

1 **Exploring the archaeome: detection of archaeal signatures in the human body**

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15 **Abstract**

16 Due to their fundamentally different biology, archaea are consistently overlooked in conventional  
17 microbiome surveys. Using amplicon sequencing, we evaluated methodological set-ups to detect  
18 archaea in samples from five different body sites: respiratory tract (nose), digestive tract (mouth,  
19 appendix, and stool) and skin. With the optimized protocols, the detection of archaeal ribosomal  
20 sequence variants (RSVs) was increased from one (found in currently used, so-called “universal”  
21 approach) to 81 RSVs in a representative sample set. In order to assess the archaeome diversity, a  
22 specific archaea-targeting methodology is required, for which we propose a standard procedure. This  
23 methodology might not only prove useful for analyzing the human archaeome in more detail but  
24 could also be used for other holobionts’ samples.

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## 28 **Introduction**

29 The importance of microbial communities to human and environmental health motivates  
30 microbiome research to uncover their diversity and function. While the era of metagenomics and  
31 metatranscriptomics has begun, 16S rRNA gene amplicon sequencing still remains one of the most  
32 used methods to explore microbial communities, mainly due to the relatively low cost, the number of  
33 available pipelines for data analysis, and the comparably low computational power required.

34 It has been recognized that methodological issues in sample processing can significantly influence the  
35 outcome of microbiome studies, affecting comparability between different studies<sup>1,2</sup> or leading to an  
36 over-and under-estimation of certain microbial clades<sup>3,4</sup>. For better comparability among different  
37 studies, standard operational procedures for sampling, storing samples, DNA extraction,  
38 amplification and analysis were set-up (e.g. the Earth Microbiome Project<sup>5</sup> and the Human  
39 Microbiome Project<sup>6</sup>). This includes the usage of so-called “universal primers”<sup>7-9</sup>, to maximally cover  
40 the broadest prokaryotic diversity.

41 The human microbiome consists of bacteria, archaea, eukaryotes and viruses. The overwhelming  
42 majority of microbiome studies is bacteria-centric, but in recent years, awareness on eukaryotes (in  
43 particular fungi) and viruses has increased<sup>10-12</sup>. However, most microbiome studies still remain blind  
44 for the human archaeome<sup>3,13</sup>. A few of the underlying reasons for the under-representation of  
45 archaea in microbiome studies are (i) primer mismatches of the “universal primers”<sup>14</sup>, (ii) the  
46 sometimes too low abundance of the archaeal DNA in the studied samples<sup>15</sup>, (iii) improper DNA  
47 extraction methods<sup>16</sup>, and (iv) the incompleteness of the 16S rRNA gene reference databases due to  
48 missing isolates, especially for the DPANN superphylum<sup>15,17</sup>. Moreover, the clinical interest on  
49 archaea is minor, due to the fact that there are no known or proved archaeal pathogens yet<sup>18</sup>.

50 Nevertheless, (methanogenic) archaea are part of the commensal microorganisms inhabiting the  
51 human body, being regularly detected in the oral cavity and the gastrointestinal tract<sup>19-22</sup>; in the  
52 latter they sometimes even outnumber the most abundant bacterial species (14%,<sup>23</sup>). Most human

53 archaea studies use either cultivation or qPCR methods<sup>24–30</sup> and only a few, 16S rRNA gene  
54 sequencing archaea-centric studies are available<sup>24,31–33</sup>. These new studies have shown that archaea  
55 are also present in the human respiratory tract<sup>24</sup> and on human skin in considerable amounts<sup>31,34</sup>.  
56 Furthermore, Koskinen et al.<sup>24</sup> have shown for the first time that archaea reveal a body site specific  
57 pattern, similar to bacteria: the gastrointestinal tract being dominated by methanogens, the skin by  
58 *Thaumarchaeota*, the lungs by *Woesearchaeota*, and the nose archaeal communities being  
59 composed of mainly methanogens and *Thaumarchaeota*. Altogether, this indicates a substantial  
60 presence of archaea in some, or even all, human tissues.

61 As a logic consequence of our previous studies, we have started to optimize the detection a methods  
62 of archaea as human commensals. We tested, *in silico* and experimentally, 27 different 16S rRNA  
63 gene targeting primer pair combinations suitable for NGS amplicon sequencing, to detect the  
64 archaeal diversity in samples from different body sites, including respiratory tract (nose samples),  
65 digestive tract (oral samples, appendix specimens and stool), and skin. Our results culminate in a  
66 proposed standard operating procedure for archaea diversity analysis in human samples.

67

68 **Results**

69 Primer pairs were evaluated with respect to the following characteristics: high *in silico* specificity for  
70 archaeal 16S rRNA genes and an amplicon length of 150 to 300 bp, suitable for NGS, and *in vitro*  
71 capability to amplify diverse archaeal 16S rRNA genes from a variety of human specimens

72 Besides archaea-specific primer pairs, two widely used “universal” primers (515F-806uR original;  
73 515FB-806RB modified; <sup>7,9</sup>) were evaluated all along to assess the potential of “universal” primers to  
74 display archaeal diversity associated with the human body.

75 *Most archaea-targeting primers reveal good coverage in silico*

76 A total of 12 different primer pairs were evaluated *in silico* (Table 1). Most primer pairs showed high  
77 coverage for the archaeal domain ranging from 46% to 89% and revealed a high domain-specificity (8  
78 of 12 primer pairs without matches outside of the archaeal domain). When one mismatch per primer  
79 was allowed, the coverage increased to values from 68% to 95%.

80

81 **Table 1.** Primer selection and results of the pre-analysis *in silico* evaluation of all primer pairs used.  
 82 Coverage of *Archaea*, *Bacteria* and *Eukarya* is given in percentages, depending on whether no or one  
 83 mismatch was allowed. Designated “universal” primers (primer pairs 10-12) are indicated in bold  
 84 letters.

Primer pair	Name	Primer name*	Sequence (5' -> 3')	Fragment size (bp)	0 mismatch			1 mismatch		
					Archaea	Bacteria	Eukarya	Archaea	Bacteria	Eukarya
<b>1</b>	344F	S-D-Arch-0344-a-S-20	ACGGGGYGAGCAGGCGCGGA	571	46.1%	0.0%	0.0%	68.6%	0.0%	0.0%
	915R	S-D-Arch-0911-a-A-20	GTGCTCCCCGCCAATTCCT							
<b>2</b>	349F	S-D-Arch-0349-a-S-17	GYGCASCAGKCGMGAAW	566	71.8%	0.0%	0.0%	87.8%	0.0%	0.0%
	915R	S-D-Arch-0911-a-A-20	GTGCTCCCCGCCAATTCCT							
<b>3</b>	344F	S-D-Arch-0344-a-S-20	ACGGGGYGAGCAGGCGCGGA	697	51.5%	0.0%	0.0%	73.0%	0.0%	0.0%
	1041R	S-D-Arch-1041-a-A-18	GGCCATGCACCWCCTCTC							
<b>4</b>	349F	S-D-Arch-0349-a-S-17	GYGCASCAGKCGMGAAW	692	71.2%	0.0%	0.0%	90.0%	0.0%	0.0%
	1041R	S-D-Arch-1041-a-A-18	GGCCATGCACCWCCTCTC							
<b>5</b>	519F	S-D-Arch-0519-a-S-15	CAGCMGCCCGGGTAA	522	79.3%	0.0%	0.0%	93.7%	0.0%	0.0%
	1041R	S-D-Arch-1041-a-A-18	GGCCATGCACCWCCTCTC							
<b>6</b>	344F	S-D-Arch-0344-a-S-20	ACGGGGYGAGCAGGCGCGGA	462	48.3%	0.0%	0.0%	71.3%	0.0%	0.0%
	806R	S-D-Arch-0786-a-A-20	GGACTACVSGGGTATCTAAT							
<b>7</b>	349F	S-D-Arch-0349-a-S-17	GYGCASCAGKCGMGAAW	457	75.2%	0.0%	0.0%	91.1%	0.0%	0.0%
	806R	S-D-Arch-0786-a-A-20	GGACTACVSGGGTATCTAAT							
<b>8</b>	519F	S-D-Arch-0519-a-S-15	CAGCMGCCCGGGTAA	287	85.6%	6.8%	0.0%	95.2%	90.9	0.1%
	806R	S-D-Arch-0786-a-A-20	GGACTACVSGGGTATCTAAT							
<b>9</b>	349F	S-D-Arch-0349-a-S-17	GYGCASCAGKCGMGAAW	170	79.3%	0.0%	0.0%	92.8%	0.0%	0.1%
	519R	S-D-Arch-0519-a-A-16	TTACCGCGCKGCTG							
<b>10</b>	<b>519F</b>	S-D-Arch-0519-a-S-15	CAGCMGCCCGGGTAA	266	88.9%	88.8%	0.6%	95.3%	95.4%	1.2%
	<b>785R</b>	S-D-Bact-0785-b-A-18	TACNVGGGTATCTAATCC							
<b>11</b>	<b>515F</b>	515F-original	GTGCCAGCMGCCCGGGTAA	291	52.9%	86.8%	0.0%	94.6%	95.0%	0.3%
	<b>806uR</b>	806R-original	GGACTACHVGGGTWTCTAAT							
<b>12</b>	<b>515FB</b>	515F-modified	GTGYCAGCMGCCCGGGTAA	291	85.7%	87.7%	0.0%	95.4%	95.1%	1.4%
	<b>806RB</b>	806R-modified	GGACTACNVGGGTWTCTAAT							

\*according to <sup>8</sup>

85  
 86 One designated archaeal primer pair was found to target additionally sequences of the bacterial and  
 87 eukaryotic domain when one mismatch per primer was allowed, namely primer pair 519F-806R, with  
 88 a coverage of the bacterial domain > 90%.

89 We further evaluated the detailed coverage of the primer pairs for specific archaeal phyla and genera  
 90 of particular interest in human archaeome studies: *Euryarchaeota*, *Thaumarchaeota*, and  
 91 *Woesearchaeota*, as well as *Nitrososphaera*, *Methanobrevibacter*, *Methanosphaera* and  
 92 *Methanomassiliicoccus*. For all subsequent *in silico* analyses we allowed one mismatch.

93 All primer pairs revealed a high coverage for the *Euryarchaeota* phylum (in total >90%), for genera  
 94 *Methanobrevibacter* (between 94.6% and 98.9%) and *Methanomassiliococcus* (between 92.9% and  
 95 100%), while the coverage for *Methanosphaera* was below 90% for most primer pairs except for  
 96 519F-806R and 349F-519R (Table 2).

97

98 **Table 2:** *In silico* analysis of the coverage of chosen primer pairs for specific archaeal taxa of interest.  
 99 One mismatch was allowed per primer. For primer full names and sequences, please refer to Table 1.

primer pair	Name	<i>Euryarchaeota</i>			<i>Thaumarchaeota</i>		<i>Nanoarchaeota</i>	
		total	<i>Methano-brevibacter</i>	<i>Methano-sphaera</i>	<i>Methano-massiliococcus</i>	total	<i>Nitrososphaera</i> ( <i>Woesearchaeota</i> )	
1	344F 915R	90.80%	95.30%	82.20%	100.00%	20.60%	87.60%	66.40%
2	349F 915R	91.50%	95.30%	84.20%	100%	92%	89.70%	70.30%
3	344F 1041R	90.80%	94.60%	79.40%	100%	20.70%	89.00%	67.90%
4	349F 1041R	91.50%	94.60%	79.40%	100%	96.40%	92.30%	74.30%
5	519F 1041R	95%	97.80%	85.40%	92.90%	96.60%	90.60%	83%
6	344F 806R	92.30%	95.50%	82.60%	100%	23.30%	88%	65.20%
7	349F 806R	93.20%	95.60%	84.20%	100%	96.50%	90.10%	72.90%
8	519F 806R	96.60%	98.90%	90%	95%	96.70%	89.50%	83.10%
9	349F 519R	93.60%	95.80%	90.70%	95%	98%	94.40%	83.10%
10	519F 785R	96.50%	98.60%	89.60%	95%	96.20%	87.80%	87.60%
11	515F 806R	96.20%	98.60%	89.60%	95%	94.70%	86.90%	89.50%
12	515FB 806RB	96.20%	98.60%	89.60%	95%	96.50%	89%	89.50%

100

101 The coverage of the *Thaumarchaeota* phylum depended on the primer pair used. Most analyses that  
 102 included the primer 344F showed a low *in silico* coverage for *Thaumarchaeota* (below 30%) while all  
 103 other primer pair combinations revealed a high coverage of this phylum (>90%; Table 2). The

104 coverage for *Nitrososphaera* in particular varied between 86.9% and 94.4%. The class  
105 *Woesearchaeota* showed variable coverage between 65.2% and 89.5%.

106 As the archaeal primer 344F has often been used for detecting archaea in a variety of environmental  
107 samples<sup>35,36</sup>, we took a closer look on its coverage capacity using the TestProbe 3.0<sup>8</sup> and the SILVA  
108 database SSU132<sup>37</sup>. Overall, the primer revealed 73.2% coverage of the archaeal domain. The *in*  
109 *silico* results showed a high coverage of the *Euryarchaeota* phylum (93.8%) and the genera within,  
110 especially *Methanobrevibacter* with 96.1%, *Methanosphaera* with 89.9% and *Methanomassiliicoccus*  
111 with 100%. It also revealed a good coverage for *Woesearchaeota* with 74.6%, but showed, despite a  
112 high coverage for the genus *Nitrososphaera* (93.6%), a generally low coverage of the  
113 *Thaumarchaeota* phylum with only 24%, indicating a potentially low capacity for studies with  
114 thaumarchaeotal diversity in focus.

115 Another primer that we analyzed in more detail was primer 519F, also known as S-D-Arch-0519-a-S-  
116 15. As the sequence of this primer (5' - CAGCMGCCGCGGTAA - 3') overlaps with the sequence of the  
117 "universal" primer S\*-Univ-0519-a-S-18 (5' - CAGCMGCCGCGGTAATWC - 3'), we were interested to  
118 compare their coverages.

119 As expected, the results from the *in silico* analysis indicated that the primer S-D-Arch-0519-a-S-15  
120 targets *Bacteria* (coverage 98%), *Archaea* (coverage 98.2%) and *Eukarya* (coverage 96.4%). The  
121 universal primer S\*-Univ-0519-a-S-18 has a similar coverage and specificity for the three domains of  
122 life: *Bacteria* (coverage 97.5%), *Archaea* (coverage 96.4%), and *Eukarya* (coverage 95.6%).  
123 Considering our *in silico* results, the primer S-D-Arch-0519-a-S-15 cannot be used to target archaea  
124 specifically and should be re-named to S-D-Univ-0519-a-S-15.

125 As most selected archaea-targeting primers revealed a good coverage of the archaeal domain in  
126 general, all primer pairs were used for subsequent wet-lab experiments.

127



128 Archaeal community composition varies according to the used primer pairs and universal primers fail  
129 to detect the archaeal diversity

130 Herein we sought to identify the optimal primer pair for amplicon sequencing of the archaeomes in  
131 human samples. For this, we selected five representative sample types from different body sites:  
132 nose (upper nasal cavity), oral (subgingival sites), stool and appendix specimens, and skin (back)  
133 (sample set 1). The stool sample represented the natural positive control.

134 Next generation sequencing was performed, after a two-step nested PCR (for archaea) or a single-  
135 step PCR (“universal” target). The nested PCR approach was selected based on the reasons given in  
136 the Materials and Methods section. In brief, the first PCR was intended to select the archaeal  
137 community of interest, the second to further amplify the archaeal signal.

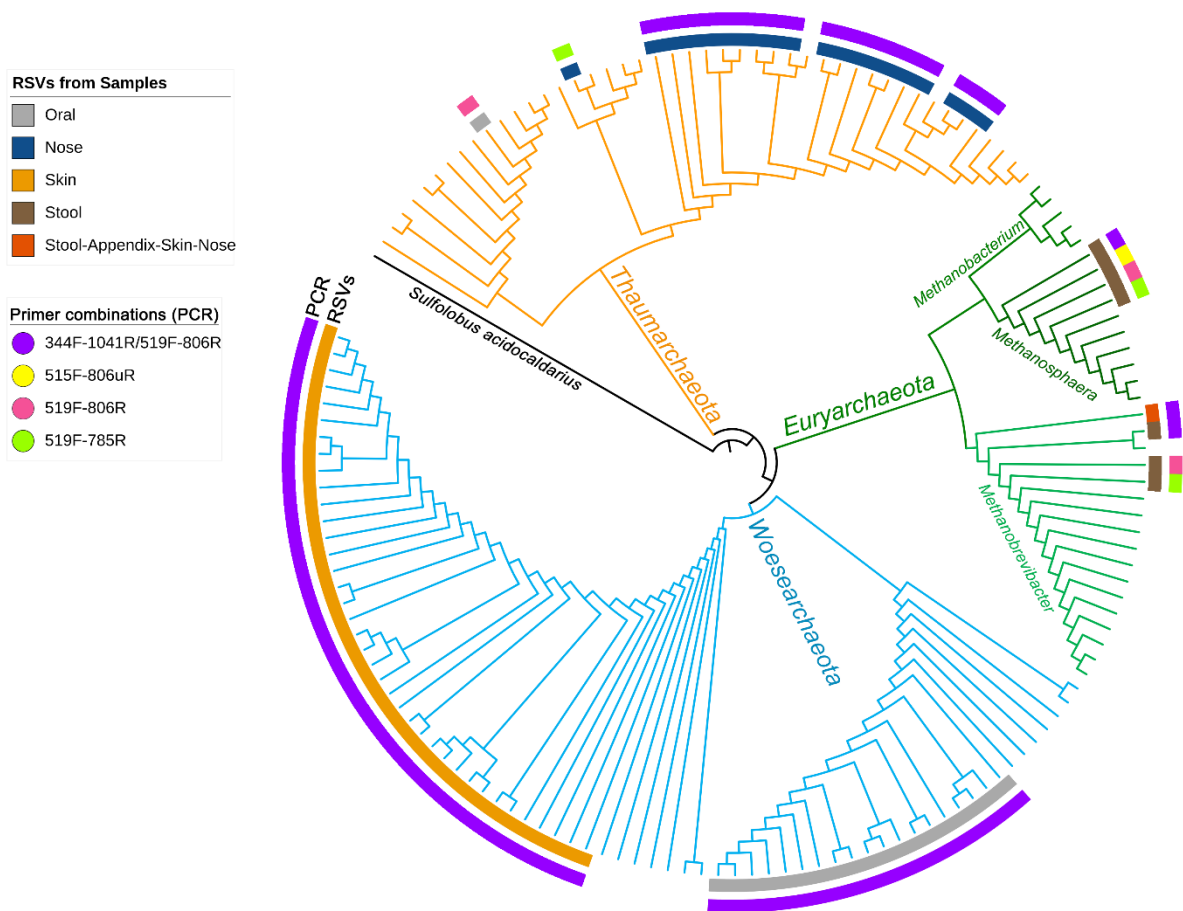
138 The use of universal primers (primer pair 515F-806uR, 515FB-806RB and 519F-785R) in the PCR  
139 reaction resulted in reads that were classified mainly within the bacterial domain with almost no  
140 reads classified within the archaea, confirming our previous observations<sup>24</sup>. In fact, when the two  
141 universal primer pairs (515F-806uR original and 515FB-806RB) were compared regarding the  
142 archaeal domain, only primer pair 515F-806uR allowed the detection of only one RSV being classified  
143 within the archaea and from only one sample, the stool sample.

144 Universal primer pair 519F-785R yielded slightly better results, allowing the detection of three  
145 different archaeal RSVs from two different samples: *Methanobrevibacter* and *Methanosphaera* in the  
146 stool sample, and one RSV from the nose sample, classified within the *Thaumarchaeota* phylum. Very  
147 similar results (detection of the same methanoarchaeal signatures in the stool sample, and one  
148 thaumarchaeal signature in the oral sample instead of the nose sample) were obtained from primer  
149 pair 519F-806R, which was originally described to be archaea-specific, but revealed wide coverage of  
150 the bacterial and archaeal domain (>90%, when one mismatch allowed) *in silico* (see previous  
151 chapter).

152 To identify whether the universal primer pairs allow the detection of the same RSVs or closely related  
153 RSVs in the analyzed samples, a phylogenetic tree was constructed (Fig. 1). Besides the obtained  
154 archaeal RSVs from the universal approaches, the RSVs retrieved from the archaeal specific primer  
155 pair combination 344F-1041R/519F-806R were included for comparison. This approach allowed the  
156 detection of 20 RSVs in the nose, 19 RSVs in the oral, one RSV in the appendix, 3 RSVs in the stool,  
157 and 39 RSVs in the skin sample. For the stool sample, the RSVs obtained from the universal and  
158 archaeal specific approach grouped together, either within *Methanobrevibacter* or *Methanosphaera*  
159 clade (Fig. 1), whereas the RSVs (universal and specific approach) from nose and oral samples  
160 diversified.

161

162



163

164

165 **Fig. 1:** Phylogenetic tree based on the retrieved RSVs from the universal approach, archaeal approach  
166 with primer 519F-806R or from the PCR based on the primer pair combination 344F-1041R/519F-  
167 806R as indicated in colors as an outermost circle (legend "Primer combinations (PCR)"). The inner  
168 circle represents the body site from where the RSVs were identified (see legend). Reference  
169 sequences from the SILVA database are shown without label. The branches of the tree were colored  
170 according to the phyla, blue: *Woesearchaeota*, green: *Euryarchaeota*, and orange: *Thaumarchaeota*.

171

172

173 Overall, 10 out of 23 primer pair combinations allowed the detection of archaeal signatures in all  
174 analyzed samples. All 23 primer pair combinations were able to detect archaeal reads in at least one  
175 of the sample types analyzed, for example all primer pair combinations detected archaeal RSVs in the  
176 stool sample; the number of RSVs, however, varied according to the used primer pair combination.

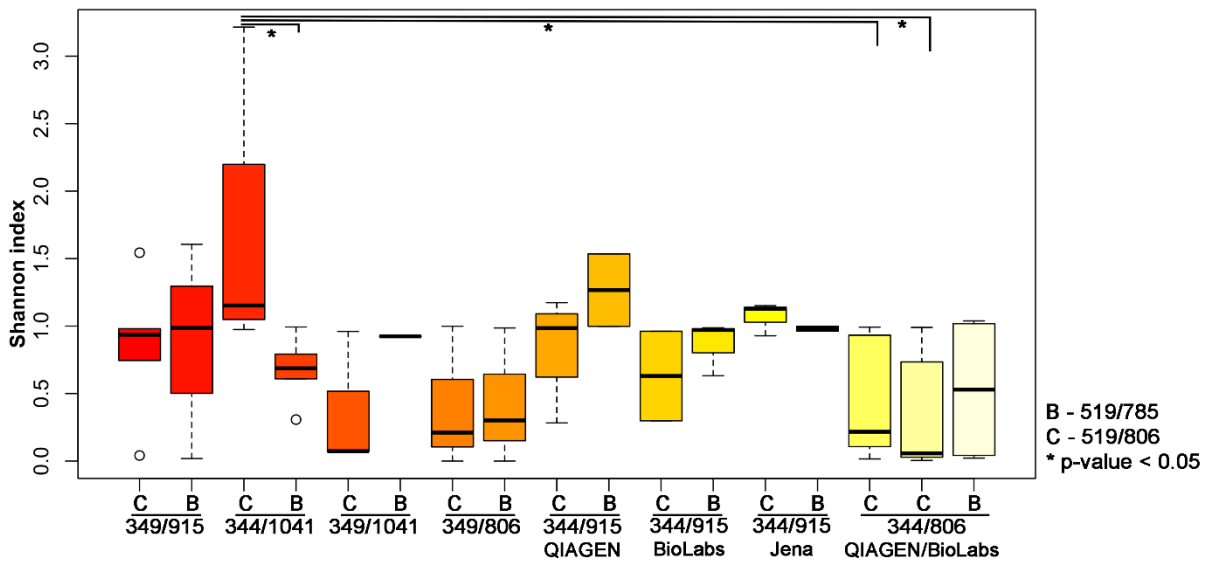
177 Depending on the used primer pair, the archaeal community composition was found to be highly  
178 variable (Suppl. Fig. 1). We observed that the detected variation in the archaeal composition was due  
179 to the used primer pair in the first PCR, the primer pair used to select the communities, while the  
180 second PCR and primer pair enhanced the signal of the first PCR (Suppl. Fig. 1). It shall be mentioned  
181 that for the second PCR only three different primer pairs have been used, 349F-519R, 519F-785R and  
182 the 519F-806R, of which the first two primer pairs had been used before to explore archaeal  
183 communities in human samples<sup>24</sup> and in confined habitats<sup>39</sup>.

184 To further explore the influence of the primer pair selection on the archaeal community composition,  
185 the alpha diversity was calculated using the Shannon index (Fig. 2). For this analysis, we excluded the  
186 results obtained from the second primer pair 349F-519R as most samples herein (except stool  
187 samples) yielded less than 500 reads.

188 The highest archaeal diversity could be detected with the primer combination 344F-1041R/519F-  
189 806R (PCR34); this result was found to be significant ( $p < 0.05$ ) compared to PCR 33 (344F-  
190 1041R/519F-785R), PCR Q7 (344F-806R/519F-806R) and PCR M7 (344F-806R/519F-806R; Table 3 and  
191 Fig. 2), whereas no other significant differences could be detected.

192

193



194

195 **Fig. 2:** Shannon index indicating the diversity received from different PCR approaches. The results  
196 have been plotted and grouped according to the first PCR used and the statistical significance (p-  
197 value <0.05; Wilcoxon Rank Test) is indicated by \*.

198

199 According to the comparison of the alpha diversity of the archaeal communities between the  
200 different primer pair combinations, we recommend the use of the nested approach with the primer  
201 pair 344F-1041R in the first PCR, followed by a second PCR with the primers 519F-806R for studying  
202 and exploring the archaeal communities in human samples.

203 The use of the different purification kits between the first and the second PCR resulted in no  
204 significant results based on the alpha diversity (Shannon index) comparison using the Wilcoxon Rank  
205 Test (p-value >0.05; Fig. 2). Due to visible bands on the gel electrophoresis for the results obtained  
206 after the purification with the Monarch® PCR & DNA Cleanup Kit (5 µg) (New England Biolabs GmbH;  
207 Ipswich, USA) we decided to further use this kit for the purification step.

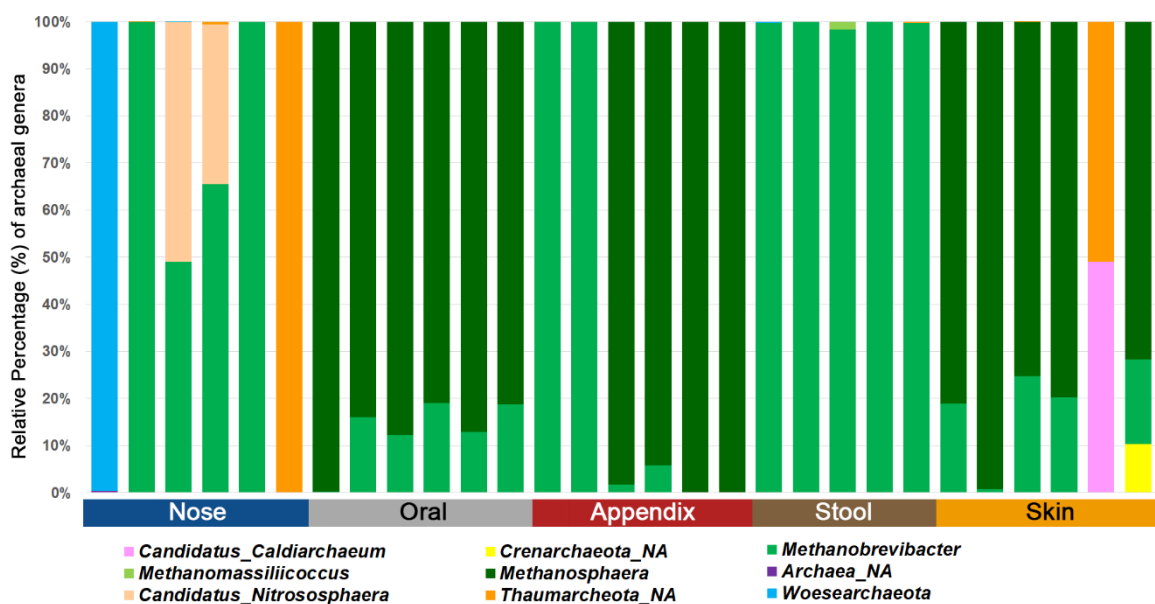
208

209 The primer combination with superior performance revealed a broad archaeal diversity in stool,  
210 appendix, nose, oral and skin samples

211 To further test and validate the use of the primer pair combination 344F-1041R/519F-806R for  
212 studying the archaeal communities within human samples, we selected additional samples from the  
213 same body sites: nose (n=5), oral (n=6), appendix (n=5), stool (n=5), and skin (n=7) (sample set 2).

214 Our selected PCR approach allowed the detection of archaea in all samples investigated with an  
215 average of 102,366 reads and 8 observed RSVs for the nose, 56,480 reads and 35 observed RSVs for  
216 oral, 46,022 reads and 8 observed RSVs for the appendix, 93,948 reads and 4 observed RSVs for the  
217 stool sample, and 76,001 reads and 30 observed RSVs for the skin samples.

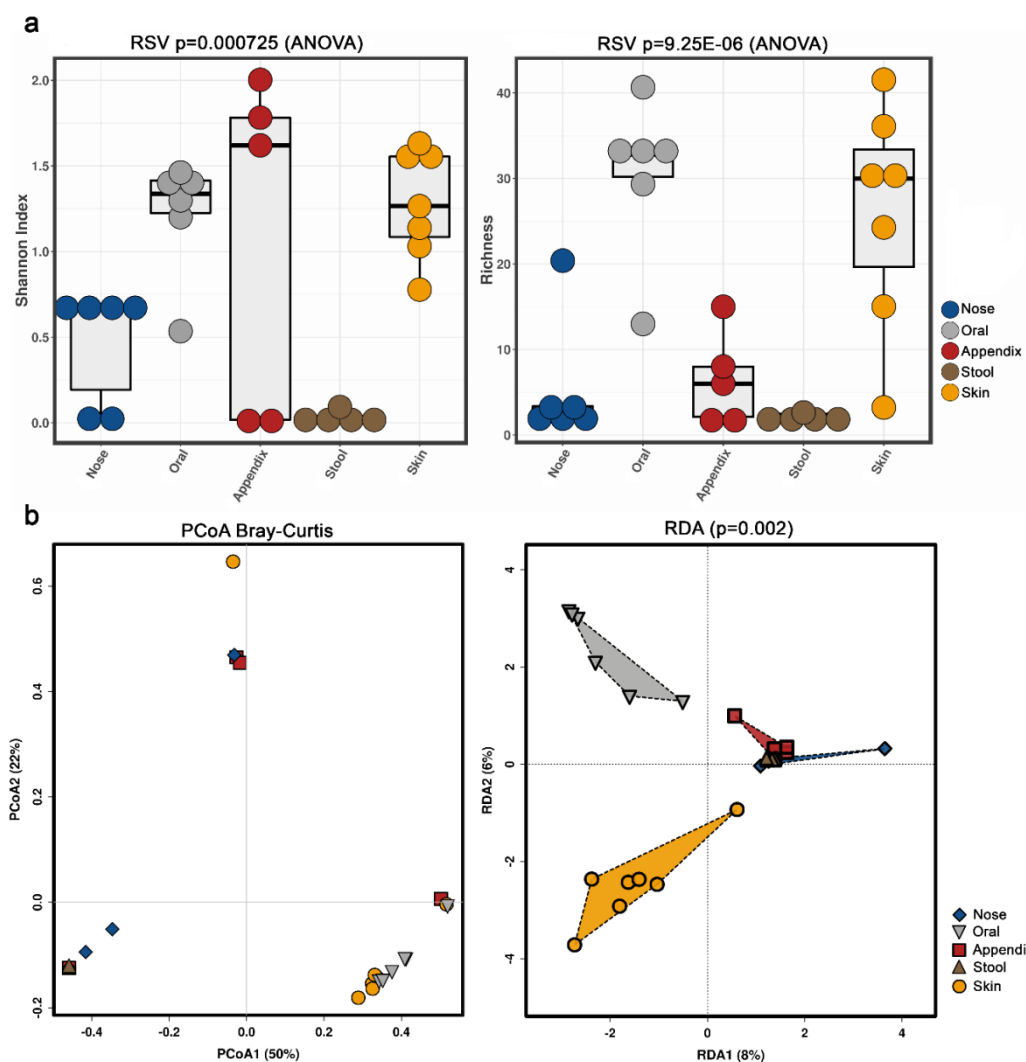
218 The results were plotted to indicate the archaeal communities present at genus level in the analyzed  
219 samples (Fig. 3).



221 **Fig. 3:** Bar chart displaying the different archaeal genera detected in different human samples using  
222 the superiorly performing primer combination 344F-1041R/519F-806R.

223

224 We further characterized the archaeal community information with respect to alpha and beta  
225 diversity. Depending on the body site a significant difference ( $p$ -value  $< 0.05$ ) could be shown for  
226 alpha (Shannon index and richness) and beta diversity (PCoA and RDA) (Fig. 4). Our results confirm  
227 the findings that archaeal communities are body site specific<sup>24</sup>.  
228 Notably, the stool samples revealed the overall lowest diversity of archaea, with only 3-5 identified  
229 archaeal RSVs, while skin and oral samples contained a higher diversity, with 5 to 49 RSVs found in  
230 the skin samples and 14 to 49 RSVs in the oral samples.



231

232 **Fig. 4:** Alpha (a; Shannon index and richness) and beta diversity (b; PCoA and RDA) analyses of the  
233 obtained archaeal community information, based on primer combination 344F-1041R/519F-806R.

234

235 **Discussion**

236 Up to now, little it is known about the composition of the human archaeome. It is unknown, whether  
237 archaeal communities are affected by dysbiosis or human disease, or how we acquire these  
238 microorganisms after birth, although several studies have shown that archaea are present in the first  
239 year of life <sup>27,40</sup>. Additionally, it is largely unexplored, how archaeal communities  
240 interact/communicate with other commensal microorganisms inhabiting the human body.  
241 Furthermore, there still remains the most burning question, if there are really no archaeal pathogens.  
242 Facing these numerous unsolved mysteries, we argue that more studies are needed with respect to  
243 the human archaeome. For these, however, standardized protocols are required, which are powerful  
244 enough to reliably assess archaeal diversity and abundance based on 16S rRNA gene signatures.

245 To address the need for archaea-targeted amplicon method for NGS in human samples, we herein  
246 tested 12 different primers previously described in literature <sup>8</sup>, in 27 primer pair combinations and  
247 evaluated their performance using *in silico* and experimental approaches on five different human  
248 sample types.

249 Despite their overall good *in silico* results, the three universal primer pairs tested failed to assess the  
250 archaeal diversity in the experiments. Two of these primer pairs represent the most-used universal  
251 primers for amplicon sequencing methods <sup>7,9</sup>, resulting in the detection of one (515F-806uR) or zero  
252 archaeal RSVs (515FB-806RB) in five sample types that evidentially possessed a variety of archaeal  
253 signatures.

254 The reasons for the failure of the universal primers to detect Archaea are unclear; however, it seems  
255 bacterial signatures outcompete archaeal signatures, just due to slightly better primer matches,  
256 depending on the diversity within the sample.

257 Furthermore, an archaeal primer pair (519F-806R) that has been used before for amplicon  
258 sequencing <sup>41</sup> detected only a small proportion of the archaeal diversity in the analyzed samples, but



259 the same primer pair performed better when used in a nested PCR together with the primer pair  
260 344F-1041R for the first PCR.

261 Nested PCR has been shown to improve sensitivity and specificity and are useful for suboptimal DNA  
262 samples<sup>42,43</sup>. Based on our experience in the past<sup>24</sup>, other reports<sup>44</sup>, and due to the fact that all  
263 attempts to use Illumina-tagged archaeal primers to directly identify archaeal 16S rRNA genes in  
264 human samples failed, we kept to this approach for the archaeal diversity assessment.

265 We used a combination of an archaea-specific first PCR (9 different primer combinations) and two  
266 archaeal specific and one universal primer pair, resulting in 23 different approaches (Table 3).

267 Notably, although the primer pair combinations 344F-915R/349F-519R and 344F-915R/519F-785R  
268 had been used earlier to detect archaeal signatures in human samples and confined environments<sup>24</sup>  
269<sup>39</sup>, our study revealed that when the second PCR contained the Illumina-tagged primers 349F-519R,  
270 almost no reads apart from the stool samples were retrieved.

271 Ten out of the 23 different primer combinations allowed the detection of archaeal signatures in all  
272 analyzed samples (sample set 1). The results of two of the primer pair combinations were  
273 outstanding regarding the number of reads and observed RSVs identified in each sample, namely  
274 primer pair 344F-1041R/519F-806R and 344F-1041R/519F-785R. The comparison of the alpha  
275 diversity (based on Shannon index) indicated that the archaeal diversity uncovered with the primer  
276 pair 344F-1041R/519F-806R was significantly higher than the one obtained with the primer pair  
277 combination 344F-1041R/519F-785R (Fig. 2), which was thus considered superior.

278 To further test and validate the use of the primer pair 344F-1041R/519F-806R, we selected 29  
279 samples from different body sites (nose, oral, appendix, stool, skin; sample set 2), resulting in overall  
280 85 archaeal RSVs from 6 different phyla. We were able to confirm body-site specificity through PCoA  
281 and RDA analysis<sup>24</sup>, with the gastrointestinal tract (stool and appendix samples) being dominated by  
282 euryarchaeal communities, the oral samples dominated by archaeal communities from the  
283 *Euryarchaeota* phylum but different from the ones found in the gastrointestinal tract and the nose

284 dominated by *Euryarchaeota* and *Thaumarchaeota* signatures. The skin revealed a mixture of  
285 *Euryarchaeota*, *Thaumarchaeota*, *Aenigmarchaeota*, and, in very low amounts also *Crenarchaeota*,  
286 confirming previous results<sup>24,31,45</sup>.

287 According to the obtained results we recommend the use of the primer pair combination 344F-  
288 1041R/519F-806R to identify and characterize archaeal communities within human samples, even  
289 though the second primer pair 519F-806R is a universal primer pair according to the *in silico* results.  
290 Although this led to retrieval of not only archaeal reads, but also reads classified within *Bacteria* and  
291 *Eukarya* which had to be filtered bioinformatically, this procedure proved superior to all the other  
292 primer pairs tested in identifying archaeal signatures in the analyzed samples.

293 In conclusion, we have shown that the choice of the archaeal primer pair influences substantially the  
294 perspective of the obtained archaeal community in the analyzed samples. Therefore, for future  
295 comparisons between studies focused on exploring and characterizing the archaeal community in  
296 human samples using amplicon sequencing approach, it should be considered to make use of the  
297 same, standardized methodology. For this we recommend the use of a nested approach with the  
298 primer pair 344f-1041R for the first PCR, followed by a second PCR with the primer pair 519F-806R.

## 299 **Conclusions**

300 The optimized and evaluated protocol for archaeal signature detection can now be used for all  
301 human samples and might also be useful for samples from other environments and holobionts, such  
302 plants or animals.

303

304 **Material and methods**

305 Selection of samples and DNA extraction

306 Representative sample types from various body sites including the respiratory tract (nose swabs), the  
307 digestive tract (oral biofilm, appendix biopsy and stool samples) and skin swabs were selected for the  
308 comparison of amplification-based protocols (See NOTE).

309 The nose swabs were obtained from healthy adults' volunteers (18-40 years old) and were taken  
310 from the olfactory mucosa located at the ceiling of the nasal cavity using ultra minitip nylon flocked  
311 swabs (Copan, Brescia, Italy; n=7)<sup>46</sup>. The oral samples have been obtained by standardized protocol  
312 for paper point sampling<sup>47</sup> from healthy children (10 years old) who participated in a microbiome  
313 study investigating the subgingival biofilm formation (n=7)<sup>48</sup>. Appendix samples have been obtained  
314 during pediatric appendectomies from either acute or ulcerous appendicitis from children (7-12 years  
315 old) (n=6). Stool samples have been obtained from healthy adults' volunteers (18-40 years old) (n=5)  
316<sup>49</sup>, and from one patient (68 years old) with above average methane production after metronidazole  
317 treatment (n=1; this sample was used for comparing different amplification protocols). Skin samples  
318 were obtained from healthy adults' volunteers (18-40 years old) from either the back (n=1; this  
319 sample was used for comparing different amplification protocols) or the left forearm, using BD  
320 Culture Swabs<sup>TM</sup> (Franklin Lakes, New Jersey, USA; n=7).

321 In all cases, the genomic DNA was extracted by a combination of mechanical and enzymatic lysis.  
322 However, depending on the sample type, different protocols were used: for the stool samples  
323 around 200mg of sample has been used for DNA extraction using the E.Z.N.A. stool DNA kit according  
324 to the manufacturer's instruction. The DNA from the appendix samples was obtained using the  
325 AllPrep DNA/RNA/Protein Mini Kit (QIAGEN), before the DNA extraction, small pieces of cryotissue  
326 were homogenized 3 times for 30s at 6500rpm using the MagNALyzer<sup>®</sup> instrument (Roche Molecular  
327 Systems) with buffer RTL and  $\beta$ -mercaptoethanol (according to the manufacturer's instructions). For  
328 the nose and skin samples from the forearm, the DNA was extracted using the FastDNA Spin Kit (MP

329 Biomedicals, Germany) according to the provided instructions. The DNA from the oral samples and  
330 from the skin samples from the back were isolated using the MagnaPure LC DNA Isolation Kit III  
331 (Bacteria, Fungi; Roche, Mannheim, Germany) as described by Santigli et al. <sup>48</sup> and Klymiuk et al. <sup>50</sup>.

332 **NOTE: Sample set 1** (one representative sample from each body site: nose, oral, appendix, stool from  
333 patient with high methane production and skin from the back) was used to initially evaluate the  
334 primers and methods, whereas **sample set 2** (6 nose samples, 6 oral samples, 5 appendices, 5 stool  
335 samples, and 7 skin samples) was then used for assessing the archaeal diversity, based on the  
336 chosen, optimized protocol.

### 337 16S rRNA gene primer selection and pre-analysis *in silico* evaluation

338 Different primer pairs targeting the archaeal 16S rRNA gene region have been selected from recent  
339 publications <sup>8,24</sup>. The main criteria for selection were: a. specificity for archaea *in-silico*, b. low or no  
340 amplification of eukaryotic DNA, and c. amplicon length between 150 to 300bp, suitable for NGS such  
341 as Illumina MiSeq. In addition, three “universal” primer pairs <sup>7-9</sup> were tested in parallel to determine  
342 their efficiency in detecting archaea in human samples. Full information on the selected primer pairs  
343 is given in Table 1.

344 *In silico* evaluation of the selected primer pairs has been performed using the online tool  
345 TestPrime1.0 <sup>8</sup> and the non-redundant SILVA database SSU132 <sup>37</sup>. Two of the primers (344F and S-D-  
346 Arch-0519-a-S-15) were also tested using TestProbe 3.0 <sup>8</sup> and the SILVA database SSU132 to assess  
347 their individual coverage for the archaeal domain. These two primers were tested either due to low  
348 coverage of the Thaumarchaeota domain (such as primer combinations including the 344F primer) or  
349 because the primers were targeting other domains of life such as Bacteria and Eukarya (primer  
350 combinations including the S-D-Arch-0519-a-S-15).

351

352

353 PCR and library preparation

354 For archaea-targeting PCR, a nested approach was chosen to increase the specificity for archaea and  
 355 to avoid the formation of primer dimers caused by the tag, necessary for Illumina sequencing,  
 356 attached to the primers<sup>24,51</sup>.

357 In addition to the nested approach, a standard PCR was performed with three different universal  
 358 primer pairs, and one archaeal primer pair for comparative reasons, and to test if a universal  
 359 approach is capable to cover archaea in human samples in sufficient depth. All primer combinations  
 360 (in total 27) used for the PCR reactions are provided in Table 3.

361 **Table 3** displays all primer pair combinations used for the first and the second PCR of the nested  
 362 approach and the “universal” PCR. If not indicated otherwise (in brackets), the first PCR was followed  
 363 by a purification of the PCR product by the MinElute PCR Purification kit (QIAGEN) kit. n.a.: not  
 364 applicable.  
 365

PCR #	Primer combination 1st PCR	Primer combination 2nd PCR
PCR21	349F-915R	Illu 349F-Illu519R
PCR22		Illu 519F-Illu785R
PCR23		Illu 519F-Illu806R
PCR31	344F-1041R	Illu 349F-Illu519R
PCR33		Illu 519F-Illu785R
PCR34		Illu 519F-Illu806R
PCR41	349F-1041R	Illu 349F-Illu519R
PCR42		Illu 519F-Illu785R
PCR43		Illu 519F-Illu806R
PCR61	349F-806R	Illu 349F-Illu519R
PCR62		Illu 519F-Illu785R
PCR63		Illu 519F-Illu806R
PCR71	519F-1041R	Illu 519F-Illu785R
PCR72		Illu 519F-Illu806R
PCR81	519F-806R	Illu 519F-Illu785R
PCR82		Illu 519F-Illu806R
PCR91	344F-519R	Illu 349F-Illu519R
PCRQ1	344F-915R (QIAGEN)	Illu 349F-Illu519R
PCRQ3		Illu 519F-Illu785R
PCRQ4		Illu 519F-Illu806R
PCRM1	344F-915R (NEB Monarch)	Illu 349F-Illu519R
PCRM3		Illu 519F-Illu785R
PCRM4		Illu 519F-Illu806R
PCRA1	344F-915R (Analytik Jena)	Illu 349F-Illu519R
PCRA3		Illu 519F-Illu785R
PCRA4		Illu 519F-Illu806R
PCRQ5	344F-806R (QIAGEN)	Illu 349F-Illu519R
PCRQ6		Illu 519F-Illu785R
PCRQ7		Illu 519F-Illu806R

PCRM5	344F-806R (NEB Monarch)	Illu 349F-Illu519R
PCRM6		Illu 519F-Illu785R
PCRM7		Illu 519F-Illu806R
PCR8-Uni	n.a.	Illu 515F-Illu806uR
PCR9-Uni		Illu 515FB-Illu806RB
PCR10		Illu 519F-Illu806R
PCR11-Uni		Illu 519F-Illu785R

366

367 For the first PCR, each reaction was performed in a final volume of 20 µl containing: TAKARA Ex Taq®  
368 buffer with MgCl<sub>2</sub> (10 X; Takara Bio Inc., Tokyo, Japan), primers 500 nM, BSA (Roche Lifescience,  
369 Basel, Switzerland) 1 mg/ml, dNTP mix 200 µM, TAKARA Ex Taq® Polymerase 0.5 U, water  
370 (Lichrosolv®; Merck, Darmstadt, Germany), and DNA template (1-50 ng/µl).

371 After the first PCR, the resulting amplicons were purified to remove primer remnants. This  
372 purification was performed with three different kits to compare the different yields and efficiencies,  
373 namely MinElute PCR Purification kit (Qiagen; Hilden, Germany), Monarch® PCR & DNA Cleanup Kit  
374 (5 µg) (New England Biolabs GmbH; Ipswich, USA), or innuPREP DOUBLEpure Kit (Analytik Jena,  
375 Germany) as indicated in Table 4. The purified PCR product was eluted in 10 µl water (Lichrosolv®;  
376 Merck, Darmstadt, Germany).

377 Two µl of the resulting, purified PCR products were transferred into a subsequent 2<sup>nd</sup> PCR containing  
378 the following mixture: TAKARA Ex Taq® buffer with MgCl<sub>2</sub> (10 X; Takara Bio Inc., Tokyo, Japan),  
379 primers 500 nM, BSA (Roche Lifescience, Basel, Switzerland) 1 mg/ml, dNTP mix 200 µM, TAKARA Ex  
380 Taq® Polymerase 0.5 U, and water (Lichrosolv®; Merck, Darmstadt, Germany) up to a volume of 25  
381 µL.

382 The PCR cycling conditions are listed in Table 4, according to the primer pairs used. For all primer  
383 pairs, annealing temperatures were either determined experimentally by gradient PCR or adopted  
384 from literature information.

385

386 **Table 4:** PCR conditions. For denaturation, annealing and elongation the corresponding time and  
 387 temperature is given.

Target	Archaea (16S rRNA gene)			"Universal" (16S rRNA gene)	
	1°	1°	2°	1°	1°
(Nested) PCR, round					
Primer pair	344F / 915R 349F / 915R 344F / 806R 349F / 806R 519F / 806R	344F / 1041R 349F / 1041R 519F / 1041R	All Illumina tagged primer pairs	Illu519F/Illu806R Illu519F/Illu785R	Illu515F/Illu806uR Illu515FB/Illu806RB
Initial denaturation	2', 95°C	5', 95°C	5', 95°C	5', 95°C	3', 94°C
Denaturation	30", 96°C (first 10 cycl.), 25" 94°C	30", 94°C	40", 95°C	40", 95°C	45", 94°C
Annealing	30", 60°C	45", 56°C	2', 63°C	2', 63°C	1', 50°C
Elongation	1', 72°C	1', 72°C	1', 72°C	1', 72°C	1' 30", 72°C
Final elongation	10', 72°C	10', 72°C	10', 72°C	10', 72°C	10', 72°C
No. of cycles	25	25	30	40	40

388

389 Sample set 2 was amplified using the primer combination 344F-1041R/519F-806R (Table 3). For the  
 390 first PCR, each reaction was performed in a final volume of 20 µl as described above. After the first  
 391 PCR, the PCR products were purified using Monarch® PCR & DNA Cleanup Kit (5 µg; New England  
 392 Biolabs GmbH). For the second PCR, the final volume was 25 µl, as described above, only the volume  
 393 of the DNA template varied: 2 µl purified PCR product for stool and nose samples, 4 µl for all other  
 394 samples.

395

#### 396 Next generation sequencing, bioinformatics and statistical analyses

397 Amplicons were sequenced at the ZMF Core Facility Molecular Biology in Graz, Austria, using the  
 398 Illumina MiSeq platform<sup>50</sup>. The MiSeq amplicon sequence data was deposited in the European  
 399 Nucleotide Archive under the study accession number PRJEB27023.

400 The data processing of the obtained MiSeq sequence data was performed using the open source  
 401 package DADA2 (Divisive Amplicon Denoising Algorithm;<sup>38</sup>) as described previously<sup>39</sup>. Shortly, the  
 402 DADA2 turns paired-end fastq files into merged, denoised, chimera-free, and inferred sample  
 403 sequences called ribosomal sequence variants (RSVs). The taxonomic affiliations were determined  
 404 using SILVA v128 database as the reference database<sup>37</sup>. In the resulting RSV table, each row  
 405 corresponds to non-chimeric inferred sample sequence with a separate taxonomic classification.

406 Negative controls (extraction controls and no-template controls) were included during PCR  
407 amplification. The RSVs overlapping the negative controls and samples were either subtracted or  
408 completely removed from the data sets.

409 Processing of sequencing data was performed using the in-house Galaxy set-up<sup>50</sup> and subsequent  
410 statistical analyses were performed in R version 3.4.3<sup>52</sup>. Samples were rarefied to 500 reads and  
411 alpha diversity was calculated using the Shannon index. In order to identify differences between the  
412 archaeal diversity, Wilcoxon Rank Test was performed. The diversity of the archaeal communities  
413 within sample set 2 was determined using two diversity matrices (Shannon and richness). Analysis of  
414 variance (ANOVA) was performed to test for differences in the archaeal diversity based on the body  
415 location. Principal Coordinates Analysis (PCoA) based on Bray-Curtis distances was used to visualize  
416 differences between the samples from different body site. Redundancy discrimination analysis (RDA)  
417 was used to analyze the association between archaeal community composition and the body site  
418 location. RDA, alpha diversity and PCoA analysis were performed using Calypso Version 8.62<sup>53</sup>. The  
419 RSV tables obtained were used to summarize taxon abundance at different taxonomic levels. The  
420 taxonomic profiles obtained at the genus level for the samples with more than 100 reads were used  
421 to generate bar graphs for all samples.

422 A phylogenetic tree was constructed with the obtained archaeal RSVs from sample set 1, from the  
423 universal approach, the archaeal primer pair 519F-806R, and from the archaeal specific primer pair  
424 combination 344F-1041R/519F-806R. The alignment was performed using the SILVA SINA<sup>54</sup> and the 5  
425 most closely related available sequences (neighbors) were downloaded together with the aligned  
426 sequences. All sequences were cropped to the same length (276 nt, from position 545 nt to 821 nt)  
427 and used to construct a tree based on maximum-likelihood algorithm using MEGA7<sup>55</sup>, using a  
428 bootstrap value of 500. The Newick output was further processed with iTOL interactive online  
429 platform<sup>56</sup>.

430 **Declarations**



431 Ethics approval and consent to participate

432 Research involving human material was performed in accordance with the Declaration of Helsinki  
433 and was approved by the local ethics committees (the Ethics Committee at the Medical University of  
434 Graz, Graz, Austria). (Bacterial) microbiome studies of some of the samples used in this study have  
435 already been published elsewhere (oral, nose, skin samples: <sup>46,48,50</sup>). Details of the ethics approvals  
436 obtained are shown there. Appendix samples and stool samples have been obtained covered by the  
437 ethics votes: 25-469 ex12/13, and 27-151 ex 14/15.

438 Availability of data and material

439 The MiSeq amplicon sequence data was deposited in the European Nucleotide Archive under the  
440 study accession number PRJEB27023.

441 Competing interests

442 The authors declare no conflicts of interests.

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446 Authors' contributions

447 The study was designed by M.R.P. and C.M.E. G.S., H.T., V.S., E.S., B.K. and C.H. provided clinical  
448 samples. C.S. and M.R.P. prepared the 16S rRNA gene amplicons for sequencing and performed the  
449 quantitative PCR. Bioinformatics and statistical analysis were done by M.R.P. Analysis, visualization  
450 and interpretation of the data was done by M.R.P. All authors read, corrected and approved the final  
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