

1 **Updating the repertoire of cultured bacteria from the human being**

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20 **Abstract**

21 The recent renewal of cultural approach has substantially enriched knowledge of the human
22 microbiota, notably through the discovery of new taxa from various anatomical sites. As an
23 increasing number of these recent species are currently considered beneficial or harmful for
24 human health, a constant updating of the repertoire of bacteria and archaea isolated from
25 humans by culture is essential. Herein, we show that the number of cultured bacterial species
26 associated with human beings increased, from 2776 in 2018, to 3253 in 2020, representing a
27 17% increase in 2 years by adding 477 species, of which 64% are new species (N=307). A
28 wide majority of the species added (i.e., 63%) were isolated using the culturomics approach,
29 while 16% were cultured as part of clinical microbiology laboratories. Human microbiota
30 studies would benefit from the completeness of the repertoire of bacteria associated with
31 human beings, which would require continued efforts to culture microbes from human
32 specimens.

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34 **Keywords: Human repertoire; Bacteria: Culturomics**

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42 **1. Introduction**

43 The Human microbiota is defined as all the bacteria, parasites, viruses, fungi and archaea
44 found in the human body. In terms of biomass, the number of bacteria colonizing the human
45 body has been estimated to be equal to the number of human cells [1]. However, the bacterial
46 diversity associated with the human host remains so far unknown. Indeed, the tools dedicated
47 to the elucidation of the human microbiota suffers from various biases that were extensively
48 discussed [2]. There is however a growing evidence of the influence of the human microbiota
49 in a variety of diseases, including obesity, diabetes and cancer [3–6].

50 In addition, the advent of bacteriotherapy and the growing use of the fecal microbiota
51 transplantation (FMT) demonstrate the clinical importance of these microorganisms [7,8].
52 While most of this data is derived from high-throughput sequencing methods by screening for
53 taxa of potential medical interest, biological objects remain essential. Unattributed sequences
54 of potential anticancer prokaryotes have been shown to have already been isolated [9].
55 Various cultivation approaches have indeed recently contributed to the renewal of culture.
56 Among these, culturomics is defined as a high-throughput culture technique combining
57 variations in culture conditions and rapid identification of microorganisms using mass
58 spectrometry type Matrix Assisted Laser Desorption Ionization - Time of Flight (MALDI-
59 TOF MS) [10]. But there are many other high-throughput cultural approaches currently
60 developed to reduce the metagenomics dark matter [11,12] resulting in an increasing number
61 of new bacterial species discovered from human being. Reference databases used for
62 taxonomic assignments currently do not include the entirety of sequences derived from these
63 novel taxa. We therefore believe that updating the previous repertoire is crucial to improve the
64 identification of sequences generated by high-throughput sequencing approaches. We have
65 already provided such repertoires in 2015 and 2018 and spontaneously propose herein to
66 update the repertoire of human bacteria isolated at least once in humans [13,14].

67 2. Bibliographical methods

68 A manual query was conducted on three major databases, including Google Scholar, PubMed
69 and Web of science. We started by defining terms and text words referring to Bacteria (Q1),
70 Archaea (Q2), Man (Q3) and Culture Technology (Q4). Then, we combined these terms in
71 order to search the different databases. We build different requests for bacteria and archaea
72 (*Supplementary Data 1*). Considering the period covered by the preceding repertoire update,
73 we focused our literature search on data published from 01/01/2018 to 25/02/2020. Using
74 request for bacteria, we retrieved 5950, 27472 and 44562 papers on Google Scholar, PubMed
75 and Web of science databases, respectively. Using request for archaea, we retrieved 92, 642
76 and 2,368 papers on Google Scholar, PubMed and Web of science databases, respectively.
77 These data were exported to the EndNote version X9 platform (<https://endnote.com>) in order
78 to sort and analyze the publications. Following removal of duplicates, we obtained 66,622
79 publications from bacterial request and 2,731 publications from archaeal request (*Figure 1A*,
80 *1B*). After analysis of the titles and abstracts, we excluded 56590 studies that did not involve
81 humans, did not use culture-dependent methods and that did not focus on bacteria. Then, we
82 analyzed the full paper and supplementary data of 10,032 publications and excluded those that
83 did not add more bacterial species when compared to the dataset from Bilen et al. in 2018
84 [14]. We checked the name of each species, filled in their taxonomic information, the origin
85 of their first isolation, whether they were found in pathological situations and whether they
86 were isolated for diagnostic or research purposes. These information were collected from the
87 websites www.ncbi.nlm.nih.gov/taxonomy, www.bacdivi.dsmz.de and the original papers
88 relating to these bacteria. The oxygen tolerance of bacterial species was determined using the
89 database [https://www.mediterranee-infection.com/acces-ressources/base-de-donnees/list-of-](https://www.mediterranee-infection.com/acces-ressources/base-de-donnees/list-of-prokaryotes-according-to-their-aerotolerant-or-obligate-anaerobic-metabolism/)
90 [prokaryotes-according-to-their-aerotolerant-or-obligate-anaerobic-metabolism/](https://www.mediterranee-infection.com/acces-ressources/base-de-donnees/list-of-prokaryotes-according-to-their-aerotolerant-or-obligate-anaerobic-metabolism/)
91 www.mediterranee-infection.com.

92 **3. Number of species added and description of their characteristics.**

93 Based on our bibliographical research, we were able to add 475 bacterial species and 2
94 archaea to the last update of the human bacterial repertoire in 2018 by Bilen *et al* [14]. This
95 represents a 17% increase of the repertoire in 2 years (*Figure 2, Supplementary Table 1*). The
96 477 species added to this repertoire represent 9 phyla with a majority of *Firmicutes* (N=238;
97 50%), followed by *Actinobacteria* (N=109; 23%), *Proteobacteria* (N=73; 15%) and
98 *Bacteroidetes* (N=46; 10%). Phyla such as *Euryarchaeota* (N=2), *Fusobacteria* (N=2),
99 *Synergistetes* (N=1), *Deferribacteres* (N=1) and *Chlamydiae* (N=1) each represented less than
100 1% of the total (*Supplementary Figure 1A*). The unique bacterial species isolated in each of
101 these three phyla are *Anaerobaculum hydrogeniformans* (isolated from gut), *Simkania*
102 *negevensis* (isolated from oral cavity) and *Mucispirillum schaedleri* (isolated from breast
103 milk), respectively. For this latter, it represents the second member of the *Deferribacteres*
104 phylum ever isolated from the human being. This distribution of phylum is consistent with
105 what was demonstrated in the previous human repertoire by Bilen *et al.* in 2018[14]. The 9
106 phyla are divided into 240 bacterial genera, the majority of which belong to the *Clostridium*
107 (N=25), *Bifidobacterium* (N=20) and *Bacillus* (N=18) genera (*Supplementary Figure 1B*).
108 Anaerobic species constitute half of the species added (N=239; 50%). Majority of the species
109 added, 64% (N=307), are new species discovered from humans samples. The remaining 36%
110 (N=170) species are bacterial species first isolated from the environment (N=75; 16%),
111 animals (N=72; 15%) and food (N=23; 5%) (*Supplementary Figure 2*).

112 **4. Contribution of Culturomics to the expansion of the human bacterial repertoire.**

113 Culturomics is defined as a high-throughput culture method that has so far led to the
114 discovery of hundreds of new taxa associated with humans [10]. In this work, we qualified as
115 culturomics all studies using a high-throughput culture method with or without reference to

116 the term culturomics and evaluated whether the species included in this study were isolated by
117 this approach or not [11,12]. As a result, 301 bacterial species were isolated as a part of
118 culturomics studies, representing a contribution of 63% of the total number of species added
119 by this work (N=301/477) (*Figure 2, Supplementary Table 1*). Most of these species are
120 strictly anaerobic (64%; N=194/301) and are new taxa (73%; N=220/301). Gut is the most
121 represented anatomical site as it is the source of 73% (N=160/220) of these new taxa,
122 followed by vagina (9%; N=19/220), skin (5%; N=11/220) and less than 5% in other sites
123 such as oral cavity (N=9), urine (N=7), respiratory tract (N=6), breast milk (N=4), blood
124 (N=3) and nasopharyngeal swabs (N=1). These new species are mostly anaerobes
125 (N=150/220; 68%). The remaining species (27%; N=81/301) isolated from humans through
126 culturomics are species of non-human origin known previously. They have been found at 99%
127 (N=80/81) in the gut and 1% (N=1/81) in breast milk with an anaerobic proportion estimated
128 at 54% (N=44/81).

129 **5. The role of clinical microbiology in updating the repertoire of human microbes**

130 Microbial culture in the diagnostic laboratory has also been the subject of several innovations
131 that make it faster and more efficient. The introduction of mass spectrometry type Matrix
132 Assisted Laser Desorption Ionization - Time of Flight (MALDI-TOF MS) in the identification
133 of bacterial species in the routine laboratory has revolutionized bacterial identification by
134 progressively abandoning morphological and biochemical methods [15]. MALDI-TOF MS
135 therefore saved a considerable amount of time and increased the colony identification rate
136 because the database used for spectrum comparison is versatile. It currently represents a
137 powerful screening tool. Indeed, it has probably led microbiologists to test an increasing
138 number of colonies cultivated from clinical specimens that have contributed to expand the
139 human bacterial repertoire. In this work, we tried to evaluate the percentage of species added
140 using the microbial culture method in the diagnostic laboratory. As a result, 16% (N=76/477)

141 of the species added to the repertoire were isolated from clinical specimen (*Figure 2*,
142 *Supplementary Table 1*). Most of these species are tolerant to oxygen (i.e., 88%, 67/76) and
143 are new taxa (64/76; 84%). The clinical specimens were respiratory samples (25%; N=16/64),
144 blood cultures (23%; N=15/64), wounds (11%, N=7/64) and other sites with low percentages.
145 The species that are not new (N=12/76; 16%) are species already known from the
146 environment, animals and food.

147 **6. Updating the human archaeal repertoire**

148 Archaea are prokaryotic organisms that were first discovered in extreme environments and
149 were gradually isolated from humans, in particular from the intestinal microbiota and, more
150 recently, from the breast milk [16,17]. Importantly, archaeal species were isolated from
151 clinical specimen, including urine [18]. The most important discovery was the detection of
152 *Methanobrevibacter smithii* in the blood of febrile patients, including subjects with
153 endocarditis. These findings were screened by PCR and confirmed by various assays,
154 including culture. Regarding the repertoire, only 10 of 715 species of archaea known
155 according to NCBI taxonomy have so far been found by culture in humans in 2018[14]. The
156 present study enables to add two species of archaea, including "*Candidatus*
157 *Methanomethylophilus Alvus*" and "*Candidatus Methanomassiliicoccus intestinalis*",
158 representing a new order of methanogens related to *Thermoplasmata*. Both species were
159 isolated from the human intestinal microbiota, thus increasing the number of archaea isolated
160 by culture from humans to a total of 12 species [19,20].

161 **7. Description of the current human bacterial repertoire**

162 The aim of this work was to update the latest repertoire of bacteria and archaea cultured at
163 least once in humans carried out in 2018 by Bilen. et al [14]. Species identified in this work
164 were isolated from humans according to our data between 1982 and 2020 with a peak in 2019

165 (N=239; 50%) (*Supplementary Figure 3*). A significant number of species (N=113; 24%)
166 were reported in 2018, the year of the last update where data collection ceased in April 2018.
167 The year 2020 represents 9% (N=41) of the added species, while 18% of the species added
168 (N=84) were found between the years 1980 and 2017, which are periods normally covered by
169 the previous repertoires. This underlines the exhaustiveness of the methodology used herein,
170 that was mostly performed and verified manually. As a result, 477 were added, an increase of
171 17% in two years. The number of bacteria and archaea isolated from humans thereby
172 increases from N=2776 to N=3253, with the proportion of new species increasing from 19%
173 to 26% (N=831) (*Figure 3, Supplementary Table 2*). Most species are *Firmicutes* 37%,
174 *Proteobacteria* 25%, *Actinobacteria* 25% and *Bacteroidetes* (*Figure 4*). A total of 711
175 bacterial genera compose the human repertoire to date with the addition of 108 genera yet
176 unknown in humans. The genera *Mycobacterium* (N=163), *Clostridium* (N=133), *Bacillus*
177 (N=108), *Corynebacterium* (N=89) and *Streptococcus* (N=78) are the most represented in
178 terms of number of species. Despite the diversification of human anatomical sites subjected to
179 high-throughput culture analysis, the intestinal tract remains the site where the most species
180 are isolated. Indeed, 52% (N=247) of the total species added by this study (N=477) came
181 from the gut of which 67% (N=166) are new taxa. IHU Méditerranée-Infection contributed up
182 to 20% (N= 639/3253) of the total human bacteria and archaea repertoire, mostly through the
183 discovery of new species (*Figure 5*). The proportion of anaerobic species increased globally
184 from 24% of the previous repertoire (N=662) to 28% in this new repertoire (N=901) with the
185 addition of the 239 anaerobic species, most of which are new species. These findings show
186 that the anaerobes constitute a substantial fraction of the microbial dark matter associated with
187 humans.

188 **8. Conclusion**

189 This present study makes it possible to significantly increase the repertoire of human-
190 associated bacteria in a very short period of time. We are currently witnessing the revival of
191 culture, by culturomics, but not only, as many works have reported the isolation of new taxa
192 through innovative cultural strategies [11,12,21,22]. Update of such repertoire is thus crucial
193 as the taxa recently discovered are implied in human health and disease[9,23]. Infectious
194 diseases are also concerned as new species discovered thanks to culturomics were further
195 detected in clinical specimen [24]. We believe that such repertoire could also help in the
196 interpretation of datasets from high sequencing approaches. Indeed, with the current increase
197 of the sequencing depth, the number of sequences amplified is exponentially growing. It can
198 therefore be tricky to determine the relevance relevance of the identification of certain taxa as
199 part of human microbiome studies. Some sequences could be due to the presence of
200 contaminants, or transient microbes that are not commensal to the studied anatomical site.
201 This is also supported by the fact that not all microbes detected are viable in a human
202 specimen [25]. This repertoire, along with other repertoires dedicated to specific human
203 ecological niches [13,26–29], may at least partly help to resolve these interpretation issues.
204 Improving culture approaches to isolate of microbes that are considered "unculturable" is
205 therefore crucial, as shown by Kaboré et al for the culture of *Gemmata* genus. Known for
206 being tedious to cultivate, it was isolated using a culture medium including the skeleton of
207 marine sponges and the aqueous heat filtrate of the skeleton sponge [30]. Belkacemi et al., for
208 the same purpose, developed a successful culture technique based on passive filtration for the
209 culture of *Treponema* species [31]. Thanks to metagenomic studies, we know that the number
210 of species of archaea in humans is much higher compared to the number cultivated [32]. To
211 date, we have listed several 12 species of archaea cultivated at least once in humans. It is
212 therefore imperative to develop culture techniques to be able to isolate them and explore their
213 role as they are often found to be associated with several bacterial infections, such as

214 periodontitis, chronic sinusitis, muscular or cerebral abscesses. *Methanobrevibacter smithii*
215 was detected in blood cultures from febrile patients, thus suggesting that these microbes
216 should not be longer neglected. Guindo et al. had, for example, developed a technique for
217 cultivating methanogens archaea through the production of hydrogen necessary for their
218 growth by a chemical technique based on the use of iron filings and acetic acid [33]. In
219 parallel, we observed that most of the newly isolated species were anaerobic species, hence
220 the need to develop anaerobic culture strategies. The fact that most of the new taxa isolated
221 from clinical specimen are, on the contrary, tolerant to oxygen, reflects the gradual
222 abandonment of anaerobic culture in clinical microbiology. Yet, Diakit  et al., in analyzing
223 the cost-effectiveness of the culture conditions used in culturomic studies, showed that
224 anaerobic culture conditions allow the isolation of 5 times more bacteria than aerobic culture
225 conditions [34]. Efforts must continue to cultivate bacterial species that do not tolerate
226 oxygen, including extremely oxygen-sensitive bacteria (EOS). To this end, special attention
227 should be paid to maintaining anaerobiosis from specimen collection to incubation [35]. The
228 important contribution of culturomics technique initiated 8 years ago (2012) to the human
229 repertoire demonstrates its effectiveness in highlighting and understanding the diversity of the
230 human microbiota. These innovative approaches to culture techniques are to be encouraged in
231 order to continue isolating bacteria and filling the dark matter of high-throughput sequencing
232 approaches.

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356 **Fig. 1:** Flow chart of bibliographical bacterial (A) and archaea (B) request.

357 **Fig. 2:** All 477 added species isolated at least once from humans by culture. Using the online
358 tool wordle (www.wordle.net), the size of the name of each species is proportional to the
359 number of times it occurs in the repertoire. The circles realized thanks to the cytoscape
360 software (www.cytoscape.org) represent the partition of the culturomics technique, clinical
361 microbiology and other culture techniques in this update of the repertoire. Red edges
362 represent the contribution of IHU Méditerranée infection and green edges represent that of
363 other laboratories. Orange dots at species level represent new taxa and the grey dots represent
364 already known species other than humans.

365 **Fig. 3:** Evolution of the number of known bacterial and archaea species in humans through
366 culture in 2015, 2018 and 2020. Percentage added in relation to the previous repertoire is
367 represented by arrow. Orange proportion represents evolution of new species isolated from
368 2015 to nowadays.

369 **Fig. 4:** Distribution in terms of phylum and genera of the 3253 species of the current human
370 bacterial and archaea repertoire. In blue the distribution of phylum from the last update of the
371 repertoire in 2018 by Bilen et al, and in orange the distribution of phylum added by this study.

372 **Fig. 5:** All 3253 species of the current human bacterial isolated at least once from humans
373 using culture. Using the online tool wordart (<https://wordart.com/>), the size of the name of
374 each species is proportional to the number of times it occurs in the database. Circle in dark

- 375 blue represent IHU Méditerranée infection contribution in the total repertoire and circle in
- 376 light blue represent species firstly isolated by IHU Méditerranée infection in environment.