**Gorilla gorilla gorilla** gut: a potential reservoir of pathogenic bacteria as revealed using culturomics and molecular tools

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Wild apes are considered to be the most serious reservoir and source of zoonoses. However, little data are available about the gut microbiota and pathogenic bacteria in gorillas. For this propose, a total of 48 fecal samples obtained from 21*Gorilla gorilla gorilla* individuals (as revealed via microsatellite analysis) were screened for human bacterial pathogens using culturomics and molecular techniques. By applying culturomics to one index gorilla and using specific media supplemented by plants, we tested 12,800 colonies and identified 147 different bacterial species, including 5 new species. Many opportunistic pathogens were isolated, including 8 frequently associated with human diseases; *Mycobacterium bolletii*, *Proteus mirabilis*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Escherichia coli*, *Staphylococcus aureus* and *Clostridium botulinum*. The genus *Treponema* accounted for 27.4% of the total reads identified at the genus level via 454 pyrosequencing. Using specific real-time PCR on 48 gorilla fecal samples, in addition to classical human pathogens, we also observed the fastidious bacteria *Bartonella* spp., *Borrelia* spp., *Coxiella burnetii* and *Tropheryma whipplei* in the gorilla population. We estimated that the prevalence of these pathogens vary between 4.76% and 85.7%. Therefore, gorillas share many bacterial pathogens with humans suggesting that they could be a reservoir for their emergence.

The African great apes, including gorillas, are a reservoir and source of human pathogens. Calvignac-Spencer et al. identified 16 viral genera from wild great apes, including 8 genera that could be transmitted between humans and apes. Among these different transmissible viruses, the Ebola and Marburg viruses are the most virulent pathogens and have caused multiple human outbreaks due to direct handling of gorillas and chimpanzees. Furthermore, there is strong evidence that the human immunodeficiency virus originated from simian immunodeficiency viruses in chimpanzees and gorillas. In addition to viruses, parasites, including *Plasmodium falciparum* and *Plasmodium vivax*, nematodes (such as *Trichuris*, *Ascaris*, *Oesophagostomum*, *Strongyloides*, and *Trichostrongylus*) and the Anoplocephalid cestode can infect both humans and great apes. However, there are limited data on pathogenic bacteria in the gorilla. These bacteria include *Staphylococcus aureus*, *Escherichia coli*, *Rickettsia felis*, *Bacillus anthracis*-like bacteria, *Salmonella* spp., *Campylobacter* spp. and *Shigella* spp. Additionally, *Mycobacterium tuberculosis* and leprosy caused by *Mycobacterium leprae* have been observed in nonhuman primates. However, *Rwego* et al. demonstrated the possibility of gastrointestinal bacterial transmission between humans and gorillas sharing the same habitat. Therefore, studying the composition of bacterial communities in the gorilla gastrointestinal tract is important.

Although pyrosequencing is a rapid and efficient method for determining the bacterial phyla, it is limited, especially for bacterial identification at the genus and species levels. Microbial culturomics (large-scale culture conditions followed by mass spectrometry or 16S rRNA identification of the isolated colonies) was proved to be an efficient method to explore the gut microbiota not only because it is able to isolate a high number of bacterial species including new species but also because it is more sensitive than metagenomic methods to detect minority populations including pathogens.

In this study, we explored the prevalence of human bacterial pathogens in wild gorillas from southern Cameroon. We exhaustively analyzed one stool sample using culturomics and pyrosequencing to detect the...
commensal bacteria that are potential human pathogens. Forty-eight additional fecal samples from wild gorillas were screened via real-time PCR to examine the prevalence of the human bacterial pathogens that were identified in the first stool sample using culturo-mics and of other human pathogens, including fastidious bacteria that are tested routinely in our lab for the diagnosis of infections in Africa.

Results

Source of fecal samples, microsatellite analyses and genetic identification of gorilla individuals. Fecal samples from western lowland gorillas (Gorilla gorilla) were collected at two sites in south-central Cameroon: near the Minton village (this sample was selected and used for exhaustive examination using culturo-mics, pyrosequencing and real-time PCRs because of its enough volume required for these analyses), and 47 fecal samples were collected near the Messok village (these samples were used for the molecular screening of human bacterial pathogens via real-time PCRs). A microsatellite analysis of 48 fecal samples enabled the identification of 21 gorilla individuals: 19 males and 2 females (Supplementary Table 1).

Bacterial diversity of gorilla gut using culturo-mics. In this study, a total of 86 culture conditions were tested: the optimal conditions applied for human gut exploration (Supplementary Table 2) and innovative media, which were developed from plants (Supplementary Table 2). From one fecal sample, a total of 12,800 colonies were tested and 147 bacterial species were observed (Table 1 and Supplementary Table 2). The bacteria belonged to 47 genera divided into 4 phyla: Firmicutes (62.6%), Actinobacteria (24.5%), Proteobacteria (12.2%) and Fusobacteria (0.7%) (Table 1 and Supplementary Table 2). A comparative analysis showed that 113/147 (76.9%) of the bacterial species observed in the gorilla gut using culturo-mics were also observed in the human gut using culturo-mics (Table 1). Among the 86 different culture conditions tested, the most effective was a culture vial supplemented with rumen fluid in an anaerobic atmosphere followed by incubation for 11 days; this isolated 19 bacterial species (Supplementary Table 2). The incubation of the stool sample in aerobic or anaerobic enriched culture vials yielded 60 bacterial species (40.8%) including 50 (34%) anaerobic species (Supplementary Table 2).

Bacterial analyses of gorilla gut using specific plant media. Because gorillas are principally herbivores and because the gut microbial diversity may be influenced by diet, we designed novel media using tobacco leaves and tropical plants including the mango, papaya and banana fruits (see Methods and Supplementary Table 2). Of the 147 bacterial species observed, 60 (40.8%) grew on media supplemented with plant extracts, including 28 strains (19.04%) that were isolated exclusively using these media (Supplementary Table 2). Of the 147 bacterial species observed, 60 (40.8%) grew on media supplemented with plant extracts, including 28 strains (19.04%) that were isolated exclusively using these media (Supplementary Table 2). Of the 147 bacterial species observed, 60 (40.8%) grew on media supplemented with plant extracts, including 28 strains (19.04%) that were isolated exclusively using these media (Supplementary Table 2). Of the 147 bacterial species observed, 60 (40.8%) grew on media supplemented with plant extracts, including 28 strains (19.04%) that were isolated exclusively using these media (Supplementary Table 2). Of the 147 bacterial species observed, 60 (40.8%) grew on media supplemented with plant extracts, including 28 strains (19.04%) that were isolated exclusively using these media (Supplementary Table 2). Of the 147 bacterial species observed, 60 (40.8%) grew on media supplemented with plant extracts, including 28 strains (19.04%) that were isolated exclusively using these media (Supplementary Table 2). Of the 147 bacterial species observed, 60 (40.8%) grew on media supplemented with plant extracts, including 28 strains (19.04%) that were isolated exclusively using these media (Supplementary Table 2). Of the 147 bacterial species observed, 60 (40.8%) grew on media supplemented with plant extracts, including 28 strains (19.04%) that were isolated exclusively using these media (Supplementary Table 2). Of the 147 bacterial species observed, 60 (40.8%) grew on media supplemented with plant extracts, including 28 strains (19.04%) that were isolated exclusively using these media (Supplementary Table 2). Of the 147 bacterial species observed, 60 (40.8%) grew on media supplemented with plant extracts, including 28 strains (19.04%) that were isolated exclusively using these media (Supplementary Table 2). Of the 147 bacterial species observed, 60 (40.8%) grew on media supplemented with plant extracts, including 28 strains (19.04%) that were isolated exclusively using these media (Supplementary Table 2).

New bacterial species and human pathogens in the gorilla gut. In addition to previously known bacteria (Table 1 and Supplementary Table 2), five new species were isolated in the fecal sample analyzed via culturo-mics, and their genomes were sequenced, including one new genus, Gorillibacterium massiliense, and 4 new species: Bacillus massiliogorillae, Microbacterium gorillae, Paenibacillus gorillae and Paenibacillus camerounensis (Table 1 and Supplementary Table 2). The latter two species were recovered only from media supplemented with mango fruit (Supplementary Table 2). Notably, several potential human pathogens, including Proteus mirabilis, Klebsiella pneumoniae, Mycobacterium bovis, S. aureus, Clostridium botulinum, Acinetobacter baumannii, E. coli, Enterococcus faecalis, Enterococcus fæcium, Clostridium perfringens and Serratia marcescens, were isolated from this stool sample (Table 1 and Supplementary Table 2). C. botulinum was isolated twice using culture vials supplemented with rumen fluid and thiglycolate, and this species identification was performed by sequencing. M. boletii was isolated using the MOD4 medium and identified by sequencing the rpoB gene.

Bacterial diversity of gorilla gut via pyrosequencing. A total of 75,595 reads were obtained from pyrosequencing the fecal sample analyzed via culturo-mics. Of these, 36,463 reads with high-quality sequencing were analyzed and distributed into 11 phyla using the RDP classifier. Firmicutes composed the largest fraction (46.8% of the total reads), followed by Actinobacteria and Bacteroidetes (20% and 18.6%, respectively). Spirochaetes and Verrucomicrobia comprised 7.4% of the total reads (approximately 3.7% each). The remaining phyla, including Chloroflexi, Cyanobacteria, Fibrobacteres, Fusobacteria, Proteobacteria and Tenericutes, were represented by rare sequences. Finally, 4.6% of the reads were ascribed to unclassified bacterial phyla in the RDP database. Approximately 13% of the reads were identified at the genus level, yielding 38 genera. Prevotella (Bacteroidetes), Treponema (Spirochaetes) and Bifidobacterium (Actinobacteria) were the most abundant genera obtained from the 454 pyrosequencing (accounting for 40.8%, 27.4% and 11.3%, respectively, of the total reads identified at the genus level). Using the BLASTn tool in the NCBI website and setting the sequence similarity threshold to 98.7%, only 316 reads were identified at the species level, leading to the identification of 16 bacterial species.

Comparing the bacterial phyla found in the gorilla gut using culturo-mics and pyrosequencing with those of previous studies. Firmicutes followed by Actinobacteria comprised the majority of the detected bacteria in the fecal sample through both methods (i.e., culturo-mics and pyrosequencing). This is similar to the bacteria cultured from fecal samples from humans in Africa and Europe.

The high abundance of Firmicutes was reported previously in wild mountain gorillas, western lowland gorillas and also in other primates including old world monkeys, chimpanzees and wild pygmy lorises. Actinobacteria was the second most predominant phylum in our sample; this result is in agreement with the study by Vickova et al. in captive western lowland gorillas. Although Proteobacteria was the third most isolated phylum via culturo-mics, it was recovered from rare reads using pyrosequencing. Additional phyla such as Bacteroidetes, Cyanobacteria, Verrucomicrobia, and Tenericutes were identified in this fecal sample exclusively through pyrosequencing. All of these microbial divisions have been previously detected in human and nonhuman primates. The remaining phyla found via metagenomics, including Spirochaetes, Chloroflexi and Fibrobacteria, are usually absent from the human gut but are present in herbivore guts such as gorillas, colobuses (Colobus guereza and Piliocolobus tephrosceles) and guenons (Cercopithecus ascanius).

Molecular detection of human pathogenic bacteria and estimation of their prevalence in the gorilla gut population tested. A total of 48 fecal samples from 21 gorillas were examined via specific real-time PCR to analyze the prevalence of pathogens identified using culturo-mics and other fastidious pathogens commonly tested in our center (Supplementary Table 3). Our molecular tests showed that the most prevalent bacteria in the gorilla gut were (in decreasing order of estimated prevalence) K. pneumoniae (80.95%), Acinetobacter spp. (76.19%), Borrelia spp. (47.6%), S. aureus (42.86%), Bartonella spp. (38%), Pseudomonas aeruginosa (38%), S. marcescens (33.33%), Mycobacterium abscessus (28.57%), E. faecalis (28.57%), Coxiella burnetii (23.8%), E. coli (19%), A. baumannii (19%) and Tropheryma whipplei (4.76%) (Table 2).
In this study, we used the culturomics approach because it has greater sensitivity than molecular methods and can be used to detect even minority bacterial populations including pathogens. 16–18. Many new bacterial species found in this study.

### Table 1 | Bacterial species isolated via culturomics in the stool sample of gorilla and their previous description in the human gut and human diseases

| Bacterial species | Bacterial species | Bacterial species | Bacterial species |
|------------------|------------------|------------------|------------------|
| Acinetobacter baumannii* | Brevibacterium epidermidis | Gemella sanguinis | Pseudomonas stutzeri |
| Acinetobacter lwoffii | Brevibacterium iodinum | Gorillibacterium massiliense | Rhodococcus equi |
| Acinetobacter schindleri | Brevibacterium linens | Granulicatella adiacens | Rothia aeria |
| Actinomyces naeslundii-test-1 | Brevibacterium ureolatum | Klebsiella pneumoniae | Rothia mucilaginosa |
| Actinomyces neuii | Brevundimonas aurantiaca | Kocuria polaris | Sarcina ventriculi |
| Actinomyces odontolyticus-1 | Clostridium barattii | Kocuria rhizophila | Serratia marcescens* |
| Aerococcus viridans | Clostridium bifermentans | Kyrtococcus Schroeteri | Staphylococcus arletiae |
| Agrococcus jenensis | Clostridium botulinum | Kyrtococcus sedentarius | Staphylococcus aureus* |
| Arthrobacter castelli | Clostridium celercrense | Lactobacillus paracasei | Staphylococcus capitis |
| Bacillus arsenicus-2 | Clostridium clostridioforme | Lactococcus garvieae | Staphylococcus cohnii |
| Bacillus barbaricus | Clostridium chauveaui | Lysinibacillus fusiformis | Staphylococcus epidermidis |
| Bacillus bataviensis | Clostridium difficile | Lysinibacillus sphericus | Staphylococcus haemolyticus |
| Bacillus cereus-3 | Clostridium orbiscindens | Microbacterium flavum | Staphylococcus hominis |
| Bacillus circulans | Clostridium parapiferringens | Microbacterium kitamiense | Staphylococcus pasteuri |
| Bacillus flexus | Clostridium paraputrefaciens | Micrococcus luteus | Staphylococcus pettenkoferi |
| Bacillus humi | Clostridium perfringens | Micrococcus lylae | Staphylococcus saprophyticus |
| Bacillus idriensis | Clostridium perfringens | Moraxella osloensis | Staphylococcus siuci |
| Bacillus licheniformis-4 | Clostridium sporogenes | Mycobacterium bovis | Staphylococcus warneri |
| Bacillus massiliigoralla | Clostridium sporshaeroides | Neisseria flavescens | Staphylococcus xylosus |
| Bacillus massiliis | Clostridium symbiosum | Neisseria mucosa | Streptococcus alactolyticus |
| Bacillus megaterium | Clostridium tertium | Neisseria perflava | Streptococcus cristatus |
| Bacillus mycoides-5 | Corynebacterium aurimucosum | Paenibacillus azoreducens | Streptococcus galolyticus |
| Bacillus niaboiensis | Corynebacterium minutissimum | Paenibacillus barcinonensis | Streptococcus iniae |
| Bacillus novialis | Corynebacterium pseudogenitalium | Paenibacillus cameronensis | Streptococcus mitis |
| Bacillus oshimensis | Corynebacterium variabile | Paenibacillus glycanilyticus | Streptococcus oralis |
| Bacillus pumilus-6 | Dietzia natronolimnaea | Paenibacillus illinoisensis | Streptococcus parasanguinis |
| Bacillus simplex-7 | Enterobacter cloacae | Paenibacillus gilliae | Streptococcus peroralis |
| Bacillus thuringiensis-8 | Enterococcus avium | Paenibacillus pabuli | Streptococcus salivarius |
| Bacillus viridii | Enterococcus casseliflavus | Paracoccus yeast | Streptococcus sanguinis |
| Bacillus weihenstephanensis | Enterococcus faecalis | Propionibacterium acnes | Streptomyces fungicida |
| Bifidobacterium dentium | Enterococcus faecium | Propionibacterium acnes | Streptomyces griseus |
| Brachybacterium paraconglomeratum | Enterococcus mundtii | Proteus mirabilis | Veillonella atypica |
| Brevibacillus agri | Enterococcus sanguinocola | Proteus vulgaris | Veillonella dispar |
| Brevibacillus brevis-9 | Enterococcus thailandicus | Pseudomonas luteola | Veillonella parvula |
| Brevibacillus choshinensis | Escherichia coli | Pseudomonas oryzihabitans | Weissella cibaria |
| Brevibacillus formosus | Eubacterium tetani | Staphylococcus pasteuri | Staphylococcus simulans |
In conclusion, we observed that many human bacterial species, including pathogens, are also present in the gorilla gut. This type of non-invasive analysis of gorilla feces could facilitate the surveillance and discovery of potential human pathogens and unknown bacteria, especially in areas where human and gorilla habitats overlap and because of the increasing presence of humans in the African equatorial forests. Furthermore, similar studies on non-human primates would contribute to the detection of emerging diseases circulating in wild fauna.

**Methods**

**Samples.** Gorillas’ fecal samples from two sites in south-central Cameroon (near the Minto village and near the Messok village) were collected as previously described (about 1 g of dung was collected in a 50-ml tube containing RNAlater™ (Ambion, Austin, TX)) and preserved at ambient temperature for a maximum of 3 weeks in the field and subsequently stored at −80 °C, except the sample used in culturoemics, for which about 25 g of dung was collected and stored directly at −80 °C without the RNAlater reagent. The time between defecation and collection was estimated at <24 h, according to the physical texture of the samples. No experimentation was conducted on these animals. The collection of fecal samples from the soil was approved by the Ministry of Scientific Research and Innovation of Cameroon. No other permit was required, as this research was non-invasive work, and the collection of the samples did not disrupt wild fauna.

**Microsatellite analyses.** The DNA extracted from 48 fecal samples was used for microsatellite analysis as previously described. Briefly, seven loci including D18S536, D4S243, D10S676, D9S922, D2S1326, D2S1333 and D4S1627 were amplified (Supplementary Table 1). A region of amelogenin was also amplified for determining the gender.40 The loci were amplified four times to exclude allelic dropouts. One microliter of the amplification product was mixed with 10 μl of formamide and 0.25 μl of size marker (ROX GeneScan 400HD, Applied Biosystems, Foster city, CA). This mixture was analyzed via pyrosequencing and pyrosequencing analyses were performed as previously reported. A sequence file was deposited to the NCBI Sequence Read Archive under the accession numbers KC193239, JX650054, JX650055, JX650056 and JX650057, respectively. The description and genome sequencing of these new species were performed for three of them and are ongoing for those remaining.

**New bacterial species.** The new species Gorillibacterium massiliense, Paenibacillus massiliensis, Bacillus massiliobacteriae, Microbacterium gorillae and Paenibacillus camerounensis were deposited in the German collection of microorganisms (DSMZ) under the accession numbers DSM 27179, DSM 26181, DSM 26159, DSM 26203 and DSM 26182, respectively, and were submitted in the GenBank database under the accession numbers KC193239, JX650054, JX650055, JX650056 and JX650057, respectively. The description and genome sequencing of these new species were performed for three of them and are ongoing for those remaining.

**454 FLX Titanium pyrosequencing analyses.** DNA was extracted from the feces as previously described (Macherey-Nagel, Hoerdedt, France). 16S rRNA amplicon pyrosequencing and pyrosequencing analyses were performed as previously reported. A sequence file was deposited to the NCBI Sequence Read Archive under the run accession number SRR1177866 and BioProject accession number PRJNA239545.

**Real-time PCR assay for the detection of human pathogens.** The primers and probes used in this study were previously described or designed in our laboratory for routine diagnosis (Supplementary Table 3). The DNA was extracted from 48 gorilla fecal samples using a kit (Macherey-Nagel, Hoerdedt, France) as previously described. In each PCR, 5 μl of DNA of each fecal sample was used in a final volume of 25 μl using the QuantitTec Probe PCR Kit (Qiagen, Courtaboeuf, France). The real-time PCR was performed using a CFX96™ Real-Time PCR Detection System (Bio-Rad, Life Science, Marnes-la-Coquette, France) with the following program: 95 °C for 15 min followed by 44 cycles of 95 °C for 0.5 min and 60 °C for 1 min. The positive samples were sequenced using the same primers.

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Author contributions D.R. and F.B. designed the experiments; M.K. conducted the experiments; F.B., M.K., J.C.L., R.M., F.D. and F.B. wrote the manuscript. All authors reviewed the manuscript.

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