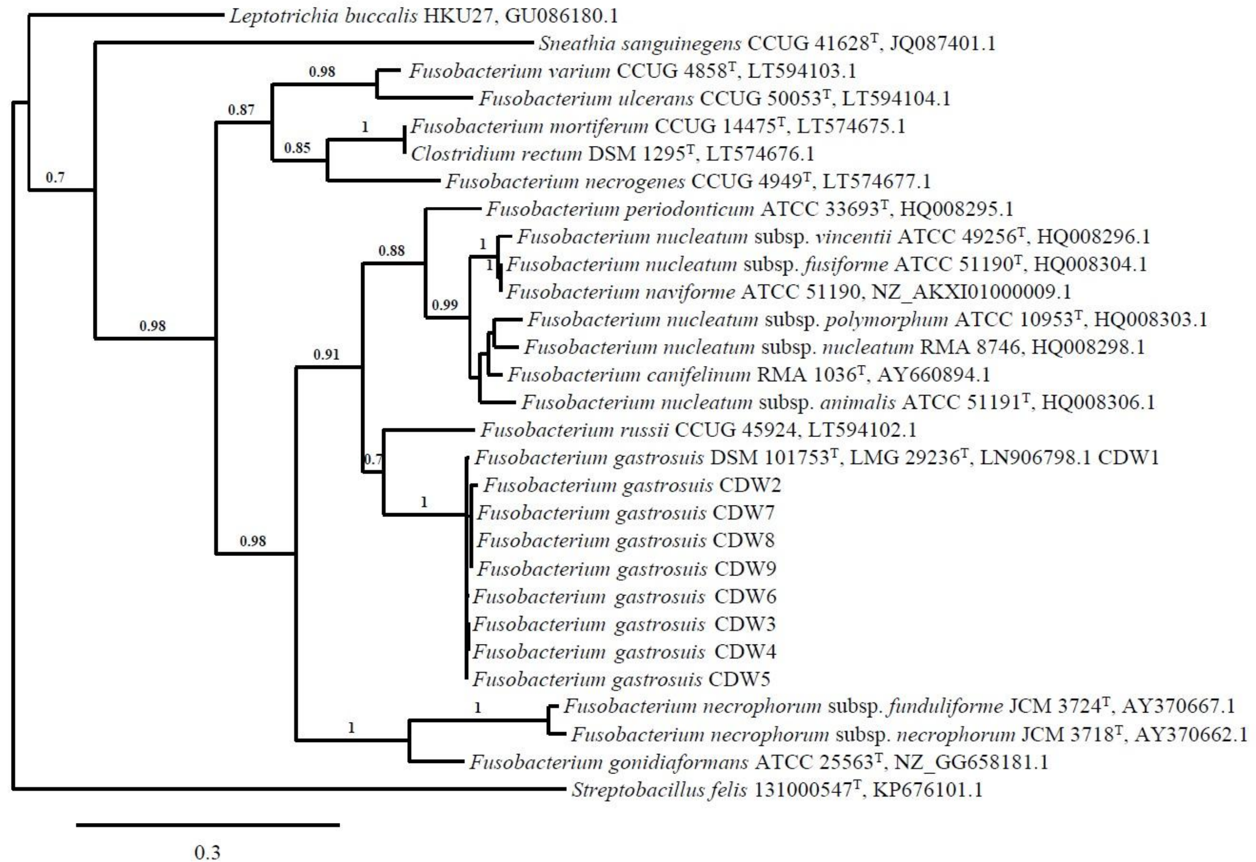

Fig. 2: Phylogenetic tree based on gyrB sequences and maximum likelihood method shows the genetic relationships between *Fusobacterium* spp. and the 9 isolates of *Fusobacterium gastrosuis* sp. nov. (CDW1-9). The scale-bar represents 30% differences in nucleotide sequences; bootstrap values (≥0.7) of 1000 replicates are displayed next to the corresponding branch and GenBank accession numbers are included. *Leptotrichia buccalis* HKU27, *Sneathia sanguinegens* CCUG 41628^T and *Streptobacillus felis* 131000547^T were used as outgroups. All present nodes were coincident in the tree generated with the neighbor algorithm.

ACCEPTED MANUSCRIPT

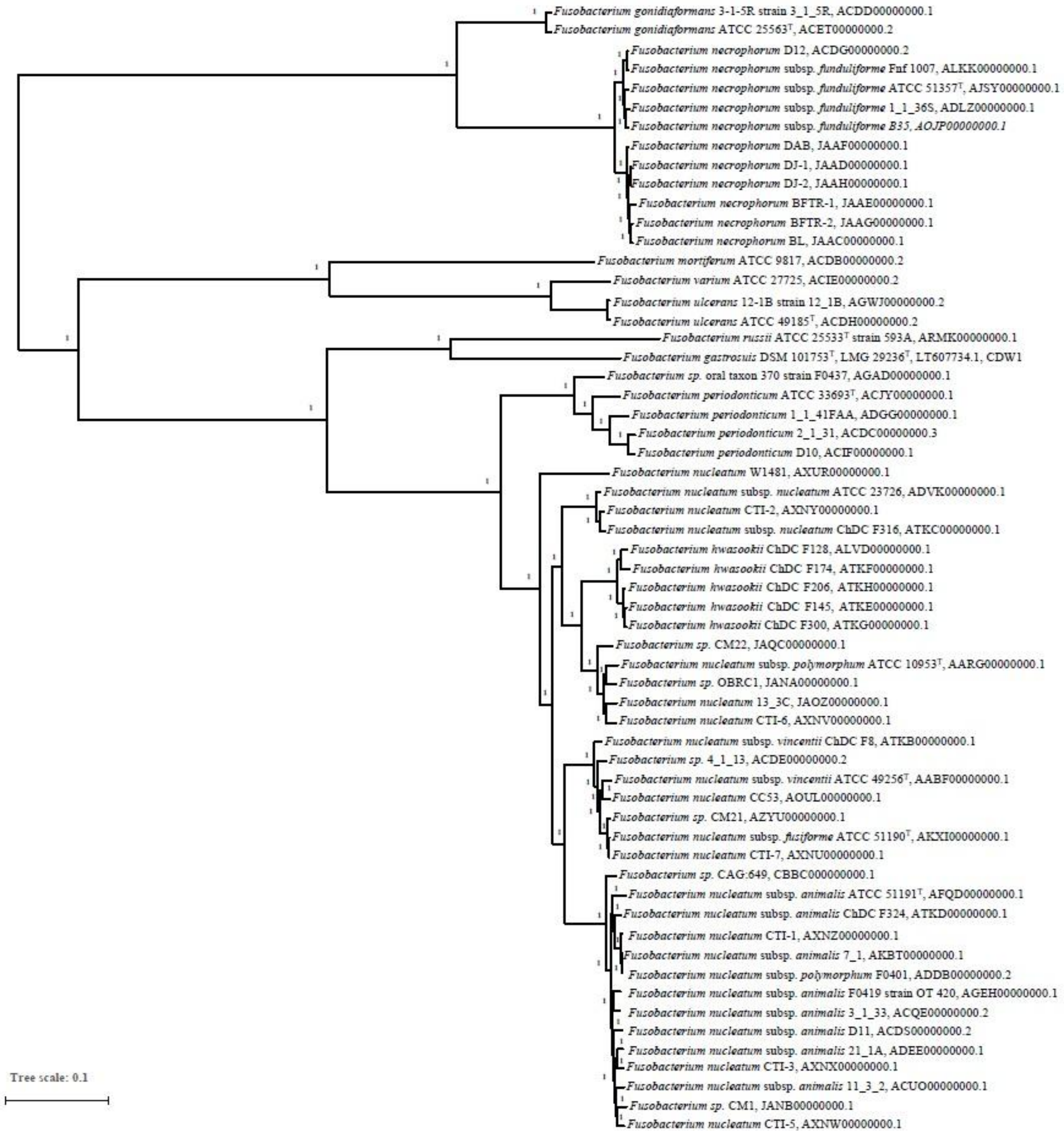

Fig. 3: Phylogenetic tree based maximum likelihood method and on 486 aligned and concatenated core genes of *Fusobacterium* spp. shows the genetic relationships between *Fusobacterium* spp. and the type strain of *Fusobacterium gastrosuis* sp. nov. (CDW1). The scale-bar represents 10% differences in nucleotide sequences; bootstrap values (≥0.7) of 1000 replicates are displayed next to the corresponding branch and GenBank accession numbers are included.

ACCEPTED MANUSCRIPT

Table 1 Phenotypic features for distinguishing *Fusobacterium gastrosuis* sp. nov. (9 isolates) from other *Fusobacterium* spp. with high sequence similarity. All data were obtained in this study. The organisms were grown under identical conditions, i.e. on Columbia agar plates® supplemented with 5% defibrinated sheep blood® for 3 days at 37°C.

Test	***Fusobacterium mortiferum***	***Clostridium rectum***	***Fusobacterium necrogenes***	***Fusobacterium russii***	***Fusobacterium ulcerans***	***Fusobacterium varium***	***Fusobacterium gastrosuis* sp. nov.**
Indole production	-	-	-	-	-	+	+
Esculin hydrolysis	+	+	+	+	+	+	-
Cystine arylamidase	-	W	-	-	-	W	$-^{W}$
Histidine arylamidase	-	-	-	+	+	+	V
Proline arylamidase	-	-	-	-	-	-	+
Pyroglumtaic acid arylamidase	+	+	-	+	+	+	-
Alanine arylamidase	-	-	-	-	+	+	-
Arginine arylamidase	-	-	-	W	+	+	-
Glutamyl glutamic acid arylamidase	-	-	-	+	-	+	-
Glycine arylamidase	-	-	-	-	-	W	-
Leucine arylamidase	-	-	-	W	+	+	-
Leucyl glycine arylamidase	-	-	-	-	-	W	-
Phenylalanine arylamidase	-	-	-	W	-	+	-
Serine arylamidase	-	-	-	-	+	+	-
Tyrosine arylamidase	-	-	-	-	W	+	-
Valine arylamidase	-	-	-	-	-	-	$-^{W}$
α-galactosidase	W	W	W	-	-	-	-
β-galactosidase	+	+	W	-	-	-	-
β-galactosidase 6 Phosphate	+	+	+	-	-	-	-
α-glucosidase	-	-	W	-	-	-	-
N-acetyl-β-glucosaminidase	-	-	-	-	-	-	$-^{W}$
Cellobiose acidification	+	-	-	-	-	-	-
Glucose acidification	+	+	+	+	+	+	-
Glycerol acidification	-	-	-	W	-	-	-
Lactose acidification	+	+	W	-	-	-	-
Mannitol acidification	-	-	W	-	-	-	-
Mannose acidification	+	+	W	-	+	+	-
Maltose acidification	+	+	-	+	+	-	-
Raffinose acidification	+	+	-	-	-	-	-
Saccharose acidification	+	+	-	-	-	-	-
Salicin acidification	+	+	W	-	-	-	-

ACCEPTED MANUSCRIPT

Trehalose acidification	W	W	W	-	-	-	-
Xylose acidification	-	-	-	-	W	W	-
Glutamic acid decarboxylase	-	-	-	+	+	+	+
Naphtol-AS-BI-phosphohydrolase	+	+	W	-	-	-	W
Esterase lipase (C8)	-	-	-	-	-	-	W
α-chymotrypsin	-	-	-	-	-	-	V

+ = positive, - = negative, W = weakly positive, -^{W} = most strains are negative and some weakly positive, V = some strains are positive and others negative.

ACCEPTED MANUSCRIPT

Table 2 Cellular fatty acid profiles, expressed as percentage of the total cellular fatty acids, of *Fusobacterium gastrosuis* sp. nov. and the species with high sequence similarity. All data were obtained in this study. The organisms were grown under identical conditions, i.e. on Columbia agar plates® supplemented with 5% defibrinated sheep blood® for 3 days at 37°C.

Cellular fatty acid	***Fusobacterium mortiferum***	***Clostridium rectum***	***Fusobacterium necrogenes***	***Fusobacterium russii***	***Fusobacterium ulcerans***	***Fusobacterium varium***	***Fusobacterium gastrosuis* sp. nov.**
Saturated							
C12 : 0	ND	T	1.6	7.6	T	T	1.5
C14 : 0	13.7	12.5	17.2	11.5	17.1	13.5	10.1
C16 : 0	27.9	22.0	13.5	15.2	ND	ND	22.0
C17 : 0	ND	T	ND	ND	ND	3.5	T
C18 : 0	5.0	6.6	6.4	1.8	1.4	ND	5.3
Unsaturated							
C13 : 1 AT 12-13	ND	0.6	1.5	ND	1.5	2.9	ND
C16 : 1ω9c	1.8	1.7	ND	3.2	2.8	2.1	6.2
C16 : 1ω5c	T	T	ND	ND	ND	ND	1.2
C17 : 1ω8c	ND	T	ND	ND	T	ND	ND
C18 : 1ω9c	13.5	15.1	11.5	9.3	7.2	1.5	12.0
C18 : 1ω7c	4.1	2.8	1.1	ND	1.0	ND	2.1
C18 : 1ω6c	ND	1.0	ND	ND	ND	ND	1.0
Hydroxyl							
C12 : 0 3-OH	1.0	T	ND	1.1	T	1.4	ND
C15 : 0 3-OH	ND	ND	ND	ND	ND	ND	T
C16 : 0 3-OH	ND	T	1.4	T	T	ND	1.2
Summed features							
Summed feature 1	T	1.5	5.5	ND	4.7	5.1	ND
Summed feature 2	14.6	15.9	22.4	40.5	27.1	52.0	18.9
Summed feature 3	12.2	10.2	8.4	7.0	13.0	9.7	14.5
Summed feature 4	2.5	3.1	5.9	ND	7.1	3.3	ND
Summed feature 5	2.0	1.5	1.9	1.5	T	ND	1.7

ND = not detected. T = values below 1% have no taxonomic evidence and were considered as traces. Summed features = groups of fatty acids that cannot be separated by the MIDI System: summed feature 1 = C13 : 0 3-OH, iso-C15 : 1 H, iso-C15 : 1 I; summed feature 2 = C14 : 0 3-OH, iso-C16 : 1 I; summed feature 3 = C16 : 1ω7c, iso-C15 : 0 2-OH; summed feature 4 = iso-C17 : 1 I, anteiso-C17 : 1 B; summed feature 5 = C18 : 2ω6,9c, anteiso-C18 : 0.

ACCEPTED MANUSCRIPT

1. Aziz, R.K., Bartels, D., Best, A.A., De Jongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., Kubal, M., Meyer, F., Olsen, G.J., Olson, R., Osterman, A.L., Overbeek, R.A., McNeil, L.K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G.D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., Zagnitko, O. (2008) The RAST server: rapid annotations using subsystems technology. BMC Genomics, doi: 10.1186/1471-2164-9-75.

2. Baele, M., Devriese, L.A., Haesebrouck, F. (2001) *Lactobacillus agilis* is an important component of the pigeon crop flora. J. Appl. Microbiol. 91, 488-491.

3. Bartlett, J.G., Finegold, S.M. (1972) Anaerobic Pleuropulmonary Infections. Medicine 51**,** 413-450.

4. Bolivar, I., Whiteson, K., Stadelmann, B., Baratti-Mayer, D., Gizard, Y., Mombelli, A., Pittet, D., Schrenzel, J. (2012) Bacterial Diversity in Oral Samples of Children in Niger with Acute Noma, Acute Necrotizing Gingivitis, and Healthy Controls. Plos Negl. Trop. Dis. 6, e1556.

5. Brazier, J.S., Citron, D.M., Goldstein, E.J. (1991) A selective medium for *Fusobacterium* spp. J. Appl. Bacteriol. 71, 343-346.

6. Cameron, R. (2012) Integumentary System: skin, hoof and claw. In: Diseases of Swine, Zimmerman, J.J., Karriker, L.A., Ramirez, A., Schwartz, K.J., Stevenson, G.W. (Eds), Tenth edition, Wiley-Blackwell, Iowa, pp. 257.

7. Chow, A.W., Guze, L.B. (1974) *Bacteroidaceae* Bacteremia - Clinical Experience with 112 Patients. Medicine 53, 93-126.

8. Citron, D.M. (2002) Update on the taxonomy and clinical aspects of the genus *Fusobacterium*. Clin. Infect. Dis. 35, S22-S7

9. Cleenwerck, I., De Vos, P., De Vuyst, L. (2010) Phylogeny and differentiation of species of the genus *Gluconacetobacter* and related taxa based on multilocus sequence analyses of housekeeping genes and reclassification of *Acetobacter xylinus* subsp. *sucrofermentans* as *Gluconacetobacter sucrofermentans* sp. nov., comb. nov. Int. J. Syst. Evol. Microbiol. 60, 2277–2283.

10. Collins, M.D., Lawson, P.A., Willems, A., Cordoba, J.J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, M., 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, 812-816.

11. Conrads, G., Citron, D. M., Goldstein, E. J. (2004) *Fusobacterium canifelinum* sp. nov., from the oral cavity of cats and dogs. Syst. Appl. Microbiol. 27, 407-413.

12. Ekseth, O.K., Kuiper, M., Mironov, V. (2014) OrthAgogue: an agile tool for the rapid prediction of orthology relations. Bioinformatics 30, 734-736, doi: 10.1093/bioinformatics/btt582.

13. Euzéby, J.P. List of Bacterial Names with Standing in Nomenclature – Genus *Fusobacterium*. Internet reference: www.bacterio.net/fusobacterium.html (consulted December 24, 2015).

14. Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989) Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane-filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int. J. Syst. Bacteriol. 39, 224–229.

15. Garrity, G. M. (2001) Bergey's Manual of systematic bacteriology. 2nd ed. New York, Springer, 603-637.

16. George, W.L., Kirby, B.D., Sutter, V.L., Citron, D.M., Finegold, S.M. (1981) Gram-negative anaerobic bacilli: Their role in infection and patterns of susceptibility to antimicrobial agents. II. Little-known *Fusobacterium* species and miscellaneous genera. Rev. Infect. Dis. 3, 599-626.

17. Gharbia, S.E., Shah, H.N., Bernard, K. (2012) International Committee on Systematics of Prokaryotes Subcommittee on the taxonomy of Gram-negative anaerobic rods: Minutes of the open meeting, 1-2 February 2011, Health Protection Agency, Colindale, London, UK. Int. J. Sys. Evol. Microbiol. 62, 467-471.

ACCEPTED MANUSCRIPT

18. Haesebrouck, F., Pasmans, F., Flahou, B., Chiers, K., Baele, M., Meyns, T., Decostere, A., Ducatelle, R. (2009) Gastric helicobacters in domestic animals and nonhuman primates and their significance for human health. Clin. Microbiol. Rev. 22, 202-223.

19. Hampson, D.J., Nagaraja, T.G., Kennan, R.M., Rood, J.I. (2010) Gram-negative Anaerobes. In: Pathogenesis of bacterial infections in animals, Gyles, C.L., Prescott, J.F., Songer, J.G., Thoen, C.O. (Eds), Fourth edition, Wiley-Blackwell, Iowa, pp. 514-517.

20. Harris, N.M. (1901). Bacillus mortiferus nov. spec. J. Exp. Med. 6, 519-547.

21. Hellemans, A., Chiers, K., Decostere, A., De Bock, M., Haesebrouck, F., Ducatelle, R. (2007) Experimental infection of pigs with Candidatus *Helicobacter suis*. Vet. Res. Commun. 31, 385-395.

22. Heller, H.H. (1922) Certain genera of the *Clostridiaceae*: studies in pathogenic anaerobes. J. Bacteriol. 7, 1-38.

23. Konstantinidis, K.T., Tiedje, J.M. (2005) Genomic insights that advance the species definition for prokaryotes. Proc. Natl. Acad. Sci. 102, 2567–2572, 10.1073/pnas.0409727102.

24. Krakowka S., Eaton K.A., Rings D.M., Argenzio R.A. (1998) Production of gastroesophageal erosions and ulcers (GEU) in gnotobiotic swine monoinfected with fermentative commensal bacteria and fed high-carbohydrate diet. Vet. Pathol. 35, 274-282.

25. Langworth, B.F. (1977) *Fusobacterium necrophorum* its characteristics and role as an animal pathogen. Bacteriol. Rev. 41, 373-390.

26. Lee, Y., Eun, C.S., Han, D.S. (2016). Clinical *Fusobacterium mortiferum* isolates cluster with undifferentiated *Clostridium rectum* species based on *16S rRNA* gene phylogenetic analysis. Ann. Clin. Lab. Sci. 46, 279-281.

27. Manson McGuire, A., Cochrane, K., Griggs, A. D., Haas, B. J., Abeel, T., Zeng, Q., Nice, J. B., MacDonald, H., Birren, B. W., Berger, B. W., Allen-Vercoe, E., Earl, A. M. (2014) Evolution of invasion in a diverse set of *Fusobacterium* species. M. Bio. 5, e01864-14.

28. Markey, B.K. (2013) Non-spore forming anaerobes. In: Markey, B.K., Leonard, F., Archambault, M., Cullinane, A., Maguire, D. (Eds). Clinical Veterinary Microbiology, 2nd edition, Elesevier, Edinburgh, pp. 205-213.

29. Moore, W.E.C., Holdeman, L.V. Fusobacterium (1970). In: Outline of Clinical Methods in Anaerobic Bacteriology, Cato, E.P., Cummins, C.S., Holdeman, L.V., Johnson, J.L., Moore, W.E.C., Smibert, R.M., Smith, L.D.S (Eds), 2nd revision, Virginia Polytechnic Institute, Anaerobe Laboratory, Blacksburg, Virginia.

30. Moore, W.E., Holdeman, L.V. (1972) Identification of anaerobic bacteria. Am. J. Clin. Nutr. 25, 1306-1313.

31. Moore-Gillon, J., Lee, T.H., Eyken, S.J., Philips, I. (1984) Necrobacillosis a forgotten disease. Br. Med. J. 288, 1526-1527.

32. Onyenwoke, R.U., Brill, J.A., Farahi, K., Wiegel, J. (2004) Sporulation genes in members of the low G+C Gram-type-positive phylogenetic branch (Firmicutes). Arch. Microbiol. 182, 182-192.

33. Olsen, I., Shah, N.H. (2003) International Committee on Systematics of Prokaryotes, Subcommittee on the taxonomy of Gram-negative anaerobic rods: Minutes of the meeting, 29 July 2002, Paris, France. Int. J. Syst. Evol. Microbiol. 53, 923–924

34. Overbeek, R., Olson, R., Pusch, G.D., Olsen, G.J., Davis, J.J., Disz, T., Edwards, R.A., Gerdes, S., Parrello, B., Shukla, M., Vonstein, V., Wattam, A.R., Xia, F., Stevens, R. (2014) The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic. Acids. Res. 42, 206-214, doi: 10.1093/nar/gkt1226.

ACCEPTED MANUSCRIPT

35. Page, A.J., Cummins, C.A., Hunt, M., Wong, V.K., Reuter, S., Holden, M.T., Fookes, M., Falush, D., Keane, J.A., Parkhill, J. (2015) Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics 31, 3691-3693, doi: 10.1093/bioinformatics/btv421.

36. Popham, D.L., Illades-Aguiar, B., Setlow, P. (1995) The Bacillus subtilis dacB gene, encoding penicillin-binding protein 5*, is part of a three-gene operon required for proper spore cortex synthesis and spore core dehydration. J. Bacteriol. 177, 4721-4729.

37. Praet, J., Meeus, I., Cnockaert, M., Aerts, M., Smagghe, G., Vandamme, P. (2015) *Bifidobacterium commune* sp. nov. Isolated from the bumble bee gut. Antonie van Leeuwenhoek 107, 1307-1313.

38. Rowland, M.D., Delbene, V.E., Lewis J.W. (1987) Factors Affecting Antimicrobial Susceptibility of *Fusobacterium* species. J. Clin. Microbiol. 25, 476-479.

39. Švec, P., Pantůček, R., Petráš, P., Sedláček, I., Nováková, D. (2010) Identification of *Staphylococcus* spp. using (GTG)5-PCR fingerprinting. Syst. Appl. Microbiol. 33, 451–456.

40. Sutter, V.L., Finegold, S.M. (1971) Antibiotic disc susceptibility tests for rapid presumptive identification of Gram-negative anaerobic bacilli. Appl. Microbiol. 21, 13-20.

41. Wayne, L.G., Brenner, D.J., Colwell, R.R., Grimont, P.A.D., Kandler, O., Krichevsky, M.I., Moore, L.H., Moore, W.E.C., Murray, R.G.E., Stackebrandt, E. (1987) Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics. Int. J. Syst. Bacteriol. 37, 463-464.

42. Whitman, W.B., Parte A.C. (2011) The *Bacteroidetes*, *Spirochaetes*, *Tenericutes* (*Mollicutes*), *Acidobacteria*, *Fibrobacteres*, *Fusobacteria*, *Dictyoglomi*, *Gemmatimonadetes*, *Lentisphaerae*, *Verrucomicrobia*, *Chlamydiae* and *Planctomycetes*. In: Bergey's manual of systematic bacteriology, Krieg, N.R., Parte, A., Ludwig, W., Whitman, W.B., Hedlund, B.P., Paster, B.J., Staley, J.T., Ward, N., Brown, D. (Eds), Volume 4, Springer, New York, pp. 747-758.

43. Wiegel, J., Tanner, R., Rainey, F.A. (2006) An introduction to the family Clostridiaceae. In: The Prokaryotes, A Handbook on the Biology of Bacteria, Bacteria: Firmicutes, Cyanobacteria, Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.H., Stackebrandt, E. (Eds), Volume 4, Springer, New York, pp. 654–678.

44. Yamamoto, S., Harayama, S. (1995) PCR amplification and direct sequencing of gyrb genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas Putida* strains. Appl. Environ. Microbiol. 61, 3768-3768.

45. Yarza, P., The All-Species Living Tree - Release LTPs123. Internet reference: https://www.arb-silva.de/fileadmin/silva_databases/living_tree/LTP_release_123/LTPs123_SSU_tree.pdf (consulted August 11, 2016).

46. Zhou, H.T., Dobbinson, S., Hickford, J.G.H. (2010) *Fusobacterium necrophorum* variants present on the hooves of lame pigs. Vet. Microbiol. 141, 390-390.