An effective culturomics approach to study the gut microbiota of mammals

André C. Pereira^{1,2}, Mónica V. Cunha^{1,2*}

¹Centre for Ecology, Evolution and Environmental Changes (cE3c), Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisboa, Portugal.

²Biosystems & Integrative Sciences Institute (BioISI), Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisboa, Portugal.

Short title: A culturomics approach to study gut microbiota

*** Correspondence:**

Mónica V. Cunha. Centre for Ecology, Evolution and Environmental Changes (cE3c), Faculdade de Ciências, Universidade de Lisboa, 1749-016 Lisboa, Portugal. e-mail: mscunha@fc.ul.pt; Phone: +351 217 500 000

André C. Pereira, Mónica V. Cunha, An effective culturomics approach to study the gut microbiota of mammals, Research in Microbiology, Volume 171, Issue 8, 2020, Pages 290-300, ISSN 0923-2508, <https://doi.org/10.1016/j.resmic.2020.09.001>.

Abstract

The microbial characterization of the mammal's gut is an emerging research area, wherein culturomics methodologies applied to human samples are transposed to the animal context without improvement. In this work, using Egyptian mongoose as a model, we explore wet bench conditions to define an effective experimental design based on culturomics and DNA barcoding with potential application to different mammal species. After testing a battery of solid media and enrichments, we show that YCFA-based media, in aerobic and anaerobic conditions, together with PDA supplemented with chloramphenicol, are sufficient to maximize bacterial and fungal microbiota diversity. The pasteurization of the sample enrichment before cultivation is central to gain insight into sporogenic communities. We suggest the application of this optimized culturomics strategy to accurately expand knowledge on the microbial diversity and abundance of mammals' gut, maximizing the application of common laboratory resources, without dramatic time and consumables expenditure but with high resolution of microbial landscapes. The analysis of ten fecal samples proved adequate to assess the core gastrointestinal microbiota of the mesocarnivore under analysis. This approach may empower most microbiology laboratories, particularly the veterinary, to perform studies on mammal's microbiota, and, in contrast with metagenomics, enabling the recovery of live bacteria for further studies.

Keywords: Culturomics; 16S rRNA; 26S rRNA, ITS; Gut microbiota

Introduction

Gut microbiota has been under the focus of research over the last few decades. While the first reports had humans and companion animals as subjects, gut microbiota studies began to be progressively extended to other domestic and wildlife species to understand one important component of their bioecology. The first microbiota studies were based on Gram staining and direct microscopy observation of fecal samples, as these were useful and cost-effective direct-observation methods. However, reports have shown that some bacterial groups are not successively Gram-stained and their Gram status remains indeterminate (e.g. *Rickettsia*, *Chlamydia*, and *Mycoplasma* genera), while some genera (e.g. *Bacillus*, *Gemella*, and *Listeria*) show aberrant Gram staining [1]. After this initial characterization, several other biochemical and physiological testes can be performed (e.g. oxidase and catalase rapid tests) [2]. The first gut microbiota studies showed a predominance of Gram-negative bacteria based on the Gram staining methods directly applied to fecal samples, however cell counts and subsequent testing of isolates obtained from bacteriological culture on solid media would identify the dominance of Gram-positive and anaerobe bacteria.

In the 2000s, culture-independent methods based on high-throughput sequencing techniques, metagenomics, or microbial profiling, were introduced to fully characterize microbiota, with studies reporting that 80% of bacteria detected through these methods were unculturable [3]. The sequencing of the 16S rRNA gene became a great breakthrough to report and describe new bacterial species and it enlarged the proficiency of bacterial identification. Despite this methodology had become the gold standard for microbiota studies, major drawbacks were frequently reported with massive bias across studies, due to different DNA extraction methods, differences in target regions and primers for 16S rRNA amplification, natural heterogeneity within some species, and lack

of selectivity to distinguish bacteria from some specific taxonomic groups, such as *Rickettsia* spp., *Brucella* spp., *Streptococcus* spp., *Corynebacterium* spp., *Bacillus* spp., among others [4].

A new approach based on culture-dependent methods integrated with high-throughput identification techniques appeared after 2012, denominated culturomics [5]. This method developed by Lagier and collaborators (2012, 2016) is based on the utilization of non-selective and selective enrichment and inoculation media, using, for instance, antibiotics and heat shock selectivity and also different incubation conditions, namely in terms of temperature range and oxygen presence/depletion. Isolation of culturable communities is thus coupled with molecular identification based on sequencing of the V1 to V9 hypervariable regions of the 16S rRNA gene and/or Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF MS) [6]. This large scale culture methodology intends to mimic the different natural conditions within the digestive tract and enables the detection of marginal populations, the evaluation of cell viability, and downstream studies using the culturable isolates [4, 6]. Additionally, culturomics made possible the cultivation of microorganisms that thus far were non-culturable [4, 6]. Several disadvantages of culturomics and the underlying enrichment procedures include the underestimation of microbial burden and diversity, since some taxa overgrow, while other are more difficult and fastidious to culture, but also because not all nutritional requirements of the microorganisms can be fulfilled, limiting the growth of the more demanding taxa and possibly leading to diversity analyses biases.

After the development of this methodology, its application has been scarcely used in domestic and wild animals. In 2015, it was used to study *Gorilla gorilla* gut microbiome by the cultivation of 48 fecal samples under more than 70 different

conditions, including new media supplemented with lyophilized plants, which resulted in the identification of 147 different bacterial species and the discovery of five new ones [7]. Moreover, the identification of the microbiota composition of the midgut of different species of mosquitoes was accomplished by culturomics [8]. In this work, it was possible to identify four different phyla, mainly *Proteobacteria*, unraveling taxonomical compositional differences between wild and laboratory strains of mosquitoes [8]. Furthermore, the aerobic culturable microbiota of the pest insect *Diabrotica speciose* was evaluated using culturomics and MALDI-TOF MS, resulting in the detection of 17 genera and 29 species, mostly belonging to the *Proteobacteria* phylum [9]. Additionally, the microbiota from the critically endangered Northern bald ibis (*Geronticus eremita*) was also analyzed by this technique, providing essential knowledge and helping the veterinary management of these birds [10].

Besides the debate on the importance and utility of metagenomics vs. culturomics approaches, the complementarity between these two methodologies is recognized as necessary, with studies reporting that only 15% of bacterial species are concomitantly detected by both methods when used in parallel [5, 11]. Moreover, culturomics was applied to the study of rumen microbiome by using two different anaerobic media, a chemically defined one (based on M10) and a complex medium with rumen fluid [12]. This allowed the recovery of 23% of the rumen microbiome, however most of the cultured strains belonged to the rare rumen biosphere, being undetectable in the culture-independent rumen microbiome [12]. Furthermore, pig gut microbiota was also investigated using YBHI medium in anaerobic conditions, resulting in the isolation of 46 bacterial species together with metagenomic analysis [13]. Several discrepancies were identified regarding taxonomical composition when culture-dependent and culture-independent methodologies were compared, however, the interesting achievement was

the sequencing of isolates and the underlying generation of high quality metagenomic bins used to supplement culture-independent metagenomic data [13]. The obtained bin genomes populated clades that were missed by metagenomics and provided genes not observed in the metagenomics data [13].

Culturomics has been mostly applied to human gut samples and still involves the application of concurrent time-consuming and expensive wet bench protocols in parallel with highly expensive mass spectrometry equipment hardly available in most microbiology research laboratories. Building on these backgrounds and bearing in mind the standard equipment existent in veterinary research laboratories, in this work we explore a systematic and simple culturomics strategy coupled with standard amplification and Sanger sequencing to define an optimal study design and experimental procedure with wide application in the characterization of the microbiota of wild and domestic mammals. We use different approaches for the initial handling of fecal samples and compare the results obtained in selective and non-selective media, when incubated in aerobic versus anaerobic conditions, in their ability to capture the most representative diversity of the cultivable gut microbiota. We also explore how many specimens are necessary to maximize the underlying diversity and to get a clear snapshot of the living bacterial and fungal communities, using a wild mesocarnivore as a model (Egyptian mongoose [*Herpestes ichneumon*]).

Materials and methods

Study design and animal specimens

Fecal samples from 20 Egyptian mongoose (*Herpestes ichneumon*, 10 males and 10 females, numbered from 01 to 20) hunted in 2018 during legal predator density control actions, were opportunistically used for this work. The animals were harvested from the same geographical region, in Portugal, within a radius of 50 km. The animal carcasses were donated by hunters for scientific research and no animals were sacrificed for this study purpose. Ethical approval was thus not applicable. Shortly, after death, animal carcasses were transported under refrigeration into the laboratory and submitted to necropsy within 48 h. Then, the solid intestinal content (colon) of each animal was collected using a sterile feces collection tube and immediately processed for microbiological analyses.

Bacteriological and mycological culture

Each collected sample was divided into two equal parts that were homogenized in buffered peptone water (1 g stool per 10 ml) and incubated each at 37°C for 24 h, with orbital shaking (150 rpm), under aerobic and anaerobic conditions. The anaerobic atmosphere was accomplished by flushing with nitrogen stream followed by completing 20% of the final volume with mineral oil.

Next, the enriched fecal sample incubated under anaerobic conditions was serially diluted (until 10^{-7}) and 100 μ L of each dilution (10^{-5} - 10^{-7}) was plated onto Yeast extract, Casitone, and Fatty Acid (YCFA) agar [14], and incubated for 72 h, at 37°C, in anaerobic conditions. YCFA is composed of: 10 g/l casitone, 2.5 g/l yeast extract, 4 g/l NaHCO₃, 2 g/l glucose, 2 g/l maltose, 2 g/l cellobiose, 1 g/l cysteine, 0.45 g/l K₂HPO₄, 0.45 g/l KH₂PO₄, 0.9 g/l NaCl, 0.09 g/l MgSO₄.7H₂O, 0.09 g/l CaCl₂, 1 mg/l resazurin, 10 mg/l haemin, 10 μ g/l biotin, 10 μ g/l cobalamin, 30 μ g/l p-aminobenzoic acid, 50 μ g folic

acid, 0.15 mg/l pyridoxamine, 33 mM acetate, 9 mM propionate, 1 mM isobutyrate, 1mM isovalerate, and 1 mM valerate, followed by the addition of 0.5 µg/l thiamine and 0.5 µg/l riboflavin after autoclave. Anaerobic conditions were accomplished using AnaeroGen™ 3.5L anaerobic atmosphere generation systems (Thermo Scientific, Massachusetts, USA).

Moreover, 1 mL of the enriched fecal sample from aerobic and anaerobic conditions was pasteurized at 80°C for 12 min, serially diluted (between 10^{-1} and 10^{-6}) and 100 µL of each dilution was plated onto YCFA agar supplemented with 0.1% of sodium taurocholate (YCFA P), and incubated for 72 h at 37°C, under aerobic and anaerobic conditions, respectively. Pasteurization was used to positively select for spores and the YCFA medium was supplemented with 0.1% of sodium taurocholate to promote spore germination [4, 14].

Additionally, the rest of the enriched fecal sample incubated under aerobic conditions was serially diluted up until 10^{-7} and 100 µl of each dilution (10^{-5} until 10^{-7}) was plated onto YCFA and also onto selective media: MacConkey solid medium (Biokar diagnostic, France); Potato Dextrose Agar supplemented with chloramphenicol (PDA) (Biokar diagnostic, France); Extended-Spectrum Beta-Lactamase (ESBL) chromogenic medium (Conda, Pronadisa, Spain), with (ESBL w/ AS) and without (ESBL w/o AS) ESBL antibiotic supplement (Conda, Pronadisa, Spain). All media were incubated at 37°C, under aerobic conditions for 24 h, except YCFA and PDA solid media, that were incubated for 72 h.

Colony-forming units per milliliter (CFU/ml) and per gram of wet fecal weight (CFU/g) were determined for all conditions and culture media.

Purification and presumptive identification of isolates

For purification of isolates, five isolated colonies from each different morphology were picked from all media inoculated with three dilutions. The colonies that were picked up were re-streaked twice in the original growth medium to confirm purity. The 1500 purified individual colonies were assessed for shape, pigmentation, and opacity. Bacteria were characterized in terms of gram character, endospore formation, and the presence of catalase and cytochrome c oxidase enzymes. Gram character was determined based on traditional Gram staining, endospore formation was determined based on the presence/absence of endospores by Schaeffer–Fulton stain method (malachite green and safranin), catalase test was performed by the slide method based on the breakdown of hydrogen peroxide resulting in bubbles formation, and cytochrome c oxidase test was performed by the filter paper test method, based on the oxidation of dimethyl-*p*-phenylenediamine dihydrochloride, resulting in dark purple coloration. All tests were performed with fresh colonies (<42 h), except endospore staining that was performed with colonies with >72 h, to ensure the depletion of nutrients in the growth medium.

The 30 isolated fungi were stained using lactophenol cotton blue and different morphological features were characterized. For multicellular filamentous fungi isolates, hyphal septation and spores color, morphology, and septation were assessed. For yeast isolates, we evaluated cell morphology and division.

Considering the previous characterization, all isolates were grouped into morpho-physiological types (MT): MT-I to MT-XI for bacterial isolates (Supplementary Table 1); MT-XII for yeasts; MT-XIII for filamentous fungi.

Molecular fingerprinting and identification of bacterial isolates

To analyze the intraspecific polymorphisms present in the overall bacterial population that would enable reducing the number of isolates for subsequent molecular identification, molecular fingerprinting of isolates was completed based on Random

209 Amplified Polymorphic DNA (RAPDs). A bacterial isolate from each MT, from each
210 solid media, from each host specimen, was selected (n=500). Total cell lysis of each
211 isolate was performed by direct boiling at 95°C, for 15 min, of 2 to 3 colonies in 250 µL
212 of TE 1 M, pH 8.0, centrifuging, collection of the supernatant into a clear microtube and
213 storing at -20°C [15].

214 For RAPD, which uses single-primed PCR fingerprinting, an initial screening to
215 select the most appropriate primer was performed by comparing four different primers.
216 Seven isolates (one from each morpho-physiological type) were tested. The tested primers
217 can be classified into three groups: primers directed towards regions containing mini-
218 satellite from M13 bacteriophage – primer M13 (5'-GAG GGT GGC GGT TCT-3') [16];
219 random primers – primer OPC19 (5'-GTT GCC AGC C-3') [17] or primer 1281 (5'-AAC
220 GCG CAA C-3') [18]; and universal primer for 16S rRNA gene – primer PH (5'-AAG
221 GAG GTG ATC CAG CCG CA-3') [19].

222 PCR amplifications were performed in a Biometra Uno II Thermal Cycler, using
223 a total volume of 15 µl and including 0.2 mM of primer (Invitrogen, Massachusetts,
224 USA), 7.5 µl of NZYTaQ II 2x Green Master Mix (NZYTech, Lisbon, Portugal), 5 µl of
225 DNA. The dilutions of cell lysates containing genomic DNA were used in each PCR
226 reaction were adjusted according to the semi-quantitation of their concentration (ranging
227 between 100 and 20 ng/µl) in 0.8% (w/v) agarose gels. After PCR reaction, the RAPD
228 amplicons were resolved by 1.5% (w/v) agarose gel (NZYTech, Lisbon, Portugal)
229 containing 0.03 µl/ml of GreenSafe Premium (NZYTech, Lisbon, Portugal) in 0.5 X TBE
230 buffer (Invitrogen, Massachusetts, USA), at 2.6 V/cm for 4 h. The gel casts were always
231 the same size to avoid variability across batches. DNA was visualized under UV light and
232 photographed with Alliance 4.7 system (UVITEC Cambridge, United Kingdom).

The fingerprints produced by OPC19 and 1281 primers showed low polymorphic profiles and badly-defined amplification patterns, with faint fragments. The selected primers M13 and PH provided suitable fingerprints, with well-defined amplification patterns. PCR cycling conditions for M13 consisted of 94°C for 5 min, followed by 40 cycles of 60 s at 94°C, 3 min at 40°C, 120 s at 72°C, plus an additional step at 72°C for 7 min, for chain elongation. The PCR cycling conditions for PH consisted of 95°C for 3 min followed by 35 cycles of 30 s at 94°C, 30 s at 35°C, 3 min at 72°C, plus an additional step at 72°C for 5 min. Nuclease-free water was used as no-template control in each sample batch.

The PCR products from RAPDs analyses with PH or M13 were resolved on agarose gels as stated above.

Molecular identification of bacteria was based on 16S rRNA gene sequence analyses. At least, one isolate from each dendrogram cluster resulting from RAPD-fingerprints was randomly selected for 16S rRNA gene sequencing (n=139), since several representatives of two clusters were sequenced and they were shown to represent the same genus within each cluster. A PCR was performed using as forward primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and as reverse primer 1387r (5'-GGG CGG WGT GTA CAA GGC-3') [20], in a final volume of 25 µl with 0.2 mM from each primer (Invitrogen, Massachusetts, USA), 12.5 µl NZYTaQ II 2x Green Master Mix, and 5 µL of DNA. This set of primers allows the amplification of all hypervariable regions (V1-V9) of 16S rRNA. The PCR amplification program consisted of 1 cycle of 5 min, 95°C, followed by 30 cycles of 45 s, 95°C; 45 s, 55°C; 120 s, 72°C, and a final step of 7 min, 72°C. PCR products with the expected size (approximately 1500 bp) were resolved as stated above.

DNA was quantified using a Qubit fluorometer (Invitrogen, Massachusetts, USA), following the manufacturer's instructions. Samples were commercially sequenced by the Sanger sequencing technique using 63f primer (GATC Biotech AG, Germany). Since the *Taq DNA polymerase* used has a mutation rate of 10^{-5} , the reproducibility of the originated sequences was assessed through the comparison of duplicate sequences that were re-sequenced (>10% of isolates) and taking into consideration that only one strand was sequenced.

Molecular identification of fungi

Fungi isolates with different morphology were selected for species identification through sequencing. Yeast were lysed using the direct boiling method previously described, and filamentous fungi DNA was extracted using NZY Plant/Fungi gDNA Isolation kit (NZYTech, Lisbon, Portugal), following the manufacturer instructions.

Amplification of the D1/D2 domain region of the 26S rRNA gene (Large subunit-LSU) in yeast [21] and the Internal Transcribed Spacer (ITS) region in filamentous fungi were performed [22]. For yeast isolates, a PCR was performed using NL-1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL-4 (5'-GGT CCG TGT TTC AAG ACG G-3') primers [23], and for filamentous fungi, a PCR was performed using ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') primers [24]. In both cases, a final volume of 25 µl was used, containing 0.2 mM of each pair of primers (Invitrogen, Massachusetts, USA), 12.5 µl NZYTaq II 2x Green Master Mix, and 5 µL of DNA. The PCR amplification program consisted of 1 cycle of 3 min at 95°C, followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, 30 s at 72°C and a final step of 10 min at 72°C. Nuclease-free water was used as no-template control in each sample batch. PCR products with expected size (approximately 650 bp and between 600 and 800 bp, respectively) were resolved as stated above and extracted from gels using

QIAquick Gel Extraction Kit (QIAGEN, Netherlands), according to manufacturer's handbook.

DNA was quantified using a Qubit fluorometer, following the manufacturer's instructions. Samples were commercially sequenced by Sanger sequencing technique, using a mix of 20 to 80 ng/ μ l of PCR product and 5 μ M of NL-4 and ITS4 primers, for yeast and filamentous fungi, respectively (GATC Biotech AG, Germany).

Homology searches for genome-based identification of isolates

Electropherograms were manually inspected and corrected whenever necessary. The 16S rRNA gene partial sequences generated were located in the early region of the gene (V1-V3), which is informative for the identification of most genera since it is a highly polymorphic moiety [25]. The 16S rRNA gene, the D1/D2 domain region, and ITS gene sequences were compared with those available in the GenBank databases using the BLASTN program through the National Center for Biotechnology Information (NCBI) server. Comparisons were performed using the default parameters. Sequences were annotated with taxonomic information from the top three best matches displaying the same nucleotide pairwise identity, ranging from 82% to 100%, 95% to 99%, and 94% to 99% in the 16S rRNA gene, D1/D2 domain region, and ITS gene sequences, respectively. The criteria used for bacteria and fungi identification are represented in Table 1 [25-27]. A failure to identify phylotypes was defined as a 16S rRNA gene sequence similarity score lower than 75% and an ITS sequence similarity score lower than 60% with sequences deposited in GenBank at the time of analysis.

Data analyses

Considering culture assays, results from CFU counts were displayed as means of values of twenty independent experiments with respective standard deviation. All variables were tested for normality using the D'Agostino-Pearson test ($\alpha=0.05$). When

comparing two conditions, a t-student test (Mann-Whitney test, $\alpha=0.05$) was performed. Results were considered non-significant if p-value ≥ 0.05 , significant if p-value = 0.01 to 0.05, very significant if p-value = 0.0001 to 0.01, and extremely significant if p-value < 0.0001. All statistical analyses were performed using GraphPad Prism software (Version 7, California, USA).

At the genus level, Margalef index ($\frac{S-1}{\ln N}$) and Menhinich index ($\frac{S}{\sqrt{N}}$) were calculated to assess species richness. These indices ignore the relative abundance of taxa taking into consideration only the total number of detected genera and the total number of isolates recovered from a given sample or medium [28].

Results and Discussion

Evaluation of microbial burden and comparison across media

The steps of the experimental culturomics workflow (considering YCFA and PDA solid media) are summarized in Fig. 1. Fecal samples were pre-cultured under different conditions: aerobically and anaerobically in buffered peptone water, after flushing the vials with nitrogen stream. After 24h, the fecal enrichments were cultured: aerobically in YCFA (YCFA w/ O₂); anaerobically in YCFA (YCFA w/o O₂); aerobically, after pasteurization, in supplemented YCFA (YCFA P w/ O₂) to promote endospore germination under aerobiosis; anaerobically, after pasteurization, in supplemented YCFA (YCFA P w/o O₂) to promote endospore germination under anaerobiosis; aerobically in Extended-Spectrum Beta-Lactamase medium without antibiotic supplement (ESBL w/o AS); aerobically in Extended-Spectrum Beta-Lactamase solid medium with antibiotic supplement (ESBL w/ AS); aerobically in MacConkey; and aerobically in PDA supplemented with chloramphenicol (PDA).

After incubation, colony forming units were counted, differentiated according to morphotype defined by a strict scheme (see Supplementary Table 1), followed by RAPD

discrimination, and molecular identification by 16S rRNA / 26S rRNA / ITS amplification and sequencing (Fig. 1).

Comparing the microbial load of spore-forming bacteria obtained in YCFA P media, we detected a mean of 5.1×10^5 and 2.1×10^7 CFU/g under aerobic and anaerobic conditions, respectively. A statistically significant higher microbial load was registered in YCFA P w/o O₂ when comparing with YCFA P w/ O₂ (p-value=0.0006), indicating higher load of spore-forming bacteria under anaerobiosis. The supplementation of media with sodium taurocholate, a well-known germination signal to the *Clostridia* members, was used to promote spore germination [29]. However, other germinant, such as selective amino acids, are known to be needed to promote sporulation of *Bacillus* members [30]. To minimize the potential taxonomical recovery bias arising from the sole addition of sodium taurocholate as germinant, microscopic analysis based on malachite green staining of all Gram positive and catalase positive isolates followed, providing a more complete description of the community of sporulating bacteria. Noteworthy, all spore-forming bacteria were successfully recovered from the sodium taurocholate supplemented media, with the exception of *Paenibacillus* and *Paeniclostridium* members. More information regarding morphophysiological type distribution along fecal samples and incubation conditions can be found in Supplementary Figures 1 and 2, respectively.

Molecular identification of isolates

Purification of 1500 bacterial isolates from all media was followed by classification into morphophysiological types. After selection of the primers best suited for molecular fingerprinting, a bacterial isolate from each morphophysiological type, from each solid media, and each host specimen (n=500), was fingerprinted by RAPD with M13 primer and in parallel with PH primer.

To obtain a measure of RAPD reproducibility, each PCR batch included a randomly selected duplicate, with a total number of 18 isolates for primer M13 and 22 isolates for primer PH. The similarity between each pair of duplicates was based on the dendrogram computed with the Pearson correlation coefficient and the unweighted pair group method with arithmetic average (UPGMA) as the agglomerative clustering algorithm (BioNumerics version 4.0 – Applied Maths, Belgium). The reproducibility value was determined as the average value for all pairs of duplicates. The reproducibility of fingerprints with these primers, estimated by the similarity average value for all pairs of duplicates, was $97.2\% \pm 3.3\%$ for M13 and $83.1\% \pm 9.6\%$ for PH. The reproducibility value enables the definition of a cutoff for cluster formation in a dendrogram.

To integrate all this information, a composite dendrogram based on M13 and PH fingerprints was generated for the differentiation of bacterial isolates. Strain relationships, based on the molecular characters presented as fingerprints, were analyzed by hierarchical numerical methods with Pearson correlation similarity and UPGMA clustering, using 70% similarity as the cutoff value for cluster formation. This value was selected to ensure a conservative approach since it is the lowest similarity value between pairs of duplicates. We obtained a total of 122 clusters, 55 of them being single-member clusters. To reduce the entropy resulting from the enormous diversity of isolates, we grouped the isolates of each MT in individual dendrograms. Additionally, a few isolates of identified genera, that were misplaced due to misleading results of the morpho-physiological tests, were also regrouped subsequently into the correct MT. Representative RAPD profiles of each identified phylotype using both M13 primer and PH primer can be found in Supplementary Figure 3.

One hundred and thirty-nine isolates selected from all clusters were subjected to 16S rRNA gene amplification and sequencing. Thirty fungal isolates were identified at

the genus level according to LSU/ITS barcodes in the case of yeast/filamentous fungi. Molecular identification of isolates obtained from the 20 fecal samples belonged to 2 kingdoms, 6 phyla, 9 classes, 12 orders, 21 families, 24 genera, and 32 species. The composition of each fecal sample at the genus level was analyzed (Fig. 2A), with five genera being common to at least 50% of total fecal samples: *Enterococcus* (100%), followed by *Bacillus* (90%), *Pseudomonas* (75%), *Ralstonia* (65%), and, finally, *Clostridium* (55%). *Pseudomonas* and *Ralstonia* have been rarely found in healthy mammalian gut microbiota and their sporadic presence is sometimes associated to contamination [31]. Working under sterile conditions (e.g. laminar flow hood) and performing routine microbiological controls in each batch of growth experiments, as well as including negative and no-template controls in molecular biology procedures, are crucial to rule out contaminant microbiota which can confound microbiome studies. Working with high biomass samples and ensuring the collection of the central core area of the fecal sample to be processed also avoid biases from environmental contamination. Previous work assessing the gut microbiota of Egyptian mongoose have also detected *Pseudomonas* and *Ralstonia* species in different mongoose specimens from other geographic regions [32], indicating that these bacteria are indeed characteristic of this carnivore's microbiota.

The species richness of each fecal samples was calculated possessing a Margalef index mean of 2.6 and a Menhinick index mean of 2.1 (Fig. 2B), showing similar richness values between fecal samples.

The microbial species isolated from a single fecal sample explained 31% of the overall microbial entities (species level) identified, while two samples accounted for as much as 50% of total microbial species (Fig. 2C), showing that about one-third of

cultivated microorganisms could be isolated from a single sample and half from two samples.

Comparison of bacterial isolation from the aerobic and anaerobic groups

The proportion of bacterial species isolated from the aerobic and the anaerobic groups were compared. For non-pasteurized enrichments, 20% of bacterial species were exclusively isolated from aerobic conditions (YCFA w/ O₂), while 45% were only isolated under anaerobic conditions (YCFA w/o O₂), with the remaining 35% of bacterial species being isolated under both conditions (Fig. 3A). Likewise, for pasteurized enrichments, 45% and 41% of bacterial species were solely isolated either in aerobic (YCFA P w/ O₂) or anaerobic (YCFA P w/o O₂) conditions, respectively, while 14% of taxa were isolated under both conditions (Fig. 3A). When plotting the number of bacterial species per number of samples analyzed, two aspects become central: 1) sample cultivation simultaneously in aerobic and anaerobic conditions leads to the cumulative isolation of extra bacterial taxa as compared to single growth under aerobiosis (Fig. 3B); (2) as the number of fecal samples increases, the number of new bacterial taxa detected increases to a certain point but then remains steady, both under aerobic and combined aerobic and anaerobic conditions, showing that it is not useful to extend the number of samples indefinitely. Hence, the best number of fecal samples to characterize was determined as $n = 6$, under aerobic conditions, for which slightly less than 20 species could be identified; and $n = 9$, when anaerobic conditions are used in parallel, which increases to almost 30 the number of identified species; at these sampling points, the curves reach the plateau (Fig. 3B). An extra sample should be included beyond the point identified as the sample leading to the highest number of detectable species, so seven and ten can be considered as the optimal number of fecal samples from mongoose for cultures grown under aerobic and anaerobic conditions, respectively.

Comparison of bacterial isolation from the pasteurized and non-pasteurized groups

The proportion of bacterial species isolated from the pasteurized group and the non-pasteurized group were compared. Under aerobic conditions, 35% of bacterial species were exclusively isolated from pasteurized enrichments (YCFA P w/ O₂), while 24% were detected solely in non-pasteurized enrichments (YCFA w/ O₂) (Fig. 4A). The remaining 41% of taxa were detected in both enrichments (Fig. 4A). Also, under anaerobic conditions, 20% and 40% of the bacterial species were isolated from pasteurized (YCFA P w/o O₂) or non-pasteurized (YCFA w/o O₂) enrichments, respectively, while 40% of bacterial species could be detected in both enrichments (Fig. 4A). Based on the number of bacterial species identified across the samples, and depending on the number of samples analyzed, it becomes clear that, in these conditions, as the number of samples increases, the more species are isolated. The concomitant use of pasteurized enrichments in addition to unpasteurized will lead to the isolation of an increased number of bacterial species (Fig. 4B). Thus, the detection of further bacterial species can be ameliorated by pasteurizing the enrichments. However, the number of new species detected slightly decreases with the increase in the number of fecal samples, under both pasteurized and non-pasteurized enrichments, possibly due to the redundancy of bacterial species across the culturable microbiota, i.e. detection of more isolates but from the same taxon. So, in this case, and, as previously noted for the aerobic/anaerobic comparison, it is not beneficial to sample indefinitely. In these conditions, the best sample number to screen was $n = 9$, i.e. when the curve reaches the plateau, which is achieved either for non-pasteurized enrichment or in the situation wherein results from pasteurized enrichments were added (Fig. 4B). An extra sample should be included, as previously stated, leading to ten as the optimal number of fecal samples to be analyzed for both types of enrichment.

Evaluation of media cultivability

The ability of a medium to grow the largest range of bacterial taxa was analyzed at the genus level (Fig. 5A), with six taxa being cultured across at least 50% of all media: *Enterococcus* (87.5%), followed by *Bacillus* and *Enterobacteriaceae* (both 75%), and, finally, *Carnobacterium*, *Pseudomonas*, and *Rummellibacillus* (all 62.5%). At the species level, YCFA in anaerobic conditions (YCFA w/o O₂) alone yielded 50% of the total taxa identified (Fig. 5B), meaning that half of the microorganisms could be isolated from a single medium. ESBL medium non-supplemented (ESBL w/o AS) showed to be a good rich media to use, enabling the detection of 14 bacterial species and reaching the plateau when the number of samples was four (Supplementary Fig. 4). However, the complementation of the panel of media with the progressive introduction of ESBL w/o AS, ESBL w/ AS, and MacConkey does not reveal an improvement of identifiable species over the results obtained on YCFA media, with only an extra species (*Ralstonia insidiosa*) being added to the panel. However, complementation of the assays with media other than YCFA led to an anticipation of the plateau as far as the number of necessary fecal samples is concerned ($n = 7$ instead of $n = 9$) (Fig. 5C).

Besides the bacterial community, the GI tract also harbors a fungal community, quite often neglected in microbiota studies. PDA medium supplemented with chloramphenicol was tested as a potentially suitable medium for fungi cultivation. The 30 isolates selected for ITS/LSU barcoding yielded four fungi species from three phyla not detectable with the remaining media (Fig. 5C).

Regarding species richness, YCFA medium supplemented with sodium taurocholate in aerobiosis (2.9, Margalef; 2.0, Menhinick) and YCFA medium non-supplemented in anaerobiosis (2.4, Margalef; 1.5, Menhinick) were the ones showing

higher richness, contrary to PDA medium that showed lower richness (0.9, Margalef; 0.7, Menhinick) (Fig. 5D).

Comparisons of microbial taxa across sex

To identify sex-related differences across the culturome, we compared results from ten males and ten females, using the most effective condition inferred from previous experiments. So, the appraisal of the number of samples from each sex necessary to get a view on the effect of sex was carried out using YCFA-based media together with PDA.

Under aerobic conditions, 29% of microbial species were exclusive from males, while 24% were only isolated from females (Fig. 6A). The remaining 47% were common across sexes (Fig. 6A). Also, under anaerobic conditions, 26% and 32% of taxa were isolated from males or females, respectively, while 42% was detected in both sexes (Fig. 6A). For non-pasteurized enrichments, 30% of microbial species were detected within males, while 35% were only isolated from females and the remaining 35% from both sexes (Fig. 6A). Likewise, for pasteurized enrichments, 33% and 29% of the microbial species were cultured from males or females, respectively, while 38% of the remaining taxa were isolated from both conditions (Fig. 6A). Considering the number of species identified per the number of samples from both sexes, it is evident that the use of samples from both sexes leads to the isolation of more bacterial groups (Fig. 6B). So, the number of new taxa added to the core microbiota can be increased by using samples from both sexes. Furthermore, it is possible to perceive that fecal samples collected from females possess lower microbial diversity than those collected from males (Fig. 6B). However, as expected by previous results, the number of new detectable bacteria declines with the increment in the number of fecal samples from both sexes. So, we also evaluated the optimal number of samples to analyze sex-related differences in the gut microbiota. When the number of fecal samples from males is $n = 9$ and $n = 6$ for female specimens, the

plateau is achieved (Fig. 6B), thus raising to ten and seven the best number of fecal samples to be analyzed from male and female in such type of studies. Since these studies normally require matched paired samples, with diminished unevenness in other variables (e.g. geographical location, age groups, diet, etc), we recommend that ten fecal samples from each sex are to be analyzed although this result might not be generalized to other mammalian species. Regarding species richness, both male and female collected samples show similar values, 3.8;1.5 (Margalef; Menhinick) and 3.4;1.4 (Margalef;Menhinick), respectively (Fig. 6C).

Conclusions

Culturomics is a culture-dependent approach that uses a large combination of different culture conditions and high-throughput means to identify the bacterial strains isolated, namely mass spectrometry or DNA barcoding. Wide-range culture-dependent methods have the potential to increase scientific knowledge, providing the ability to culture and characterize *a posteriori* bacteria that previously were unculturable, also enabling the detection of species that had never been reported within microbiota communities [4-6, 33-35]. In addition, culturomics provides the opportunity to discover novel species. Nonetheless, several bacterial groups or strains require very specific *in vitro* growth conditions, and, for those cases, the culturomics methodology needs to be customized. Hence, experimental study design and optimization are extremely important. Previous studies indicated that increasing the concentration of several medium components (e.g. vitamins), the use of fecal extracts, the application of several filtration and selective techniques (e.g. pasteurization) can lead to increased isolation of bacterial diversity [4, 5]. In particular, in the recent study by Lagier et al. (2015), 212 growth conditions were tested for the isolation of fecal bacteria from human samples, with 18 of those conditions being selected as optimal to successfully identified novel bacteria, namely by using enrichment conditions through the addition of sheep's blood, rumen fluid, and stool extract, but also by pasteurization of the fecal sample [4]. Despite the utility of enrichment steps to maximize the isolation of different species, comparisons on relative species abundances to infer physiological relevance cannot be drawn because the densities of the bacteria recovered are dependent on their specific growth rate in the enrichment medium. Different bacteria and fungi would be differently enriched according to their metabolic capabilities and their proportion does not reflect that found in the animal intestine.

Culturomics has been mainly used for the study of human microbiota [6, 36-38], but increasingly extended to characterize the microbiota of other mammal species, including wildlife [7, 10, 12, 39]. However, the characterization of the gut of wild animals is an emerging research area, wherein methodologies applied to human samples are directly transposed without further improvement. Furthermore, culturomics of human specimens often takes place in highly equipped laboratories, with available mass spectrometry resources and MALDI-TOF databases optimized for bacteria of human origin, that only occasionally are encountered in veterinary settings. In this work, we tried to optimize a culturomics approach to study the gut microbiota of mammalian specimens in a time and cost-effective manner, using a panel of solid media and exploring different enrichment and growth conditions followed by DNA barcoding to capture the most representative diversity of bacterial and fungal communities.

A significant higher microbial load was registered in pasteurized enrichments under anaerobiosis, yielding higher values of microbial diversity compared to pasteurized enrichments incubated under aerobiosis. A significant lower microbial diversity was registered in antibiotic supplemented ESBL medium when compared with the non-supplemented cognate medium. These results came as no surprise since the addition of the antibiotic supplement was expected to inhibit the growth of non-ESBL-producing bacteria. The microbial diversity in the non-supplemented ESBL medium was higher than in non-pasteurized enrichments that were aerobically cultured onto YCFA. Besides the scarce information regarding medium composition given by the manufacturer of ESBL chromogenic medium, the presence of several growth factors, in addition to nitrogen, vitamins, minerals, and amino acids essential for bacterial growth, may enhance the range of the bacterial taxa recovered over the broad-range bacteriological medium YCFA.

Our results also demonstrate that, concerning fecal sample richness, some variation can be found between samples, however, no difference between sexes was detected, with most YCFA-based media and ESBL medium without antibiotic supplementation being the ones allowing greater richness. The use of richness indices instead of diversity indices is due to the absence of true biological meaning of abundances in this work since an enrichment and a selective picking procedure were used, as previously stated.

We found that 33% and 43% of bacteria were exclusively isolated from aerobic or anaerobic conditions, respectively, indicating that both conditions are complementary and should be used in combination. As indicated by the plotted curves, the number of bacterial species attained by incubating in the aerobic condition increased when results from the cognate anaerobic condition were added.

We also detected that 28% and 32% of bacterial taxa were only isolated from pasteurized or non-pasteurized enrichments, respectively, indicating that pasteurization before culture strongly promotes taxa range. Additionally, this also means that these two enrichment conditions are complementary and should also be used in combination. As indicated by the plotted curves, the number of bacterial species identified from the non-pasteurized enrichment increased when results from pasteurized enrichments were added.

The use of ESBL medium supplemented or not, and MacConkey in addition to the YCFA-based media was proven unnecessary to detect novel bacteria. However, the use of a selective medium for fungi was crucial to isolate yeast and filamentous fungi, that none of the other media was able to do. Thus, YCFA-based media and PDA supplemented with chloramphenicol should be used together to assess both the bacterial and fungal gastrointestinal community.

One of the disadvantages that could arise from culturomics is the great amount of time that is involved in the process of cultivation and isolation of all bacterial strains. In this regard, we proposed a simple scheme to group the isolates according to morphophysiological type (MT). Next, to try to reduce the number of isolates within each MT subject to molecular identification, we introduced a step of molecular fingerprinting by RAPD to select representative isolates from each cluster. This allowed us to reduce the panel of isolates from 1500 to 139 (less than 10%). DNA barcoding of all isolates within a single RAPD cluster was performed and molecular identification across isolates was concordantly showing that this framework is adequate to reduce experimental effort and underlying costs. To further optimize the flowchart, we determined how many fecal samples should be characterized to reach an extended, maximum microbial diversity. Since the use of all YCFA-based media in both aerobic and anaerobic conditions, with non-pasteurized and pasteurized enrichments, revealed to be the most appropriate condition, the analysis of ten fecal samples was shown to be highly informative. This final number of samples should be sufficient for any population of mongoose under analysis since a previous study focusing on Egyptian mongoose specimens for several geographical regions in Portugal showed no effect of geographic location on the microbiota richness [32]. However, the number of samples that should be analyzed could depend on the mammalian species under study.

To understand if this methodology could be used in studies aiming to disclose sex-related differences in the gut microbiota, we assessed how many fecal samples from each sex are deemed necessary to correctly identify particularities across sex. We found that 30% of taxa were exclusively detected in males or females, indicating that both sexes should be analyzed in conjunction when trying to characterize the core microbiota of a mammal species. As indicated by the plotted curve, the number of taxa identified from

females increased when male data was added. Using this optimized methodology that disclosed the use of all YCFA-based media, in both aerobic and anaerobic conditions, with non-pasteurized and pasteurized enrichments, together with the PDA supplemented with chloramphenicol, the analysis of ten fecal samples of each sex was sufficient.

Culture-dependent methods are inevitably biased by the ability to offer in vitro conditions for the growth of different taxa. They can underestimate microbial abundance and diversity, namely due to limitations on the detection of unculturable or fastidious microorganisms [4]. Using the methodology proposed herein, by picking morphologically different colonies, we enabled the selection of a large diversity for molecular barcoding of cultivated isolates and the assembly of an informative and extensive collection of microbial isolates that represent the core ecosystem of a carnivore's gut. We also show that, when adequate culture conditions are used, a significant number of microbial taxa may be isolated, without exclusion of those that occur at low abundances. The bias of culturomics over metagenomics is greatly surpassed by the possibility of downstream analyses of the cultivated isolates to detect new metabolic functions, discover antimicrobial resistance determinants and patterns, and disclose whole genomes, among many other knowledge opportunities.

Microbial profiling based on culture-independent sequencing has previously shown that the core gut microbiome of adult Egyptian mongoose is dominated by *Firmicutes*, followed by *Fusobacteria*, *Actinobacteria*, and *Proteobacteria* [40]. The comparison between culturomics and microbial profiling data from this species was also the subject of a previous publication [41]. In agreement with microbial profiling, culturomics showed that the core gut cultivable microbiota of the mongoose is dominated by *Firmicutes* and, as the former approach, was able to distinguish sex- and age class-specific genera. Additional information could be obtained through culturomics, with six

new genera unveiled, showing that, when used in a complementary perspective to metagenomics, knowledge can be expanded by culture approaches. Richness indices and the Shannon index were concordant between culture-dependent and culture-independent strategies, highlighting significantly higher values when using microbial profiling. Also, the influence of abiotic and biotic factors on the microbial community composition of mongoose' gut could be drawn by microbial profiling, while findings arising from culturomics were inconclusive. In conclusion, we propose a culturomics approach to be used in the characterization of mammals' microbiota, using different media and incubating under aerobic and anaerobic conditions. Additionally, we stress the need to use both non-pasteurized and pasteurized enrichments to detect a higher number of bacterial taxa. We highlight the complementarity of several *in vitro* conditions and their value to providing a clearer picture of the microbial diversity. Finally, we clearly show that a rational reduction in the number of fecal samples and solid media to assess both bacterial and fungal gastrointestinal core microbiota of mammals can be successfully adopted without loss of biological information but with resource gains.

Author contributions

MVC conceived the study and defined methodological and analytical approaches. ACP performed the experimental work and formal analyses. ACP wrote the first draft of the manuscript. MVC revised and gave critical feedback on all drafts. Both authors approved the final version for submission.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgments

MVC acknowledges the regular collaboration of hunters and hunter associations, particularly FENCAÇA, in the scope of scientific studies using wildlife specimens. Strategic funding from Fundação para a Ciência e a Tecnologia (FCT), Portugal, to cE3c and BioISI Research Units (UID/BIA/00329/2020 and UID/Multi/04046/2020, respectively) is gratefully acknowledged. ACP is the recipient of a PhD fellowship by FCT (SFRH/BD/136557/2018).

670 **References**

- 671 [1] Hugon P, Lagier J-C, Robert C, Lepolard C, Papazian L, Musso D, et al. Molecular Studies
672 Neglect Apparently Gram-Negative Populations in the Human Gut Microbiota. *Journal of Clinical*
673 *Microbiology* 2013;51:3286-93.
- 674 [2] Miller JM OHC. Substrate utilization systems for the identification of bacteria and yeasts.
675 Murray PR, Baron EJ, Jorgensen JH, Landry ML, Pfaller MA (ed), *Manual of clinical microbiology*,
676 ASM Press, Washington, DC. 2007;9th ed.:103-9.
- 677 [3] Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, et al. Diversity of the
678 human intestinal microbial flora. *Science (New York, N.Y.)* 2005;308.
- 679 [4] Lagier J-C, Hugon P, Khelaifia S, Fournier P-E, La Scola B, Raoult D. The Rebirth of Culture in
680 Microbiology through the Example of Culturomics To Study Human Gut Microbiota. *Clinical*
681 *microbiology reviews* 2015;28:237-64.
- 682 [5] Lagier JC, Armougom F, Million M, Hugon P, Pagnier I, Robert C, et al. Microbial culturomics:
683 paradigm shift in the human gut microbiome study. *Clinical microbiology and infection : the*
684 *official publication of the European Society of Clinical Microbiology and Infectious Diseases*
685 2012;18:1185-93.
- 686 [6] Lagier JC, Khelaifia S, Alou MT, Ndongo S, Dione N, Hugon P, et al. Culture of previously
687 uncultured members of the human gut microbiota by culturomics. *Nature microbiology*
688 2016;1:16203.
- 689 [7] Bittar F, Keita MB, Lagier J-C, Peeters M, Delaporte E, Raoult D. Gorilla gorilla gut: a
690 potential reservoir of pathogenic bacteria as revealed using culturomics and molecular tools.
691 *Scientific reports* 2014;4:7174-.
- 692 [8] Tandina F, Almeras L, Koné AK, Doumbo OK, Raoult D, Parola P. Use of MALDI-TOF MS and
693 culturomics to identify mosquitoes and their midgut microbiota. *Parasites & Vectors* 2016;9:495.
- 694 [9] Perlatti B, Luiz AL, Prieto EL, Fernandes JB, da Silva MFdGF, Ferreira D, et al. MALDI-TOF MS
695 identification of microbiota associated with pest insect *Diabrotica speciosa*. *Agricultural and*
696 *Forest Entomology* 2017;19:408-17.
- 697 [10] Spargser J, Loncaric I, Tichy A, Fritz J, Scope A. The cultivable autochthonous microbiota of
698 the critically endangered Northern bald ibis (*Geronticus eremita*). *PLOS ONE* 2018;13:e0195255.
- 699 [11] Ferrario C, Alessandri G, Mancabelli L, Gering E, Mangifesta M, Milani C, et al. Untangling
700 the cecal microbiota of feral chickens by culturomic and metagenomic analyses. *Environmental*
701 *microbiology* 2017;19:4771-83.
- 702 [12] Zehavi T, Probst M, Mizrahi I. Insights Into Culturomics of the Rumen Microbiome. *Frontiers*
703 *in Microbiology* 2018;9:1999.
- 704 [13] Fenske GJ, Ghimire S, Antony L, Christopher-Hennings J, Scaria J. Integration of culture-
705 dependent and independent methods provides a more coherent picture of the pig gut
706 microbiome. *FEMS Microbiology Ecology* 2020;96.
- 707 [14] Browne HP, Forster SC, Anonye BO, Kumar N, Neville BA, Stares MD, et al. Culturing of
708 'unculturable' human microbiota reveals novel taxa and extensive sporulation. *Nature*
709 2016;533:543-6.
- 710 [15] Dashti A, Jadaon M, Abdulsamad A, Dashti H. Heat Treatment of Bacteria: A Simple Method
711 of DNA Extraction for Molecular Techniques. *Kuwait Medical Journal* 2009;41.
- 712 [16] Huey B, Hall J. Hypervariable DNA fingerprinting in *Escherichia coli*: minisatellite probe from
713 bacteriophage M13. *Journal of Bacteriology* 1989;171:2528-32.
- 714 [17] Chambel L, Sol M, Fernandes I, Barbosa M, Zilhao I, Barata B, et al. Occurrence and
715 persistence of *Listeria* spp. in the environment of ewe and cow's milk cheese dairies in Portugal
716 unveiled by an integrated analysis of identification, typing and spatial-temporal mapping along
717 production cycle. *International journal of food microbiology* 2007;116:52-63.
- 718 [18] Akopyanz N, Bukanov NO, Westblom TU, Kresovich S, Berg DE. DNA diversity among clinical
719 isolates of *Helicobacter pylori* detected by PCR-based RAPD fingerprinting. *Nucleic Acids Res*
720 1992;20:5137-42.

- [19] Massol-Deya AA, Odelson D, Hickey RF, Tiedje JM. Bacterial community fingerprinting of amplified 16S and 16S-23S ribosomal DNA gene sequences and restriction endonuclease analysis (ARDRA). Akkermans, A.D.L., van Elsas, J.D., de Bruijn, F.J. (Eds.), *Molecular Microbial Ecology Methods* 1995.
- [20] Marchesi JR, Sato T, Weightman AJ, Martin TA, Fry JC, Hiom SJ, et al. Design and Evaluation of Useful Bacterium-Specific PCR Primers That Amplify Genes Coding for Bacterial 16S rRNA. *Applied and Environmental Microbiology* 1998;64:795-9.
- [21] Kurtzman CP, Robnett CJ. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie van Leeuwenhoek* 1998;73:331-71.
- [22] Larena I, Salazar O, Gonzalez V, Julian MC, Rubio V. Design of a primer for ribosomal DNA internal transcribed spacer with enhanced specificity for ascomycetes. *Journal of biotechnology* 1999;75:187-94.
- [23] O'Donnell K. *Fusarium and its near relatives* In: Reynolds DR & Taylor JW. *The Fungal Holomorph: Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics* 1993;225-33.
- [24] White TM, Bruns, T., Lee, S., Taylor, J. Amplification and direct sequencing of fungal ribosomal RNA for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. *PCR protocols: a guide to methods and applications* 1990;315-21.
- [25] Yarza P, Yilmaz P, Pruesse E, Glockner FO, Ludwig W, Schleifer KH, et al. Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. *Nature reviews. Microbiology* 2014;12:635-45.
- [26] Stackebrandt E, Ebers J. Taxonomic parameters revisited: tarnished gold standards. *Microbiol Today* 2006;33:152-5.
- [27] White JR, Maddox C, White O, Angiuoli SV, Fricke WF. CloVR-ITS: Automated internal transcribed spacer amplicon sequence analysis pipeline for the characterization of fungal microbiota. *Microbiome* 2013;1:6-.
- [28] Hill TC, Walsh KA, Harris JA, Moffett BF. Using ecological diversity measures with bacterial communities. *FEMS Microbiol Ecol* 2003;43:1-11.
- [29] Sorg JA, Sonenshein AL. Bile Salts and Glycine as Cogermnants for *Clostridium difficile* Spores. *Journal of Bacteriology* 2008;190:2505.
- [30] Setlow P. Germination of Spores of *Bacillus* Species: What We Know and Do Not Know. *Journal of Bacteriology* 2014;196:1297.
- [31] Eisenhofer R, Minich JJ, Marotz C, Cooper A, Knight R, Weyrich LS. Contamination in Low Microbial Biomass Microbiome Studies: Issues and Recommendations. *Trends in microbiology* 2019;27:105-17.
- [32] Cunha MV, Albuquerque T, Themudo P, Fonseca C, Bandeira V, Rosalino LM. The Gut Microbiota of the Egyptian Mongoose as an Early Warning Indicator of Ecosystem Health in Portugal. *International Journal of Environmental Research and Public Health* 2020;17.
- [33] Lagier JC, Million M, Hugon P, Armougom F, Raoult D. Human gut microbiota: repertoire and variations. *Frontiers in cellular and infection microbiology* 2012;2:136.
- [34] Hugon P, Lagier JC, Colson P, Bittar F, Raoult D. Repertoire of human gut microbes. *Microbial pathogenesis* 2017;106:103-12.
- [35] Lagier J-C, Dubourg G, Million M, Cadoret F, Bilen M, Fenollar F, et al. Culturing the human microbiota and culturomics. *Nature Reviews Microbiology* 2018;16:540-50.
- [36] Si J, Lee C, Ko G. Oral Microbiota: Microbial Biomarkers of Metabolic Syndrome Independent of Host Genetic Factors. *Frontiers in cellular and infection microbiology* 2017;7.
- [37] Iszatt N, Janssen S, Lenters V, Dahl C, Stigum H, Knight R, et al. Environmental toxicants in breast milk of Norwegian mothers and gut bacteria composition and metabolites in their infants at 1 month. *Microbiome* 2019;7:34.
- [38] Chang Y, Hou F, Pan Z, Huang Z, Han N, Bin L, et al. Optimization of Culturomics Strategy in Human Fecal Samples. *Frontiers in Microbiology* 2019;10.

772 [39] Crhanova M, Karasova D, Juricova H, Matiasovicova J, Jahodarova E, Kubasova T, et al.
773 Systematic Culturomics Shows that Half of Chicken Caecal Microbiota Members can be Grown
774 in Vitro Except for Two Lineages of Clostridiales and a Single Lineage of Bacteroidetes.
775 Microorganisms 2019;7:496.
776 [40] Pereira AC, Bandeira V, Fonseca C, Cunha MV. Egyptian Mongoose (*Herpestes ichneumon*)
777 Gut Microbiota: Taxonomical and Functional Differences across Sex and Age Classes.
778 Microorganisms 2020;8:392.
779 [41] Pereira AC, Bandeira V, Fonseca C, Cunha MV. Crosstalk Between Culturomics and Microbial
780 Profiling of Egyptian Mongoose (*Herpestes ichneumon*) Gut Microbiome. Microorganisms
781 2020;8.

782

Figure Captions

Figure 1 – Schematic representation of the main steps underlying the proposed experimental workflow: fecal samples are pre-cultured in buffered peptone water under both aerobic and anaerobic conditions. After, the fecal enrichments are cultured under different conditions: aerobically in YCFA (YCFA w/ O₂); anaerobically in YCFA (YCFA w/o O₂); aerobically, after pasteurization, in supplemented YCFA (YCFA P w/ O₂); anaerobically, after pasteurization, in supplemented YCFA (YCFA P w/o O₂); aerobically in Extended-Spectrum Beta-Lactamases without antibiotic supplement (ESBL w/o AS); aerobically in Extended-Spectrum Beta- Lactamases with antibiotic supplement (ESBL w/ AS); aerobically in MacConkey; and aerobically in PDA supplemented with chloramphenicol (PDA). After incubation, colonies are counted, morpho-physiologically evaluated and grouped, molecularly differentiated by RAPD, and identified by 16S rRNA gene sequencing.

Figure 2 – (A) Heatmap regarding the presence and absence of each taxon (genus level) in each mongoose fecal sample. (B) Margalef and Menhinick indices regarding genus richness in each fecal sample. (C) Percentage of microbial species cumulatively identified by analyzing fecal samples from one to seven mongoose hosts.

Figure 3 – Comparison between aerobic and anaerobic growth conditions. (A, left moiety) Bacterial species isolated exclusively from the aerobic non-pasteurized enrichment, solely from the anaerobic non-pasteurized enrichment, and from both non-pasteurized enrichments. (A, right moiety) Bacteria isolated exclusively from the aerobic pasteurized enrichment, exclusively from the anaerobic pasteurized enrichment, and from both pasteurized enrichments. (B) Number of identifiable microbial species depending on the number of fecal samples analysed under aerobic (w/ O₂) and complementarily by both aerobic and anaerobic (w/ O₂ + w/o O₂) growth conditions.

Figure 4 – Comparison between non-pasteurized and pasteurized enrichments. (A, left moiety) Bacteria isolated exclusively from the aerobic non-pasteurized enrichment (YCFA w/ O₂), exclusively from the aerobic pasteurized enrichment (YCFA P w/ O₂), and from both aerobic enrichments (i.e. pasteurized and non-pasteurized). (A, right moiety) Bacteria isolated exclusively from the anaerobic non-pasteurized enrichment (YCFA w/o O₂), exclusively from the anaerobic pasteurized enrichment (YCFA P w/o O₂), and from both anaerobic enrichments. (B) Number of identifiable microbial species according to the number of fecal samples analysed subjected to non-pasteurized (NP) and both non-pasteurized and pasteurized (NP + P) enrichments.

Figure 5 – (A) Heatmap regarding the presence and absence of each taxon (genus level) in each growth condition: aerobically in YCFA (YCFA w/ O₂); anaerobically in YCFA (YCFA w/o O₂); aerobically, after pasteurization, in supplemented YCFA (YCFA P w/ O₂); anaerobically, after pasteurization, in supplemented YCFA (YCFA P w/o O₂); aerobically in Extended-Spectrum Beta-Lactamases without antibiotic supplement (ESBL w/o AS); aerobically in Extended-Spectrum Beta- Lactamases with antibiotic supplement (ESBL w/ AS); aerobically in MacConkey; and aerobically in PDA supplemented with chloramphenicol (PDA). (B) Percentage of identifiable microbial species with the cumulative addition of novel growth conditions. (C) Margalef and Menhinick indices regarding genus richness in each medium. (D) Number of identifiable microbial species depending on the number of fecal samples analysed for the YCFA-based media, all bacterial media, and all solid media (PDA included).

Figure 6 – Comparison between male and female samples. Bacteria isolated only from aerobic conditions, bacteria isolated only from anaerobic conditions, bacteria isolated only from non-pasteurized enrichments, and bacteria isolated only from pasteurized conditions. (B) Number of identifiable microbial species depending on the number of

833 fecal samples analysed from female (F), male (M), and both sexes (M + F). (C) Margalef
834 and Menhinick indices regarding genus richness according to the number of fecal samples
835 analysed from female and male.

836

837 **Tables**

838 Table 1 – Taxonomic threshold similarity values (%) for bacteria and fungi.

	Taxon	Species	Genus	Family	Order	Class	Phylum
Threshold similarity value (%)	Bacteria (16S rRNA gene) ^a	98.7	94.5	86.5	82	78.5	75
	Fungi (ITS region) ^b	90	85	75	70	60	-

839 ^aas in [19, 20].

840 ^bas in [21]; the same criteria were used for yeast identification using the D1/D2 domain region of the 26S rRNA gene.

841

842 **Figures**

843
844 **Figure 1**

845

846

847 Figure 2

848

849 Figure 3

850

851 Figure 4

Figure 5

854

855 Figure 6