

1 **A comparative and functional genomics analysis of the genus *Romboutsia***  
2 **provides insight into adaptation to an intestinal lifestyle**

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## 24 **Abstract**

25 Cultivation-independent surveys have shown that the recently described genus *Romboutsia* within the  
26 family *Peptostreptococcaceae* is more diverse than previously acknowledged. The majority of  
27 *Romboutsia*-associated 16S rRNA gene sequences have an intestinal origin, but the specific roles that  
28 *Romboutsia* species play in the digestive tract are largely unknown. The complete genomes of the  
29 human intestinal isolate *Romboutsia hominis* FRIFI<sup>T</sup> (DSM 28814) and the soil isolate *Romboutsia*  
30 *lituseburensis* A25K<sup>T</sup> (DSM 797) were sequenced. An evaluation of the common traits of this recently  
31 defined genus was done based on comparative genome analysis of the two strains together with the  
32 previously elucidated genome of the type species *Romboutsia ilealis* CRIB<sup>T</sup>. These analyses showed  
33 that the genus *Romboutsia* covers a broad range of metabolic capabilities with respect to  
34 carbohydrate utilization, fermentation of single amino acids, anaerobic respiration and metabolic end  
35 products. Main differences between strains were found in their abilities to utilize specific  
36 carbohydrates, to synthesize vitamins and other cofactors, and their nitrogen assimilation capabilities.  
37 In addition, differences were found with respect to bile metabolism and motility-related gene clusters.

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## 39 **Keywords**

40 *Romboutsia*, small intestine, soil, genome assembly, comparative genomics

## 41 **Short title**

42 Comparative genomics of the genus *Romboutsia*

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## 48 Introduction

49 The family *Peptostreptococcaceae* has recently been undergoing significant taxonomic changes. One  
50 of them involved the creation of the new genus *Romboutsia*, which currently contains the recognized  
51 species *Romboutsia ilealis*, *Romboutsia lituseburensis* (formerly known as *Clostridium lituseburensis*)  
52 and *Romboutsia sedimentorum* (1, 2). Sequences related to the genus *Romboutsia* have been  
53 predominantly reported from samples of (mammalian) intestinal origin. Clone-library studies have  
54 shown the occurrence of *Romboutsia*-like 16S ribosomal RNA (rRNA) sequences in intestinal content  
55 samples (duodenum, jejunum, ileum and colon) from dogs (3) and cows (4), and in faecal samples  
56 from rats (5), polar bears (6) and porpoises (7). In addition, more recent sequencing-based studies  
57 have identified similar phylotypes in faecal samples from humans and other mammals (8), human ileal  
58 biopsies (9), mouse faecal samples (10), ileal biopsies from pigs (11), and ileal contents from rats (12)  
59 and deer (13). The isolation of strains from intestinal sources that possibly belong to other novel  
60 species within the genus *Romboutsia* has been reported as well. For example, strain TC1 was isolated  
61 from the hide of a cow, where it was found likely as the result of a faecal contamination of the hide  
62 (14). Furthermore, *Romboutsia timonensis* was isolated from human colon (15), and '*Clostridium*  
63 *dakarensis*' was isolated from the stool of a 4-month-old Senegalese child (16). Recently, our search  
64 for a *Romboutsia* isolate of human small intestinal origin has resulted in the isolation of strain FRIFI  
65 from ileostoma effluent of a human adult and the subsequent proposal of *Romboutsia hominis* sp.  
66 nov. (17).

67 Although these findings suggest that members of the *Romboutsia* genus are mainly gut  
68 inhabitants, *Romboutsia* strains have been isolated from other environmental sources as well (2, 18,  
69 19). The type strain of the second validly described species within the genus *Romboutsia*, *R.*  
70 *lituseburensis* A25K<sup>T</sup>, is not of intestinal origin, but was originally isolated from soil and humus from  
71 Côte d'Ivoire (20). Based on 16S rRNA gene identity, strains very similar to *R. lituseburensis* A25K<sup>T</sup> have  
72 been isolated in recent years: strain H17 was isolated from the main anaerobic digester of a biogas

73 plant (GI: EU887828.1), strain VKM B-2279 was isolated from a p-toluene sulfonate degrading  
74 community (21), strain 2ER371.1 was isolated from waste of biogas plants (22), and strain E2 was  
75 isolated from a cellulose degrading community enriched from mangrove soil (23). Furthermore, the  
76 type strain of the recently described novel species *R. sedimentorum* has been isolated from sediment  
77 samples taken from an alkaline-saline lake located in Daqing oilfield.

78 Altogether these studies suggest that the genus *Romboutsia* is probably more diverse than  
79 previously appreciated, and it is the question whether intestinal strains have adapted to a life outside  
80 a host or whether strains originating from other, non-host associated environments have adapted to  
81 a life in the intestinal tract. Because of the still limited availability of cultured representatives and their  
82 genomes, we know little about the specific roles that members of the genus *Romboutsia* play in the  
83 ecosystems in which they are found. To gain more insight into the metabolic and functional  
84 capabilities of the genus *Romboutsia*, we present here the genomes of the human intestinal isolate *R.*  
85 *hominis* FRIFI and the soil isolate *R. lituseburensis* A25K<sup>T</sup>, together with an evaluation of the common  
86 traits of this recently defined genus based on comparative genome analysis, including a comparison  
87 with the previously elucidated genome of the rat small intestinal isolate and type species of the genus,  
88 *R. ilealis* CRIB<sup>T</sup>.

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## 90 **Materials and Methods**

### 91 **Growth conditions and genomic DNA preparation**

92 For genomic DNA extraction, *R. hominis* FRIFI<sup>T</sup> and *R. lituseburensis* A25K<sup>T</sup> (DSM 797) were grown  
93 overnight at 37°C in liquid CRIB-medium (pH 7.0) (1). DNA extraction was performed as described  
94 previously (24). DNA quality and concentrations were determined by spectrophotometric analysis  
95 using NanoDrop (Thermo Scientific) and by electrophoresis on a 1.0 % (w/v) agarose gel. DNA was  
96 stored at 4°C until subsequent sequencing.

## 97 **Genome sequencing and assembly**

98 Genome sequencing of *R. hominis* FRIFI<sup>T</sup> was carried out at the University of Helsinki (Finland) on a  
99 PacBio RS II, resulting in 134.366 PacBio reads and a total amount of 464.930.600 bases. Assembly was  
100 performed with PacBio SMRT analysis pipeline v2.2 and the HGAP protocol (25). Default settings were  
101 used except for: minimum sub-read length 500, minimum polymerase read length quality 0.80,  
102 minimum seed read length 7000, split target into chunks 1, alignment candidate per chunk 24, genome  
103 Size 3,000,000, target coverage 30, overlapper error rate 0.06, overlapper mini length 40, overlapper  
104 K-mer 14.

105 Genome sequencing of *R. lituseburensis* A25K<sup>T</sup> was performed at GATC Biotech (Konstanz,  
106 Germany). One MiSeq library was generated on an Illumina MiSeq Personal Sequencer with 250 nt  
107 paired-end reads and an insert size of 500 nt, which resulted in 772.051 paired-end reads. Additionally  
108 one PacBio library was generated on a PacBio RS II, which resulted in 441.151 subreads and in total  
109 998.181.178 bases. A hybrid assembly was carried out with MiSeq paired-end and PacBio CCS reads.  
110 For the MiSeq paired-end reads first all rRNA reads were removed with SortMeRNA v1.9 (26) using all  
111 included databases. Next, adapters were trimmed with Cutadapt v1.2.1 (27) using default settings  
112 except for an increased error value of 20 % for the adapters, and also using the reverse complement  
113 of the adapters. Quality trimming was performed with PRINSEQ Lite v0.20.0 (28) with a minimum  
114 sequence length of 40 and a minimum quality of 30 on both ends and as mean quality of the read. The  
115 assembly was done in parallel using two different assemblers. Ray v2.3 was used for the MiSeq paired-  
116 end dataset and the PacBio CCs dataset, using default settings except for a k-mer value of 75. The  
117 PacBio SMRT analysis pipeline v2.2 was run on the SMRT-cell subreads with the protocol  
118 RS\_HGAP\_Assembly\_2, using default settings except for that the number of seed read chunks was set  
119 to 1, minimum seed read length was set to 7000, alignment candidate per chunk was set to 24 and  
120 the estimated genome size was reduced to 4 Mb. Both assemblies were merged, and duplications  
121 were identified based on BLASTN hits. Duplicate contigs were discarded if they had a hit with at least  
122 99 % sequence identity within a bigger contig, which spanned at least 98 % of contig query length.

123 Furthermore contigs with a length of less than 500 bp were discarded. The remaining contigs were  
124 merged with CAP3 v.12/21/07 (29), with an overlap length cut-off of 5000 bp and a minimum identity  
125 of 90 %. A circular element was detected within this assembly, based on BLASTP results of the  
126 predicted proteins (e-value 0.0001), and this was excluded from the further assembly process, but  
127 added to the final assembly result. Scaffolding of the contigs was done with SSPACE-LongRead (30)  
128 and the PacBio CCS reads using default settings. Further scaffolding was done with Contiguator v2.7.4  
129 (31) using the genome of *R. hominis* FRIFI<sup>T</sup> as reference genome and applying default settings.  
130 Copy numbers of the 16S rRNA gene from published genomes were derived from the rrnDB v4.0.0  
131 (32). Average nucleotide identity (ANI) values were calculated with JSpecies v1.2.1 (33) by pairwise  
132 comparisons of available genomes within the family *Peptostreptococcaceae*.

## 133 **Genome annotation**

134 Annotation was carried out with an in-house pipeline (adapted from (34)), with Prodigal v2.5 for  
135 prediction of protein coding DNA sequences (CDS) (35), InterProScan 5RC7 for protein annotation (36),  
136 tRNAscan-SE v1.3.1 for prediction of tRNAs (37) and RNAmmer v1.2 for the prediction of rRNAs (38).  
137 Additional protein function predictions were derived via BLAST searches against the UniRef50 (39) and  
138 Swissprot (40) databases (download August 2013). Subsequently the annotation was further  
139 enhanced by adding EC numbers via PRIAM version March 06, 2013 (41). Non-coding RNAs were  
140 identified using rfam\_scan.pl v1.04, on release 11.0 of the RFAM database (42). COGs (43) were  
141 determined via best bidirectional blast (44), with an e-value of 0.0001. CRISPRs were annotated using  
142 CRISPR Recognition Tool v1.1 (45). A further step of automatic curation was performed, by weighting  
143 the annotation of the different associated domains, and penalizing uninformative functions (e.g.  
144 “Domain of unknown function”), and prioritizing functions of interest (e.g. domains containing “virus”,  
145 “phage”, “integrase” for phage related elements; similar procedure for different other functions).

146 Homology between the CDS of the *Romboutsia* strains was determined via best bidirectional  
147 BLAST hit (44) at the amino acid level with an e-value cut-off of 0.0001. To evaluate the core and pan

148 metabolism of the *Romboutsia* strains, the three annotated genomes were supplied to Pathway tools  
149 v18 (46), and a limited amount of manual curation was performed to remove obvious false positives.  
150 Next the pathway databases were exported via the built-in lisp interface and the exported data was  
151 merged. A reaction was considered to be in the core metabolism if it was present in all three  
152 databases, else it was considered to be in the pan metabolism. Both parts were then reimported  
153 separately and combined into Pathway tools for further analyses.

154 Genes were matched to the list of essential and non-essential sporulation-related genes  
155 compiled by Galperin *et al.* (47) via different methods. Firstly, the protein-coding genes of *Bacillus*  
156 *subtilis* subsp. *subtilis* 168 were annotated via InterProScan and the respective *B. subtilis* sporulation-  
157 related proteins were matched to the proteins encoded by the three *Romboutsia* genomes, if they  
158 contained at least 50 % of the same domains. In case multiple matches were possible, the match with  
159 the highest domain similarity was picked. The matches were manually curated, and arbitrary proteins  
160 and/or false hits were excluded. For every protein, which did not have any match via the domains, the  
161 best bidirectional BLAST hit (e-value cut-off of 0.0001) was used instead. Secondly, the genome of *R.*  
162 *ilealis* CRIB<sup>T</sup> was manually curated with respect to putative sporulation-related genes. In case the  
163 genomes of the other *Romboutsia* strains did not have any corresponding match for one of the  
164 proteins whereas a manually curated hit was present in *R. ilealis* CRIB<sup>T</sup>, the best bidirectional hit was  
165 assigned. Genomes were manually checked for further missing essential sporulation- and  
166 germination-related genes as defined by Galperin *et al.* (47). Function curation was performed with  
167 assistance of the *B. subtilis* wiki (<http://subtiwiki.uni-goettingen.de/>).

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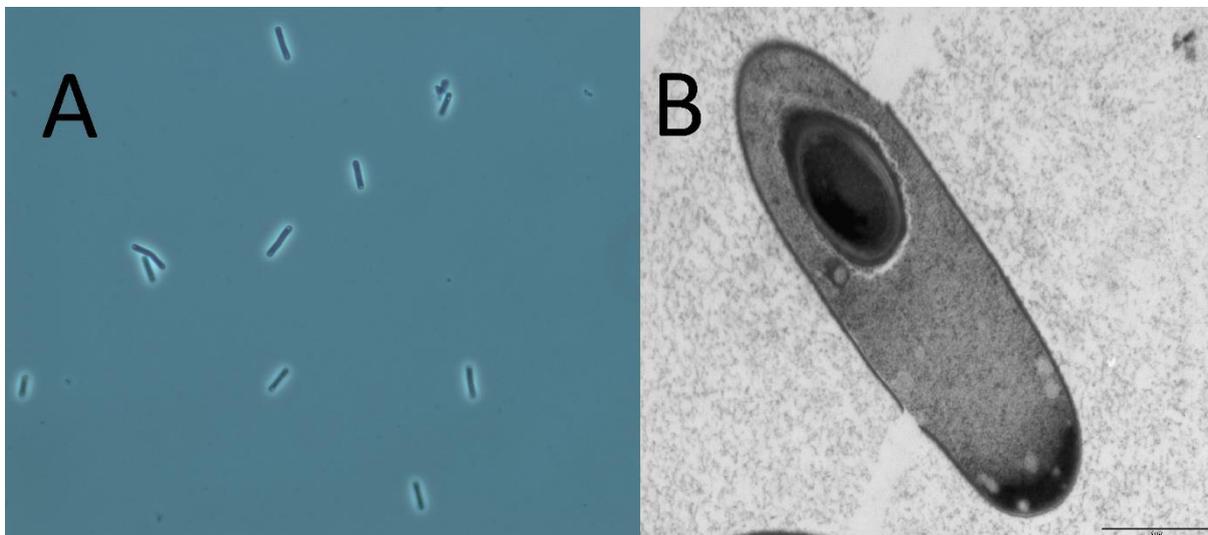
## 169 **Results and Discussion**

170 To gain more insight in the metabolic and functional capabilities of members of the genus *Romboutsia*  
171 within an intestinal environment, we set out to elucidate the genome of a *Romboutsia* strain of human  
172 intestinal origin. To this end, the genome of *R. hominis* FRIF1<sup>T</sup>, isolated from ileostoma effluent of a

173 human adult, was sequenced (17). For comparative analysis, we also aimed to determine the genome  
174 sequence of an isolate from another habitat, and thus the soil isolate *R. lituseburensis* A25K<sup>T</sup> was  
175 obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig,  
176 Germany). Here we present the genome sequences of both organisms, together with an evaluation of  
177 the common traits of this recently defined genus based on comparative genome analysis, including  
178 the recently elucidated genome of the type species *R. ilealis* CRIB<sup>T</sup> (1, 48). The genomes of *R. hominis*  
179 *FRIFI*<sup>T</sup> and *R. lituseburensis* A25K<sup>T</sup> (raw data and annotated assembly) have been deposited at the  
180 European Nucleotide Archive under project numbers PRJEB7106 and PRJEB7306, respectively.

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182 Both *Romboutsia hominis* *FRIFI*<sup>T</sup> (DSM 28814) and *R. lituseburensis* A25K<sup>T</sup> (DSM 797) are anaerobic,  
183 Gram-positive, motile rods, belonging to the genus *Romboutsia*. *R. lituseburensis* A25K<sup>T</sup> possesses the  
184 ability to sporulate. Pictures of both organisms can be seen in (Fig. 1).

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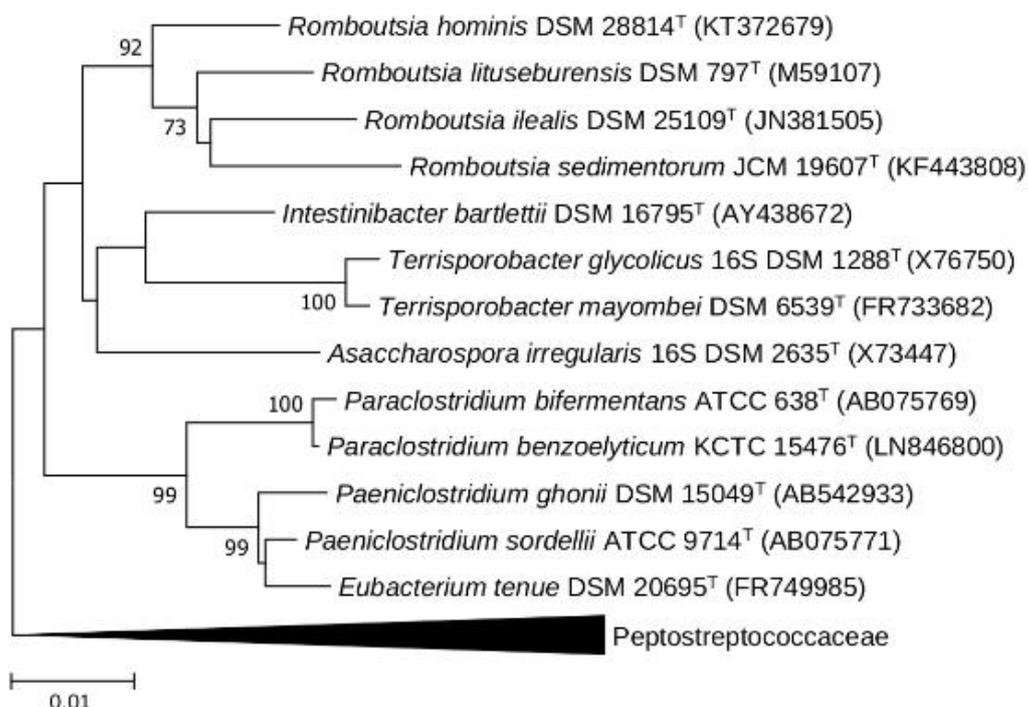
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189 **Fig. 1. Electron micrographs of both Romboutsia species.**

190 A) Micrograph of *Romboutsia hominis* *FRIFI*<sup>T</sup> B) Micrograph of *Romboutsia lituseburensis* A25K<sup>T</sup>.

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192 To investigate the relationships between these isolates and their closest relatives, a 16S rRNA  
193 gene based neighbour joining-tree was constructed with a representative copy of the 16S rRNA gene  
194 of the type strains of the three species *R. ilealis*, *R. lituseburensis* and *R. hominis* (Fig. 2). Based on their  
195 16S rRNA gene sequence these three species, together with the recently characterized species  
196 *Romboutsia sedimentorum*, form a monophyletic group.  
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199 **Fig. 2. Neighbour-joining tree based on 16S rRNA gene sequences of *Romboutsia* species and closely-**  
200 **related species.**

201 The 16S rRNA gene sequences were aligned using the SINA aligner (49). The tree was constructed  
202 using MEGA6 software (50) with Kimura's two-parameter model as substitution model. Only bootstrap  
203 values >70 % are shown at branch nodes. Bootstrap values were calculated based on 1000 replications.  
204 The reference bar indicates 1 % sequence divergence. GenBank accession numbers are given in  
205 parentheses. "The tree was rooted using 16S rRNA gene sequences of type strains of more distantly-  
206 related species within the family *Peptostreptococcaceae*.

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*R. hominis* FRIFI<sup>T</sup> contains a single, circular chromosome of 3.090.335 bp with an overall G+C content of 27.8 % (Table 1). The chromosome encodes 2.852 predicted coding sequences (CDS), of which 83 % have a function assigned. *R. lituseburensis* strain A25K<sup>T</sup> contains one circular chromosome of 3.776.615 bp and one circular plasmid of 97.957 bp, with an overall G+C content of 28.2 % (Table 1). The chromosome encodes 3.535 CDS and the plasmid 123 CDS, of which 82 % have a function assigned. In addition, one segment of 4.101 bp, containing one 5S rRNA and four CDS (one two-component system and two subunits of an ABC transporter), could not be placed. The plasmid of *R. lituseburensis* A25K<sup>T</sup> encodes two plasmid replication proteins, transporters, transcription factors, hydrolases and acyltransferases.

The numbers of genes associated with general COG functional categories are shown in Table 2. The biggest differences between both genomes were found within the genes not assigned to any COG category. Within the COG categories, the most noticeable difference was observed within category J, with more than 1% difference. Most other categories are present in comparable abundances, despite the fact that both organisms were isolated from different habitats.

234 **Table 1.** General features of the *Romboutsia* genomes

	<i>Romboutsia</i> sp. strain FRIFI	<i>R. lituseburensis</i> A25K <sup>T</sup>
Status current assembly of the chromosome	1 scaffold, 1 gap	2 scaffolds, 11 gaps
Genome size (Mb)	3.09	3.88
Chromosome size (Mb)	3.09	3.78
Plasmid size (Mb)	-	0.98
G+C content (%)	27.8	28.2
Total no. of CDS	2852	3662
No. of rRNA genes		
16S rRNA genes	16	--*
23S rRNA genes	17	--*
5S rRNA genes	15	--*
No. of tRNAs	107	118
No. of ncRNAs	82	116
CRISPR repeats	-	-

235 \* Number of rRNA genes cannot accurately be estimated since some of the rRNA genes are situated  
236 next to an assembly gap of unknown size and might therefore be duplicates (*R. lituseburensis* A25K<sup>T</sup>)

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248 **Table 2.** Number of genes associated with general COG functional categories.

<i>Code</i>	<i>FRIF1<sup>T</sup></i>		<i>A25K<sup>T</sup></i>		<i>Description</i>
	<i>Value</i>	<i>%age</i>	<i>Value</i>	<i>%age</i>	
J	206	7.22	214	5.85	Translation, ribosomal structure and biogenesis
A	1	0.04	0	0	RNA processing and modification
K	217	7.61	283	7.74	Transcription
L	109	3.82	129	3.53	Replication, recombination and repair
B	1	0.04	1	0.03	Chromatin structure and dynamics
D	45	1.58	47	1.28	Cell cycle control, Cell division, chromosome partitioning
V	76	2.66	88	2.41	Defense mechanisms
T	147	5.15	220	6.01	Signal transduction mechanisms
M	143	5.01	186	5.08	Cell wall/membrane biogenesis
N	56	1.96	72	1.97	Cell motility
U	26	0.91	25	0.68	Intracellular trafficking and secretion
O	85	2.98	107	2.93	Posttranslational modification, protein turnover, chaperones
C	145	5.08	180	4.92	Energy production and conversion
G	145	5.08	182	4.98	Carbohydrate transport and metabolism
E	161	5.65	202	5.52	Amino acid transport and metabolism
F	87	3.05	95	2.60	Nucleotide transport and metabolism
H	125	4.38	145	3.96	Coenzyme transport and metabolism
I	79	2.77	99	2.71	Lipid transport and metabolism
P	139	4.87	153	4.18	Inorganic ion transport and metabolism
Q	39	1.37	39	1.07	Secondary metabolites biosynthesis, transport and catabolism
R	203	7.12	247	6.75	General function prediction only
S	171	6.00	201	5.49	Function unknown
		28.2			
-	805	3	1184	32.37	Not in COGs

249 The total is based on the total number of protein coding genes in the genome.

## 250 **Impact of high number of ribosomal operons on sequencing efforts**

251 Gaps in whole genome assemblies are usually located in repetitive regions that include ribosomal  
252 operons, which can appear multiple times in the genome. Also for the *Romboutsia* genomes, the  
253 presence of a high number of rRNA operons has been problematic for genome assembly. The assembly  
254 of *R. hominis* FRIFI<sup>T</sup> contains one gap situated in a long stretch of ribosomal operons. The assembly of  
255 the chromosome of *R. lituseburensis* A25K<sup>T</sup> contains eleven gaps, of which six are generated due to  
256 scaffolding with the use of a reference. Nine of the eleven gaps are situated within or neighbouring  
257 rRNA operons or tRNA clusters. A total of 16 copies of the 16S rRNA gene were identified in the  
258 genome of *R. hominis* FRIFI<sup>T</sup>. This is one of the highest copy numbers reported for the 16S rRNA gene  
259 in prokaryotic species up to this date. For *R. lituseburensis* A25K<sup>T</sup>, the total number of 16S rRNA genes  
260 could not be accurately estimated since some of the rRNA genes are situated next to assembly gaps,  
261 but at least 15 rRNA operons seem to be present. Pairwise sequence identity of the 16S rRNA  
262 sequences showed that within the genome of strain FRIFI<sup>T</sup> there was an average sequence identity of  
263 99.3 % and the lowest identity between individual copies was 98.4 %. Sequence divergence in the 16S  
264 rRNA gene is not uncommon within individual prokaryotic genomes (51, 52). However, for *R. hominis*  
265 FRIFI<sup>T</sup> the divergence is located in the V1-V2 region of the 16S rRNA gene, one of the regions that is  
266 commonly used for sequence-based bacterial community analyses (53). In this region the average  
267 sequence identity was only 96.5 % and the lowest identity was only 92.3 %. Consequence of this  
268 divergence is that during identity clustering in operational taxonomic units (OTUs) the different copies  
269 of the 16S rRNA gene of *R. hominis* FRIFI<sup>T</sup> end up in different OTUs, even at the level of 97 % identity,  
270 resulting in an overestimation of the diversity in *Romboutsia* phylotypes. In comparison, the type  
271 species of the genus, *R. ilealis* CRIB<sup>T</sup>, contains little variation in the 16S rRNA gene sequence (>99 %  
272 sequence identity), despite that also in this genome 14 copies of the 16S rRNA gene were identified.

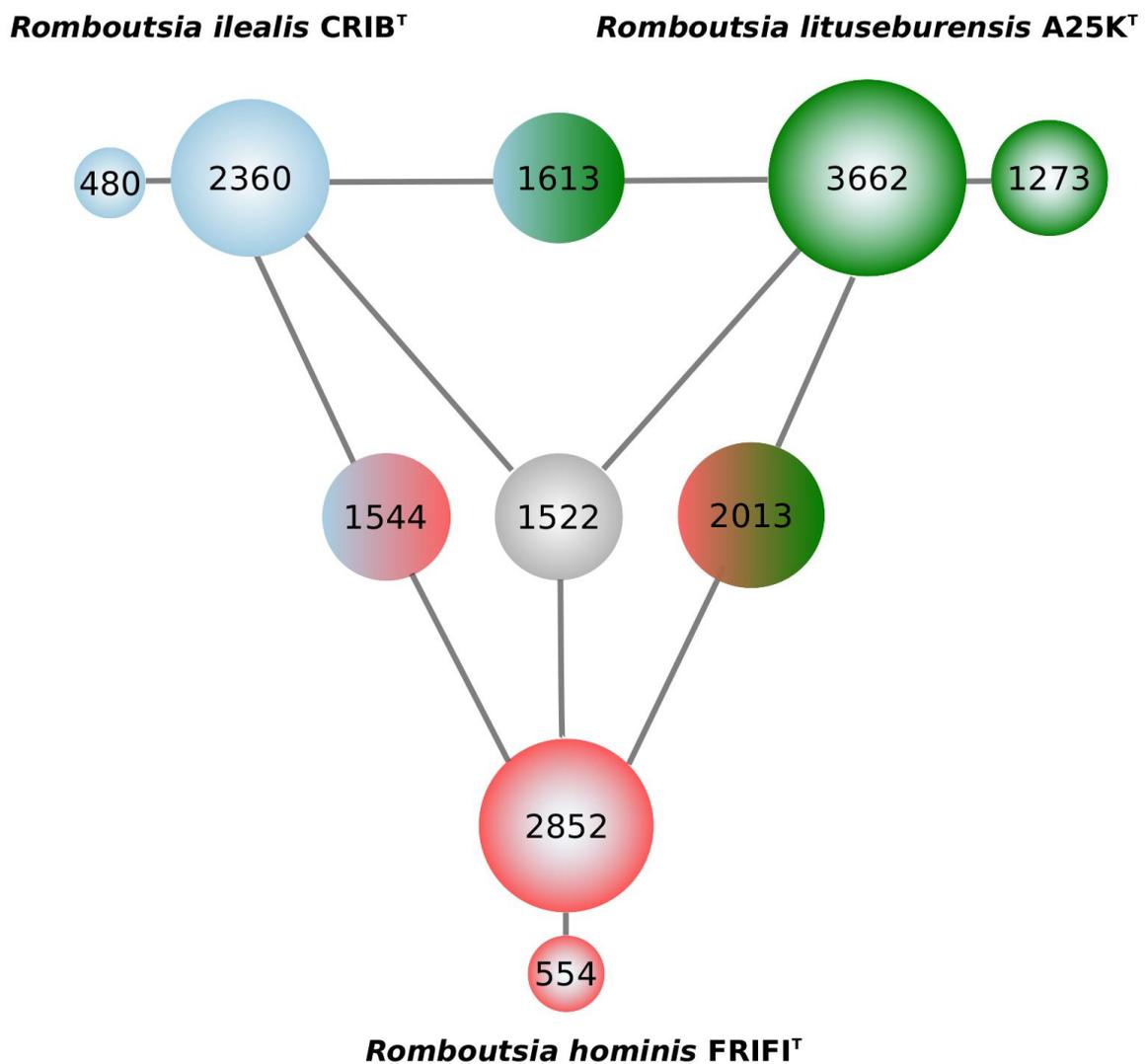
## 273 **Comparison of the genome of *Romboutsia lituseburensis* to** 274 **other genomes of this species**

275 The genome sequence of *R. lituseburensis* A25K<sup>T</sup> was compared to the genome sequence of the same  
276 strain (Bioproject PRJEB16174) that has been sequenced by the JGI and that has become publicly  
277 available during the course of this project (54). This comparison showed only minor sequence  
278 differences. Both genome sequences, including the plasmid sequences, are nearly identical (99.9%),  
279 with most differences arising from the gaps within our assembly or from contig ends (~500bp of each  
280 contig) within the JGI assembly. One difference was observed within the repetitive gene RLITU\_1618,  
281 which was shorter assembled in the JGI genome. The surface antigen encoding gene RLITU\_0237 was  
282 also assembled shorter within the JGI assembly. Duplicated Lysine, Serine and Arginine tRNA genes  
283 (location 1433719 - 1434325) were omitted in the JGI assembly, probably due to misassembly in this  
284 complicated region, which is not resolvable with Illumina short reads. Furthermore the JGI contig GI:  
285 1086420641 seems to be assembled differently, since the rRNA region present in this contig was not  
286 connected to the protein coding sequences in our assembly, but both were located at different places  
287 within the genome. We were unable to locate the first 8kb of JGI contig GI: 1086420759 within our  
288 assembly. The remaining parts of this contig match to an area following an assembly gap, and it  
289 therefore cannot be excluded that it was missed in our assembly. The only unplaced contig within our  
290 assembly was also nearly completely contained in a single contig within the JGI assembly, and  
291 therefore did not help to resolve this situation. Overall, it seems that most of the observed differences  
292 were due to technical reasons, and not due to underlying differences in the genomes of both strains.  
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## 294 **Comparative genomic analysis of the new *Romboutsia*** 295 **genomes to the type species of the genus**

296 The genome sequences of the two newly sequenced strains were compared to the type strain of the  
297 type species of the genus, *Romboutsia ilealis* CRIB<sup>T</sup> (48). The number of protein coding genes per

298 genome within the various strains was quite variable, ranging from 2359 to 3658 (Table 1). The  
299 number of putative homologous genes among the three *Romboutsia* genomes was determined via  
300 amino acid level best bidirectional hits (Fig. 3). In total 1522 genes were shared between all three  
301 strains, the core genome, accounting for 42 % to 64 % of the total gene count in the individual  
302 genomes, providing a first insight in the genomic heterogeneity within the genus. The bigger the  
303 genome, the more unique genes were present, ranging from 19 % to 34 % of the total gene count.  
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308 **Fig. 3. Overview of the number of homologous genes shared between the three *Romboutsia***  
309 **genomes.**

310 The circles are colour-coded by the *Romboutsia* strains they represent: blue, *R. ilealis* CRIB<sup>T</sup>; green; *R.*  
311 *lituseburensis* A25K<sup>T</sup>; red *R. hominis* FRIFI<sup>T</sup>. Also the total number of genes and the number of unique  
312 genes are indicated for each genome. The area of the circle is representative for the number of genes.

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315 The comparative genome analysis showed a general conservation of the genomic structure of  
316 the genus *Romboutsia* around the replication start site, while synteny appears to be lost towards the  
317 replication end point. For most pairwise comparisons, synteny was lost at a quarter of the genome in  
318 both up- and downstream directions, making roughly half of the genomes syntenic. Breaks of synteny  
319 appear to be related to specific recombination events. For example, compared to the other genomes  
320 synteny is absent in *R. ilealis* CRIB<sup>T</sup> due to the insertion of a prophage, whereas the regions both up-  
321 and downstream are syntenic. At another spot in the genome of *R. ilealis* CRIB<sup>T</sup> synteny is lost due to  
322 phage-related genes found around the tmRNA gene, which has been reported to be a common  
323 insertion site for phages (55). The position of the tmRNA itself is roughly equal in all three genomes,  
324 but no synteny could be observed in its vicinity. Strain/species-specific gene clusters, like the CRISPR-  
325 Cas system or the fucose degradation pathway present in *R. ilealis* CRIB<sup>T</sup>, appear to be situated more  
326 towards the less conserved replication end point. One point of conservation in the less conserved area  
327 is an inversion of one part of the butyrate fermentation pathway, which is absent in *R. ilealis* CRIB<sup>T</sup>,  
328 but inverted between *R. hominis* FRIFI<sup>T</sup> and *R. lituseburensis* A25K<sup>T</sup>. Some significant deletion events  
329 appear to have occurred, since they can be observed in the conserved areas of the genomes. For  
330 example, the pili encoding gene cluster, which is found in the genome of *R. lituseburensis* A25K<sup>T</sup> close  
331 to the replication start site, is absent in the genomes of *R. ilealis* CRIB<sup>T</sup> and *R. hominis* FRIFI<sup>T</sup> except for  
332 a twitching motility protein encoding gene. Another example is the biosynthesis cluster for vitamin  
333 B12, which is also located in all strains close to the replication start site. While this cluster is complete

334 in *R. lituseburensis* A25K<sup>T</sup> and *R. hominis* FRIF1<sup>T</sup>, only remnants of the cluster are visible in *R. ilealis*  
335 CRIB<sup>T</sup> as there is a deletion of nine genes, which prevents the biosynthesis of cob(II)yrinate a,c-  
336 diamide. This cluster is also situated next to an rRNA operon, of which only the one in *R. ilealis* CRIB<sup>T</sup>  
337 has an integrase inserted.

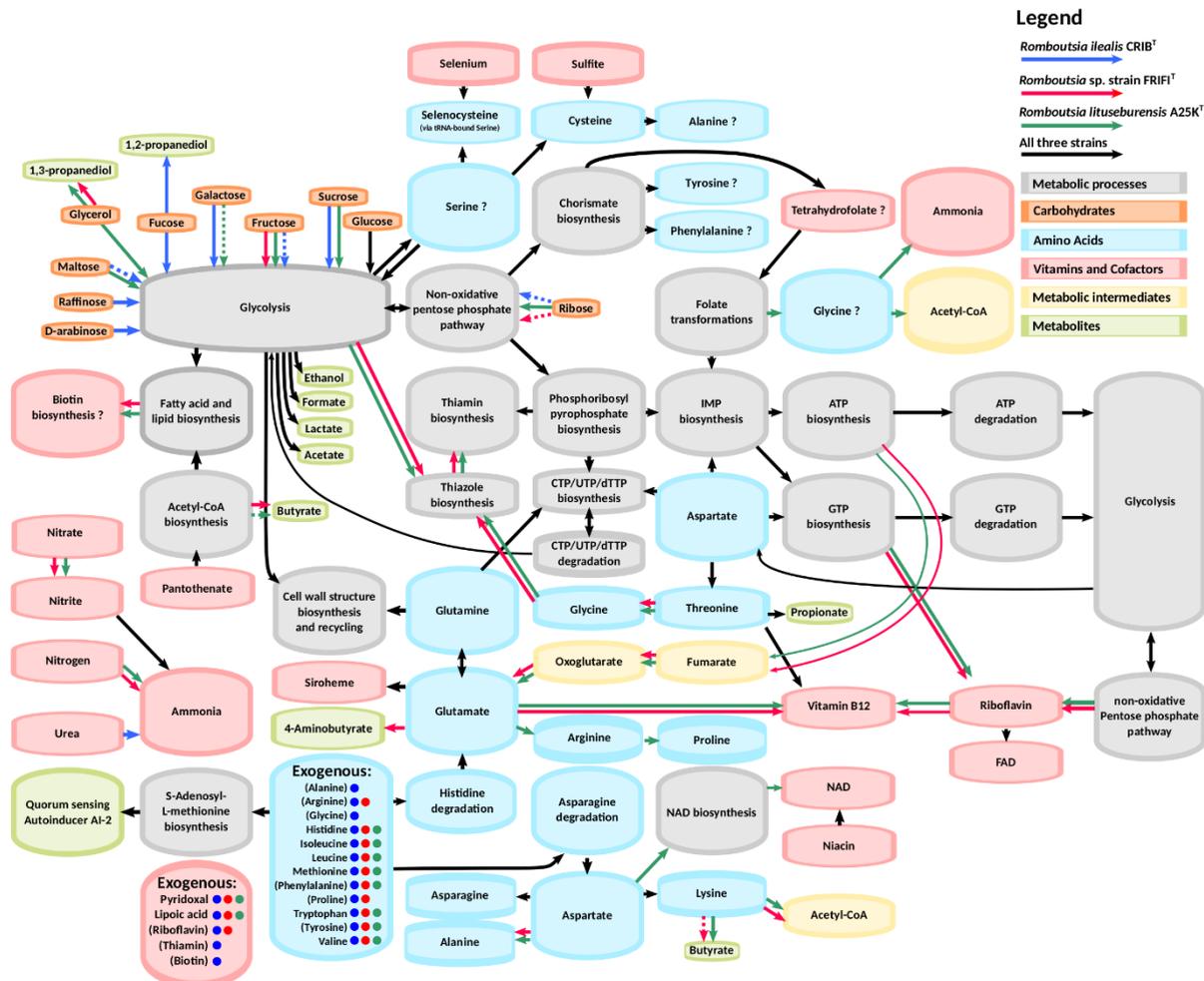
## 338 **Core and pan metabolism of the genus *Romboutsia***

339 An overview of the core metabolism of the *Romboutsia* strains and strain-specific metabolic features  
340 is provided in Fig. 4. All three *Romboutsia* strains can ferment carbohydrates via the glycolysis, and  
341 possess the non-oxidative pentose phosphate pathway. Moreover, from the genomes it was predicted  
342 that all strains have the capability to synthesize (and degrade) all nucleotides, cell wall components,  
343 fatty acids and siroheme. In addition, it was predicted that all three *Romboutsia* spp. strains can only  
344 produce a limited non-identical set of amino acids. In turn they are, however, also able to ferment  
345 numerous amino acids. Furthermore various pathways for assimilation of nitrogen were predicted, as  
346 well as a pathway for production of the quorum sensing compound autoinducer AI-2. Some of the  
347 metabolic highlights will be discussed in the following paragraphs.

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351

352 **Fig. 4. Overview of the predicted core metabolism and strain-specific metabolic features of three**  
 353 ***Romboutsia* spp. strains.**

354 Dotted lines indicated instances where reported experimental observations do not match genome-  
 355 based predictions. Question marks indicate processes for which not all enzymes could be identified in  
 356 the genome. Brackets around compounds indicate that the enzymes necessary for de novo production  
 357 of the compounds might be present in the genome.

358

359

360

## 361 **Fermentation and anaerobic respiration**

362 Similar fermentation end-products have been observed for *R. ilealis* CRIB<sup>T</sup>, *R. hominis* FRIFI<sup>T</sup> and *R.*  
363 *lituseburensis* A25K<sup>T</sup> during growth on glucose, including formate, acetate and a small amount of  
364 lactate (17). The pathways leading to formate, acetate and lactate production, which have previously  
365 been described for *R. ilealis* CRIB<sup>T</sup> (48), were also found in the two other *Romboutsia* strains suggesting  
366 that all strains are indeed able to produce formate, acetate and lactate. Butyrate (and iso-valerate)  
367 production has been observed for *R. lituseburensis* A25K<sup>T</sup> during *in vitro* growth on undefined medium  
368 components such as beef extract, peptone and casitone (but not on yeast extract). The addition of a  
369 carbohydrate (e.g. glucose) resulted in a redirection of the fermentation pathways towards other end  
370 products such as formate (data not shown). Two pathways leading to butyrate synthesis, the acetyl-  
371 CoA and the lysine pathways, could be predicted from the genome of *R. lituseburensis* A25K<sup>T</sup>. The  
372 pathways are co-located in the genome, suggesting that the acetoacetyl-CoA formed during lysine  
373 fermentation can be directly used as substrate in the acetyl-CoA pathway for additional energy  
374 conservation (56, 57). A lysine-specific permease was predicted in the genome as well, suggesting that  
375 exogenous lysine can serve as energy source for this strain. Since an acetyl-CoA acetyltransferase was  
376 found in the gene cluster as well, a fully functioning carbohydrate-driven acetyl-CoA pathway is  
377 expected. For the final step in butyrate production, a phosphate butyryl transferase/butyrate kinase  
378 (*buk*) gene cluster was identified in the genome. Similar gene clusters (although with some gene  
379 inversions) were found in the genome of *R. hominis* FRIFI<sup>T</sup> as well, but butyrate production has not  
380 (yet) been observed (17).

381 In the genomes of both *R. hominis* FRIFI<sup>T</sup> and *R. lituseburensis* A25K<sup>T</sup> a reductive pathway for  
382 the metabolism of glycerol was predicted, comprising a glycerol dehydratase and 1,3-propanediol  
383 dehydrogenase (58). This suggests that these strains are able to ferment glycerol and produce 1,3-  
384 propanediol as one of the fermentation end-products. Production of 1,3-propanediol has indeed been  
385 reported for *R. lituseburensis* (22). Furthermore, in the genome of *R. lituseburensis* A25K<sup>T</sup> the oxidative  
386 pathway for glycerol metabolism, including glycerol dehydrogenase and dihydroxyacetone kinase,

387 could be identified as well, suggesting that this strain should be able to use glycerol as sole carbon and  
388 energy source. For both *R. hominis* FRIFI<sup>T</sup> and *R. lituseburensis* A25K<sup>T</sup> growth on glycerol has indeed  
389 been observed (17), although the responsible genes could not be predicted in *R. hominis* FRIFI<sup>T</sup>.

390 The genomes of all three *Romboutsia* strains studied here contain genes encoding for enzymes  
391 of the Wood-Ljungdahl pathway. A formate dehydrogenase was predicted for all strains except *R.*  
392 *ilealis* CRIB<sup>T</sup>. The presence of formate dehydrogenase together with a complete Wood-Ljungdahl  
393 pathway categorizes them as potential acetogens, microbes that can grow autotrophically using CO<sub>2</sub>  
394 and H<sub>2</sub> as carbon and energy source. This provides them with metabolic flexibility in addition to  
395 heterotrophic growth on organic compounds. The role of acetogens in the intestinal tract is not well  
396 studied. They have been proposed to play an important role in hydrogen disposal, in addition to  
397 methanogens and sulfate reducers (59, 60).

398 Genomes of all three *Romboutsia* strains contain genes predicted to encode a sulfite  
399 reductase of the AsrABC type. Inducible sulfite reductases are directly linked to the regeneration of  
400 NAD<sup>+</sup>, which plays a role in energy conservation and growth, as well as to detoxification of sulfite (61).  
401 *R. hominis* FRIFI<sup>T</sup>, however, appears to lack the formate/nitrite transporter family protein that was  
402 found in the vicinity of the predicted sulfite reductase in the other strains similarly to *Clostridioides*  
403 *difficile* (previously known as *Clostridium difficile*) where it was characterized as a hydrosulfide ion  
404 channel which exports the toxic metabolites from the cell (62). The genes coding for a complete  
405 membrane-bound electron transport system were identified in both genomes of *R. hominis* FRIFI<sup>T</sup> and  
406 *R. lituseburensis* A25K<sup>T</sup>, similar to the Rnf system identified in microbes such as *Clostridium tetani*,  
407 *Clostridium ljungdahlii* and *C. difficile*. In these species the system is suggested to be used to generate  
408 a proton gradient for energy conservation in microbes without cytochromes. In *C. tetani*, the system  
409 is proposed to play a role in the electron flow from reduced ferredoxin, via NADH to the NADH-  
410 consuming dehydrogenase of the butyrate synthesis pathway (63). In addition, the Rnf system is  
411 proposed to be used by *C. ljungdahlii* during autotrophic growth (64). In the genome of *R. ilealis* CRIB<sup>T</sup>

412 only remnants of an Rnf electron transport system could be found, which might be a result of genome  
413 reduction since also no complete butyrate synthesis pathway or acetogenic pathways were found.

## 414 **Fermentation of individual amino acids**

415 Species belonging to the class *Clostridia* are known for their capabilities to ferment amino acids. Of  
416 the three *Romboutsia* strains, *R. lituseburensis* A25K<sup>T</sup> appears to be the most resourceful. All three  
417 *Romboutsia* strains are predicted to be able to ferment L-histidine via glutamate using a histidine  
418 ammonia lyase. In addition, fermentation of L-threonine was predicted using a L-threonine  
419 dehydratase resulting in propionate production, which has been described for *R. lituseburensis* (20).  
420 Fermentation of L-serine into pyruvate using an L-serine dehydratase was predicted for all three  
421 strains as well. As aforementioned, *R. hominis* FRIFI<sup>T</sup> and *R. lituseburensis* A25K<sup>T</sup> are predicted to be  
422 able to ferment L-lysine. In addition, *R. lituseburensis* A25K<sup>T</sup> is predicted to ferment glycine using the  
423 glycine reductase pathway found in other related species including *C. difficile* (65, 66). A corresponding  
424 complex has also been identified in *R. hominis* FRIFI<sup>T</sup>, but it is likely to be non-functional, due to a loss  
425 of two of the three subunits. Furthermore, the ability to ferment L-arginine (using an arginine  
426 deiminase) and L-glutamate (using a Na<sup>+</sup>-dependent glutamoyl-CoA decarboxylase) was predicted for  
427 *C. lituseburensis* A25K<sup>T</sup> as well. A glutamate decarboxylase was predicted for *R. hominis* FRIFI<sup>T</sup>,  
428 suggesting the ability to decarboxylate glutamate to 4-aminobutyrate (GABA) for this strain only.

## 429 **Amino acid and vitamin requirements**

430 Pathways for (*de novo*) synthesis of amino acids were identified in the three *Romboutsia* strains (Table  
431 3). All three strains show similar dependencies on exogenous amino acid sources. Based on genome  
432 predictions, *R. lituseburensis* A25K<sup>T</sup> is able to synthesize lysine from aspartate, whereas the last  
433 enzyme in this pathway is missing in the genomes of *R. hominis* FRIFI<sup>T</sup> and *R. ilealis* CRIB<sup>T</sup>. In addition,  
434 *R. hominis* FRIFI<sup>T</sup> and *R. lituseburensis* A25K<sup>T</sup> are predicted to synthesise alanine from aspartate and  
435 glycine from threonine. Common to all organisms is that the prephenate dehydratase for the

436 biosynthesis of phenylalanine and tyrosine is missing, although all other enzymes for the biosynthesis  
 437 of chorismate and for the further conversion to both amino acids are present.

438

439 **Table 3.** Overview of genome-based predictions for amino acid requirements of the three *Romboutsia*  
 440 strains. In case only one or two enzymes are missing in either salvage or *de novo* pathway leading to  
 441 the production of an amino acid, this is indicated in parentheses.

442

	<i>Romboutsia</i>	<i>R. lituseburensis</i>	<i>R. ilealis</i>
	<i>hominis</i> FRIFI <sup>T</sup>	A25K <sup>T</sup>	CRIB <sup>T</sup>
<b>Alanine</b>	+	+	-
<b>Arginine</b>	-	+	-
<b>Asparagine</b>	+	+	+
<b>Aspartate</b>	+	+	+
<b>Cysteine</b>	+	+	+
<b>Glutamate</b>	+	+	+
<b>Glutamine</b>	+	+	+
<b>Glycine</b>	+	+	-
<b>Histidine</b>	-	-	-
<b>Isoleucine</b>	-	-	-
<b>Leucine</b>	-	-	-
<b>Lysine</b>	- (-1)	+	- (-1)
<b>Methionine</b>	-	-	-
<b>Phenylalanine</b>	- (-1)	- (-1)	- (-1)
<b>Proline</b>	-	- (-1)	-
<b>Serine</b>	- (-1)	- (-1)	- (-1)

<b>Threonine</b>	- (-1)	- (-1)	- (-1)
<b>Tryptophan</b>	-	-	-
<b>Tyrosine</b>	- (-1)	- (-1)	- (-1)
<b>Valine</b>	-	-	-

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446 The urease gene cluster, previously identified in *R. ilealis* CRIB<sup>T</sup> (48), could not be identified in  
447 the two other *Romboutsia* strains. However, a nitrogenase encoding gene cluster was identified in the  
448 genomes of *R. hominis* FRIFI<sup>T</sup> and *R. lituseburensis* A25K<sup>T</sup>, suggesting that these two strains are able to  
449 fix N<sub>2</sub>.

450 All three strains encode one or several oligopeptide transporters of the OPT family (67). In  
451 addition, two oligopeptide transport systems (*Opp* and *App*) (68, 69) were predicted in *R. hominis*  
452 FRIFI<sup>T</sup> and *R. lituseburensis* A25K<sup>T</sup> (strain FRIFI<sup>T</sup> misses the *OppA*), whereas they were absent in *R. ilealis*  
453 CRIB<sup>T</sup>. Based on the predicted amino acid dependencies, it can be concluded that these *Romboutsia*  
454 strains are adapted to an environment rich in amino acids and peptides.

455 The metabolic capabilities of the three *Romboutsia* species are comparable regarding the  
456 ability to produce certain vitamins and other cofactors (Fig. 4). None of them is predicted to be able  
457 to synthesize vitamin B6, lipoic acid or pantothenate, but it is likely that they are all able to produce  
458 siroheme from glutamate and CoA from pantothenate. As previously described for *R. ilealis* CRIB<sup>T</sup> (48),  
459 the pathway for *de novo* folate biosynthesis via the pABA branch is present, however, a gene encoding  
460 dihydrofolate reductase could not be identified in any of the three *Romboutsia* strains. However, since  
461 this enzyme is essential in both *de novo* and salvage pathways of tetrahydrofolate, it is highly likely it  
462 is present in the genomes. The biosynthetic capabilities of *R. lituseburensis* A25K<sup>T</sup>, and *R. hominis*  
463 FRIFI<sup>T</sup> are larger than that of *R. ilealis* CRIB<sup>T</sup>, as they are both predicted to produce biotin, thiamin and  
464 vitamin B12. The gene clusters for biotin and thiamin biosynthesis are located in the more variable

465 regions of the genomes as discussed above, and the vitamin B12 biosynthesis pathway is incomplete  
466 in *R. ilealis* CRIB<sup>T</sup> due to a deletion, as mentioned earlier. Only *R. lituseburensis* A25K<sup>T</sup> is predicted to  
467 have the capacity to produce riboflavin *de novo*. Furthermore, *R. lituseburensis* A25K<sup>T</sup> is, as the only  
468 non-host derived organism in this comparison, the only strain that can synthesize NAD *de novo*.

## 469 **Bile resistance**

470 One of the challenges for microbes living in the intestinal tract is that they have to deal with the host-  
471 secreted bile acids. The bile acid pool size and composition modulates the size and composition of the  
472 intestinal microbiota and vice versa (70, 71). Bile acids can undergo a variety of bacterial  
473 transformations including deconjugation, dehydroxylation and epimerization. In both intestinal  
474 isolates, *R. ilealis* CRIB<sup>T</sup> and *R. hominis* FRIFI<sup>T</sup>, a choloylglycine hydrolase encoding gene was identified.  
475 Bile salt hydrolases (BSHs), also known as conjugated bile acid hydrolases, from the choloylglycine  
476 hydrolase family are widespread among Gram-positive and Gram-negative intestinal microbes (72).  
477 They are involved in the hydrolysis of the amide linkage in conjugated bile acids, releasing primary bile  
478 acids. There is a large heterogeneity among BSHs, including with respect to their substrate specificity  
479 such as specificity towards either taurine or glycine conjugated bile salts (70). The choloylglycine  
480 hydrolases of *R. ilealis* CRIB<sup>T</sup> and *R. hominis* FRIFI<sup>T</sup> differ significantly from each other (32 % identity at  
481 amino acid level), suggesting a different origin. The BSH of *R. ilealis* CRIB<sup>T</sup> and *R. hominis* FRIFI<sup>T</sup> show  
482 at the amino acid level 52 % and 33 % identity, respectively, with the choloylglycine hydrolase CBAH-  
483 1 from *Clostridium perfringens* (73).

484 In addition to the possible BSH, a bile acid 7 $\alpha$ -dehydratase encoding gene could be identified  
485 in *R. hominis* FRIFI<sup>T</sup>. This enzyme is part of the multi-step 7 $\alpha$ / $\beta$ -dehydroxylation pathway that is  
486 involved in the transformation of primary bile acids into secondary bile acids. So far, this pathway has  
487 been found exclusively in a small number of anaerobic intestinal bacteria all belonging to the  
488 *Firmicutes* (72, 74). The presence of this pathway enables microbes to use primary bile acids as an  
489 electron acceptor, allowing for increased ATP formation and growth. High levels of secondary bile  
490 acids are associated with diseases of the host such as cholesterol gallstone disease and cancers of the

491 GI tract (75, 76). However, the evidence that bacteria capable of 7 $\alpha$ -dehydroxylation are directly  
492 involved in the pathogenesis of these diseases is still limited. The pathway has been extensively  
493 studied in the human isolate *Clostridium scindens* VPI 12708 (formerly known as *Eubacterium* sp. strain  
494 VPI 12708 (77)). In addition, 7 $\alpha$ -dehydroxylation activity was also reported for *Clostridium hiranonis*  
495 (78) and *Paeniclostridium sordellii* (previously known as *Clostridium sordellii*) (79), and other close  
496 relatives of the genus *Romboutsia* (74). Extensive characterization of the 7 $\alpha$ / $\beta$ -dehydroxylation  
497 pathway in *C. scindens* VPI 12708 has demonstrated that the genes involved are encoded by a large  
498 bile acid inducible (*bai*) operon (72). For *R. hominis* FRIFI<sup>T</sup> several other genes were identified in the  
499 vicinity of the bile acid 7 $\alpha$ -dehydratase gene that showed homology to the genes in the *bai* operon,  
500 but some other (essential) genes seem to be missing. From gene presence/absence it was therefore  
501 not possible to predict whether *R. hominis* FRIFI<sup>T</sup> has 7 $\alpha$ / $\beta$ -dehydroxylation activity and that has to be  
502 confirmed experimentally.

## 503 **Toxins and virulence-related genes**

504 The class *Clostridia* contains some well-known pathogens, including *C. difficile*, *C. botulinum* and *C.*  
505 *perfringens*, for which several toxins have been characterized in depth (80). No homologues of the  
506 genes coding for the toxins of *C. difficile* (toxin A, toxin B, binary toxin) or *C. botulinum* could be found  
507 in the genomes of the three *Romboutsia* strains. The genome of *R. ilealis* CRIB<sup>T</sup> encodes a predicted  
508 protein that was annotated as a putative septicolysin (CRIB\_2392) since it shares 56 %identity to a  
509 protein that has been characterized as an oxygen-labile hemolysin in *Clostridium septicum* (81).  
510 However, the exact role of septicolysin in potential pathogenesis is not known. Homologues are not  
511 found in other related species. A homologue for the alpha toxin of *Clostridium perfringens* (80, 82), a  
512 phospholipase C protein involved in the aetiology of gas gangrene caused by *C. perfringens* (83), was  
513 found by BLAST search in the genomes of *R. hominis* FRIFI<sup>T</sup> and *R. lituseburensis* A25K<sup>T</sup> (49.4 - 54.3 %  
514 identity at the amino acid level). In addition, *R. lituseburensis* A25K<sup>T</sup> is predicted to contain a protein  
515 homologous to the perfringolysin O (theta toxin) of *C. perfringens*, which is a thiol-activated cytolysin  
516 that forms large homo-oligomeric pore complexes in cholesterol-containing membranes, which is also

517 involved in gas gangrene aetiology. By BLAST search similar proteins could also be found in the  
518 genomes of *P. sordellii* and *Paraclostridium bifermentans* (previously known as *Clostridium*  
519 *bifermentans*), which are close relatives of the genus *Romboutsia*. There are many homologous  
520 enzymes produced by other bacteria that do not have similar toxigenic properties as the *C. perfringens*  
521 proteins (83). For example, the phospholipase C proteins produced by *P. bifermentans* and *P. sordellii*  
522 were found to have significantly less haemolytic activity than the homologous protein of *C.*  
523 *perfringens* (51 and 53.4 % similarity on amino acid level, respectively) (84, 85). The predictions for  
524 the presence of potential toxin-encoding genes in the *Romboutsia* strains was done based on  
525 homology; the enzymatic activity of the gene products will have to be determined in the future to see  
526 whether some of the *Romboutsia* strains have toxigenic properties.

## 527 **Motility**

528 Motility was observed for *R. hominis* FRIFI<sup>T</sup> and *R. lituseburensis* A25K<sup>T</sup> but not for *R. ilealis* CRIB<sup>T</sup>, as  
529 previously reported (17). In general, different appendages can be found on bacterial surfaces that  
530 provide bacteria with the ability to swim in liquids or move on surfaces via gliding or twitching motility  
531 (86, 87). In the genomes of *R. hominis* FRIFI<sup>T</sup> and *R. lituseburensis* A25K<sup>T</sup> a large gene cluster for the  
532 synthesis of flagella could be identified. The organization of the flagella gene cluster is very similar to  
533 that in the genome of *C. difficile*. The formation of flagella involves a whole array of different  
534 components, including the core protein flagellin. Post-translational modification of flagellin by  
535 glycosylation is an important process both for the flagellar assembly and biological function, and genes  
536 involved in these modifications are often found in the vicinity of the structural flagellin genes (88).  
537 This is also the case for *R. hominis* FRIFI<sup>T</sup> and *R. lituseburensis* A25K<sup>T</sup>, and these genes are found in an  
538 intra-flagellar synthesis locus similar to the situation in *C. difficile* 630 (89). In *R. ilealis* CRIB<sup>T</sup> no genes  
539 encoding flagellar proteins or genes involved in chemotaxis could be identified in line with the lack of  
540 motility (90). Flagella are dominant innate immune activators in the intestinal tract as flagellin  
541 molecules can be recognized by host cell-surface and cytoplasmatic pattern recognition receptors (91-  
542 93). The role for flagella in virulence and pathogenicity of *C. difficile* is a topic of interest, however,

543 their exact contribution is still unknown (94). The flagellin proteins of some of the most abundant  
544 motile commensal microbes that are found in the human intestinal tract (*Eubacterium* and *Roseburia*  
545 species) have recently also been shown to possess pro-inflammatory properties (95).

546 One type of gliding motility involves the extension, attachment and retraction of type IV pili  
547 (TFP), which pull the bacterium towards the site of attachment (96). In the genome of *R. lituseburensis*  
548 A25K<sup>T</sup> a complete set of genes for the assembly of Type IV pili could be identified. In contrast, in the  
549 genomes of *R. ilealis* CRIB<sup>T</sup> and *R. hominis* FRIF<sup>T</sup> only remnants could be identified.

## 550 **Sporulation**

551 Sporulation is a trait found only in certain low G+C Gram-positive bacteria belonging to the *Firmicutes*  
552 (97). The formation of metabolically dormant endospores is an important strategy used by bacteria to  
553 survive environmental challenges such as nutrient limitation. These endospores are resistant to  
554 extreme exposures (e.g. high temperatures, freezing, radiation and agents such as antibiotics and  
555 most detergents) that would kill vegetative cells. The ability to form endospores was also studied for  
556 the three *Romboutsia* strains. *R. lituseburensis* A25K<sup>T</sup> readily forms mature spores, especially during  
557 growth in Duncan-Strong medium and Cooked meat medium, that both contain large quantities of  
558 proteose peptone, and spore formation was observed in almost every cell (data not shown).  
559 Previously, the endospore forming capabilities of *R. ilealis* CRIB<sup>T</sup> and *R. hominis* FRIF<sup>T</sup> have been  
560 studied (90). Using different media and incubation conditions it was observed that the process of  
561 sporulation appears to be initiated, however, no free mature spores could be observed.

562 The whole process of sporulation and subsequent spore germination involves the expression  
563 of hundreds of genes in a highly regulated manner. At a molecular level the process is best understood  
564 in the model organism *Bacillus subtilis* (98). For species belonging to the class *Clostridia*, the process  
565 of sporulation is mainly studied in microbes in which sporulation has been shown to play a big role in  
566 other processes such as virulence (*C. perfringens*, *C. difficile*, *C. botulinum* and *C. tetani*) or solvent  
567 production (*Clostridium acetobutylicum*). Studying these microbes has made it clear that there are  
568 significant differences in the sporulation and germination process in species belonging to the class

569 *Clostridia* compared to members of the *Bacilli* (47, 99, 100). The *B. subtilis* proteins involved in the  
570 early stages of sporulation, i.e. onset (stage I), commitment and asymmetric cell division (stage II), and  
571 engulfment (stage III), are largely conserved in *Clostridia* species. However, many of the proteins that  
572 play a role in later stages, i.e. cortex formation (stage IV), spore coat maturation (stage V), mother cell  
573 lysis and spore release (stage VII), appear to be less conserved. For example, limited spore outer layer  
574 conservation was observed in *C. difficile* compared to *B. subtilis* (101). Comparative genomic based-  
575 studies have tried to define the minimal set of genes essential for sporulation in clostridial species,  
576 but this has appeared to be challenging (47, 100, 102). In all spore-formers, initiation of sporulation is  
577 controlled by the transcription factor Spo0A, a highly conserved master regulator of sporulation.  
578 Phosphorylation of Spo0A leads to the activation of a tightly regulated cascade involving several sigma  
579 factors that regulate the further expression of a multitude of genes involved in sporulation. There are,  
580 however, significant differences in the regulation of the sporulation pathway between different  
581 clostridial species (103) of which we do not completely understand the impact on sporulation itself,  
582 highlighting that there is still a big gap in our knowledge on the complex process of sporulation.

583         The genomes of the three *Romboutsia* genomes were mined for homologues of sporulation  
584 specific genes according to the publication of Galperin *et al.* (47). All three *Romboutsia* strains have  
585 similar sets of sporulation-related genes, with *R. ilealis* CRIB<sup>T</sup> having the least number of genes (147  
586 genes) and *R. lituseburensis* A25K<sup>T</sup> having the most (183 genes) (**Table S1**). The only protein that is  
587 deemed essential for sporulation, but which was only found in the genome of *R. lituseburensis* A25K<sup>T</sup>,  
588 is the Stage V sporulation protein S which has been implicated to increase sporulation (104). For *R.*  
589 *lituseburensis* A25K<sup>T</sup>, the sporulation regulator Spo0E was predicted to be absent, due to a point  
590 mutation in the start codon of the corresponding gene, changing it to an alternative start codon. This  
591 regulator is suggested to be involved in the prevention of sporulation under certain circumstances  
592 (105); impact of the point mutation on the presence/absence of the protein and on regulation of  
593 sporulation in *R. lituseburensis* A25K<sup>T</sup> will have to be determined. Interestingly, the stage V sporulation  
594 proteins AA and AB, encoded by *spoVAA* and *spoVAB*, that are essential for sporulation in *Bacilli* since

595 mutants lead to the production of immature spores (106), are absent in sporulating *Clostridium*  
596 species, but are present in all three *Romboutsia* strains. Furthermore, *R. lituseburensis* A25K<sup>T</sup> is the  
597 only strain that contains the *sps* operon that has been shown to be involved in spore surface adhesion  
598 (107). Absence of this operon in *B. subtilis* resulted in defective germination, and more hydrophobic  
599 and adhesive spores, however, given that these proteins are also absent in nearly all clostridial species,  
600 their role in sporulation and germination in the *Romboutsia* strains still has to be determined. As also  
601 noted by Galperin *et al.* (47), there are other species that have been demonstrated to be spore-  
602 forming but which also lack some of the genes that are deemed to be essential, e.g. *spolIB*, *spolIM*,  
603 and other proteins from the second sporulation stage in *Lysinibacillus sphaericus* C3-41v (47). In  
604 comparison, it is interesting to note that the genome of *C. hiranonis*, a close relative of the genus  
605 *Romboutsia* (and *C. difficile*), appears to contain only 21 of the essential sporulation genes, missing  
606 for example most of the proteins related to the second and third stage of sporulation, while *C.*  
607 *hiranonis* is known to form spores ((108), and own observations). Altogether, based on gene  
608 presence/absence it is not possible to predict whether these *Romboutsia* strains are indeed able to  
609 successfully complete the process of sporulation and release endospores. An asporogenous  
610 phenotype could be credited to the absence or mutation of a single gene.

611 Initiation of sporulation is still a topic of interest. Accessory gene regulatory (*agr*)-dependent  
612 quorum sensing, and thus most likely cell density, has been proposed to play an important role in  
613 efficient sporulation (109). For *C. difficile*, however, quorum-sensing has been shown not to play a role  
614 in initiation of sporulation, and recently a more direct link between nutrient availability and  
615 sporulation was suggested (110). The uptake of peptides by the Opp and App oligopeptide transport  
616 systems appears to prevent initiation of sporulation in nutrient rich environments (69). Both transport  
617 systems are absent in *R. ilealis* CRIB<sup>T</sup>, but are present in the two other *Romboutsia* strains.

618 During sporulation, a number of species produce inclusion bodies and granules that are visible  
619 by phase contrast and electron microscopy. This is also true for *R. lituseburensis* A25K<sup>T</sup> in which  
620 electron translucent bodies are visible in TEM pictures (Fig. 1), similar to the carbohydrate or

621 polyhydroxybutyrate inclusions observed in for example *Clostridium pasteurianum* (111), *C.*  
622 *acetobutylicum* (112) and *C. botulinum* (113). The development of these inclusion bodies appears to  
623 coincide with the initiation of sporulation. Based on this observation, it can be speculated that by  
624 intracellular accumulation of a carbon and energy source these microbes ensure they can complete  
625 the sporulation process with only limited dependence on external carbon and energy sources.

## 626 **Conclusions**

627 Based on the comparative genome analysis presented here we can conclude that the investigated  
628 genomes of the genus *Romboutsia* encode a versatile array of metabolic capabilities with respect to  
629 carbohydrate utilization, fermentation of single amino acids, anaerobic respiration and metabolic end  
630 products. A relative genome reduction is observed in the isolates from intestinal origin. In addition,  
631 the presence of bile converting enzymes and pathways related to host-derived carbohydrates, point  
632 towards adaption to a life in the (small) intestine of mammalian hosts. For each *Romboutsia* strain  
633 unique properties were found. However, since currently only one genome was available for each  
634 species, it is impossible to unequivocally predict which properties might apply to each species and  
635 which properties are strain-specific. Isolation and genome sequencing of additional strains from  
636 diverse environments is needed to provide a more in-depth view of the metabolic capabilities at the  
637 species- as well as the genus level and to reveal specific properties that relate to adaptation to an  
638 intestinal lifestyle.

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## 644 **Availability of data and material**

645 All data has been uploaded to the European Nucleotide Archive under project numbers PRJEB7106  
646 and PRJEB7306

## 647 **Competing interests**

648 Jacoline Gerritsen is an employee of Winlove Probiotics. The company had no influence on this  
649 manuscript.

## 650 **Financial disclosure**

651 Research was partially funded by a grant of SenterNovem (FND-07013) and ERC advanced grant  
652 Microbes Inside (250172), and the SIAM Gravitation Grant (024.002.002) of the Netherlands  
653 Organization for Scientific Research (NWO) to WMdV.

654 B. Hornung was supported by Wageningen University and the Wageningen Institute for Environment  
655 and Climate Research (WIMEK) through the IP/OP program Systems Biology (project KB-17-003.02-  
656 023). The funders had no influence on this study.

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## 663 **Acknowledgements**

664 The authors would like to thank Wilma Akkermans-van Vliet for help with DNA extractions, Aleksander  
665 Umanetc for providing the microscopic picture of *R. hominis* FRIFI<sup>T</sup>, and Laura van Niftrik for providing

666 the TEM picture of *R. lituseburensis* A25K<sup>T</sup>. We would also like to thank Jasper Koehorst for help with  
667 the annotation, Bart Nijse for assisting with the different software packages, and Jesse van Dam for  
668 help with the Pathway Tools lisp interface. In addition, we would like to thank William Trimble from  
669 the Argonne National Laboratory for helpful discussions concerning genome assembly with MiSeq  
670 data sets.

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## 953 **Supplementary information:**

### 954 **File #1:**

- 955
- File name: supplementary\_table\_1.xls
- 956
- File format including the correct file extension: Excel document, .xls

- 957       • Title of data: Supplementary Table 1
- 958       • Description of data: Overview of sporulation-related genes in the three *Romboutsia*
- 959       *genomes*. Genes from *Bacillus subtilis* subsp. *subtilis* 168, to which no homologues could be
- 960       identified, are omitted. In case multiple candidate loci were detected, all are mentioned.
- 961       Loci which are assigned to more than one gene from *B. subtilis* subsp. *subtilis* 168 are
- 962       marked with an asterisk

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