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Understanding the cross-talk between microbiota, host fitness, and the environment using Egyptian mongoose (*Herpestes ichneumon*) as a model

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ABSTRACT

Gut microbiota is the complex and diverse community of bacteria, archaea, fungi, protozoa, and viruses present in the gastrointestinal tract of animals. Once established, this ecosystem is relatively stable, but responsive to a variety of effects, namely host diet, health, genetics, sex, and reproductive status, and also the habitat. Increasing importance has been attributed to host gut-microbe interactions due to implications in the immune system and ecological features, such as behavior, however, the microbiota of many carnivores remains unknown. In this work, the gut microbiota of Egyptian mongoose, a medium-size mammalian carnivore, with opportunistic feeding behavior, ranging in distribution throughout the African continent, but also in Mediterranean Middle East, southern Turkey, and the Iberian Peninsula, was thoroughly investigated using a wide range culture-based approach. The aims of this work were: [1] the characterization of the core gut microbiota of Egyptian mongoose population; [2] the investigation of sex- and age class-related differences of gut microbiota; and [3] the analysis of the relationship between bio-environmental features and gut microbiota of these specimens.

Fecal samples from ten males and ten females sampled in mainland Portugal were enriched in Buffered Peptone Water, in both aerobic and anaerobic conditions. Part of the enriched samples was pasteurized and inoculated into YCFA P solid medium, under both aerobic and anaerobic conditions. The remaining part was inoculated into YCFA under anaerobic conditions and into YCFA, MacConkey, PDA supplemented with chloramphenicol solid media, ESBL chromogenic medium, with and without antibiotic supplement, and Brilliance ESBL solid medium. Selected isolates were grouped into different morpho-physiological types (MT) based on Gram character, catalase and oxidase activities, and endospore formation. A bacterial isolate belonging to each MT, in each media, for each mammal host, was selected for molecular fingerprinting using Random Amplified Polymorphic DNA (RAPD) with M13 and PH primers. Strain relationships were analyzed by hierarchical numerical methods with Pearson correlation coefficient and Unweighted Pair Group Method with Arithmetic mean (UPGMA) clustering. One isolate from each cluster was randomly selected for 16S rDNA gene sequencing. Fungi isolates with different morphology were selected for genomic identification through Internal Transcribed Spacer (ITS) region sequencing.

In this study, we generated for the first-time extended baseline information on the microbiome of mongoose, enabling the exploitation of microbial community differences between sexes and exploring the influence exerted by the biological and environmental context of each host in its microbiota signature. Looking at each individual host as a habitat with its own community, the MT-II, MT-VI, MT-VII, MT-IX and MT-XI types may be considered the core gut microbiota community and the remaining morpho-physiological types can be considered part of the intra-specific individual microbiota community. Additionally, we perceived that the majority of individuals possess MT-II ESBL-producing bacteria.

Higher microbial load was present in fecal samples from female hosts in rich medium under anaerobic conditions, both for total and sporobiota bacterial community. The bacterial microbiota of both males and females was dominated by Gram-positive bacteria, mainly of the phylum Firmicutes, with bacilli isolates prevailing, in particular, *Enterococcus* spp. and *Bacillus* spp. The growth of putative *Escherichia coli* was only registered in female host samples. The specimens analyzed in this study revealed high Proteobacteria/Bacteroidetes ratio, a feature that may be related to a carnivorous or scavenger dietary regime and with highly efficient energy harvest. Filamentous fungi were exclusively detected in fecal samples from male hosts and their genus identified as *Pseudozyma* and *Naganishia* (Basidiomycota phylum), *Penicillium* (Ascomycota phylum) and *Mucor* (Mucoromycota phylum). Although the number of surveyed specimens is limited, considerable similarity between adult and juvenile microbiota was found, which contrasted with sub-adult's, probably due to higher proximity and interaction between the first two groups, since this species social behavior includes protection of the cubs and juveniles, leading to similar diet and easier host-to-host transmission of microbiota.

This work sets the ground for more comprehensive studies on the microbiota of Mediterranean wild carnivores, including sympatric threatened species. Future studies using culture-independent methods will improve our knowledge of this species microbiome and lead to a better understanding of its bio-ecology.

Key-works: Egyptian mongoose, Gut Microbiota, Microbial Profiling, Carnivores, Host fitness.

RESUMO

O trato gastrointestinal (GI) dos vertebrados é um ecossistema complexo que serve de habitat para uma enorme variedade e diversidade de microrganismos, maioritariamente bactérias, mas também árqueas, fungos, protozoários e vírus. Carnívoros, têm um sistema gastrointestinal complexo, onde a maior concentração bacteriana se encontra no intestino grosso, dominada por aeróbios restritos. Em vertebrados, uma vez desenvolvido o microbiota intestinal, a sua composição é relativamente estável, sendo possível sofrer variações devido a dieta, sistema imunitário, genética, sexo e estado reprodutivo do hospedeiro, bem como devido ao habitat. Independentemente destas variações, a maioria dos vertebrados tem um microbiota intestinal composto por membros dos filos Firmicutes, Bacteroidetes e Proteobacteria.

O sacarrabos, *Herpestes ichneumon* (Linnaeus, 1758), é um mamífero carnívoro da família *Herpestidae*. Este mamífero tem uma alimentação oportunista, mas primordialmente constituída por coelhos. Tem como distribuição geográfica o continente africano, tendo-se expandido até ao Mediterrâneo Oriental. Na Península Ibérica, é considerado tradicionalmente uma espécie introduzida durante as Invasões Muçulmanas, mas um estudo recente baseado em DNA mitocondrial sugere que sofreu uma dispersão natural durante as flutuações marítimas do Pleistocénico Tardio. Esta espécie era restrita ao sul do rio Tejo, tendo-se vindo a expandir por todo o território nacional, havendo registo da sua presença a norte do rio Douro, estando ausente apenas no noroeste do território de Portugal continental. Esta expansão foi motivada por mudanças do uso da terra em ecossistemas dominados por vegetação arbustiva, limpezas florestais, práticas agrícolas e alterações climáticas. Em indivíduos adultos amostrados em Portugal, foram encontradas evidências de dimorfismo sexual, particularmente no que concerne ao tamanho corporal, tendo-se também encontrado diferenças no tamanho corporal de indivíduos de diferentes regiões.

O estudo do microbiota de animais selvagens apenas recentemente ganhou importância, tendo sido negligenciado devido a falta de financiamento específico e a dificuldades técnicas na obtenção e manutenção das amostras. Uma vez que o microbiota tem sido progressivamente reconhecido como fundamental na ecologia dos mamíferos, neste trabalho investigou-se o microbiota intestinal de 20 espécimes de sacarrabos, incluindo 10 machos e 10 fêmeas, amostrados em Portugal continental, explorando abordagens de cultura microbiológica, usando-se para o efeito um espetro alargado de condições de crescimento. Os objetivos deste trabalho foram: (1) caracterizar o microbial nuclear (*core microbiota*) da população de sacarrabos; (2) investigar as diferenças do microbiota intestinal relativamente ao sexo e à classe etária; (3) analisar a relação entre as características bio-ecológicas e o microbiota intestinal desta espécie. As amostras foram enriquecidas em água peptonada, divididas e incubadas em paralelo em condições de aerobiose e anaerobiose. Parte destes enriquecimentos foi pasteurizado e inoculado em meio sólido YCFA P. A parte restante do enriquecimento foi inoculada nos meios sólidos YCFA, MacConkey, PDA suplementado com cloranfenicol, e ainda nos meios cromógenicos ESBL com (ESBL w/ AS) e sem (ESBL w/o AS) suplemento de antibióticos, e Brilliance

ESBL, ambos utilizados para deteção de bactérias produtoras β-lactamases de largo espectro (ESBL). O número de unidades formadoras de colónias por mililitro foi determinado para todas as condições. Cinco colónias bacterianas de diferentes morfologias obtidas nos diferentes meios foram repicadas e caracterizadas através de testes morfo-fisiológicos, nomeadamente, coloração de Gram, coloração de endósporos e testes da catalase e oxidase. Leveduras e fungos filamentosos foram observados macroscopicamente, ao nível da cor, e microscopicamente, ao nível das hifas e esporos. Os indivíduos foram organizados em diferentes tipos morfo-fisiológicos. Um isolado pertencente a cada tipo morfo-fisiológico (MT), proveniente de cada meio de cultura, e obtido de cada hospedeiro, foi selecionado para genotipagem, utilizando os *primers* M13 e PH. A relação entre os diferentes isolados foi analisada utilizando métodos hierárquicos numéricos com o coeficiente de correlação de Pearson e o método de aglomeração *Unweighted Pair Group Method with Arithmetic mean* (UPGMA), estabelecendo-se uma percentagem de semelhança de 70 para a formação de clusters. Um isolado de cada cluster foi selecionado para sequenciação do gene que codifica para 16S rRNA e um isolado de cada levedura e fungo filamentoso com diferentes morfologias foram selecionados para sequenciação da região entre o domínio D1/D2 do gene que codifica para 26S rRNA e a região *Internal Transcribed Spacer* (ITS), respetivamente. As sequencias obtidas foram comparadas com sequencias publicamente disponíveis na base de dados GenBank através do programa BLASTN no servidor do NCBI. A diversidade das amostras foi analisada através do cálculo de índices de diversidade e de estimadores de riqueza especifica não-paramétricos. Procurou-se identificar igualmente associações entre o microbiota e dados bio-ecológicos do hospedeiro e caraterísticas do seu habitat.

De modo a se recuperar o maior numero de bactérias cultiváveis, utilizou-se dois meios de cultura não-seletivos (YCFA e ESBL w/o AS). Em condições de aerobiose, registou-se maior numero de CFU/g de peso fresco de fezes e maior diversidade bacteriana em YCFA e ESBL w/o AS, tendo-se detetado, respetivamente, uma média de $2{,}8\times10^{9}$ e $3{,}3\times10^{12}$ CFU/g de peso fresco de fezes, de bactérias aeróbias em cada um dos meios e sete e treze tipos morfo-fisiológicos. Os resultados registados em YCFA são semelhantes aos reportados noutros estudos focados em amostras de humanos, ruminantes (vaca, ovelha e cabra), suínos (porco doméstico) e carnívoros (urso pardo). Os resultados obtidos em ESBL w/o AS são semelhantes aos reportados em estudos com humanos. As ligeiras diferenças reportadas nos diversos estudos devem-se, possivelmente, a diferenças decorrentes das porções do GI selecionadas para estudo e de caraterísticas individuais dos espécimes amostrados, mas também a diferenças nos meios de cultura utilizados. Em anaerobiose, verificou-se o crescimento médio de $5{,}5\times10^{9}$ CFU/g de peso fresco de fezes em YCFA. Um estudo anterior que fez uso do mesmo meio de cultura (YCFA) para caraterização do microbiota de humanos, e nas mesmas condições de incubação, reportou o crescimento de cerca de 72% da população bacteriana detetada por métodos independentes de cultura (metagenómica), o que sugere que este meio de cultura é adequado para capturar a riqueza e diversidade bacterianas presentes no trato gastrointestinal.

Para se estudar o esporobiota, foi utilizado o meio YCFA agar suplementado com glucose, maltose, cellobiose e taurocholato de sódio (YCFA P), tendo sido detetado, em média, $5,1x10^5$ e $4,2x10^8$ CFU/g de peso fresco de fezes, de bactérias formadoras de endósporos, em condições de aerobiose e anaerobiose, respetivamente. Tanto quanto se conseguiu apurar, nenhum outro estudo reportou a comunidade esporulante do trato GI.

Relativamente aos meios de cultura seletivos usados, o meio de cultura MacConkey permite o crescimento seletivo de bactérias gram-negativas. Detetou-se, em média, $8,0x10^9$ CFU/g de peso fresco de fezes, de bactérias da família *Enterobacteriaceae* neste meio de cultura, sendo estes resultados ligeiramente diferentes de estudos anteriores focados noutras espécies de carnívoros, nomeadamente urso pardo, para o qual foi reportado cerca de 10^8 cópias de genes/g de peso fresco de fezes. Em amostras do íleo de porcos domésticos e, em humanos, foi detetado 10^7 CFU/g de peso fresco de fezes. Os fabricantes dos dois meios de cultura cromogénicos seletivos utilizados descrevem a possibilidade de deteção e isolamento de bactérias gram-negativas produtoras de ESBL. Os resultados obtidos no presente estudo sugerem uma redução clara do crescimento bacteriano na presença dos suplementos com atividade antimicrobiana fornecidos, cuja atividade é exercida sobre várias espécies bacterianas. Não foi assim possível confirmar a presença de *E. coli* produtoras de ESBL, uma vez que o seu crescimento, em condições seletivas por adição do suplemento, foi inibido. Verificou-se também falta de seletividade destes meios de cultura para isolamento específico de bactérias gram-negativas, uma vez que se registou um crescimento de cerca de 99% de bactérias gram-positivas nestas condições. No meio de cultura Brilliance, registou-se a menor taxa de crescimento microbiano, com um crescimento de 100% de isolados pertencentes ao género *Pseudomonas*, de acordo com os resultados de sequenciação do gene 16S rRNA.. De acordo com estes resultados, não foi detectado nenhum género de *Enterobacteriaceae* que pareça possuir capacidade de produzir ESBL. Estudos anteriores demonstraram a existência de *Enterobacteriaceae* produtoras de ESBL em animais selvagens de Portugal continental, bem como de outros paises, em particular em mamíferos da familia *Herpestidae*. A existência de contaminações cruzadas de bactérias fecais que circulam entre a população humana e animais selvagens e a possibilidade de transmissão de bactérias resistentes entre estas duas comunidades são também realçadas por vários estudos.

No que diz respeito à caraterização do micobiota intestinal, utilizou-se PDA suplementado com cloranfenicol, tendo sido registado, em média, cerca de $1,08x10^8$ CFU/g de peso fresco de fezes, de fungos, dos quais cerca de $5,0x10^7$ CFU/g de peso fresco de fezes correspondem a leveduras e $5,8x10^7$ CFU/g de peso fresco de fezes a fungos filamentosos.. Do que foi possível aferir da consulta da bibliografia disponível, este resultado é o mais alto registado no tracto GI, tendo os outros estudos registado um máximo de 10^6 CFU/g de peso fresco de fezes e 10^7 cópias de genes/g de peso fresco de fezes noutras espécies de mamiferos.

Relativamente ao efeito do sexo no microbiota, registou-se maior carga microbiana em fêmeas em YCFA e em YCFA P, e em anaerobiose. Registou-se também em fêmeas a presença presuntiva de *E.*

coli em meio ESBL sem suplemento de antibiótico, não tendo sido observado o crescimento desta espécie em amostras de machos. Seis das oito amostras que registaram crescimento bacteriano em Brilliance são provenientes de fêmeas. Em termos de diversidade microbiana, registou-se um maior número de isolados do MT-II em meio ESBL com antibiótico e de isolados MT-IX, aquando da soma das percentagens de todos os meios, também em amostras de fêmeas. Em contraste, registou-se a presença de fungos filamentosos somente em amostras provenientes de machos. Estudos anteriores realizados com amostras fecais de chimpazés, macacos e lémures, demostraram diferenças significativas entre sexos nas comunidades bacterianas respetivas. Por outro lado, um estudo realizado em humanos demonstrou maior riqueza e diversidade de fungos em amostras de fêmeas.

A sequenciação do gene 16S rDNA de 139 isolados selecionados demonstrou que o microbiota bacteriano do sacarrabos é dominado por bactérias gram-positivas (76%), do filo Firmicutes (68%), nomeadamente da classe Bacilli (50%), dos géneros *Enterococcus* (18%) e *Bacillus* (14%). Os membros do filo Firmicutes são normalmente os mais abundantes no trato GI dos vertebrados, sendo responsáveis, sobretudo, pela degradação de proteínas. Membros da família *Bacillaceae* são frequentemente associados a amostras de solo e ar, sendo por vezes considerados transientes no trato GI originários de plantas e raízes utilizadas como alimento. O trato GI de vertebrados é possivelmente o maior reservatório de *Enterococcus*, sendo considerados patogénicos oportunistas. Estudos recentes identificaram, em Portugal, *Enterococcus* spp. provenientes de diversos ambientes, incluindo o trato GI de animais selvagens, estando este género associado a mamíferos com uma alimentação predominantemente carnívora. A elevada percentagem de bactérias do filo Proteobacteria e a baixa percentagem de Bacteroidetes no nosso estudo, indica um rácio elevado de Proteobacterias/Bacteroidetes, o qual é normalmente associado a animais com um regime alimentar carnívoro ou detritívoro, de que são exemplos a chita, o diabo da Tasmânia, a hiena e o urso polar. Este rácio está também associado a um armazenamento energético altamente eficiente.

A sequenciação da região entre o domínio D1/D2 do gene que codifica para 26S rRNA e a região ITS dos 6 diferentes fungos isolados permitiu identificar os géneros *Penicillium, Naganishia, Pseudozyma* e *Mucor*.

Relativamente aos índices de diversidade calculados (índice de Shannon, índice de Simpson e índice de equitabilidade das espécies derivado do índice de Shannon), registou-se, ao nível do género bacteriano, valores muito semelhantes entre as três comunidades (fêmeas, machos e total da população de sacarrabos), sendo todas elas comunidades bem balanceadas e com elevado nível de equitabilidade. Adicionalmente, os estimadores não-paramétricos de riqueza especifica demonstram uma taxa de complementaridade de 100%, sugerindo que todos os 21 géneros detetados no estudo, correspondem ao total de géneros teoricamente existentes na comunidade. Tendo todos estes parâmetros em conta, conclui-se que o painel e número de isolados selecionados para sequenciação do 16S rRNA é adequado ao propósito deste estudo de caracterizar o microbiota nuclear da população de sacarrabos.

A análise de variações individuais no microbiota nuclear (*core microbiota*) foi possibilitada pela seleção prévia ao estudo de individuos com o mesmo regime alimentar (com base na análise do conteúdo estomacal presente no momento da morte), localização geográfica, condições edafoclimáticas e habitat (avaliado pelo uso da terra). Registaram-se variações do microbiota nuclear entre indivíduos, com valores de riqueza e diversidade compreendidos entre $4,6x10^{11}$ e $8,8x10^{12}$ CFU/g e 3 e 7 MT, respetivamente, à semelhança do que já foi previamente reportado noutros animais selvagens e em humanos. Analisando cada hospedeiro como um habitat com a sua própria comunidade, os tipos morfo-fisiológicos MT-II, MT-VI, MT-VII, MT-IX e MT-XI podem ser considerados o microbiota intestinal nuclear e os restantes tipos morfo-fisiológicos podem ser considerados parte da comunidade microbiota individual intraespecífica. Também, detetamos que a maioria dos hospedeiros possuía bactérias produtoras de ESBL pertencentes ao tipo morfo-fisiológico II.

Registou-se similaridade do microbiota intestinal de indivíduos adultos e juvenis, em contraste com o microbiota de indivíduos sub-adultos, provavelmente devido a fatores de carácter comportamental, uma vez que esta espécie possui padrões de proteção e alimentação dos indivíduos mais jovens, zonas de marcação de território através da excreção anal de fluidos e defecação comunitária em latrinas. Estes fatores podem promover a transmissão fecal-oral intraespecífica de microrganismos entre indivíduos da mesma comunidade (adultos e juvenis).

Este estudo visou, pela primeira vez, a caracterização extensiva da composição microbiana do trato GI de sacarrabos, permitindo a análise das diferenças na comunidade microbiota entre sexos e a análise da influencia exercida pelo contexto biológico e ambiental em cada hospedeiro na sua assinatura microbiota Apesar da elevada diversidade microbiana capturada por recurso a métodos clássicos de cultura, estudos futuros baseados em métodos independentes de cultura (metagenómica) poderão complementar a informação aqui reunida, conduzindo a um melhor entendimento da comunidade microbiana presente no sacarrabos e da bioecologia da espécie; uma espécie altamente adaptativa, em franca expansão no território e cuja presença exerce efeitos em cascata na estrutura e organização das comunidades dos ecossistemas mediterrânicos. Acresce que a natureza comparativa deste estudo, relativamente aos contributos que o sexo do hospedeiro pode ter sobre o seu microbiota, ajudam a melhorar o entendimento sobre os aspetos ecológicos e adaptativos desta espécie, reforçando a importância de se considerar o microbioma como um componente fundamental da biologia do hospedeiro e um elemento chave necessário para compreender a ecologia dos mamíferos.

Palavras-chave: Sacarrabos, Microbiota Intestinal, Identificação & Diferenciação, Carnívoros, Aptidão do Hospedeiro.

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ABBREVIATIONS

°C – degrees Celsius

µL – Microliter

ANOVA – Analysis of Variance

BLASTN – nucleotide-nucleotide Basic Local Alignment Search Tool

bp – Base pair

BW – Body Weight

$CaCl_2$ – Calcium Chloride

CFU – Colony Forming Unit

DNA – Deoxyribonucleic Acid

ESBL – Extended-Spectrum β-Lactamases

ESBL w/ AS – ESBL chromogenic medium with antibiotic supplement

ESBL w/o AS – ESBL chromogenic medium without antibiotic supplement

FF – Filamentous Fungi

g – Grams

GI – Gastrointestinal Tract

h – hour

HBL – Head and Body Length

HD – Head Diameter

HW – Hearth Weight

IgA – Immunoglobulin A

ITS – Internal Transcribed Spacer

K_2HPO4 – Dipotassium Phosphate

KESC – *Klebsiella, Enterobacter, Serratia, Citrobacter* group

KW – Kidney Weight

L – Liter

LF – lactose Fermenting

LNF – Lactose Non-Fermenting

M – Mol

mg – Milligrams

$MgSO_4.7H_2O$ – Magnesium Sulfate Heptahydrate

min – Minutes

mL – Milliliter

mM – Millimolar

MT – Morpho-physiological Type

NaCl – Sodium Chloride

$NaHCO_3$ – Sodium Bicarbonate

NCBI – National Center for Biotechnological Information

ng – Nanograms

NP – Neck Perimeter

OTU – Operational Taxonomic Unit

PC -Principal Component

PCA – Principal Component Analysis

PCR – Polymerase Chain Reaction

PDA w/ CHLO – PDA medium supplemented with chloramphenicol

PFI – Perivisceral Fat Index

RAPD – Random Amplified Polymorphic DNA

RHFL – right hind foot length

RHLL – Right Hind Leg Length

rpm – Revolutions per minute

rRNA – Ribosomal Ribonucleic Acid

s – Seconds

SCFA – Short Chain Fat Acids

SFI – Subcutaneous Fat Index

SH – Shoulder Height

STL – Snout-Tail Length

SW – Spleen Weight

TBE – Tris-Boric acid-EDTA

TE – Tris-EDTA

TL – Tail Length

UPGMA – Unweighted Pair Group Method with Arithmetic mean

V – Volt

v/v – Volume/volume

w/ O_2 – in aerobiosis

w/o O_2 – in anaerobiosis

w/v – Weight/volume

YCFA – YCFA agar medium

YCFA P – YCFA agar medium supplemented with sodium taurocholate

CHAPTER I – INTRODUCTION

1.1. Gut microbiota: introduction

The vertebrate gastrointestinal (GI) tract is a complex ecosystem that is the habitat of an enormous density and diversity of microorganisms, containing mostly bacteria, but also archaea, fungi, protozoa, and viruses (1, 2). In carnivores, microbial density and diversity differ within gut sections, with the main concentration of bacteria being found in the large intestine (approximately $5x10^{10}$ CFU/g wet weight of feces), dominated by strict anaerobes (3). At birth, vertebrates begin to be colonized with microorganisms and, for humans it is known that individual microbiota is acquired during the first year of life, stabilizing its composition later on (4), being usually dominated by members of Firmicutes, Bacteroidetes and Proteobacteria phyla (5). However, there are a variety of effects that can alter this equilibrium (6).

1.1.1. Gut microbial community and diet

Host diet has such a deep effect on the gut microbiota that resulted in an evolutionary divergence between carnivores and herbivores, leading to two distinct gut types, with an increase in microbiota diversity from carnivores to herbivores (5, 7). Nevertheless, gut physiology is, as well, a predictor of the gut microbiota landscape, influencing distribution along the GI tract, such as the dichotomy that is observed between foregut fermenters vs. hindgut fermenters herbivores (5, 7).

For most mammals, diet can vary drastically, in time and space, across season and habitat, so that the composition of the gut microbiota, as well as it functionality, are likely to fluctuate across season and habitat in direct response to these dietary changes (8). Gut microbiota that can quickly shift activity, by changing its composition in response to changes in host dietary intake can lead to improved nutritional flexibility and increased fitness host (9). However, if a poor microbiota response to short-term changes in the diet occurs, the limited food and nutrient availability can affect host health and immune system (8), leading to decreased host fitness.

Microbial communities from animals are mostly composed of r-selected organisms, that can rapidly use the accessible nutrients in the gut and quickly multiply (7), giving a selective advantage to the host, increasing host fitness, ultimately leading to the persistence and survival of this microbiota in the host, and increasing microbiota fitness. Because of this mutually beneficial interaction, gut microbiota and the host coevolved, this is, they reciprocally adapted to each other as interacting species (1, 10). Thus, host adaptation to a defined diet gives chance to gut microbes to evolve and to adapt to the host gut and environment (11). This coevolution has been shown by the discovery of patterns of community similarity that match the mammalian phylogeny, with some lineages co-diversifying with their mammal hosts (5).

Additionally, gut microbiota plays a role in host energy uptake, breaking down fibers, carbohydrates, and proteins otherwise not digestible by the host, producing short-chain fatty acids

(SCFA) that provide up to 70% of an animal's daily energy intake (12). In addition, they also reduce the pH of the intestinal lumen, facilitating nutrient absorption and preventing accumulation of toxic metabolic by-products, producing vitamins, and regulating xenobiotic metabolism (6). Alterations in gut microbial community composition thus alter the interactions between microbes, subsequently affecting energy production and host nutrition (6), and ultimately affecting the host fitness.

1.1.2. Gut microbial community and host health

The existence of an adaptive immune system in vertebrates permits a greater level of complexity of their microbiota (13). The gut microbiota has been shown to modulate the host immune system by attenuating inflammatory responses and increasing resistance to pathogenic bacteria, in fish, rodents, mice, piglets and humans, assisting in the development and maturation of the host intestinal mucosal and systemic immune systems, in mice and humans, in the development and function of the brain and modulating behavior, in humans (6).

The symbiosis, developed from coevolution, is based on a molecular exchange linking microbial signals that are recognized by host receptors to arbitrate valuable outcomes for both host and microbes (14). Furthermore, the adaptive immune system is recognized to shape microbial community composition in the gut. This system mediates tolerance to the gut microbiota through IgA production (15). Additionally, Toll-like receptors are important in their ability to evaluate the composition of the microbiota, and they also mediate the host tolerance to symbiotic microbes and the immune responses to pathogens (2).

1.1.3. Gut microbial community, host habitat, and genetics

Microbiota attained primarily in life are inherited from the mother or from social contacts, but the microbial composition is also influenced by host genetics (16). Host species that live in more interconnected social groups, with high frequencies of social contact, are expected to have less inter-individual variation in gut microbial community structure than those that are geographically isolated (17). Populations that become geographically isolated should develop distinct gut microbial communities because they are exposed to distinct microbial taxa pools, thus they are colonized by distinct gut microbial communities, normally with lower taxonomic and/or functional diversity (6).

More recently, host genetics has been associated with the taxonomic structure of the gut microbiota, since intraspecific differences have been observed (18), however individual phylotypes are also promiscuous, as they have been found in multiple host species (5, 7).

1.1.4. Gut microbial community, host sex, and reproductive status

Information about the influence of host sex and reproductive status on microbial community composition is still lacking, however, in mammals, these two parameters have been linked to variations

in gut microbial community composition, for example in chimpanzees (19), black howler monkeys (20), rufous mouse lemurs (16), Verreaux's sifakas (17), and humans (21, 22).

Regarding microbiota, two essential concepts can be considered: core microbiota and individual microbiota. The core microbiota is the number and the identity of bacteria that are shared among different individuals of the same species; in contrast, the individual microbiota is the transient gut inhabitants that fluctuate, depending on the genetics, habitat, health, diet, among other factors (23). The common core bacteria are conserved during the mutual coevolution of the species and its intestinal microbes (23). These consortia of microorganisms are important to investigate the mechanisms underlying microbe–microbe and microbe-host interactions.

1.2. Herpestes ichneumon: biology and ecology

The study of wildlife microbiota has recently gained attention, but few studies are still available due to technical difficulties in obtaining appropriate samples and the lack of specific funding (3). To study the microbiota and their interaction with host fitness and the environment, we used Egyptian mongoose, *Herpestes ichneumon* (Linnaeus, 1758), as a model (Figure 1.1). This species is a medium-sized mammalian carnivore from the Herpestidae family, with an opportunistic feeding behavior, consuming mostly rabbits, but also reptiles, other small mammals, amphibians, birds, crayfish, eggs or carrion (24). Despite being mostly African, ranging extensively throughout the continent, it has expanded into the Mediterranean Middle East and southern Turkey (25). In Iberia Peninsula, it was conventionally considered as an introduced species during the Muslim Invasions (26, 27). However, a recent study based on mitochondrial DNA suggested that the Egyptian mongoose naturally dispersed into the Iberian Peninsula during the Late Pleistocene sea-level fluctuations (28). It was restricted to the south of the Tagus River (29), nonetheless, in the last three decades, it gradually expanded into central and north-eastern regions (30). This expansion was mostly driven by a land-use change in shrub-dominated ecosystems, forest clearing, agricultural practices, and climate change (31).

Egyptian mongoose has a home-range of about 3 km², habiting locals with understory vegetation in coastal, lacustrine, and riparian habitats, avoiding humid forests and extreme deserts. In Europe, it is found in Mediterranean maquis. Listed as Least Concern, the species is widespread, common, and present in many protected areas. There are no major threats to this species across its range, although, on the Iberian Peninsula, incidental and deliberate poisoning is a localized threat; also, in Portugal, mongoose hunting is legal (32). Evidence of both sexual and

Figure 1.1 – Egyptian mongoose (*Herpestes ichneumon*) adult (A) and cub (B), in Mediterranean maquis.

regional dimorphism in body size of Egyptian mongoose adults in Portugal have been found. This dimorphism probably results from differences in feeding habits leading to southern male adults being larger and heavier (33). *H. ichneumon* exhibit variability in social organization, ranging from solitary individuals to groups, which show cooperative tendencies, particularly in areas with abundant food resources. The exclusive home-range use of males in high-density populations suggests the existence of a polygynous mating system, with is accomplished due to the spatial distribution of females, in combination with the absence of paternal care behavior (24, 29, 34-36).

1.3. Objectives of the present work

Several aspects of mongoose' biology remain ill-defined. In this study, we thoroughly investigated the gut microbiota of Egyptian mongoose sampled in South Portugal, since the gut microbiota is being progressively acknowledged as a fundamental component of mammals' ecology. The aims of this work were: [1] the characterization of the core gut microbiota of Egyptian mongoose population; [2] the investigation of sex- and age class-related differences of gut microbiota; and [3] the analysis of the relationship between bio-environmental features and gut microbiota of these specimens. To accomplish these objectives, the fecal samples were cultured in selective and non-selective media, under aerobiosis and anaerobiosis conditions, in order to capture the most representative diversity of gut microbiota. Purification and presumptive identification of microbial isolates followed, using morpho-biochemical tests and grouping the isolates in morpho-physiological types. The purified bacterial isolates were submitted to 16S rRNA- and Internal Transcribed Spacer-based molecular identification and molecular fingerprinting based on Random Amplification of Polymorphic DNA (RAPD). At the bacterial genus level, a diversity analysis of the samples under study was performed. We compared sex- and age class-related differences (genus-based phylotypes) and tested the effect of individual bio-environmental features on the microbiota of each individual specimen.

CHAPTER II - MATERIALS AND METHODS

2.1. Egyptian mongoose specimens

Twenty Egyptian mongoose carcasses, both male (n=10) and female (n=10), from predator control hunted by shotgun, were donated for scientific purposes and were selected based on sex, age class (inferred from dentition), stomach content at the time of death, and land-use (Table 2.1). The carcasses were subjected to necropsy by a veterinarian; no sign of putrefaction or disease were detected. This information was obtained from University of Aveiro, which developed ecological studies with the same specimens (33). The selected specimens had an age class distribution of 16 adult, two subadult, and two juvenile.

These animals were originated from south of Tagus River (Baixo Alentejo), wherein similar land-use, predominated by agroforestry, mixed forests, shrubs and agriculture, was confirmed. The selected animals had the same stomach content at death, namely mammal and egg items.

2.2. Bacteriological culture

After collection at necropsy, samples from the terminal portion of the large intestine of Egyptian mongoose specimens were kept frozen at -20°C until utilization, to preserve both aerobic and anaerobic species and to avoid potential loss of bacterial viability and composition changes. The samples were divided into two equal parts, that were homogenized in buffered peptone water (1 g stool per 10 mL) and incubated at 37°C for 24 h with orbital shaking (150 rpm), under aerobic and anaerobic conditions. Anaerobic atmosphere was accomplished by completing with 20% of the final volume of mineral oil.

Next, 1 mL of enriched fecal sample from aerobic and anaerobic conditions were pasteurized at 80°C for 12 min, serially diluted and plated onto YCFA agar (12.5 g/L agar, 10 g/L casitone, 2.5 g/L yeast extract, 4 g/L $NaHCO_3$, 1 g/L cysteine, 0.45 g/L K_2HPO_4, 0.45 g/L KH_2PO_4, 0.9 g/L NaCl, 0.09 g/L $MgSO_4.7H_2O$, 0.09 g/L $CaCl_2$, 1 mg/L resazurin, 10 mg/L haemin, 10 µg/L biotin, 10 µg/L cobalamin, 30 µg/L p-aminobenzoic acid, 50 µg/L folic acid, 150 µg/L pyridoxamine, 33 mM acetate, 9 mM propionate, 1 mM isobutyrate, 1 mM isovalerate, 1 mM valerate, 50 µg/L thiamine and 50 µg/L riboflavin), supplemented with 2 g/L each of glucose, maltose, and cellobiose and 0.1% of sodium taurocholate (YCFA P) (37), and incubated for 72 h at 37°C, under aerobic and anaerobic conditions, respectively. The rest of the enriched fecal sample, incubated under anaerobic conditions, was serially diluted and 100 µL was plated onto YCFA agar supplemented with 2 g/L each of glucose, maltose, and cellobiose (YCFA) (37), and incubated for 72 h, at 37°C, in anaerobic conditions. Anaerobic conditions were accomplished using AnaeroGen™ 3.5L anaerobic atmosphere generation systems (Thermo Scientific). Additionally, the rest of the enriched fecal sample incubated under aerobic conditions was serially diluted and 100 µL was plated onto YCFA and also onto selective media: MacConkey solid medium (12 g/L agar, 5 g/l bile salts, 10 g/l lactose, 0.075 g/l neutral red, 20 g/l peptone, 5 g/l sodium chloride); Potato Dextrose Agar supplemented with chloramphenicol (PDA+CHLO) (Biokar diagnostic, Noack Group); ESBL chromogenic medium (Conda, Pronadisa), with (ESBL w/ AS) and without (ESBL w/o AS) ESBL supplement of antibiotics (Conda, Pronadisa); and Brilliance ESBL medium (Brilliance) (Oxoid). 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) were determined for all conditions and media.

Table 2.1 – List of Egyptian mongoose specimens studied in this work. Sex, age class, stomach content at death, georeferenced location, and land-use data are indicated.

ID	Sex	Age[1]	Stomach content at death[2]				Georeferenced location			Land-use[3]						
			Mammals	Reptiles	Invertebrates	Eggs	District	Latitude	Longitude	Urban	Agroforestry	Shrubs	Vineyards and Orchards	Coniferous	Mix forests	Agriculture
HI383	Male	Adult	100	0	0	0	Beja	37,824	-7,377	0	233	0	0	0	125	42
HI388	Female	Subadult	100	0	0	0	Beja	37,824	-7,377	0	233	0	0	0	125	42
HI396	Female	Adult	100	0	0	0	Beja	37,824	-7,377	0	135	93	0	0	172	0
HI399	Female	Adult	100	0	0	0	Beja	37,720	-8,097	0	135	93	0	0	172	0
HI460	Male	Juvenile 2	100	0	0	0	Beja	37,824	-7,377	0	0	250	0	4	0	132
HI462	Female	Adult	96	4	0	0	Beja	37,824	-7,377	0	135	93	0	0	172	0
HI463	Male	Adult	0	0	29	0	Beja	38,107	-7,205	93	17	0	170	0	0	110
HI466	Male	Adult	100	0	0	0	Beja	38,107	-7,205	0	127	116	0	0	0	157
HI467	Male	Adult	77	20	3	0	Beja	37,824	-7,377	0	127	116	0	0	0	157
HI471	Male	Adult	0	0	0	100	Beja	37,824	-7,377	0	127	116	0	0	0	157
HI501	Female	Adult	100	0	0	0	Beja	37,824	-7,377	0	135	93	0	0	172	0
HI502	Male	Juvenile 2	100	0	0	0	Beja	37,824	-7,377	0	0	0	0	0	0	400
HI504	Female	Subadult	95	0	0	0	Beja	37,824	-7,377	0	233	0	0	0	125	42
HI505	Female	Adult	65	35	0	0	Beja	37,824	-7,377	0	127	116	0	0	0	157
HI508	Female	Adult	97	3	0	0	Beja	38,287	-8,224	0	127	116	0	0	0	157
HI509	Male	Adult	89	0	0	0	Beja	38,107	-7,205	76	0	4	0	123	8	189
HI516	Male	Adult	100	0	0	0	Beja	37,824	-7,377	76	0	4	0	123	8	189
HI519	Female	Adult	100	0	0	0	Beja	37,824	-7,377	0	135	93	0	0	172	0
HI636	Female	Adult	88	0	12	0	Beja	37,824	-7,377	0	135	93	0	0	172	0
HI675	Male	Adult	100	0	0	0	Setúbal	38,107	-7,205	0	127	116	0	0	0	157

[1]Each specimen was assigned to one of four age classes: adults over one year of age, sub-adults between nine and twelve months, juveniles type II between five-and-a-half and nine months. Skulls with completely developed definitive dentition were assigned to the adult class. Skulls with 40 definitive teeth, but with some still growing, were assigned to the sub-adult age class. Animals whose skulls presented all 40 teeth, but including at least one milk tooth, were assigned to the juvenile type II class (33).

[2](personal information from Victor Bandeira)

[3]Number of hectares of each habitat type (urban, rice fields, agroforestry, shrubs, inland water bodies, vineyards and orchards, coniferous, broadleaved and mix forests and agriculture areas) were retrieved from Corine Land Cover (2006) with spatial resolution of 250m. This variable was represented by mean values of the 2×2 km grid cell, considering the home-range of the Egyptian mongoose (33).

2.3. Purification and presumptive identification of isolates

For purification of isolates, five isolated colonies from each different morphology were picked from different dilutions of all media. The colonies that were picked up were re-streaked twice to confirm purity. The purified individual colonies were assessed in terms of shape, pigmentation, and opacity. Bacteria were characterized in terms of gram character, endospore formation and the presence of catalase and cytochrome c oxidase enzymes.

ESBL Chromogenic medium can differentiate between *E. coli* that grow as pink colonies and bacteria belonging to the *Klebsiella, Enterobacter, Serratia,* and *Citrobacter* (KESC) group that grow as dark blue colonies (information available from the manufacturer, Figure 2.1.). Brilliance ESBL medium can differentiate between *E. coli* that grow as blue or pink colonies and KESC bacteria group that grow as green, green/blue or even brownish-green colonies (38). Moreover, *Proteus, Morganella*, and *Providencia* group grow as tan-colored colonies with brown halo (38). Finally, *Pseudomonas aeruginosa* colonies can also be differentiated, once they exhibit pyocyanin-related green-brown pigmentation (39). Colorless colonies may be *Salmonella* spp., *Acinetobacter* spp. or others (information provided by the manufacturer).

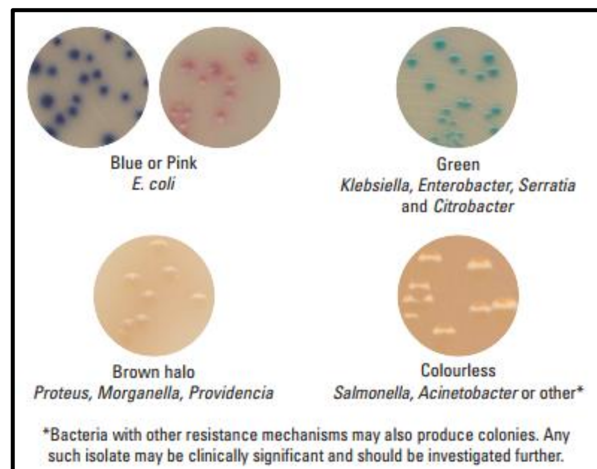


Figure 2.1 – Brilliance ESBL medium differentiating pigmentation characteristics (manufacturer available information).

The isolated fungi were stained using lactophenol cotton blue and different morphological aspects were characterized. For 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 (Figure 2.2); MT-XII for yeasts; MT-XIII for filamentous fungi. Additionally, MT-Vibrium and MT-Others were created, the first to place a vibrium-like isolate and the second to place unidentifiable isolates.

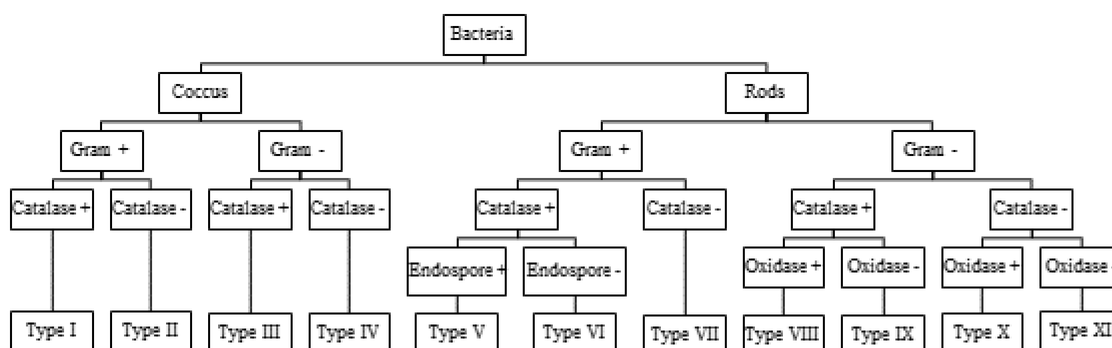


Figure 2.2 – Flowchart used for differentiation of the purified bacterial isolates into different morpho-physiological types using morphological (cell morphology, Gram and endospore staining) and biochemical tests (catalase and oxidase tests).

2.4. Molecular identification and molecular fingerprinting of bacterial isolates

With the objective to analyze the intraspecific polymorphism present in the bacterial population, molecular fingerprinting was based on random amplified polymorphic DNA (RAPDs). A bacterial isolate from each MT, from each solid media, from each mongoose host, was selected. Total DNA extraction was performed by direct boiling at 95°C for 15 min of 2 to 3 colonies in 250 µL of TE 1 M, pH 8.0, centrifuged, the supernatant was collected to a clear microtube and stored at -20°C.

For single primed PCR fingerprinting, an initial screening to select the most appropriate primer was performed using four different primers. Seven isolates (one from each morpho-physiological type) were tested, including two isolates of MT-II and one isolate of MT-VII, MT-V, MT-VIII, MT-IX, and MT-IV. The tested primers can be classified into three groups: primers directed to regions containing mini-satellite from M13 bacteriophage – M13 (5’-GAG GGT GGC GGT TCT-3’) (40); random primers – OPC19 (5’-GTT GCC AGC C-3’) (41) and 1281 (5’-AAC GCG CAA C-3’) (42); and universal primers for 16S rRNA gene – PH (5’-AAG GAG GTG ATC CAG CCG CA-3’) (43).

PCR amplifications were performed in a Biometra Uno II Thermal Cycler, using a total volume of 15 µl and including 0.2 mM of primer (Invitrogen), 7.5 µL of NZYTaq II 2x Green Master Mix (NZYTech), 5 µL of DNA (from different dilutions of the DNA extract). DNA dilutions were decided from empirical analyses of DNA extract concentration run in 0.8% (w/v) agarose gel.

After preliminary analyses, the selected primers for molecular fingerprinting were M13 and PH. 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.

The PCR products were resolved by 1.5% (w/v) agarose gel (NZYTech) containing 0.03 µL/mL of GreenSafe Premium (NZYTech) in 0.5 X TBE buffer (44.5 mM Tris, 44.5 mM boric acid, and 1 mM

EDTA) (Invitrogen), at 90 V for 4 h. DNA was visualized under UV light and photographed with Alliance 4.7 system (UVITEC Cambridge).

To obtain a measure of reproducibility, each PCR batch included a randomly selected duplicate, with a total number of 18 isolates for M13 and 22 isolates for PH. The similarity between each pair of duplicates was based on the dendrogram computed with Pearson correlation coefficient and the unweighted pair group method with arithmetic average (UPGMA) as the agglomerative clustering algorithm (software package BioNumerics version 4.0 – Applied Maths). The reproducibility value was determined as the average value for all pairs of duplicates. 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.

Molecular identification was based on 16S rRNA gene sequence analysis. At least, one isolate from each dendrogram cluster resulting from RAPD-fingerprints was randomly selected for 16S rRNA gene sequencing. 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′) (44), in a final volume of 25 µL with 0.2 mM from each primer (Invitrogen), 12.5 µL NZYTaq II 2x Green Master Mix and 5 µL of DNA (from a 1:100 dilution of DNA extract). This set of primers allow the amplification of all hypervariable region (V1-V9). 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, 72ºC. PCR products with expected size (approximately 1500 bp) were observed in a 1.5% agarose gel, in 1 X TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA), run at 90 V for 1.5 h, using GreenSafe Premium (0.003% (v/v)). DNA was quantified using a Qubit fluorometer (Invitrogen), following manufacturer's instructions. Samples were commercially sequenced by Sanger sequencing technique using 63f primer (GATC Biotech AG). 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 and taking into consideration that, for financial reasons, only one strand was sequenced.

2.5. Molecular identification of fungi

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

Amplification of the D1/D2 domain region of the 26S rRNA gene in yeast (45) and the Internal Transcribed Spacer (ITS) region in filamentous fungi were performed (46). 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 (47), 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 (48). In both cases, a final volume of 25 µL was used containing 0.2 mM of each pair of primers (Invitrogen), 12.5 µL NZYTaq II 2x Green Master Mix and 5 µL of DNA (from a 1:100 dilution of DNA extract). 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 72ºC. PCR products with expected size (approximately 650 bp and between 600 and 800 bp, respectively) were observed in a 1.2% agarose gel, in 1 X TBE buffer, at 90 V for 1 h, using GreenSafe Premium and extracted using QIAquick Gel Extraction Kit, according to manufacturer's handbook.

DNA was quantified using a Qubit fluorometer, following manufacturer's instructions. For Sanger sequencing, samples were prepared adding with 20 to 80 ng/µL of PCR product to 5 µM of NL-4 and ITS4 primers, for yeast and filamentous fungi, respectively, and commercially sequenced.

2.6. Homology searches for genome-based identification of isolates

Electropherograms were manually analyzed and corrected when necessary; undetermined nucleotides were designated as N. The 16S rRNA gene, 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. The criteria used for bacteria and fungi identification are represented in Table 2.2 (49-51). 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.

Table 2.2 – 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	-

[a]as in (42, 43).

[b]as in (44); same criteria were used for yeast identification using D1/D2 domain region of the 26S rRNA gene.

2.7. Diversity analysis of the samples under study

Diversity analyses were performed through the calculation of diversity indices (Shannon index, Simpson index and Species evenness index derived from Shannon index) and the determination of nonparametric estimators (Chao 1 and Chao 2) of species richness (Table 2.3) (52).

Table 2.3 – Diversity measurements: diversity indices and non-parametric estimators of species richness.

Measure	Formula
Shannon index (H')	$H' = -\sum pi \ln pi$
Simpson's index (D)	$D = \sum pi^2$
Shannon evenness (E)	$E = \frac{H'}{\ln S}$
Chao 1 (and Chao 2)	$Chao = S + \frac{a^2}{2b}$

Where pi=ni/N; S is the number of OTUs in the sample; N is the total number of isolates from a given OUT in the sample; ni is the number of isolates in an OTU; a is the number of OTUs recorded only once; b is the number of OTUs recorded only twice. Chao 1 analyses species abundance data and Chao 2 analyses species incidence data.

2.8. Data analysis

Considering culture assays, results from CFU counts are displayed as means of values of at least ten independent experiments with respective standard deviation. All variables were tested for normality using D'Agostino-Pearson test (α=0.05). When comparing two conditions, a t-student test (Mann-Whitney test, α=0.05) was performed. When comparing multiple conditions, a non-parametric ANOVA (Kruskal-Wallis test, α=0.05) with a Dunn's Multiple Comparison post-test was performed. When comparing multiple host communities, a two-way ordinary ANOVA (α=0.05) with a Tukey's Multiple Comparison post-test was performed. All statistical analyses were performed using GraphPad Prism software.

For microbiota and bio-environmental data integration, we performed a Principal Component Analysis (PCA) using available information for all 20 Egyptian mongoose specimens. A matrix using 72 microbiota operational taxonomic units (OTUs), based on presence/absence of every hierarchical bacterial level (Supplementary Table 7.1), was normalized using the standard score. The normalized matrix was used to perform an initial PCA. Scatter projection diagrams were obtained both for sex and age class. A dendrogram was then made based on the normalized Euclidean distance derived from the projection matrix using UPGMA. The cophenetic correlation coefficient was calculated and a 2-way Mantel test (Mantel, 1967) was performed to measure the faithfulness of the dendrogram compared to

the pairwise distances of the original unmodeled matrix. Two others PCAs were made using a matrix with 26 biological and 17 environmental variables (Supplementary Table 7.2 and 7.3). These matrices were normalized using the standard score and PCAs were performed. Scatter projection diagrams were obtained for the clusters originated from the previous microbiota dendrogram. The explanatory variables of each Principal Component (PC) were selected if the correlation coefficient between the variable and the PC were |0.5|. If a variable had this behavior with more than one PC, this variable was used as an explanatory variable for the PC with the higher correlation coefficient. All these analyses were made using NTSYSpc software (version 2.20d; Exeter Software, Setauket, NY, USA).

CHAPTER III – RESULTS

3.1. Comparison of bacterial burden and diversity of morpho-physiological types between mongoose host sexes

Culture-dependent methods were used to investigate gut microbiota diversity in 20 fecal samples of Egyptian mongoose specimens. A rich medium for bacterial growth was used, with (YCFA P) and without (YCFA) 0.1% de sodium taurocholate supplementation. In both cases, samples were incubated under aerobic (w/ O_2) and anaerobic (w/o O_2) conditions.

Microbial load in these four media/conditions (Figure 3.1 A) were compared; in male samples, an average of $2.6x10^8$ CFU/mL, $1.6x10^4$ CFU/mL, $2.9x10^8$ CFU/mL, and $8.5x10^5$ CFU/mL were found in YCFA w/ O_2, YCFA P w/ O_2, YCFA w/o O_2 and YCFA P w/o O_2, respectively. In female samples, we registered a mean of $3.0x10^8$ CFU/mL, $8.7x10^4$ CFU/mL, $8.0x10^8$ CFU/mL, and $4.1x10^7$ CFU/mL in YCFA w/ O_2, YCFA P w/ O_2, YCFA w/o O_2 and YCFA P w/o O_2, respectively.

Comparing the microbial load in the four media/conditions (Figure 3.1 A), a significant higher microbial load was registered in YCFA w/ O_2 and YCFA w/o O_2, when comparing with YCFA P w/ O_2 and YCFA P w/o O_2, respectively. Additionally, a significant lower microbial load was registered in YCFA P w/ O_2 than in YCFA P w/o O_2 and in YCFA w/ O_2 when comparing with YCFA w/o O_2.

Regarding sex-related differences (Figure 3.1 A), no significant differences were found in the microbial load using YCFA w/ O_2 and YCFA P w/ O_2 as growth conditions, but female hosts had a higher microbial load than males in YCFA w/o O_2 (p-value=0.0410) and YCFA P w/o O_2 (p-value=0.0288).

A panel of selective growth media was used to detect specific groups of bacteria. The microbial load in MacConkey medium had no significant difference between sexes, both for lactose non-fermenting (LNF) bacteria, lactose fermenting (LF) bacteria, and the sum of both types (Figure 3.1 B), with a mean of $2.1x10^8$, $1.8x10^8$, and $3.9x10^8$ CFU/mL registered for male mongooses, respectively, and a mean of $8.1x10^8$, $3.9x10^8$, and $1.2x10^9$ CFU/mL, respectively, in female mongooses.

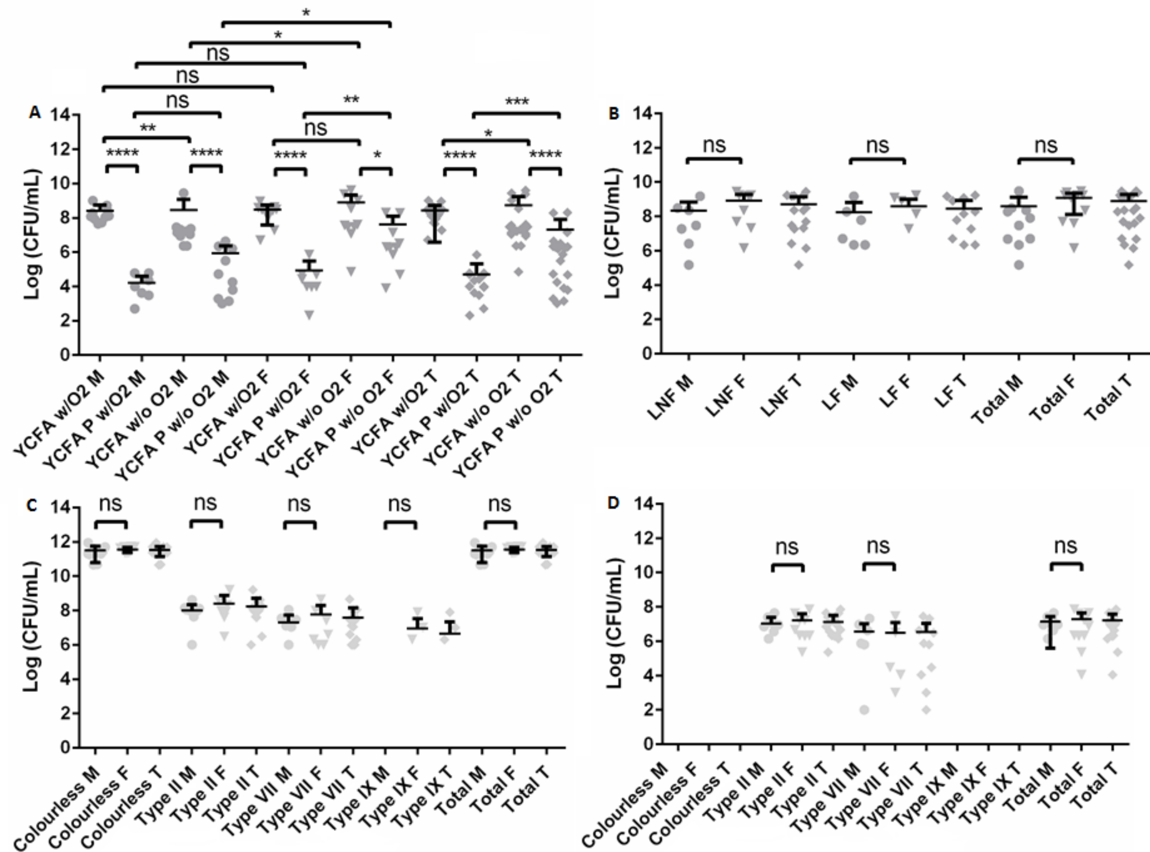


Figure 3.1 – Microbial load (expressed as Log CFU/mL) of mongoose cultivable bacteria grown in selective and non-selective media. (A) Comparison between four different media/conditions: YCFA incubated under aerobiosis (YCFA w/O_2), YCFA supplemented with sodium taurocholate and incubated under aerobiosis (YCFA P w/O_2), YCFA incubated under anaerobiosis (YCFA w/o O_2), and YCFA supplemented with sodium taurocholate and incubated under anaerobiosis (YCFA P w/o O_2). **(B)** Comparison between lactose non-fermenting (LNF) bacteria and lactose-fermenting (LF) bacteria in MacConkey medium. **(C/D)** Comparison between Extended-spectrum beta-lactamases (ESBL) Chromogenic medium **(C)** without (ESBL w/o AS) and **(D)** with (ESBL w/ AS) ESBL antibiotic supplement with the results present by colony color/type. Results from male (M), female (F), and in total (T) are presented. Horizontal bars represent the mean and error bars represent the standard deviation from 10 (male and female) and 20 (total) independent values. Statistical analysis was performed using a t-student test (Mann-Whitney test, α=0.05). ns – non-significant (p-value≥ 0.05), * - significant (p-value=0.01 to 0.05), ** - very significant (p-value=0.001 to 0.01), *** - extremely significant (p-value=0.0001 to 0.001), **** - extremely significant (p-value< 0.0001).

Additionally, two selective media for detection of extended-spectrum beta-lactamases (ESBL) producing gram-negative bacteria, namely the ESBL Chromogenic medium with ESBL antibiotic supplement (ESBL w/AS) and the ready-to-use Brilliance ESBL medium (Brilliance), were used. As well, we decided to compare the differences resulting from the addition of the ESBL antibiotic supplement, incubating in ESBL Chromogenic medium without this supplement (ESBL w/o AS).

In ESBL chromogenic medium, metallic blue colonies belonged to morpho-physiological type (MT) II, light blue colonies belonged to MT-VII, and pink colonies belonged to MT-IX, being putative *E. coli*. Colorless colonies were very diverse, fitting to different MT, namely MT-I, M-III to MT-VI, MT-VIII to MT-XI, MT-Vibrium, and MT-Others.

In ESBL w/o AS, bacterial recovery was, on average, 3.35×10^{11} CFU/mL; most colonies were colorless (3.3×10^{11} CFU/mL), although MT-II (1.8×10^{8} CFU/mL), MT-VII (3.9×10^{7} CFU/mL), and *E. coli*-like (4.5×10^{6} CFU/mL) colonies were also present (Figure 3.1 C). *E. coli*-like colonies were only observed in female samples (n=3). Moreover, ESBL w/AS presented an average bacterial load of 1.6×10^{7} CFU/mL, with an even distribution between MT-II (1.3×10^{7} CFU/mL) and MT-VII (3.4×10^{6} CFU/mL) colonies (Figure 3.1 D). In both ESBL Chromogenic media, there were no significant differences between sexes in the number of colonies of any color and morpho-physiological type (Figure 3.1 C/D).

Additionally, a comparison between ESBL Chromogenic media was made (Figure 3.2). Microbial diversity and abundance are lower in the medium with antibiotic supplement, when compared to the non-supplemented one.

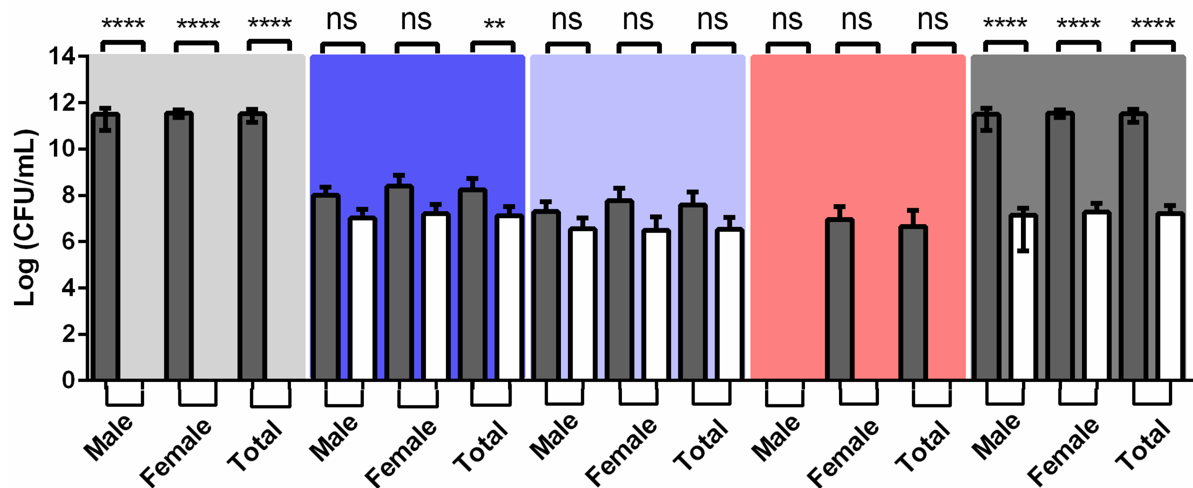


Figure 3.2 – Comparison of microbial load (expressed as log CFU/mL) in female and male mongoose samples inoculated in ESBL Chromogenic medium without (gray bars) and with (white bars) ESBL antibiotic supplement. Each media/condition is presented by colony color: colorless colonies (light gray), metallic blue colonies (dark blue), light blue colonies (light blue), pink colonies (pink), and the total number of colonies (dark gray). Horizontal bars represent the mean and error bars represent the standard deviation from 10 (male and female) and 20 (total) independent values. Statistical analysis was performed using a t-student test (Mann-Whitney test, α=0.05). ns – non-significant (p-value≥ 0.05), *significant (p-value 0.01 to 0.05), **very significant (p-value 0.001 to 0.01), ****extremely significant (p-value< 0.0001) differences.

Bacterial growth in Brilliance ESBL medium was only registered in eight fecal samples, mostly originated from female hosts (n=6) with a bacterial load average of 4.2×10^{3} CFU/mL (Figure 3.3 A). No significant differences were noticed between sexes. The distinct colonies grown on the medium were similar, characterized by lack of pigmentation and putatively identified as *Salmonella* spp. and/or *Acinetobacter* spp.

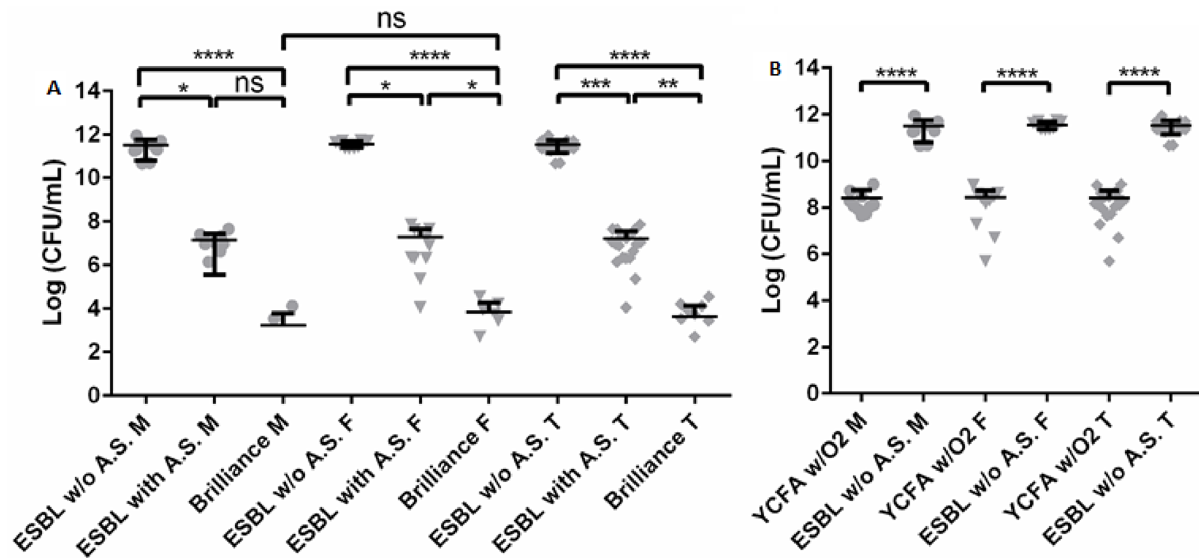


Figure 3.3 – Comparison of bacterial load (expressed as Log CFU/mL) between (A) chromogenic media and (B) non-selective media. Brilliance ESBL medium (Brilliance), ESBL Chromogenic medium without ESBL supplement of antibiotics (ESBL w/o AS), ESBL Chromogenic medium with ESBL supplement of antibiotics (ESBL w/AS), and YCFA medium incubated in aerobiosis (YCFA w/ O_2). Results from male (M), female (F), and in total (T) are represented. Horizontal bars represent the mean and error bars represent the standard deviation from 10 (male and female) and 20 (total) independent values. Statistical analysis was performed using a non-parametric ANOVA (Kruskal-Wallis test, α=0.05) with a Dunn's Multiple Comparison post-test for media comparison and using a t-student test (Mann-Whitney test, α=0.05) for gender comparison in Brilliance ESBL medium and non-selective media comparison. ns – non-significant (p-value≥ 0.05), * - significant (p-value=0.01 to 0.05), ** - very significant (p-value=0.001 to 0.01), *** - extremely significant (p-value=0.0001 to 0.001), **** - extremely significant (p-value< 0.0001).

As expected, supplemented ESBL selective media led to lower bacterial viability ($1.6x10^7$ CFU/mL), when compared to $3.3x10^{11}$ CFU/mL in ESBL w/o AS (Figure 3.3 A). A more marked reduction was recorded in Brilliance ($4.2x10^3$ CFU/mL) medium that registered differences of 10^8 and 10^4 CFU/mL, when compared to ESBL Chromogenic medium, without and with antibiotic supply, respectively (p-value<0.0001 and p-value<0.01) (Figure 3.3 A).

Interestingly, under aerobiosis, microbial load in ESBL w/o AS was higher than in YCFA (p-value<0.0001) (Figure 3.3 B).

3.2. Detection of mycobiota community

In an attempt to study the intestinal fungi community (mycobiota) of the Egyptian mongoose, PDA medium supplemented with chloramphenicol (PDA w/CHLO) was inoculated, allowing the selective growth of fungi (Figure 3.4).

The average number of viable fungi found in fecal samples were $1.1x10^7$ CFU/mL, with an even distribution of yeasts and filamentous fungi (FF) ($5.0x10^6$ CFU/mL and $5.8x10^6$ CFU/mL, respectively). Examining yeast colonies, no significant differences were shown between sexes ($8.6x10^6$ CFU/mL and $1.3x10^6$ CFU/mL, for male and female mammals, respectively). However, comparing FF, a very significant difference (p-value=0.0031) between male and female samples were registered, with microbial loads in the former of approximately $1.2x10^7$ CFU/mL, and no detection in the latter samples.

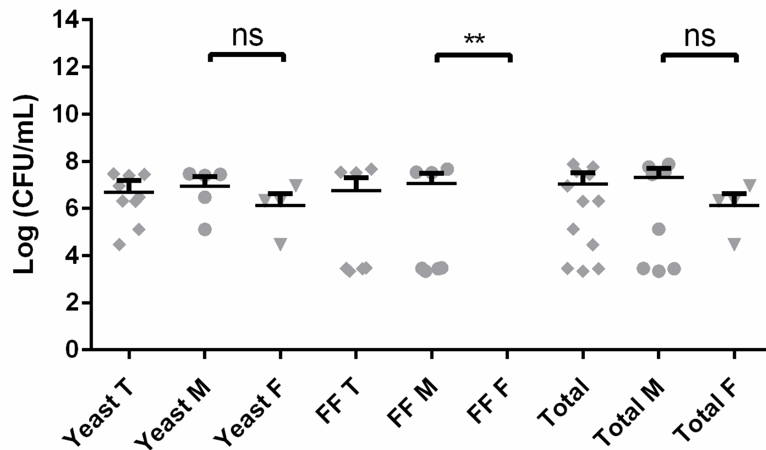


Figure 3.4 – Comparison of microbial load (expressed as Log CFU/mL) between yeast and filamentous fungi (FF) in the PDA medium with chloramphenicol. Results from male (M), female (F), and in total (T) are presented. Horizontal bars represent the mean and error bars represent the standard deviation from 10 (male and female) and 20 (total) independent values. Statistical analysis was performed using a t-student test (Mann-Whitney test, α=0.05). ns – non-significant (p-value≥ 0.05), * - significant (p-value=0.01 to 0.05), ** - very significant (p-value=0.001 to 0.01), *** - extremely significant (p-value=0.0001 to 0.001), **** - extremely significant (p-value< 0.0001).

3.3. Differences between media selectivity and detection of diversity

Comparing the percentage of isolates belonging to each MT that was registered in different growth media (Figure 3.5), some differences could be noticed between rich and selective media. As expected, rich media enabled the growth of a great variety of morpho-physiological types depending on the incubation condition: more than seven MT in YCFA media, while in ESBL w/o AS a greater diversity of MT was obtained (n=13). In contrast, all selective media led to a lower diversity of morpho-physiological types (1 to 3 MT).

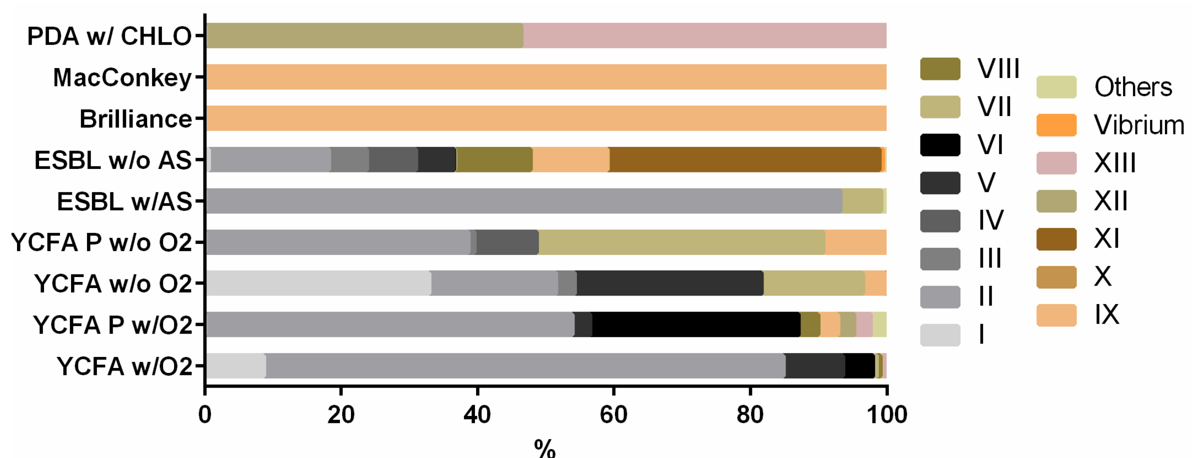


Figure 3.5 – Percentage of isolates belonging to each morpho-physiological type in each media/condition in Egyptian mongoose individuals. PDA w/ CHLO – PDA medium with chloramphenicol, MacConkey – MacConkey medium, Brilliance – Brilliance ESBL medium, ESBL w/o AS – ESBL Chromogenic medium without ESBL supplement of antibiotics, ESBL w/AS - ESBL Chromogenic medium with ESBL supplement of antibiotics, YCFA P w/o O_2 – YCFA medium supplemented with sodium taurocholate and incubated under anaerobiosis, YCFA w/o O_2 - YCFA medium incubated under anaerobiosis, YCFA P w/O_2 - YCFA medium supplemented with sodium taurocholate and incubated under aerobiosis, YCFA w/ O_2 - YCFA medium incubated under aerobiosis. Morpho-physiological types are represented as discussed in Material and Methods chapter. Results are the sum of percentages of all 20 host individuals.

As expected, MacConkey medium and Brilliance ESBL medium only registered growth of MT-IX isolates, with MacConkey medium registering the higher number than other media (p-value<0.0001), and PDA w/CHLO only registered growth of MT-XII and MT-XIII isolates. Surprisingly, MT-XI (p-value<0.0001) and MT-Vibrium were only isolated from ESBL chromogenic medium without antibiotic supplementation. ESBL chromogenic medium with antibiotic supplementation registered the higher number of MT-II isolates than the remaining media (p-value<0.001, except YCFA w/ O_2, wherein non-significant differences were found).

In YCFA medium, MT-XII and MT-Others only grew in YCFA P w/ O_2 and MT-IV only grew in YCFA w/o O_2. MT-XI and MT-Vibrium were not isolated in any of YCFA-growth conditions. Comparing aerobiosis and anaerobiosis conditions, aerobiosis favored the isolation of MT-II (YCFA - 76.2%; YCFA P – 53.9%). Bacteria from MT-VI, MT-VIII and MT-XII only grew in aerobic conditions while MT-III and MT-X only grew in anaerobic conditions. In addition, MT-VII was almost exclusive of anaerobic conditions, being able to also grow in YCFA in the presence of oxygen (0.6%). MT-I was absent in pasteurized samples, with the exception of YCFA P w/o O_2 (0.01%).

3.4. Host individuals as representatives of single communities

Comparing the percentage of isolates belonging to each MT, in each host individual, in all YCFA media (in both aerobic and anaerobic conditions) (Figure 3.6), we did not find any significant difference between sexes but did find some significant differences across host individuals (Supplementary Table 7.4).

The total aerobic microbiota (YCFA under aerobic conditions) is composed of diverse morpho-physiological types, namely, MT-I, MT-II, MT-V, MT-VI, MT-VII, MT-VIII, MT-IX, and MT-XIII, with MT-II isolates being the most detected type, appearing in at least 75% of individuals. Moreover, the total anaerobic microbiota (YCFA under anaerobic conditions) is composed of MT-I, MT-II, MT-III, MT-V, MT-VII, MT-IX, MT-X, and MT-XII, with detection of MT-II isolates in at least 75% of individuals, and MT-VII in at least 60% of individuals. Furthermore, the total aerobic sporobiota (YCFA P under aerobic conditions) is composed of diverse morpho-physiological types, namely, MT-II, MT-V, MT-VI, MT-VIII, MT-IX, MT-XII, MT-XIII, and MT-Others, with MT-VI isolates being the most detected type, appearing in at least 50% of individuals. Also, the total anaerobic sporobiota (YCFA P under anaerobic conditions) is composed of MT-I, MT-II, MT-III, MT-IV, MT-VII, MT-IX, and MT-X, with detection of MT-II and MT-VII in at least 75% of individuals.

Thus, in these conditions, the MT-II, MT-VI, and MT-VII may be considered the core gut microbiota community and the remaining morpho-physiological types can be considered part of the intra-specific individual microbiota community.

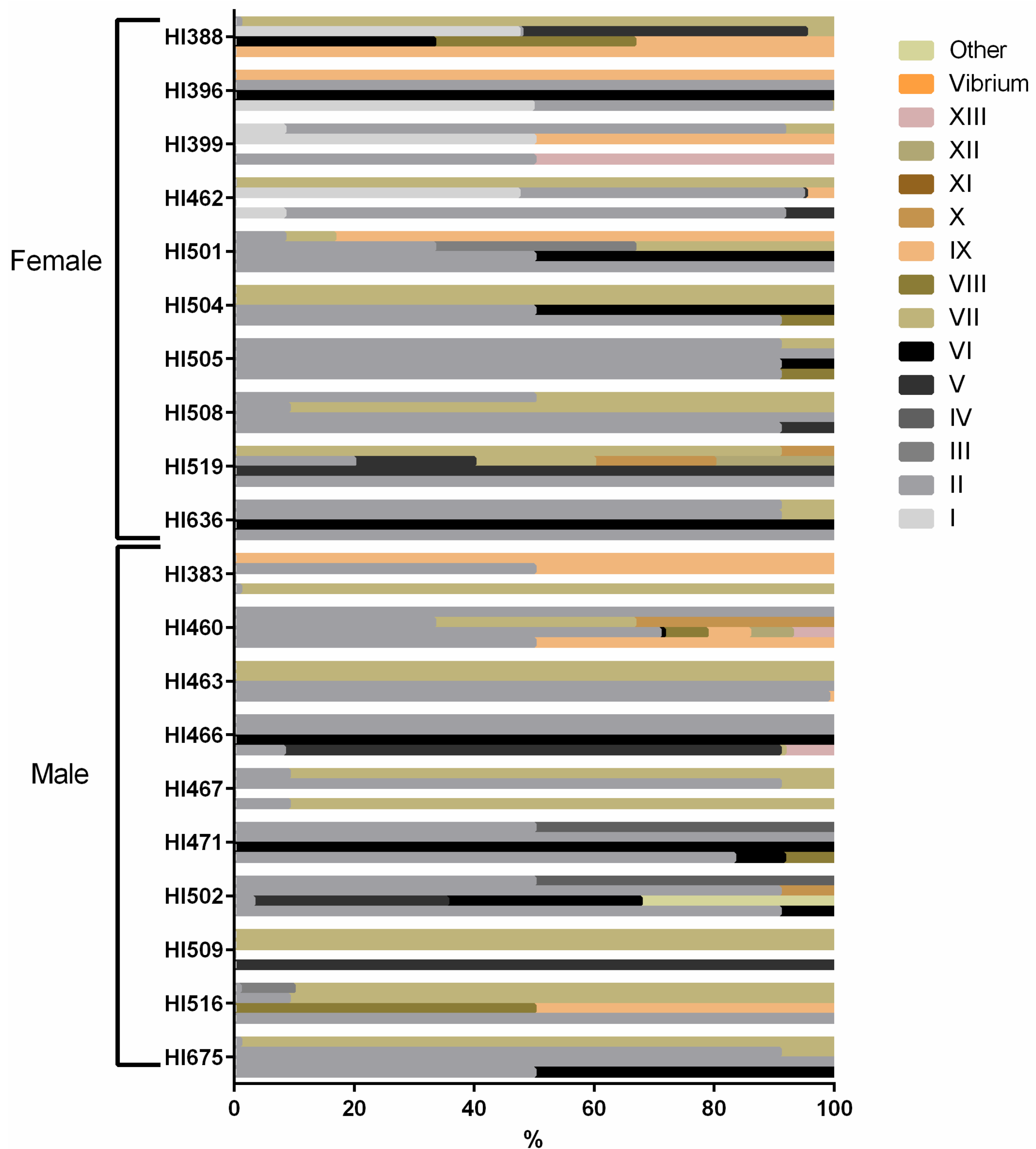


Figure 3.6 – Percentage of isolates belonging to each morpho-physiological type in each Egyptian mongoose individual in 4 different media/conditions: YCFA incubated under aerobiosis (first/bottom bar), YCFA supplemented with sodium taurocholate and incubated under aerobiosis (second bar), YCFA incubated under anaerobiosis (third bar), and YCFA supplemented with sodium taurocholate and incubated under anaerobiosis (fourth/top bar). Numbers are internal references and represent each individual. Morpho-physiological types are represented as discussed in Material and Methods chapter.

Comparing the percentage of isolates belonging to each MT, in each host individual (Figure 3.7), we found a higher number of MT-II isolates in female samples (p-value<0.05) when compared to male samples, in both ESBL chromogenic media supplemented with antibiotics. We also found some significant differences across host individuals (Supplementary Table 7.4).

The total aerobic microbiota (ESBL w/o AS) is composed of diverse morpho-physiological types, namely, MT-I, MT-II, MT-III, MT-IV, MT-V, MT-VI, MT-VII, MT-VIII, MT-IX, MT-X, MT-

XI, MT-Vibrium, and MT-Others, with MT-II isolates being the most detected type, appearing in at least 75% of individuals, and MT-VII and MT-XI in at least 65% of individuals. Moreover, the total ESBL-producing microbiota (ESBL w/ AS) is composed of MT-II, MT-VII, and MT-Other, with detection of MT-II isolates in at least 75% of individuals.

Thus, in these conditions, the MT-II, MT-VII, and MT-XI types may be considered the core aerobic gut microbiota community and the remaining morpho-physiological types can be considered part of the intra-specific individual microbiota community. Additionally, we perceived that the majority of individuals possess MT-II ESBL-producing bacteria.

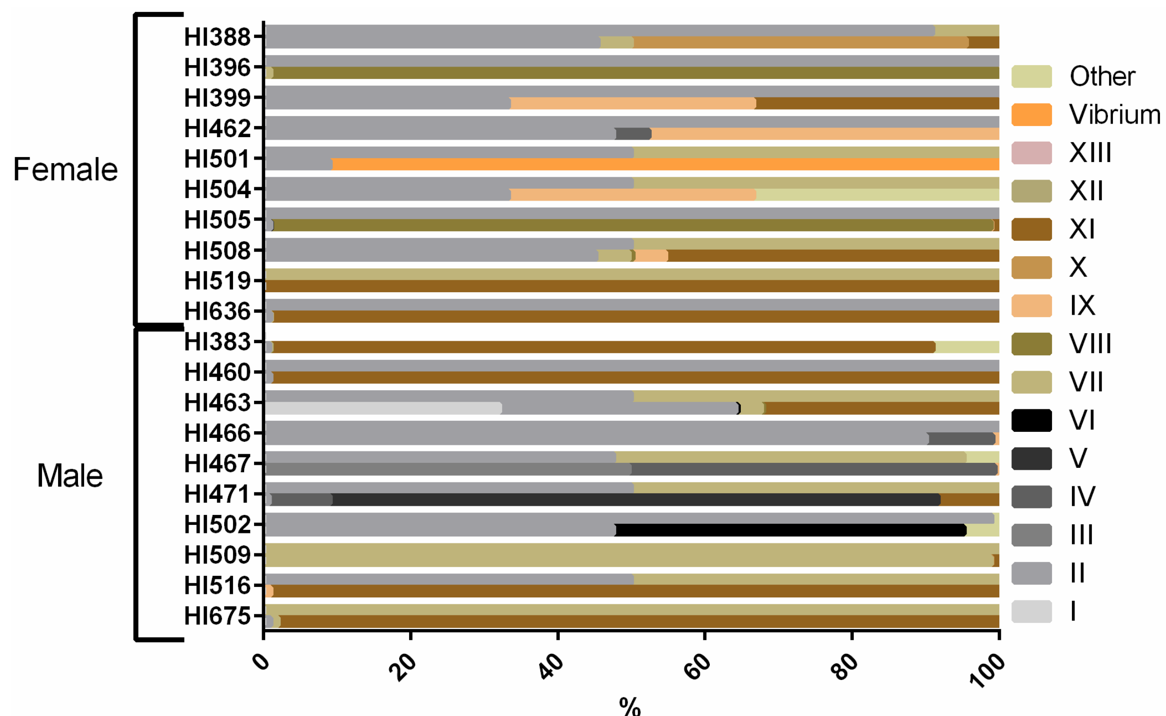


Figure 3.7 – Percentage of isolates belonging to each morpho-physiological type in each Egyptian mongoose individual between ESBL Chromogenic medium without (bottom bars) and with (top bars) ESBL supplement of antibiotics. Numbers are internal references and represent each individual. Morpho-physiological types are represented as discussed in Material and Methods chapter.

Comparing the percentage of the sum of isolates belonging to each MT, discovered in all media, in each host individual (Figure 3.8), we found a higher number of MT-IX isolates in female samples (p-value<0.01) when compared to male samples. We also found some significant differences across host individuals (Supplementary Table 7.4).

Based on morpho-physiological tests, the gut microbiota is dominated by Gram-positive bacteria (76%) and rod-shaped bacteria (77%). The total microbiota (sum of all media) is composed of diverse morpho-physiological types (n=15), with MT-IX appearing in 100% of individuals, MT-II and MT-VII in at least 95% of individuals, MT-VIII and MT-XI, in at least 60% of individuals.

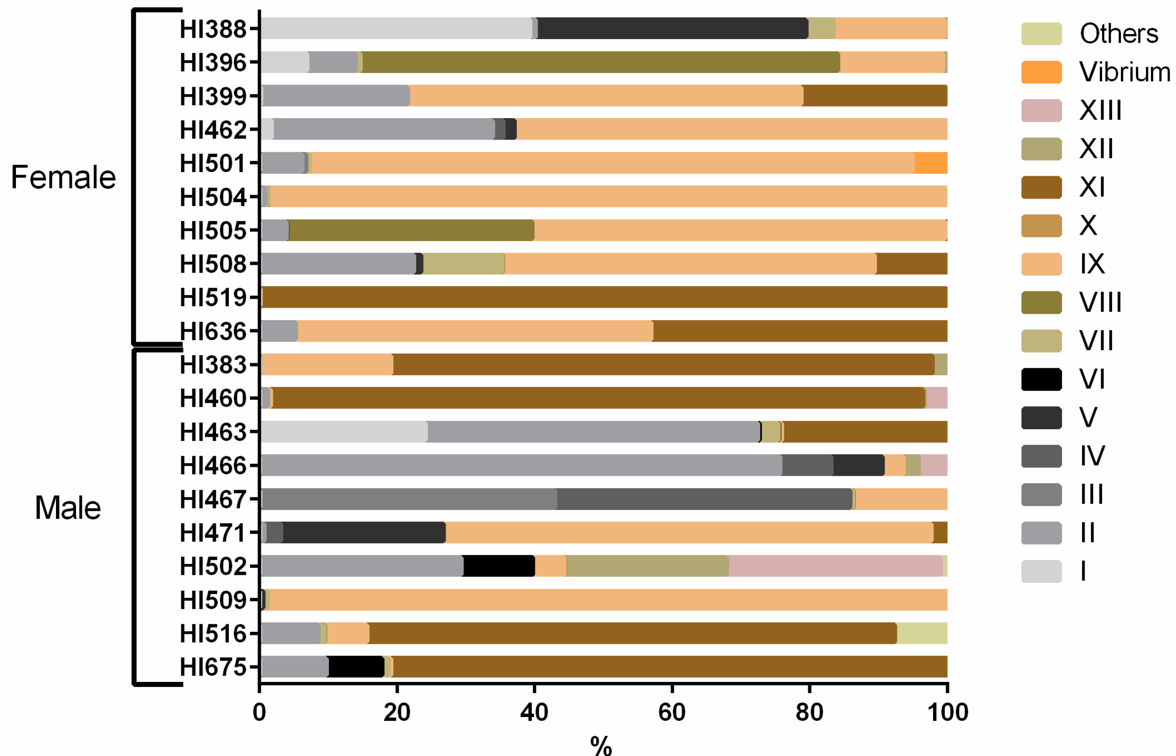


Figure 3.8 – Percentage of the sum of isolates belonging to each morpho-physiological type discovered in all media in each Egyptian mongoose individual. Numbers are internal references and represent each individual. Morpho-physiological types are represented as discussed in Material and Methods chapter. Results are the sum of the percentages of all 9 media/conditions.

Thus, the MT-II, MT-VII, MT-VIII, MT-IX, and MT-XI may be considered the core gut microbiota community and the remaining morpho-physiological types can be considered part of the intra-specific individual microbiota community.

3.5. Molecular identification and molecular fingerprinting of bacterial isolates

The differentiation of isolates was based on RAPD fingerprints, after an initial screening with seven isolates using four different primers. The fingerprints produced by OPC19 and 1281 showed low polymorphic profiles and badly-defined amplification patterns, with faint fragments (data not shown). The selected primers M13 and PH provided suitable fingerprints, with well-defined amplification patterns. The reproducibility of fingerprints with these primers, estimated by the similarity average value for all pairs of duplicates, was 97.2% ± 3.3% for M13 and 83.1% ± 9.6% for PH. To integrate all this information, a composite dendrogram based on M13 and PH fingerprints was generated for differentiation of bacterial isolates. Using 70% similarity as the cutoff value for cluster formation, one isolate of each cluster was selected and identified through 16S rRNA gene sequencing.

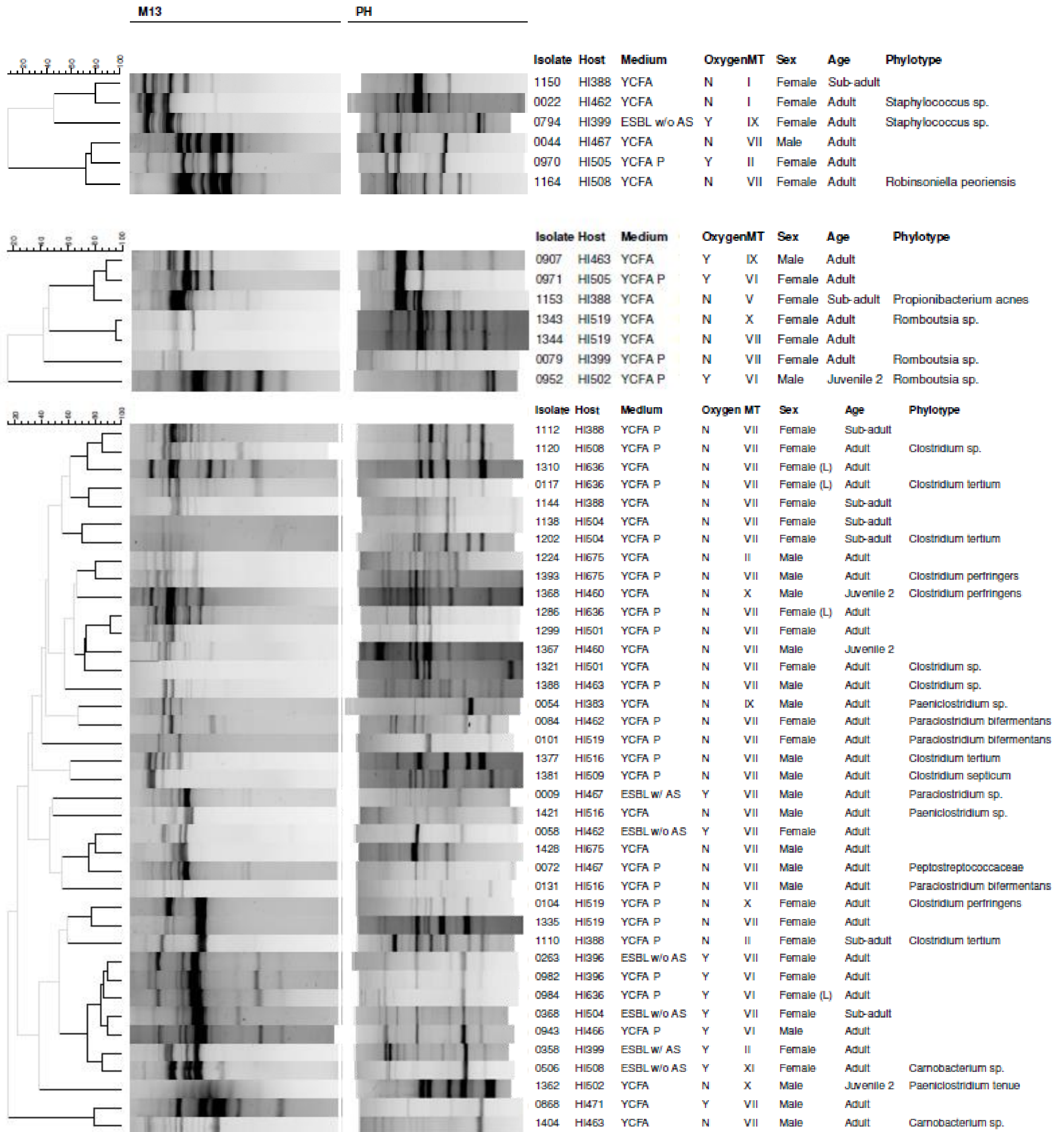
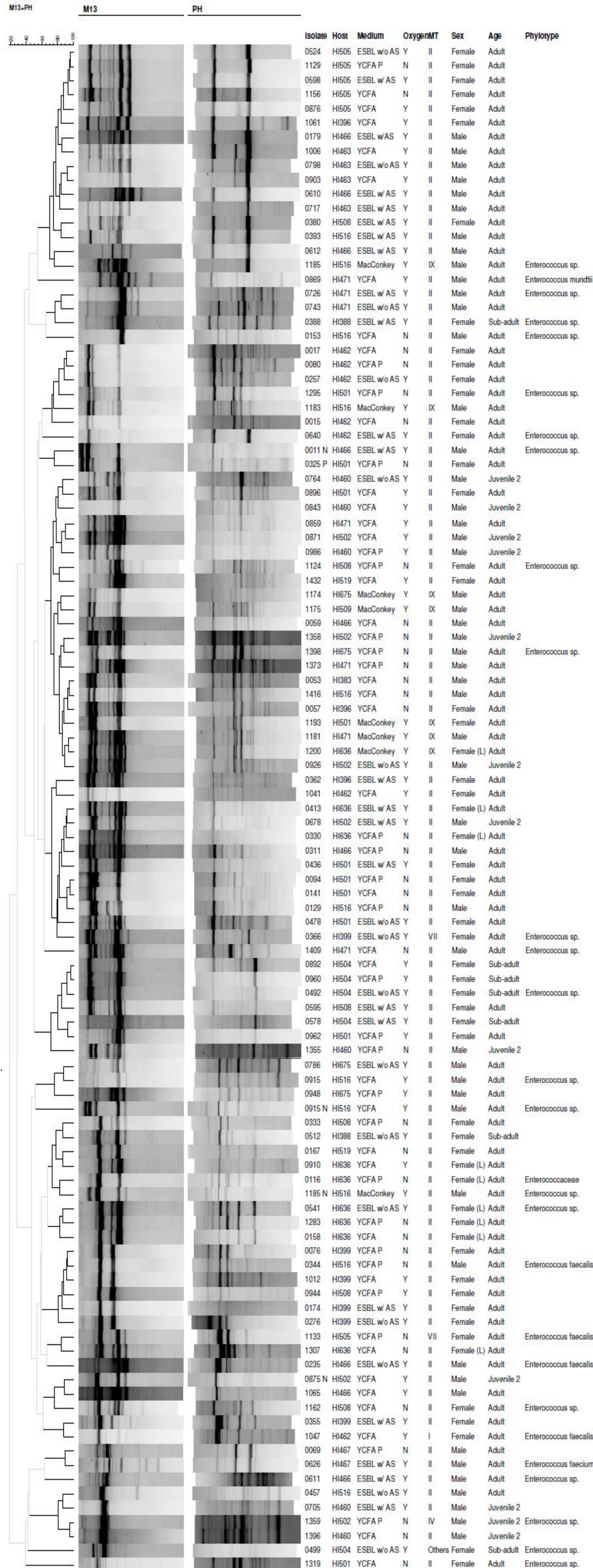
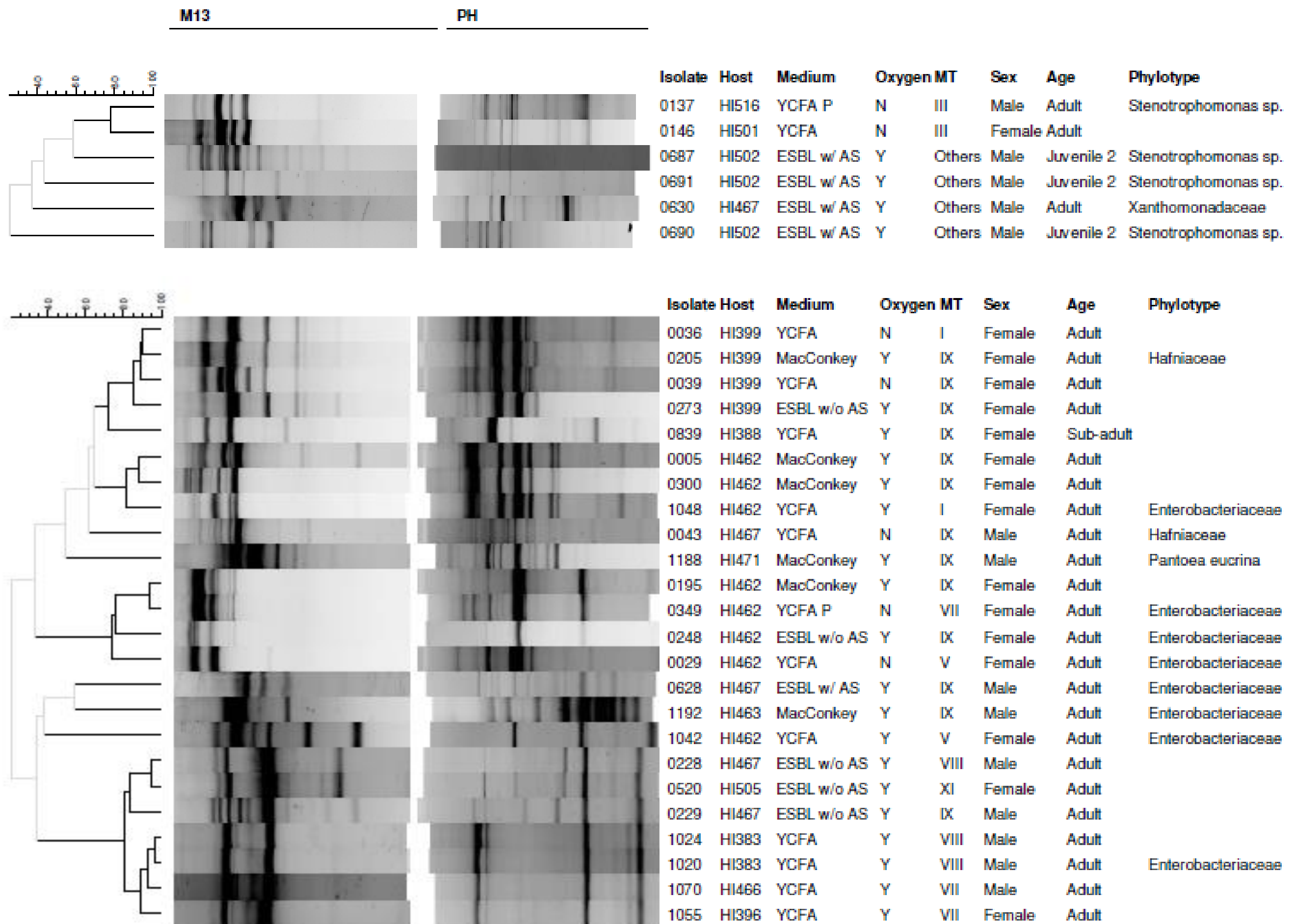


Figure 3.9 - Bacterial isolates identification and differentiation by a hierarchical numerical analysis. The PCR fingerprints obtained for M13 and PH were integrated, similarity was calculated by Pearson correlation coefficient and clustering was performed with UPGMA. The scale corresponds to global percentage of similarity. Cut-off value for cluster formation at 70% similarity. N – No; Y – Yes; (L) - Lactating



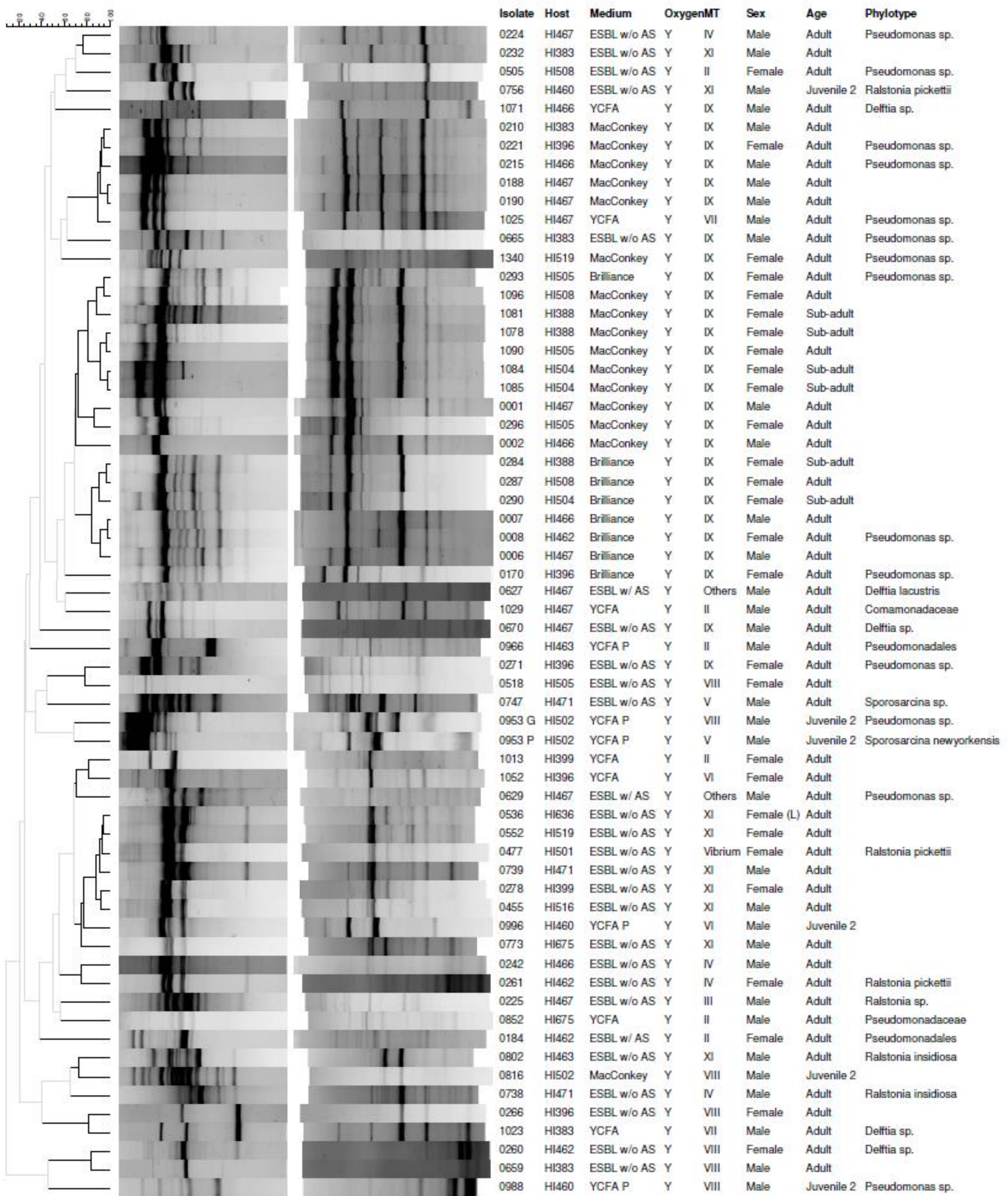
(Continuation) **Figure 3.9 - Bacterial isolates identification and differentiation by a hierarchical numerical analysis**. The PCR fingerprints obtained for M13 and PH were integrated, similarity was calculated by Pearson correlation coefficient and clustering was performed with UPGMA. The scale corresponds to global percentage of similarity. Cut-off value for cluster formation at 70% similarity. N – No; Y – Yes; (L) - Lactating



(Continuation) **Figure 3.9 - Bacterial isolates identification and differentiation by a hierarchical numerical analysis**. The PCR fingerprints obtained for M13 and PH were integrated, similarity was calculated by Pearson correlation coefficient and clustering was performed with UPGMA. The scale corresponds to global percentage of similarity. Cut-off value for cluster formation at 70% similarity. N – No; Y – Yes; (L) - Lactating

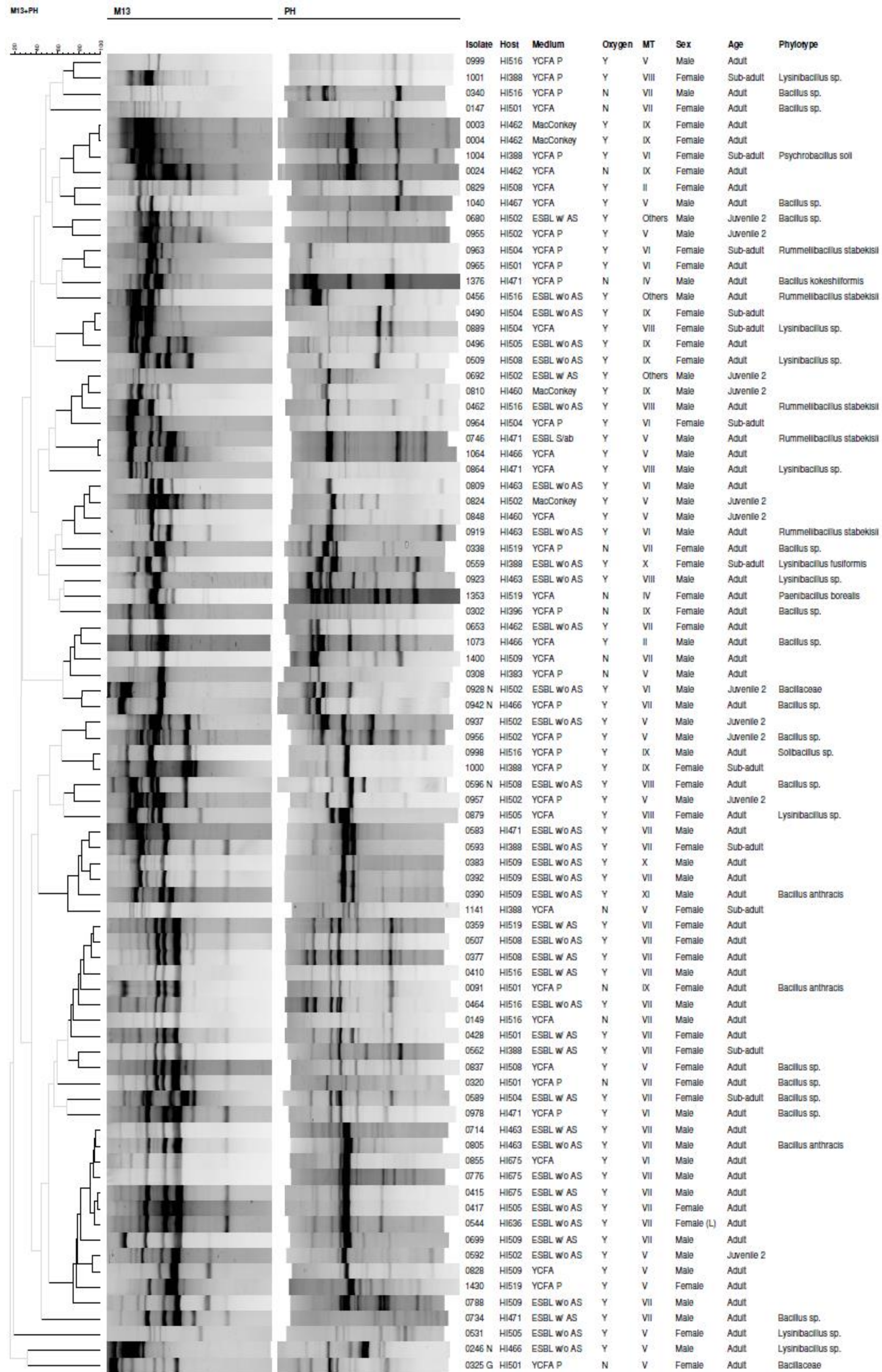
We obtained a total of 122 clusters, 55 of them being single-member clusters (data not shown). To reduce the entropy resulted in the enormous diversity of isolates, we grouped the isolates of each MT in individual dendrograms (Figure 3.9). Additionally, the isolates of identified genera, that were misplaced due to erroneous results on the morpho-physiological testes, were also regrouped into the right MT. One isolate of each newly formed unidentified clusters was then selected for 16S rRNA gene sequencing.

The identification of isolates was accomplished when both primers were used. This identification capability was tested and proved by coherent results when sequencing more than one isolate of the same cluster. The differentiation of isolates of the same taxonomic group was also accomplished, however, no clear pattern was observed between the formed clusters and host features.



Isolate	Host	Medium	Oxygen	MT	Sex	Age	Phylotype
0224	HI467	ESBL w/o AS	Y	IV	Male	Adult	Pseudomonas sp.
0232	HI383	ESBL w/o AS	Y	XI	Male	Adult	
0505	HI508	ESBL w/o AS	Y	II	Female	Adult	Pseudomonas sp.
0756	HI460	ESBL w/o AS	Y	XI	Male	Juvenile 2	Ralstonia pickettii
1071	HI466	YCFA	Y	IX	Male	Adult	Delftia sp.
0210	HI383	MacConkey	Y	IX	Male	Adult	
0221	HI396	MacConkey	Y	IX	Female	Adult	Pseudomonas sp.
0215	HI466	MacConkey	Y	IX	Male	Adult	Pseudomonas sp.
0188	HI467	MacConkey	Y	IX	Male	Adult	
0190	HI467	MacConkey	Y	IX	Male	Adult	
1025	HI467	YCFA	Y	VII	Male	Adult	Pseudomonas sp.
0665	HI383	ESBL w/o AS	Y	IX	Male	Adult	Pseudomonas sp.
1340	HI519	MacConkey	Y	IX	Female	Adult	Pseudomonas sp.
0293	HI505	Brilliance	Y	IX	Female	Adult	Pseudomonas sp.
1096	HI508	MacConkey	Y	IX	Female	Adult	
1081	HI388	MacConkey	Y	IX	Female	Sub-adult	
1078	HI388	MacConkey	Y	IX	Female	Sub-adult	
1090	HI505	MacConkey	Y	IX	Female	Adult	
1084	HI504	MacConkey	Y	IX	Female	Sub-adult	
1085	HI504	MacConkey	Y	IX	Female	Sub-adult	
0001	HI467	MacConkey	Y	IX	Male	Adult	
0296	HI505	MacConkey	Y	IX	Female	Adult	
0002	HI466	MacConkey	Y	IX	Male	Adult	
0284	HI388	Brilliance	Y	IX	Female	Sub-adult	
0287	HI508	Brilliance	Y	IX	Female	Adult	
0290	HI504	Brilliance	Y	IX	Female	Sub-adult	
0007	HI466	Brilliance	Y	IX	Male	Adult	
0008	HI462	Brilliance	Y	IX	Female	Adult	Pseudomonas sp.
0006	HI467	Brilliance	Y	IX	Male	Adult	
0170	HI396	Brilliance	Y	IX	Female	Adult	Pseudomonas sp.
0627	HI467	ESBL w/ AS	Y	Others	Male	Adult	Delftia lacustris
1029	HI467	YCFA	Y	II	Male	Adult	Comamonadaceae
0670	HI467	ESBL w/o AS	Y	IX	Male	Adult	Delftia sp.
0966	HI463	YCFA P	Y	II	Male	Adult	Pseudomonadales
0271	HI396	ESBL w/o AS	Y	IX	Female	Adult	Pseudomonas sp.
0518	HI505	ESBL w/o AS	Y	VIII	Female	Adult	
0747	HI471	ESBL w/o AS	Y	V	Male	Adult	Sporosarcina sp.
0953 G	HI502	YCFA P	Y	VIII	Male	Juvenile 2	Pseudomonas sp.
0953 P	HI502	YCFA P	Y	V	Male	Juvenile 2	Sporosarcina newyorkensis
1013	HI399	YCFA	Y	II	Female	Adult	
1052	HI396	YCFA	Y	VI	Female	Adult	
0629	HI467	ESBL w/ AS	Y	Others	Male	Adult	Pseudomonas sp.
0536	HI636	ESBL w/o AS	Y	XI	Female (L)	Adult	
0552	HI519	ESBL w/o AS	Y	XI	Female	Adult	
0477	HI501	ESBL w/o AS	Y	Vibrium	Female	Adult	Ralstonia pickettii
0739	HI471	ESBL w/o AS	Y	XI	Male	Adult	
0278	HI399	ESBL w/o AS	Y	XI	Female	Adult	
0455	HI516	ESBL w/o AS	Y	XI	Male	Adult	
0996	HI460	YCFA P	Y	VI	Male	Juvenile 2	
0773	HI675	ESBL w/o AS	Y	XI	Male	Adult	
0242	HI466	ESBL w/o AS	Y	IV	Male	Adult	
0261	HI462	ESBL w/o AS	Y	IV	Female	Adult	Ralstonia pickettii
0225	HI467	ESBL w/o AS	Y	III	Male	Adult	Ralstonia sp.
0852	HI675	YCFA	Y	II	Male	Adult	Pseudomonadaceae
0184	HI462	ESBL w/ AS	Y	II	Female	Adult	Pseudomonadales
0802	HI463	ESBL w/o AS	Y	XI	Male	Adult	Ralstonia insidiosa
0816	HI502	MacConkey	Y	VIII	Male	Juvenile 2	
0738	HI471	ESBL w/o AS	Y	IV	Male	Adult	Ralstonia insidiosa
0266	HI396	ESBL w/o AS	Y	VIII	Female	Adult	
1023	HI383	YCFA	Y	VII	Male	Adult	Delftia sp.
0260	HI462	ESBL w/o AS	Y	VIII	Female	Adult	Delftia sp.
0659	HI383	ESBL w/o AS	Y	VIII	Male	Adult	
0988	HI460	YCFA P	Y	VIII	Male	Juvenile 2	Pseudomonas sp.

(Continuation) **Figure 3.9 - Bacterial isolates identification and differentiation by a hierarchical numerical analysis**. The PCR fingerprints obtained for M13 and PH were integrated, similarity was calculated by Pearson correlation coefficient and clustering was performed with UPGMA. The scale corresponds to global percentage of similarity. Cut-off value for cluster formation at 70% similarity. N – No; Y – Yes; (L) - Lactating



(Continuation) **Figure 3.9 - Bacterial isolates identification and differentiation by a hierarchical numerical analysis**. The PCR fingerprints obtained for M13 and PH were integrated, similarity was calculated by Pearson correlation coefficient and clustering was performed with UPGMA. The scale corresponds to global percentage of similarity. Cut-off value for cluster formation at 70% similarity. N – No; Y – Yes; (L) - Lactating

Sequencing of 16S rDNA amplicon from 139 representative bacterial species (Supplementary Table 7.5), defined according to morpho-physiological tests and molecular profiling, generated partial sequences 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 (50). Homology searches of obtained sequences with publicly available sequences generated pairwise nucleotide identities between 82% and 100%. We based phylotype assignment on information from top three best matches displaying the higher nucleotide pairwise identity.

We successfully identified bacteria isolates belonging to three phyla: Firmicutes (67% of bacterial species), Proteobacteria (32%) and Actinobacteria (1%) (Figure 3.10). Bacteria affiliated with Bacilli class predominated (50%), followed by Gammaproteobacteria (23%), Clostridia (18%), Betaproteobacteria (8%), and Actinobacteria (1%). Isolates fitting to Bacillales order dominated (30%), followed by Lactobacillales (20%) and Clostridiales (18%). Pseudomonadales (12%), Burkholderiales (8%), Enterobacteriales (8%) and Xanthomonadales (3%) were only sporadically isolated, while Actinomycetales (1%) was rare.

Bacillaceae (23%) and *Enterococcaceae* (18%) were the most represented families. *Pseudomonadaceae* (11%), *Clostridiaceae* (9%), *Peptostreptococcaceae* (8%), *Enterobacteriaceae* (7%), *Xanthomonadaceae*, *Comamonadaceae*, *Burkholderiaceae* and “*Rummeliibacillus*-family” (4% each) were occasionally detected. *Carnobacteriaceae*, *Planococcaceae*, *Staphylococcaceae*, *Lachnospiraceae*, *Paenibacillaceae*, *Propionibacteriaceae* and *Hafniaceae* (1% each) were rare. We were not able to determine the family of 1% of the sequenced isolates.

We found bacterial isolates from twenty genera, with *Enterococcus* spp. (18%) and *Bacillus* spp. (14%) being the most abundant, while *Pseudomonas* spp. (10%), *Clostridium* spp. (9%), *Lysinibacillus* spp. (6%), *Delftia* spp., *Ralstonia* spp. and *Rummeliibacillus* spp. (4% each) were less denoted. Some genera were rare: *Paraclostridium* spp. and *Stenotrophomonas* spp. (3% each), *Paeniclostridium* spp. and *Romboutsia* spp. (2% each), *Carnobacterium* spp., *Sporosarcina* spp., *Staphylococcus* spp., *Paenibacillus* spp., *Pantoea* spp., *Propionibacterium* spp., *Psychrobacillus* spp., *Solibacillus* spp. and *Robinsoniella* spp. (1% each). We were not able to determine the genus of 13% of the sequenced isolates.

Paeniclostridium spp., *Pantoea* spp., *Sporosarcina* spp., *Solibacillus* spp., and *Stenotrophomonas* spp. were only detected in female individuals. In contrast, *Paenibacillus* spp., *Propionibacterium* spp., *Robinsoniella* spp. and *Staphylococcus* spp. were only detected in male individuals.

We only successfully identified the species of 30% of the sequenced bacterial isolates. *Rummeliibacillus stabekissi* represented four percent each, followed by *Enterococcus faecalis* and

Clostridium tertium that represented three percent each, *Clostridium perfringens, Paraclostridium bifermentans*, *Bacillus anthracis* and *Ralstonia pickettii* that represented two percent each. The remaining species were only detected once: *Bacillus kokeshiiformis, Clostridium septicum, Clostridium baratii, Delftia lacustris, Enterococcus faecium, Enterococcus mundtii, Lysinibacillus fusiformis, Paenibacillus borealis, Paeniclostridium tenue, Pantoea eucrina, Propionibacterium acnes, Psychrobacillus soli, Ralstonia insidiosa, Robinsoniella peoriensis* and *Sporosarcina newyorkensis*.

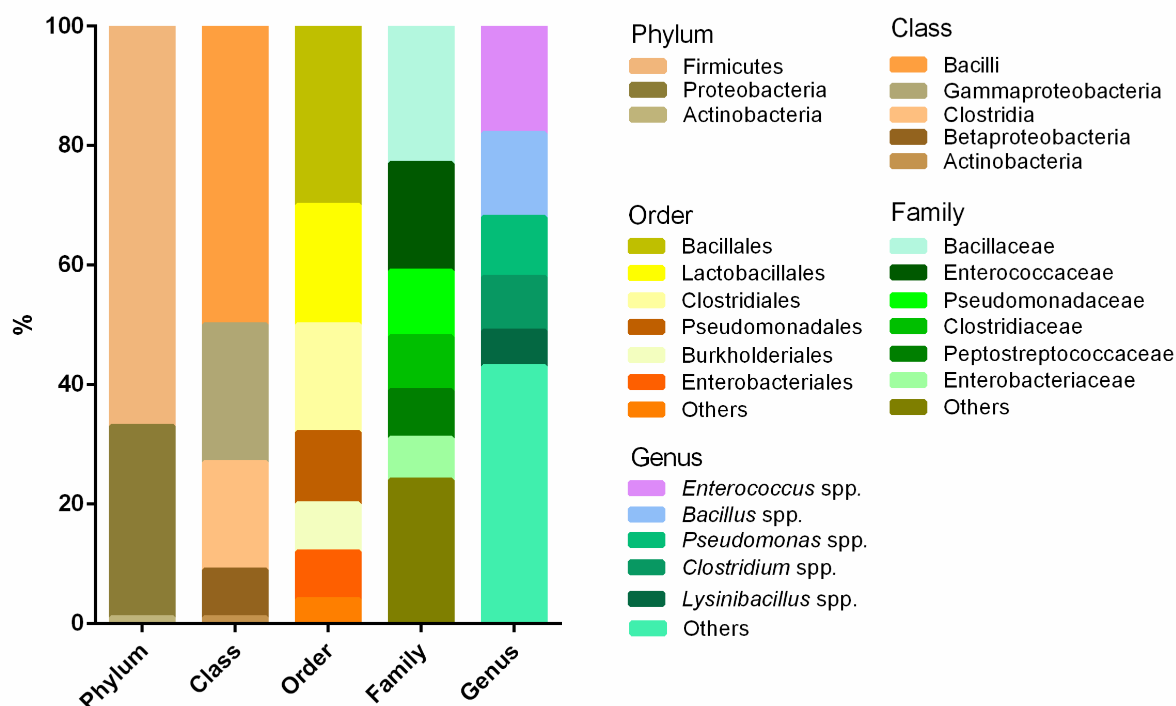


Figure 3.10 - Gut microbiota abundance of Egyptian mongoose population at different taxonomical levels. The major six groups of each taxonomical level are represented. Results are the sum of all 20 host individuals.

Sequencing of ITS and D1/D2 amplicons from six representative fungi species (Supplementary Table 7.6), according to morpho-physiological tests, generated partial sequences usable for identification of most genera. Homology searches of obtained sequences with publicly available sequences generated pairwise nucleotide identities between 94% and 99%. We based phylotype assignment on information from top three best matches displaying the higher nucleotide pairwise identity.

We successfully identified fungi isolates from four genera: *Pseudozyma* and *Naganishia* (Basidiomycota phylum), *Penicillium* (Ascomycota phylum) and *Mucor* (Mucoromycota phylum). *Mucor circinelloides*, *Naganishia albida* (=*Cryptococcus albidus*) and *Penicillium amaliae* were the three identifiable species.

At the bacterial genus level, a diversity analysis was performed for both female and male population (separately), but also for the mongoose population as a whole. Three diversity indices were calculated: Simpson's index, Shannon index, and Species evenness index derived from Shannon index

(Table 3.1), allowing the appraisal of the balance between the number of individuals and the number of species under analysis in each community. These diversity indices were very similar across males, females and the overall population. Moreover, nonparametric estimators of species richness were calculated, namely Chao 1, which analyses species abundance data, and Chao 2, that considers species incidence data (Table 3.1), with numerical values of 21, in both cases.

Table 3.1 – Diversity measurements for male, female, and the total population of Egyptian mongoose.

	Male	Female	Total
N	67	54	121
S	16	16	21
H'	2.48	2.38	2.55
D	0.90	0.88	0.89
E	0.89	0.86	0.84
Chao 1			21
Chao 2			21

N is the total number of clones in the sample; S is the number of OTUs in the sample; H' is the Shannon index; D is the Simpson's index; E is the Shannon evenness; Chao 1 analyses species abundance data; Chao 2 analyses species incidence data.

3.6. Interaction between microbiota and bio-environmental features

For microbiota and bio-environmental data integration, we performed a Principal Component Analysis (PCA) using available information for all 20 Egyptian mongoose specimens. The microbiota matrix was composed of 72 OTUs representing the presence/absence of every hierarchical bacterial level. Additionally, the biological matrix was composed of 26 biological variables related to sex, age class, reproductive status, stomach content at the time of death, and different body measurements. Also, the environmental matrix was composed of 17 environmental variables related to georeferenced location, land-use, climatic data, road net, river net, and population data.

We used a PCA methodology to reduce the number of variables, redistributing the original variables in principal components. We started by performing a PCA using the normalized microbiota matrix. This matrix registered a cumulative variance of 42% (PC1 – 17%; PC2 – 13%; PC3 – 12%). The dispersal areas of the microbiota data obtained for each age class (Figure 3.11) and sex (Figure 3.12) were compared.

Observing the microbiota data and comparing the individuals forming three clusters according to their age group, we can see that juvenile individuals overlap in total with adult individuals, but sub-adult individuals are separated from the others (Figure 3.11). Sub-adults tend to have a microbiota composed of Clostridiaceae and Actinobacteria phylum.

When we compare individuals by forming two clusters according to their sex, we can see that the two clusters have partial overlaps (Figure 3.12), but female specimens tend to have a microbiota

composed of Clostridia class (of Firmicutes phylum) and Actinobacteria phylum; and in contrast, male specimens tend to have a majority of Proteobacteria, in particular, Burkholderiales, Enterobacteriales, and Pseudomonadaceae groups, but also of Bacilli class.

The dendrogram originated from the normalized Euclidean distance derived from the projection matrix using UPGMA produced eight clusters, five of them, being single member clusters when a cut-off value of 0.182 of normalized Euclidean distance was used (Figure 3.13). The 2-way Mantel test shows a matrix correlation of 0.912.

Similarly, the dispersal areas of biological (Figure 3.14) and environmental (Figure 3.15) data obtained for the clusters originated from the previous microbiota dendrogram were compared. These matrices registered a cumulative variance of 55% (PC1 – 29%; PC2 – 14%; PC3 – 11%) and 68% (PC1 – 34%; PC2 – 18%; PC3 – 16%), respectively. We can see that cluster II is a very diverse group in terms of biological and environmental characteristics (Figure 3.14 and 3.15).

Regarding the biological characteristics and taking into consideration the first two PCs (Figure 3.14 A), it is clear that all clusters overlap, except for cluster IV and cluster VII. Cluster IV has the individual HI508, which has high values of biometric features related to the size and low values of biometric features related to the weight, but also a stomach content at the time of death composed of a smaller percentage of mammals and invertebrates. Cluster VII comprises the HI471 individual, who has high values of biometric features related to size, but unlike the previous one, high values of biometric features related to weight and a stomach content at the time of death composed of a greater percentage of mammals and invertebrates. When analyzing PC1 and PC3 (Figure 3.14 B), cluster IV overlaps with the rest, but cluster III, formed by the HI502 and HI516 individuals, is clearly separated from the rest, probably because the stomach contents at the time of death had a higher percentage of reptiles. Cluster VII remains separate from the remaining clusters. Considering the three PCs, cluster VII is distant from the rest of the clusters and cluster III is only partially overlapping with the other clusters (data not shown).

Regarding the environmental characteristics, and starting with the first two PCs (Figure 3.15 A), it is evident that all clusters overlap, apart from cluster VIII, and cluster I is partially overlapped with the rest of the clusters. Cluster VIII includes the individual HI388, whose land-use is mainly composed of agroforestry and mixed forest, and high river network; a high annual rainfall is characteristic of its habitat, while on the contrary, it has a low annual average temperature, annual thermal amplitude, and a reduced road network. Cluster I encompass the HI462 and HI383 individuals, with the same general characteristics as cluster VIII. When analyzing PC1 and PC3, all clusters are superimposed (Figure 3.15 B), showing that shrub and altimetry do not affect the gut microbiota composition. Considering the 3 PCs, cluster VIII is distant from the rest of the clusters and the remaining clusters are overlapping (data not shown).

Microbiota, biological, and environmental PCA explanatory variables for each PC and their explanatory value are shown in Tables 3.2 and 3.3, respectively.

Table 3.2 – Microbiota PCA explanatory variables for each principal component (PC) and their explanatory value.

Variables	PC	Explanatory value	Variables	PC	Explanatory value
Clostridium_tertium	1	+	*Bacillus_kokeshiiformis*	2	+
Lysinibacillus_fusiformis	1	+	*Enterococcus_mundtii*	2	+
Propionibacterium_acnes	1	+	*Pantoea_eucrina*	2	+
Psychrobacillus_soli	1	+	*Ralstonia_insidiosa*	2	+
Clostridium	1	+	*Rummeliibacillus_stabekisii*	2	+
Propionibacterium	1	+	*Enterococcus*	2	+
Psychrobacillus	1	+	*Lysinibacillus*	2	+
Clostridiaceae	1	+	*Pantoea*	2	+
Propionibacteriaceae	1	+	*Rummeliibacillus*	2	+
Actinomycetales	1	+	*Sporosarcina*	2	+
Actinobacteria (class)	1	+	*Enterococcaceae*	2	+
Actinobacteria (phylum)	1	+	*Planococcaceae*	2	+
Pseudomonas	1	-	Lactobacillales	2	+
Pseudomonadaceae	1	-	Clostridia	2	-
Burkholderiales	1	-	Clostridiales	2	-
Enterobacteriales	1	-	*Bacillus*	3	+
Proteobacteria	1	-	*Delftia*	3	-
Betaproteobacteria	1	-	*Comamonadaceae*	3	-
Gammaproteobacteria	1	-			

The explanatory variables of each Principal Component (PC) were selected if the correlation coefficient between the variable and the PC were |0.5|. If a variable had this behavior with more than one PC, this variable was used as an explanatory variable for the PC with the higher correlation coefficient. The variables with positive correlation with a given PC are represented with (+), and with negative correlation with (–).

Table 3.3 – Biological and environmental PCA explanatory variables for each PC and their explanatory value.

Biological PCA			Environmental PCA		
Variables	PC	Explanatory value	Variables	PC	Explanatory value
Adult	1	+	Urban	1	+
Sub_adult	1	+	Vineyardsorchads	1	+
BW	1	+	Coniferous	1	+
HBL	1	+	Population	1	+
RHLL	1	+	RoadNet	1	+
SH	1	+	Agroforestry	1	-
NP	1	+	RiversNet	1	-
HD	1	+	Mixforests	2	+
HW	1	+	Annual_rainfall	2	+
KW	1	+	Agriculture	2	-
STL	1	-	Average_annual_temperature	2	-
TL	1	-	Average_temperature_range	2	-
Mammals	2	+	Shrubs	3	+
Invertebrates	2	+	Altimetry	3	+
SW	2	+			
SFI_3	2	+			
PFI_2	2	+			
PFI_3	2	+			
Sex	3	-			
Reptiles	3	-			
RHFL	3	-			

The explanatory variables of each Principal Component (PC) were selected if the correlation coefficient between the variable and the PC were |0.5|. If a variable had this behavior with more than one PC, this variable was used as an explanatory variable for the PC with the higher correlation coefficient. The variables with positive correlation with a given PC are represented with (+), and with negative correlation with (–). BW – body weight; HBL – head and body length; RHLL – right hind leg length; SH – shoulder height; NP – neck perimeter; HD – head diameter; HW – hearth weight; KW – kidney weight; STL – snout-tail length; TL – tail length (terminal hairs not included); SW – spleen weight; SFI – subcutaneous fat index; PFI – perivisceral fat index; RHFL – right hind foot length.

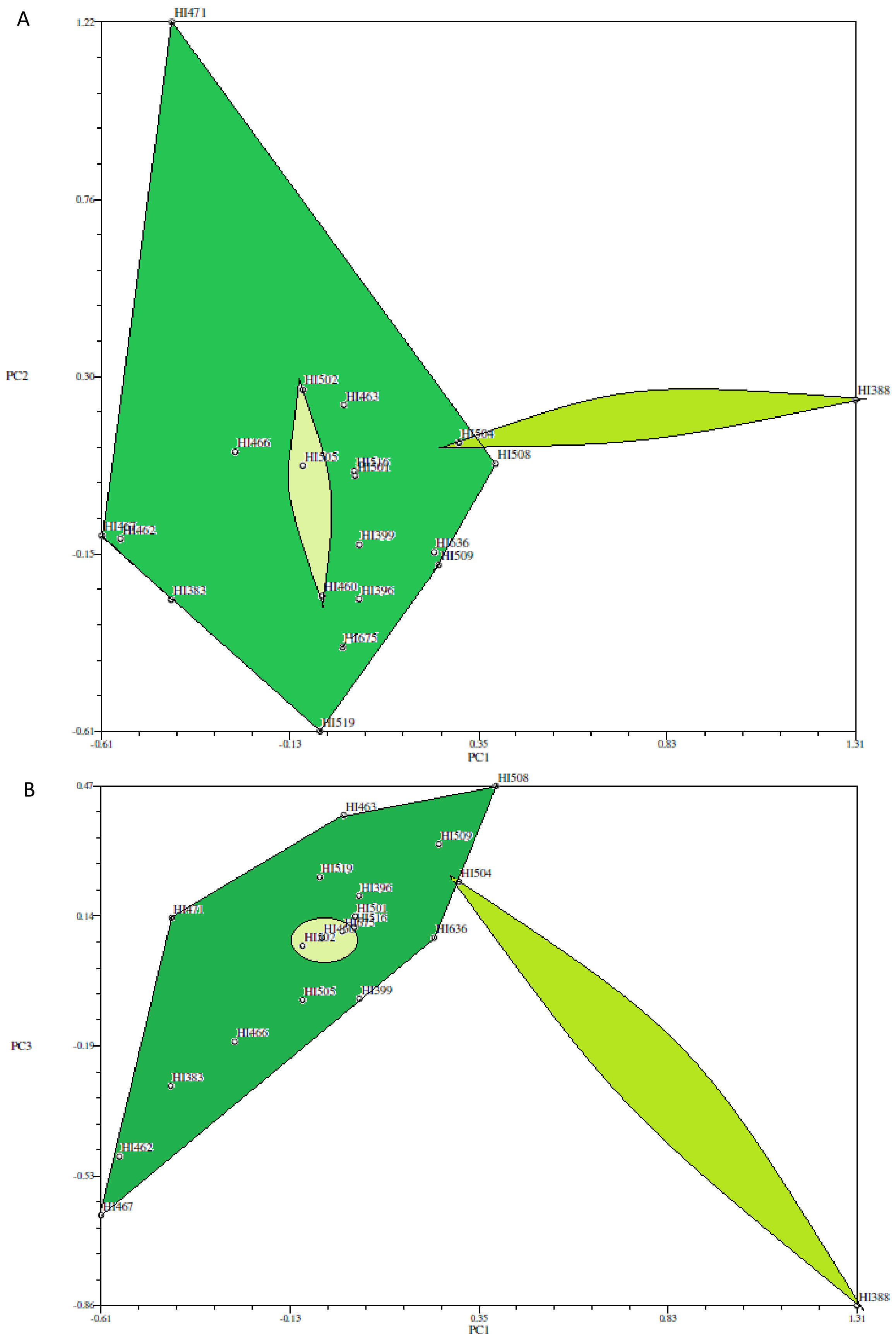


Figure 3.11 – Two principal component (PC) plots depicting the dispersal area of microbiota data of each Egyptian mongoose specimens. The 20 specimens are projected in the **(A)** PC1xPC2 and **(B)** PC1xPC3 planes resulting from a Principal Component Analysis performed on the Boolean matrix of the presence/absence of the different hierarchical bacterial levels. The vertices of each polygon correspond to the microbiota data observed for each specimen. Each polygon corresponds to age class (juvenile in light green, sub-adults in green, and adults in dark green) clustering.

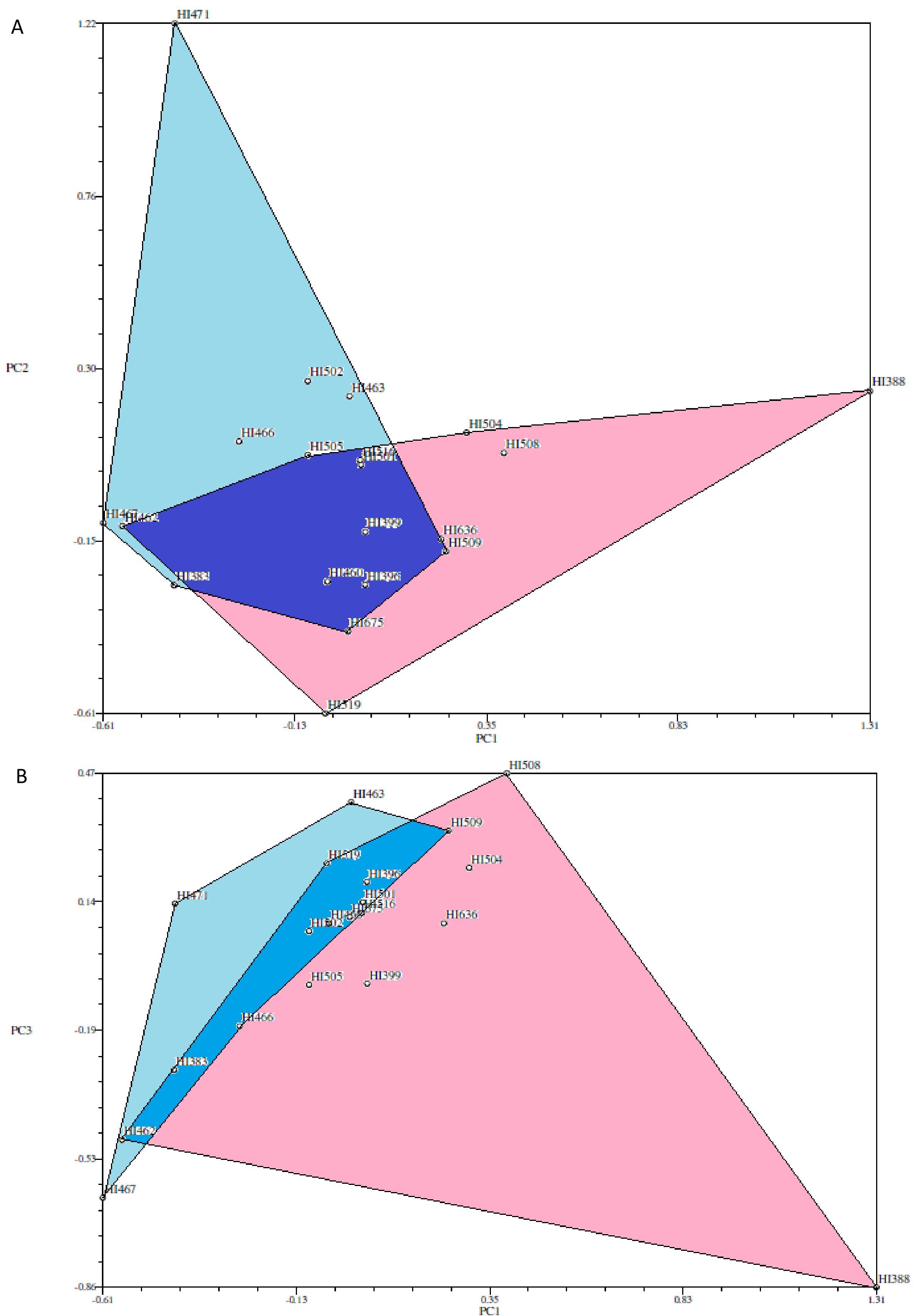


Figure 3.12 – Two principal component (PC) plots depicting the dispersal area of microbiota data of each Egyptian mongoose specimens. The 20 specimens are projected in the **(A)** PC1xPC2 and **(B)** PC1xPC3 planes resulting from a Principal Component Analysis performed on the Boolean matrix of the presence/absence of the different hierarchical bacterial levels. The vertices of each polygon correspond to the microbiota data observed for each specimen. Each polygon corresponds to sex (male in blue and female in pink) clustering.

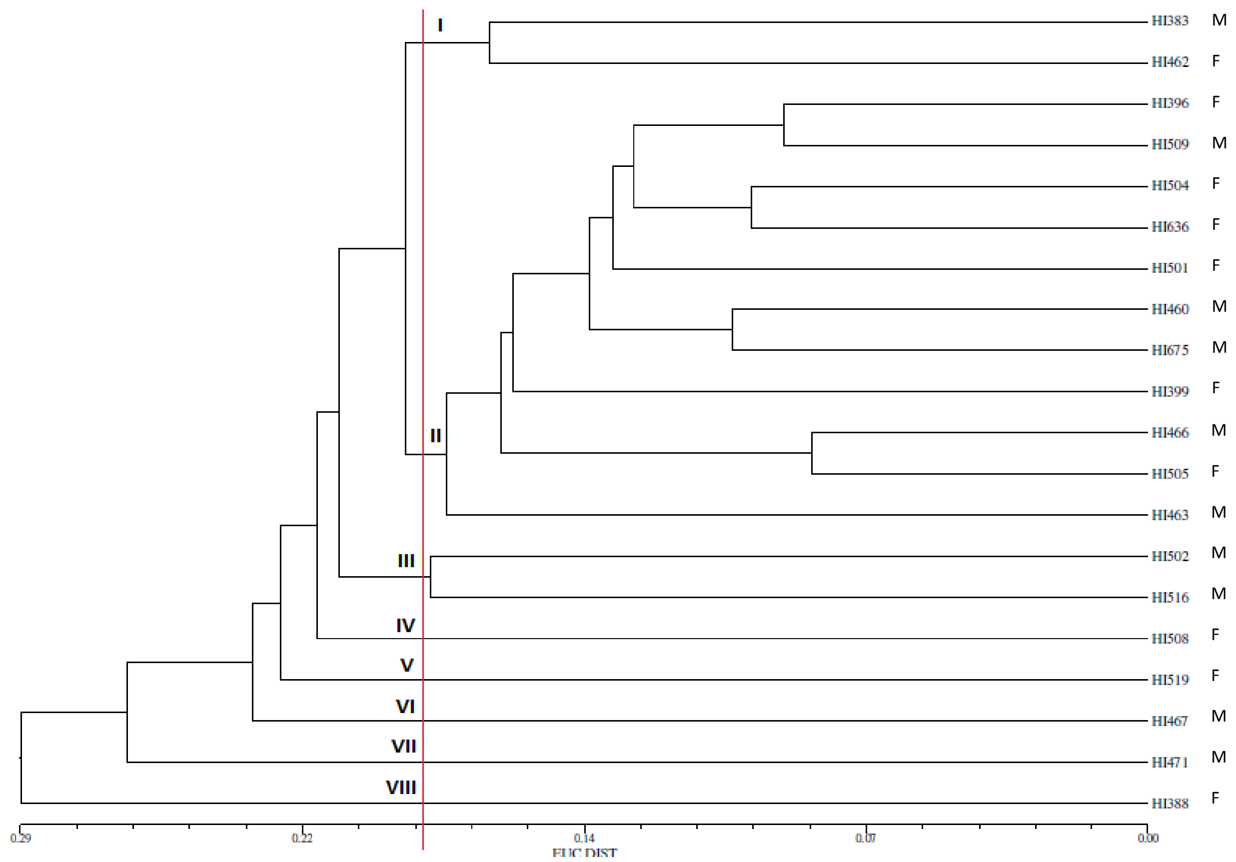


Figure 3.13 – Dendrogram representing the relationship between the Egyptian mongoose specimens in terms of microbiota. The 20 specimens were clustered using the normalized Euclidean distance derived from the projection matrix of the PCA obtained from the microbiota data and clustered using UPGMA. The cut-off value was determined at 0.18 of normalized Euclidean distance, producing 8 clusters, 5 of them single member clusters. M – Male; F- Female.

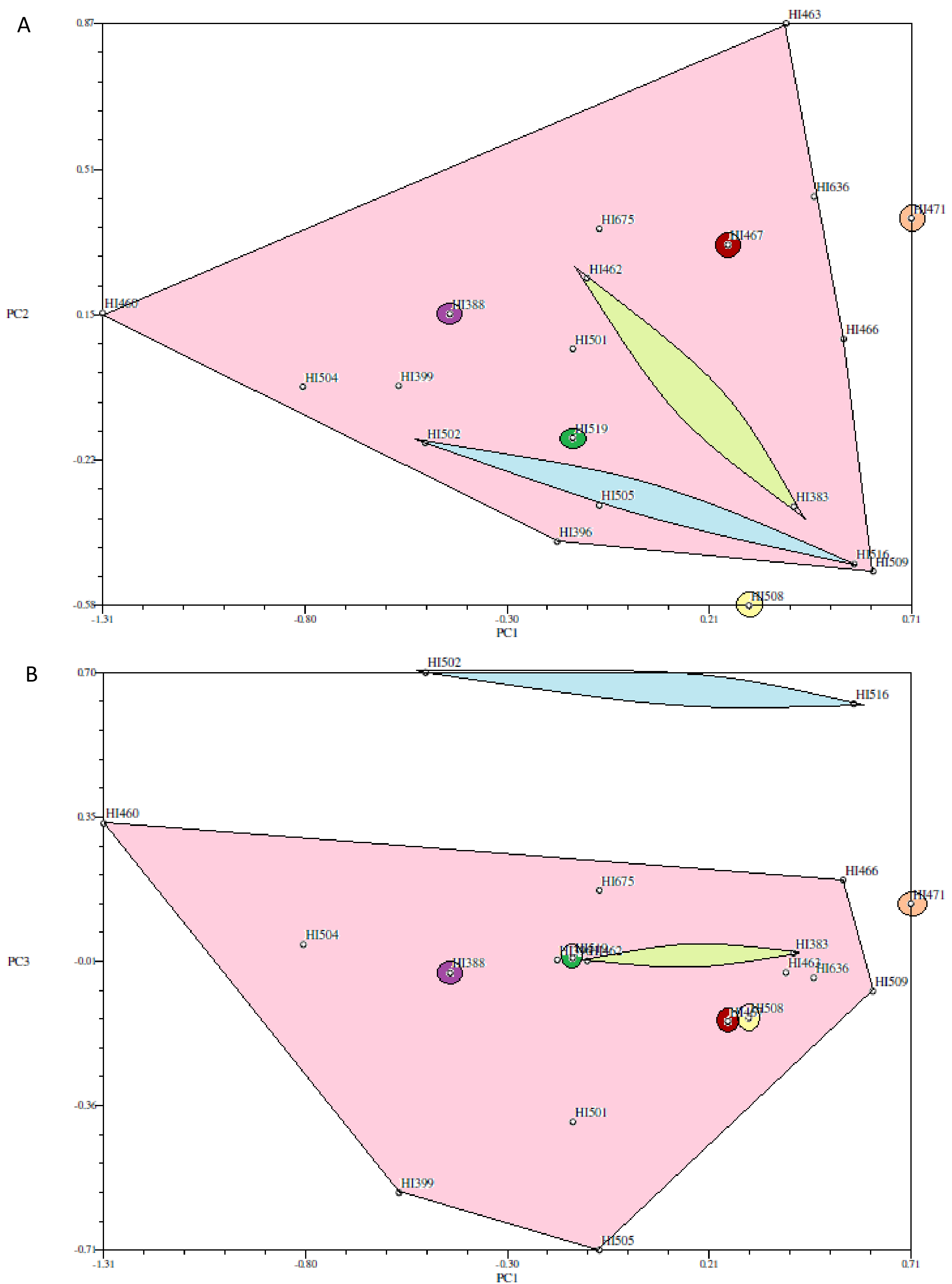


Figure 3.14 – Two principal component (PC) plots depicting the dispersal area of biological data of each Egyptian mongoose specimens. The 20 specimens are projected in the **(A)** PC1xPC2 and **(B)** PC1xPC3 planes resulting from a Principal Component Analysis performed on the matrix of the biological data. The vertices of each polygon correspond to the biological data observed for each specimen. Each polygon corresponds to the clusters originated from the microbiota data dendrogram. Cluster I – HI383 and HI462 (light green); Cluster II – HI396, HI509, HI504, HI636, HI501, HI460, HI675, HI399, HI466, HI505, and HI463 (pink); Cluster III – HI502 and HI516 (blue); Cluster IV – HI508 (yellow); Cluster V – HI519 (dark green); Cluster VI – HI467 (red); Cluster VII – HI471 (orange); Cluster VIII - HI388 (purple).

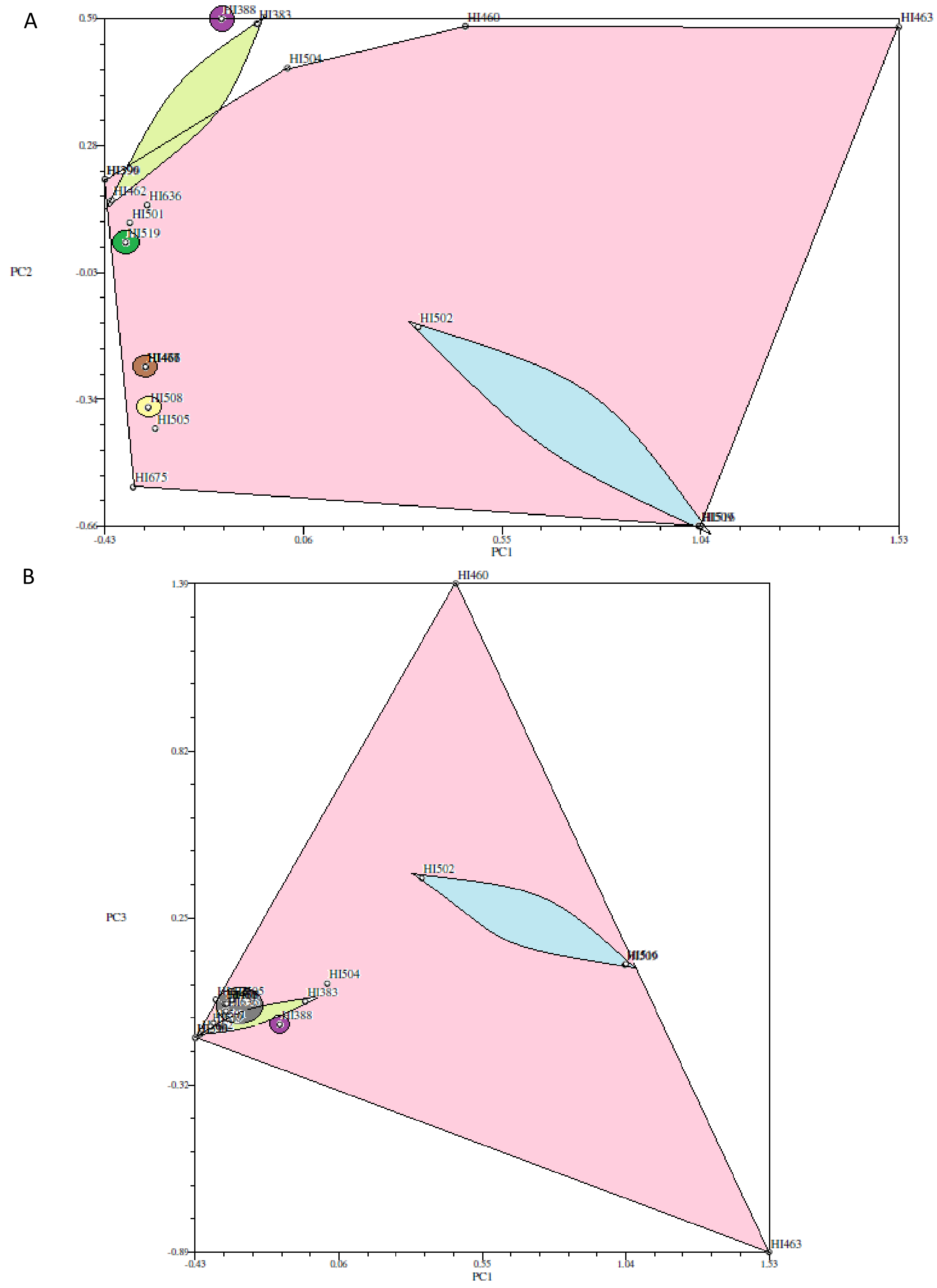


Figure 3.15 – Two principal component (PC) plots depicting the dispersal area of environmental data of each Egyptian mongoose specimens. The 20 specimens are projected in the **(A)** PC1xPC2 and **(B)** PC1xPC3 planes resulting from a Principal Component Analysis performed on the matrix of the environmental data. The vertices of each polygon correspond to the environmental data observed for each specimen. Each polygon corresponds to the clusters originated from the microbiota data dendrogram. Cluster I – HI383 and HI462 (light green); Cluster II – HI396, HI509, HI504, HI636, HI501, HI460, HI675, HI399, HI466, HI505, and HI463 (pink); Cluster III – HI502 and HI516 (blue); Cluster IV – HI508 (yellow); Cluster V – HI519 (dark green); Cluster VIII - HI388 (purple); Cluster VI (HI467) + Cluster VII (HI471) are shown in brown in (A) and Cluster IV + Cluster V + Cluster VI + Cluster VII are shown in grey in (B) due to a complete overlap of the clusters in both cases.

CHAPTER IV – DISCUSSION

Gut microbiota is nowadays an important topic of investigation in biological and medical science due to its recognized importance in host biology and ecology. In this study, we generated, for the first-time, extended baseline information on the microbiome of mongoose, enabling the exploitation of microbial community differences between sexes and exploring the influence exerted by the biological and environmental context of each host in its microbiota signature.

For gut microbiota evaluation, fecal samples were used as a proxy of the entire gut community. Two main groups of methods may be utilized for microbiota analysis: culture-dependent and culture-independent methods. In this work, to enable a wide range culture-dependent approach, different media and growth conditions were used in order to capture the most representative diversity of gut microbiota.

To enable recovery of culturable bacteria from the fecal samples, two rich media were used, YCFA and ESBL w/o AS. In aerobic conditions, both media registered the higher bacterial abundance and diversity. We accounted for an average of 2.8×10^9 CFU and 3.3×10^{12} CFU, respectively, of total aerobic bacteria per gram of feces. The abundance results from YCFA medium are similar to those reported by other microbiota studies, using different media, for carnivores mammals such as grizzly and polar bears (3, 56, 57), dogs (58), and cats (59), but also for mammals with other food regimen, such as humans (53, 54), pigs (55), cattle, sheep and goats (55). Moreover, the abundance results obtained in ESBL w/o AS are similar to those reported by other studies, using different media, with human samples (55, 60, 61). Besides inter-specific differences, different results reported by different studies may arise from different media composition, but may also be the result of individual-specific differences.

YCFA medium was previously demonstrated as a media that enables a great variety of bacteria to grow under anaerobic conditions, with 72% of coverage comparing to metagenomics (37). In this study, the average amount of anaerobic bacteria detected was 5.5×10^9 CFU/g of feces. Other study using a different rich medium also recorded analogous amounts of viable anaerobic bacteria in human fecal samples (53).

Comparing the bacterial diversity obtained in YCFA medium under differential oxygen availability, MT-VI, MT-VIII and MT-XII were only detected in aerobic conditions while, in contrast, MT-III and MT-X were only detected under anaerobiosis. In addition, MT-VII isolates were almost exclusively detected in anaerobic conditions. Therefore, these results may indicate that the isolates capable of growing in this medium, belonging to MT-III and MT-X, are obligate anaerobes and, in contrast, the isolates belonging to MT-VI, MT-VIII, MT-XII and MT-XIII are obligate aerobes. MT-VII isolates are probably aerotolerant bacteria since they growth preferentially in anaerobic conditions. The remaining MTs are probably facultative anaerobic bacteria.Surprising, YCFA medium allowed the growth of fungi, both yeasts and filamentous fungi, which has not been reported in previous studies (37). This may be justified by a bacteriological focus of this previous study.

In mammal species, gut microbiota is dominated by strict anaerobic bacteria, with poorly known mechanisms to survive environmental exposure during cross-transmission between individuals (3). A possible mechanism relies on the production of dormant structures with extremely resistant capacities, the endospores. These spores facilitate the persistence of the host and also in the environment (62). This resistance property is responsible for the wide dispersion of endospore-forming bacteria (sporobiota) in the environment and enables a recurrent exchange between numerous distinct ecological niches and between living organisms, suggesting that sporobiota plays a particular role in the microbiota and, consequently, in host biology (63). However, only a few number of bacteria associated with the gut sporobiota community have been cultured. Metagenomic data suggest a highly abundant population of potentially sporulating bacteria: an average of 60% of the genera contain endospore-forming bacteria, which represent 30% of the total gut microbiota (37, 62). To study this particular community, we used YCFA medium supplemented with 0.1% de sodium taurocholate, a biliary acid which triggers the germination mechanism (37) and incubated in both aerobic and anaerobic conditions, after pasteurization of the inoculum. The pasteurization process allows killing vegetative cells, while keeping viable the remaining resistant structures (64).We detected an average of 5.1×10^5 CFU/g of endospore-forming bacteria in aerobiosis and 4.2×10^8 CFU/g in anaerobiosis. To our knowledge, no other study reported the viable number of spore-forming bacteria in gastrointestinal samples. Furthermore, the pasteurization process only eliminated MT-I isolates from the samples, possibly due to the absence of resistant structure formation or to the presence of sodium taurocholate which may have inhibited this morpho-physiological type growth.

Enterobacteriaceae are a very important group of bacteria since they are part of the normal gut microbial community of mammals, although they may also be involved in human, cattle, and wildlife health-related problems (65). MacConkey is a selective medium that allows gram-negative bacteria growth, permitting, in addition, the determination of lactose fermentation metabolism. An average of 8.0×10^9 CFU/g of putative *Enterobacteriaceae* were detected, contrasting with previous studies, in which the amount of this family had been reported as 10^8 copies genes/g from grizzly and polar bears (3, 56, 57). Studies based in animals with other food regime, namely, a study that analyzed ileal samples detected 10^7 CFU/g from pigs (66) and 10^7 CFU/g of coliform bacteria from humans (53). This difference may be due to the growth of *Pseudomonas*-like bacteria, which can be misidentified as *Enterobacteriaceae*.

ESBLs are a very important issue in human and animal health since they confer resistance to most β-lactam antibiotics, including penicillins, monobactams, first-, second-, third-, and fourth-generation cephalosporins (67). Two chromogenic media were used to detect and isolate ESBL-producing gram-negative bacteria, namely, the ESBL Chromogenic medium with ESBL antibiotic supplement and the ready-to-use Brilliance ESBL medium. According to the manufacturer, ESBL antibiotic supplement possesses both ESBL-producing bacteria selectivity and gram-negative bacteria

selectivity. As expected, our results show that ESBL w/AS medium have a clear reduction of bacterial growth of all types of colonies. Putative *E. coli* and colorless colonies belonging to different MT had their growth inhibited in the presence of this antibiotic supplement. An astonishing lack of selectivity to gram-negative bacteria is also reported since we perceived bacterial growth of gram-positive microorganisms in approximately 99% of culturable bacteria ([gram-positive bacteria]:[total bacteria]*100 = $1.2x10^8$ CFU/g : $1.21x10^8$ CFU/g*100). Cefpodoxime, a third-generation cephalosporin, is currently the β-lactam antibiotic of choice incorporated in Brilliance ESBL medium (38). This medium enabled lower bacterial load among antibiotic selective media, supporting the growth of an average of $4.2x10^4$ CFU/g of putative ESBL-producing *Salmonella* spp. and/or *Acinetobacter* spp.

The presence of both LF and LNF bacteria isolates in MacConkey and putative *E. coli* isolates in ESBL w/o AS indicates a variety of *Enterobacteriaceae* genera in the samples but in Brilliance only *Pseudomonas* spp. were detected. So, all *Enterobacteriaceae* present in the samples presumably were not-ESBL-producing bacteria. Thus, the differences observed between growth in both ESBL media may be due to an almost exclusive selectivity of ESBL antibiotic supplement to ESBL-producing bacteria. The phenotype and genotype of ESBL-growing isolates recovered from ESBL w/ AS and also from Brilliance ESBL medium, in terms of resistance to antibiotics, especially, to β-lactams should be addressed in future work. Moreover, previous studies reported the existence of fecal-oral cross-transmission of bacteria between the human population and wildlife (68), in particular, with individuals from the *Herpestidae* family (69), demonstrating the possibility of transmission of antibiotic-resistant bacteria to wildlife living in close contact with human areas. Refining the phenotype and genotype associated with ESBL-resistant bacteria isolated (0.005%) in this work will enable estimating how much ESBL-producing bacteria circulate in wild mammals of *Herpestidae* family, in Portugal.

Besides bacterial community, the GI tract also harbors a fungi community, many times neglected in microbiota studies. To access the gut mycobiota of the Egyptian mongoose, PDA medium supplemented with chloramphenicol was inoculated. This medium allows the selective growth of both yeasts and FF, inhibiting almost all bacterial growth. We recorded 100% growth of fungi, with a mean of $1.1x10^8$ CFU/g. This result, to our knowledge, is the higher ever detected in a GI sample, with previous studies reporting a maximum of 10^6 CFU/g (70) and 10^7 copies gene/g (71). About $5.0x10^7$ CFU/g of yeasts and $5.8x10^7$ CFU/g of FF were recorded. Individual reports of amounts of yeast are rare, with a single report describing 10^4 CFU/g in human samples (72); publicly available data was not found for FF.

The influence of host sex and reproductive status on microbial community composition is nearly unknown. A higher microbial load of fecal samples from female hosts was quantified in YCFA w/o O_2 and YCFA P w/o O_2 media. Also, we only registered the growth of putative *E. coli* in ESBL w/o AS medium from female host samples. Additionally, six of the eight samples that had culturable bacteria in

Brilliance medium were from female hosts. In terms of diversity, we saw a higher number of MT-II isolates in ESBL w/AS medium and MT-IX isolates when summing percentages from all media. In contrast, we detected FF exclusively in fecal samples from male hosts. Studies on chimpanzees, black howler monkeys, and rufous mouse lemurs (16, 19, 20) have shown some differences in bacterial microbiota community between sexes, probably related to differences in the feeding behaviors (19, 20), and in host physiology, such as hormones excretion and immune system (20), but also due to the start of the gestation period in the time of trapping (16). Relatively to mycobiota, a study on human samples, show differences between sexes, reporting higher bacterial richness and diversity (number of species recovered from samples of female hosts) (73).In contrast to these examples, sex does not influence microbiota composition in baboons and wild mice (4, 74, 75). In wild baboons, host sex was significant in infants, probably due to differences in maternal care, since these differences vanish in adulthood (74).

Reproductive status, such as pregnancy and lactation, may also be associated with alteration of the gut microbiota composition (17, 21, 22), as the manipulation of the gut community by the host to promote metabolic changes that are vital to their fitness is possible, as it may support healthy pregnancy and lactation. In our study, we analyzed one sample recovered from a lactating female and did not find differences when comparing it with the remaining samples (both males and non-lactating females). However, this study needs to continue in the future with more fecal samples from lactating female to enable a robust statistical analysis.

Looking at each individual host as a habitat with its own community, the MT-II, MT-VI, MT-VII, MT-IX and MT-XI types may be considered the core gut microbiota community and the remaining morpho-physiological types can be considered part of the intra-specific individual microbiota community. These differences probably result in inter-individual variability, since all samples have stomach content, geographic, and land-use similarities. Inter-individual variability has been shown to occur in different wildlife animals (6), and also in humans (5). Additionally, we perceived that the majority of individuals possess MT-II ESBL-producing bacteria.

RAPD relies on the random amplification of polymorphic DNA through PCR, using generic primers with 6 to 10 base pairs long, under low stringency conditions, allowing unspecific binding and amplification, creating multiple amplicons that can be separated by agarose gel electrophoresis to generate a bacterial fingerprint used to compare the relatedness of bacterial strains (76). The major disadvantage of this technique relies on the alteration of reproducibility when slight changes in reagents, amplification conditions or analysis parameters, occur (77). The principal advantage is the short amount of time to conduct the analysis and the unnecessary prior knowledge of the bacterial genome sequence to identify specific target sites (77).In this study, we used this technique to assist in bacteria identification and to perform intra-species differentiation. Using a 70% similarity cut-off value, the clustering allowed bacterial identification at the genus level. In a differentiation perspective, we do not find any clear

pattern in cluster formation related to host sex or age class, despite the existence of a social behavior that may increase microbiota similarities among age classes in this species (34).

The core gut bacterial microbiota community from the analyzed Egyptian mongoose specimens was dominated by Gram-positive bacteria (76%), mainly of the phylum Firmicutes (68%), with Bacilli isolates (50%) prevailing, in particular, *Enterococcus* spp. (18%) and *Bacillus* spp. (14%).The Firmicutes members are normally the most abundant bacterial phylum present in the vertebrate GI tract, being mostly responsible for protein degradation (78). Most of them are commensal and are narrated as important in the preservation of gut homeostasis and host immunity development (78).

Bacillaceae members (23%) are frequently associated with air and soil samples initially thought to be transient in the GI tract originated from the ingested plant and root materials (79). Additionally, the ingestion of soil is frequent in wildlife mammals and has been described for grizzly bears, wild otters, and chimpanzee (3). Nowadays, these genera are considered to be part of the resident GI microbiota of vertebrates (9). *Bacillus* spp. are also associated with an extended lifespan influence on host gene regulation of immune factors and cell proliferation, as well as the availability of key vitamins and cofactors (80).

In this study, Lactobacillales members are well represented in Egyptian mongoose specimens (20%), in particular, *Enterococcus* spp. (18%). Animal GI tracts likely represent the greatest reservoir for enterococci, being opportunistic pathogens for both humans and animals. Recent studies in Portugal identified enterococci in a broad range of environments, including wild animals, which have particularly been highly associated with carnivores (68, 81, 82). In this study, this hypothesis is reinforced. We detected three different species of *Enterococcus* spp., namely, *E. faecalis* and *E. faecium,* well-known human pathogens, but can also cause infection in livestock and wildlife, and *E. mundtii,* normally associated with natural environments but can also cause infection in animals (82). However, evidence of gastroenteritis were not observed in any of the animals under analysis.

Clostridia members were also isolated in this work (17%); these are considered one of the most important groups of bacteria present in the vertebrate GI tract. This group is composed of obligate anaerobes that only perform fermentation metabolism, being an important intervenient in the breakdown of carbohydrates and proteins, but also in nutrient absorption (57). These bacteria are usually detected in a wide range of animals, including humans (83). High-protein contents have been reported to select for proteolytic bacteria, such as *Clostridium* spp. (9%) (57, 84), which is concordant with the carnivorous diet of Egyptian mongoose specimens (33, 85). In addition, this class is known to contain some important pathogens that can release toxins and cause intestinal diseases, besides the species that are commensal of GI tract microbiota (86). The presence and potential excretion of *Clostridium* pathogenic strains, whose virulence is attributed to numerous exotoxins, is a known risk for lethal enteritis and enterotoxaemia infections in livestock and wildlife (87, 88). In this work, *C. perfringens*

was isolated, being previously positively associated with protein intake and negatively correlated with dietary fiber content in grizzly bears (57), with is consistent with dietary patterns of Egyptian mongoose specimens. *Peptostreptococcaceae* family members (of Clostridia class) are highly related to *Clostridium* species and were also found in this study (8%). *Paraclostridium* (3%) and *Paeniclostridium* (2%) are genera previously identified as part of human's microbiota and as opportunistic pathogens. *Romboutsia* spp. (2%) are obligate anaerobes normally found in the digest and fecal samples of humans and rats, but also in mud and alkaline saline lake sediment (89, 90). Besides bacteria belonging to Firmicutes phylum, we also detected bacterial isolates belonging to Proteobacteria phylum (31%). Proteobacteria members are abundant in most mammal GI tract, being the most extensively studied phylum due to ease culturability. All proteobacteria are gram-negative but are extremely diverse in terms of metabolism (78). This phylum is classified into five sub-divisions: Alpha-, Beta-, Gamma-, Delta- and Epsilon- Proteobacteria. In this work, Gammaproteobacteria were detected (23%), having previously been reported to be the most common in mammal GI tract, being responsible for the breakdown and fermentation of complex sugars and production of vitamins (78). *Enterobacteriaceae* are the most representative family of this class (7%), its members are endotoxin-producing opportunistic pathogens and have putatively shown a relation in the development of obesity in humans (67, 91). This group was prominent in the fecal samples from grizzly bears and giant panda (56, 57, 92). *Pseudomonaceae* were also detected in our study (11%), particularly *Pseudomonas* genus (10%). Several *Pseudomonas* species are opportunistic pathogens including *P. aeruginosa* and *P. fluorescens* (93). Some, such as *P. aeruginosa* and *P. putida*, are also minor members of the normal gastrointestinal microbiota (93).

The individual microbiota was characterized by a great diversity of bacteria. We found other *bacillus*-like species, such as *Psychrobacillus soli*, a known oil-degrading bacterium (94), *Paenibacillus borealis,* a known nitrogen-fixing species (95), *Lysinibacillus fusiformis,* a known cause of human bacteremia (96), and *Rummeliibacillus stabekisii,* previously isolated from soils and from insect and bird gut (97). From Bacillales order, we also identified *Sporosarcina* spp., in particular, *S. newyorkensis*, previously isolated from human infected blood and raw cow's milk (98), and *Staphylococcus* spp., a known member of mammals' gut microbiota with opportunistic pathogenic behavior (99). *Carnobacterium* spp. was also present, being usually found in a range of foods, including fish, meat, and some dairy products, but also in natural environments, such as sediments and water bodies (100). To our knowledge, this is the first study to report *Carnobacterium* spp. in the GI tract microbiota of mammals. We also detected *Lachnospiraceae* family members, such as *Robinsoniella* spp.. This family is involved in the maintenance of gut health through their role in plant-derived material degradation in the gut (101) and has been linked to obesity and protection from colon cancer in humans, mainly due to the association of many species within the group with the production of butyric acid, a substance that is important for both microbial and host epithelial cell growth (101). *Pantoea* genus was detected. This

genus inhabits various habitats such as plants, soil, water, and animals, with known capacity of causing plant and human disease (102).

Xanthomonadaceae family members, such as *Stenotrophomonas* spp. were detected. This genus is a known plant and human opportunistic pathogen ubiquitously found in natural and anthropogenic environments (103). We also detected Betaproteobacteria isolates, such as *Ralstonia* spp. and *Delftia* spp. Both genera were previously reported agents of human nosocomial infections (104, 105). Actinobacteria phylum members were also identified in this study. This bacteria are heterotrophic typically associated to soil microbiota, but can also be found in animals, being a minor component of the GI tract bacterial community (78). We detected *Propionibacterium acnes* that were previously isolated from human skin and GI tract (106). To our knowledge, no clear relationship between this genus and any GI function has been made, however, a recent study may indicate a relationship between *P. freudenreichii* and the reduction of inflammation markers and gonadal adipose tissue (107).

The Egyptian mongoose specimens analyzed in this study revealed a high level of Proteobacteria (32%) and a low level of Bacteroidetes (not detected in this study), leading to a high ratio Proteobacteria/Bacteroidetes. Such pattern has also been observed in GI tract microbiota of other carnivorous mammals, such as cheetah, Tasmanian devil, spotted hyena, and polar bear (108). Actually, this feature seems to be related to a carnivorous or scavenger dietary regime. Likewise, this ratio pattern has been associated with the high efficient harvest of energy and an increased possibility of obesity development, in both humans and mice (109). Besides, a high ratio promotes the degradation of polysaccharides to short chain fatty acids, increasing acetate and decreasing butyrate production (110).

We only detected four fungi genera, all previously found to be gut commensal of mammals. *Penicillium* and *Naganishia* genera are commensal fungi previously found in Pygmy Loris (111), Yunnan snub-nosed monkeys (112), and humans (113). *Penicillium* spp. form a lot of microscopic spores that are frequently found in air and soil and can easily be inhaled or ingested during the feeding process, being able to grow on GI tract of mammals (113). *Pseudozyma* members are normally found associated with plants, is also part of the normal gut microbiota of giant pandas (114). This genus is also rarely associated with human fungemia, in particular, *P. aphidis* (115). *Mucor* spp. was previously found in GI tract of human individuals, but his function on the gut microbiota is still unknown (113).

The Shannon, Simpson, and Shannon evenness diversity indices allow the appraisal of the balance between the number of individuals and the number of species under analysis in each community (52). These indices were chosen since they represent a coherent system for diversity estimates, and they are also the most common indices used in diversity analysis in microbial ecology studies (52). Regarding the calculated diversity indices, the higher the value, the high is the diversity of the community in the analysis (52). In our study, at the genus level, all three communities (male, female, and total population) have similar values; all can be considered well-balanced communities, with high evenness.

Nonparametric estimators of species richness Chao 1, which analyses species abundance data, and Chao 2, that considers species incidence data, were also calculated (52), evidencing a completeness of the sampling method of 100%, in both cases, suggesting that all 21 genera detected in this study correspond to the total theoretical amount of genera present in the community. All parameters considered, we conclude that the panel of sampled isolates and number of isolates used for 16S rRNA sequencing is adequate for the purposes of this study of characterizing the core microbiota of the Egyptian mongoose population.

PCA is a simple non-parametric method with the main objective of reducing the number of adjacent variables of a complex group of data. This is accomplished by redistributing the original variables in a new group of axes throughout the transformations of the original variables, being these new axes named principal components. As standard procedure in this methodology, 75% of cumulative variance in the first three principal components is the minimum to assume a representability of variables in our analysis. This was not accomplished, so the results must be carefully considered. However, with this analysis, we perceived a gut microbiota community similarity between adult and juvenile specimens. This similarity may be due to the higher proximity and interaction between these two groups since mongoose has a social behavior that implies protection and feeding of the cubs, scent marking and social latrines, increasing diet similarity and fecal-oral transmission of microbiota (34). This type of social behavior may ease host-to-host transmission of microbiota, as was already been observed in captive and wild animals, such as mice, birds, and humans (5). Unfortunately, a clear association between the microbiota and the bio-environmental characteristics was not evident by the statistical analysis of data.

CHAPTER V – CONCLUDING REMARKS AND PERSPECTIVES

Culture-dependent methods can underestimate microbial abundance and diversity, namely due to limitations on the detection of unculturable and fastidious microorganisms. The origin of our samples can also lead to a reduced abundance and diversity, since the time between death and recovery of the carcasses is unknown and sometimes could be very high, leading to a partial autolysis of the sample and a reduction in nutrient supply available for microbial maintenance. Using our methodology, based on the picking of morphologically different colonies, in both rich and selective media and in the morpho-physiological test, allowing for a selection of the most diversity possible for molecular identification and molecular fingerprinting, allowing as to achieve an extensive and comprehensive collection of microbial isolates. The recovered genera were concordant at a population level and the diversity indices calculated were supportive of this idea of a compilation of isolates that represent the core microbiota of the GI tract of the Egyptian mongoose population. However, several studies demonstrated the importance of microbial cultivation, namely Lagier et al. (116) who showed that, when adequate culture conditions are used, a significant number of species may be isolated, particularly those that occur at low abundances, which are not detected in the same samples when using sequencing approaches. Wide-

range culture-dependent methods have the potential to increase our knowledge of this recent field-of-study, giving us the ability to culture bacteria previously unculturable and detect species never reported in the gut communities, and also discover new species (64, 116-119).

For microbial differentiation, we used classic phenotypic methods, namely gram staining combined with enzymatic profiles of energy metabolism enzymes (oxidase and catalase tests) associated with molecular methods. Direct observation of microorganisms has frequently been the first step in identification and differentiation. Gram staining is a useful and cost-effective technique that is commonly used in bacteriology to differentiate between Gram-positive and Gram-negative bacteria, and that was used in initial studies of the gut microbiota (53, 61). However, Gram staining is not a robust method to identify bacteria from the genera *Bacillus, Gemella, Listeria, Mycoplasma, Rickettsia, Chlamydia*, *Mycobacterium*, among others, since these bacteria can display aberrant Gram staining and appear Gram variable (64). Therefore, for more rigorous bacteria identification, we used 16S rDNA amplification and sequencing, which have offered a large opportunity to describe new bacterial species among cultured bacteria and can increase the efficiency of bacterial identification (120, 121).

Most studies of wildlife microbiota highlight the relationship between our knowledge of the microbiota and our ability to help in conservation of endangered species, in both zoos and reintroduction facilities (108). This is obviously an important component of microbiota studies since microbiota can influence several aspects of host biology and ecology (6). Also, several opportunistic pathogens have been driving many endangered species to decline or even to extinction (122). This problem can potentially escalate due to the ability that many of these pathogens have to infect threatened wildlife species through their contact with domestic, livestock and invasive species (122).

However, most studies neglect the undeniable threat of extinction of microbial communities that are associated with critically endangered host (5). Presently one-quarter of mammals species face extinction, and combining this number with other vertebrate species under threat, the probable loss of inherent microbial diversity is overwhelming (5).

Despite the methodological limitations mentioned above, this study represents an extended comprehensive attempt to characterize the microbial composition of GI tract of Egyptian mongoose, in particular, those from South of Portugal, which until now was poorly characterized. Future studies using culture-independent methods will improve our knowledge of this species and lead to a better understanding of its gut microbial community. Moreover, the comparative nature of our study relative to sex-related differences contributes to an increased knowledge of the indirect effect of host biological features, such as behavior, diet, and reproduction, on gut microbiota. Finally, our results reinforce the need to consider the microbiota as a fundamental component of host biology and a key element necessary to understand mammal ecology.

CHAPTER VI – BIBLIOGRAPHY

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CHAPTER VII - APPENDIXES

Supplementary Table 7.1 – Matrix of microbiota variables (OTUs) based in presence/absence of every hierarchical bacterial level.

	HI383	HI388	HI396	HI399	HI460	HI462	HI463	HI466	HI467	HI471	HI501	HI502	HI504	HI505	HI508	HI509	HI516	HI519	HI636	HI675
Bacillus_anthracis	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	1	0	0	0	0
Bacillus_kokeshiiformis	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Clostridium_perfringens	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1
Clostridium_septicum	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
Clostridium_tertium	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0
Delftia_lacustris	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Enterococcus_faecalis	0	0	0	0	0	1	0	1	0	0	0	0	0	1	0	0	1	0	0	0
Enterococcus_faecium	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Enterococcus_mundtii	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Lysinibacillus_fusiformis	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Paenibacillus_borealis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Paeniclostridium_tenue	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Pantoea_eucrina	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Peptoclostridium_bifermentans	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0
Propionibacterium_acnes	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Psychrobacillus_soli	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ralstonia_insidiosa	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0
Ralstonia_pickettii	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0
Robinsoniella_peoriensis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Rummeliibacillus_stabekisii	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	1	0	0	0
Sporosarcina_newyorkensis	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Bacillus	0	0	1	0	0	0	1	1	0	1	1	1	1	0	1	1	1	1	0	0
Carnobacterium	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0

	HI383	HI388	HI396	HI399	HI460	HI462	HI463	HI466	HI467	HI471	HI501	HI502	HI504	HI505	HI508	HI509	HI516	HI519	HI636	HI675
Clostridium	0	1	1	0	1	0	1	0	0	0	1	0	1	0	1	1	1	1	1	1
Delftia	1	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0
Enterococcus	0	1	0	1	0	1	0	1	1	1	1	1	1	1	1	0	1	0	0	0
Lysinibacillus	0	1	0	0	0	0	1	1	0	1	0	0	1	1	1	0	0	0	0	0
Paenibacillus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Paeniclostridium	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0
Pantoea	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Peptoclostridium	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0
Propionibacterium	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas	1	0	1	0	0	1	0	1	1	0	0	0	0	1	0	0	0	1	0	0
Psychrobacillus	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ralstonia	0	0	0	0	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0
Robinsoniella	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Romboutsia	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Rummeliibacillus	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	0	1	0	0	0
Sporosarcina	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
Staphylococcus	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Stenotrophomonas	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0
Bacillaceae	0	1	1	0	0	0	1	1	0	1	1	1	1	1	1	1	1	1	0	0
Burkholderiaceae	0	0	0	0	1	1	1	0	1	1	1	0	0	0	0	0	0	0	0	0
Carnobacteriaceae	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0
Clostridiaceae	0	1	1	0	1	0	1	0	0	0	1	0	1	0	1	1	1	1	1	1
Comamonadaceae	1	0	0	0	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0
Enterobacteriaceae	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0
Enterococcaceae	0	1	0	1	0	1	0	1	1	1	1	1	1	1	1	0	1	0	1	0
Lachnospiraceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0
Xanthomonadaceae	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0

	HI383	HI388	HI396	HI399	HI460	HI462	HI463	HI466	HI467	HI471	HI501	HI502	HI504	HI505	HI508	HI509	HI516	HI519	HI636	HI675
Paenibacillaceae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
Peptostreptococcaceae	1	0	0	1	0	1	0	0	1	0	0	1	0	0	0	0	1	1	0	0
Planococcaceae	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0
Propionibacteriaceae	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudomonadaceae	1	0	1	0	0	1	0	1	1	0	0	0	0	1	0	0	0	1	0	1
Staphylococcaceae	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hafniaceae	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
Actinomycetales	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bacillales	0	1	1	1	0	1	1	1	0	1	1	1	1	1	1	1	1	1	0	0
Burkholderiales	1	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Clostridiales	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	1	1	1	1
Enterobacteriales	1	0	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Lactobacillales	0	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	1	0	1	0
Xanthomonadales	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0
Pseudomonadales	1	0	1	0	0	1	1	1	1	0	0	0	0	1	0	0	0	1	0	1
Bacilli	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Clostridia	1	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	1	1	1	1
Actinobacteria	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Betaproteobacteria	1	0	0	0	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0
Gammaproteobacteria	1	0	1	0	0	1	1	1	1	1	0	1	0	1	0	0	1	1	0	1
Actinobacteria	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Proteobacteria	1	0	1	0	1	1	1	1	1	1	1	1	0	1	0	0	1	1	0	1

Supplementary Table 7.2 – Matrix of 26 biological variables related to sex, age class, reproductive status, stomach content at time of death, and different body measurements.

	HI383	HI388	HI396	HI399	HI460	HI462	HI463	HI466	HI467	HI471	HI501	HI502	HI504	HI505	HI508	HI509	HI516	HI519	HI636	HI675
Sex	1	0	0	0	1	0	1	1	1	1	0	1	0	0	0	1	1	0	0	1
Adult	1	0	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	1	1
Subadult	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1
Lactating	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
Mammal	100	100	100	100	100	96	0	100	77	0	100	100	95	65	97	89	100	100	88	100
Reptil	0	0	0	0	0	4	0	0	20	0	0	0	0	35	3	0	0	0	0	0
Inverteb	0	0	0	0	0	0	29	0	3	0	0	0	0	0	0	0	0	0	13	0
Eggs	0	0	0	0	0	0	0	0	0	100	0	0	0	0	0	0	0	0	0	0
BW	2943	2127	2262	2021	1476	2602	2462	3279	2425	2691	2627	2048	2079	2490	2741	2981	2864	2439	2714	2590
STL	95,5	96,1	97,9	90,5	82	95,2	99,1	104	100,5	104,5	86,7	101,6	87	93,8	99,2	99,2	102,7	101	98,5	94,5
TL	41,7	42,2	42,1	37,2	39,1	45,1	46,4	47	44,2	45,5	41,5	43,2	44,2	41	43,3	43,7	47,5	43,5	46,5	42,6
HBL	53,8	53,9	55,8	53,3	42,9	50,1	52,7	57	56,3	59	45,2	58,4	42,8	52,8	55,9	55,5	55,2	57,5	52	51,9
RHLL	24,2	22,4	22,3	18,9	20,2	21,3	22,7	23,7	24	23,7	23	22,9	21,6	20,2	22,7	23,2	25,4	21,1	23,8	22,2
RHFL	10	9	9,3	8,5	9	9,8	9,5	10,1	9	9,9	9,2	9,9	9	8,4	9,3	9,5	9,9	9,5	9,8	10
SH	19,2	16,1	17,1	15,5	15,5	17,6	17,2	17	18	19	16	16,6	14,5	18,4	18,6	19,8	19	16,5	18	18,2
NP	22,2	18,7	19,8	22	16,4	19,9	20,5	20,8	21,3	20,5	21,5	18,8	18,5	21,2	20	21	20,3	19,7	20,5	19
HD	9	8	8,2	8	7,5	8,5	10	9,5	8,7	9,1	8,7	7	8,1	8,7	9,1	9,4	10	8,5	10	8,4
HW	23	18,5	17,6	19,7	11	19,1	21	26	24,2	28,4	21,6	15,7	9,1	20,1	28	29	16,4	17,2	29,3	15
SW	0,407747	0,667607	0,229885	0,435428	0,223577	0,269024	0,779854	0,445258	0,412371	0,557414	0,437762	0,292969	0,3848	0,453815	0,248085	0,342167	0,240922	0,414104	0,427413	0,525097
KW	10	7,6	6,4	6,9	6,1	7,9	7,4	10	10,1	10,5	12,2	5,4	7	9,4	8,6	11,6	7,6	4,8	10	99999
SFI_2	1	1	1	1	0	0	1	1	0	1	1	1	1	1	1	1	1	1	0	0
SFI_3	1	0	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	0	0	0
SFI_4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
PFI_2	1	1	1	1	1	0	0	1	0	1	1	1	1	1	1	1	1	1	1	0

PFI_3	1	0	1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	0	0
PFI_4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0

Sex: 0 – Female; 1 – Male; Inverteb – Invertebrates; BW – body weight; HBL – head and body length; RHLL – right hind leg length; SH – shoulder height; NP – neck perimeter; HD – head diameter; HW – hearth weight; KW – kidney weight; STL – snout-tail length; TL – tail length (terminal hairs not included); SW – spleen weight; SFI – subcutaneous fat index; PFI – perivisceral fat index; RHFL – right hind foot length. This information was obtained from University of Aveiro, which developed ecological studies with the same specimens (33).

Supplementary Table 7.3 – Matrix of 17 environmental variables related to georeferenced location, land-use, climatic data, road net, river net, and population data.

	HI383	HI388	HI396	HI399	HI460	HI462	HI463	HI466	HI467	HI471	HI501	HI502	HI504	HI505	HI508	HI509	HI516	HI519	HI636	HI675
District	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
Season	1	1	1	1	2	2	2	1	1	1	3	3	3	4	3	3	3	4	2	3
Urban	0,00	0,00	0,00	0,00	0,00	0,00	93,39	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	75,63	75,63	0,00	0,00	0,00
Agroforestry	233,21	233,21	134,86	134,86	0,00	134,86	17,38	126,88	126,88	126,88	134,86	0,41	233,21	126,88	126,88	0,00	0,00	134,86	134,86	126,88
Shrubs	0,00	0,00	92,93	92,93	249,65	92,93	0,00	116,18	116,18	116,18	92,93	0,00	0,00	116,18	116,18	3,59	3,59	92,93	92,93	116,18
Vineyards orchards	0,00	0,00	0,00	0,00	0,00	0,00	169,66	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
Coniferous	0,00	0,00	0,00	0,00	3,55	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	123,37	123,37	0,00	0,00	0,00
Mixforests	125,19	125,19	172,21	172,21	0,00	172,21	0,00	0,00	0,00	0,00	172,21	0,00	125,19	0,00	0,00	7,99	7,99	172,21	172,21	0,00
Agriculture	41,59	41,59	0,00	0,00	131,56	0,00	109,97	156,94	156,94	156,94	0,00	399,59	41,59	156,94	156,94	189,42	189,42	0,00	0,00	156,94
Altimetry	205,97	205,97	201,91	201,91	333,59	201,91	82,23	189,38	189,38	189,38	201,91	217,17	205,97	189,38	189,38	201,03	201,03	201,91	201,91	189,38
Population	0,23	0,23	0,69	0,69	17,15	0,69	736,61	0,91	0,91	0,91	0,69	0,28	0,23	0,91	0,91	475,74	475,74	0,69	0,69	0,91
RoadNet	5187,56	5187,56	0,00	0,00	22489,47	0,00	30776,41	1245,14	1245,14	1245,14	0,00	2588,37	5187,56	1245,14	1245,14	22917,77	22917,77	0,00	0,00	1245,14
RiversNet	1534,80	1534,80	2725,91	2725,91	0,00	2725,91	1696,32	3534,81	3534,81	3534,81	2725,91	2171,90	1534,80	3534,81	3534,81	0,00	0,00	2725,91	2725,91	3534,81
Average_annual_temperature	162,67	162,67	166,17	166,17	156,00	166,17	162,33	168,25	168,25	168,25	166,17	162,67	162,67	168,25	168,25	167,00	167,00	166,17	166,17	168,25
Average_temperature_range	235,33	235,33	256,00	256,00	257,50	256,00	207,67	268,50	268,50	268,50	256,00	244,17	235,33	268,50	268,50	271,33	271,33	256,00	256,00	268,50

Annual_rainfall	633,33	633,33	531,00	531,00	898,75	531,00	761,17	527,00	527,00	527,00	531,00	605,83	633,33	527,00	527,00	534,50	534,50	531,00	531,00	527,00
NDVI	0,61	0,40	0,39	0,39	0,50	0,39	0,47	0,48	0,48	0,48	0,46	0,70	0,71	0,41	0,41	0,59	0,60	0,39	0,60	0,48

District: 0- Setúbal; 1 – Beja. Seasson: 1 - Spring; 2 – Summer; 3 – Autumn; 4 – Winter. Number of hectares of each habitat type (urban, rice fields, agroforestry, shrubs, inland water bodies, vineyards and orchards, coniferous, broadleaved and mix forests and agriculture areas) were retrieved from Corine Land Cover (2006) with spatial resolution of 250m. Mean altimetry value (data SRTM, NASA, resolution of 30 m) was gathered using the ASTER Global Digital Elevation Model platform (http://gdem.ersdac.jspacesystems.or.jp/) (ASTER, 2016). Number of inhabitants per km^2 in each grid was investigated using data from Eurostat per kilometer (http://epp.eurostat.ec.europa.eu/) (European Commission, 2016), distance in meters of road and hydrographic network, respectively with data from Instituto Geográfico Português (www.igeo.pt/) and SNIRH (http://snirh.apambiente.pt/) (SNIRH, 2016). Average annual temperature in degrees multiplied by 10 and annual rainfall in mm were gathered from BioClim (http://www.worldclim.org/bioclim) at 30arc-second resolution (Hijmans et al., 2005). Finally, primary productivity based on Normalized Difference Vegetation Index (NDVI) of each collected sample location was used as a proxy of primary productivity (Pettorelli, 2013). The NDVI value for each record was calculated from satellite images supplied by MODIS (2016) (Moderate Resolution Imaging Spectroradiometer; http://modis.gsfc.nasa.gov) at a spatial resolution of 250 m. These variables were represented by mean values of the 2×2 km grid cell, considering the home-range of the Egyptian mongoose. This information was obtained from University of Aveiro, which developed ecological studies with the same specimens (33).

Supplementary Table 7.4 – Statistical significant results from data represented in Figures 3.6, 3.7 and 3.8.

<table>
<tr><th>Growth Medium</th><th>Host</th><th>Microbial load</th><th>MT</th><th>Significantly different from (Number of hosts)</th></tr>
<tr><td rowspan="4">YCFA w/ O_2</td><td>HI383
HI388
HI509</td><td>↓</td><td>II</td><td>5</td></tr>
<tr><td>HI509</td><td>↑</td><td>V</td><td>16</td></tr>
<tr><td>HI583</td><td>↑</td><td>VII</td><td>18, except HI467</td></tr>
<tr><td>HI388</td><td>↑</td><td>IX</td><td>18, except HI460</td></tr>
<tr><td rowspan="6">YCFA P w/ O_2</td><td>HI383
HI399
HI462
HI467
HI509</td><td colspan="3">No microbial growth</td></tr>
<tr><td>HI463
HI508
HI675</td><td>↑</td><td>II</td><td>13</td></tr>
<tr><td>HI519</td><td>↑</td><td>V</td><td>18, except HI502</td></tr>
<tr><td>HI396</td><td rowspan="3">↑</td><td rowspan="3">VI</td><td>9</td></tr>
<tr><td>HI466
HI471</td><td>11</td></tr>
<tr><td>HI636</td><td>13</td></tr>
<tr><td rowspan="3">YCFA w/o O_2</td><td>HI396
HI466
HI471
HI505</td><td>↑</td><td rowspan="2">II</td><td>5</td></tr>
<tr><td>HI388
HI399
HI463
HI504
HI509</td><td>↓</td><td>3</td></tr>
<tr><td>HI463
HI504</td><td>↑</td><td>VII</td><td>9</td></tr>
</table>

Growth Medium	Host	Microbial load	MT	Significantly different from (Number of hosts)
YCFA P w/o O_2	HI509	↑	II	10
	HI460			
	HI466			
	HI388	↑	VII	6
	HI462			
	HI463			
	HI504			
	HI509			
	HI675			
	HI383	↓		
	HI396			
	HI460			
	HI466			
	HI471			
	HI502			
	HI383	↑	IX	18, except HI501
	HI396			
ESBL w/o AS	HI466	↑	II	11
	HI509*	↑	VII	All
	HI396*	↑	VIII	All
	HI505*			
	HI383	↑	XI	9
	HI460			11
	HI516			
	HI519			
	HI636			
	HI675			
	HI501	Only with MT-Vibrium		
ESBL w/ AS	HI383	No microbial growth		
	HI509	↓	II	9
	HI519			
	HI675			
	HI509	↑	VII	10
	HI519			
	HI675			
Sum of all media	HI501	↑	IX	9
	HI504			8
	HI509			5
	HI460	↑	XI	12
	HI519			

These results were obtained from a statistical analysis of data represented in Figures 3.6, 3.7, and 3.8, using a two-way ordinary ANOVA ($\alpha=0.05$), with a Tukey's Multiple Comparison post-test and had a $p\text{-value}<0.05$. ↑ - higher microbial load; ↓ - lower microbial load. * These results had a $p\text{-value}<0.01$.

Supplementary Table 7.5 – Information on the 16S rDNA nucleotide sequences of a selected group of bacterial isolates.

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
8	462	IX	1087	Pseudomonas plecoglossicida strain NBRC 103162 16S ribosomal RNA gene, partial sequence	99%	0.0	99%	NR_114226.1	Pseudomonas
9	467	VII	638	[Clostridium] bifermentans strain JCM 1386 16S ribosomal RNA gene, partial sequence	97%	0.0	99%	NR_113323.1	Paraclostridium
11	466	II	246	Enterococcus faecium strain NBRC 100486 16S ribosomal RNA gene, partial sequence	95%	0.0	99%	NR_113904.1	Enterococcus
22	462	I	1136	Staphylococcus saprophyticus subsp. bovis strain GTC 843 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	NR_041324.1	Staphylococcus
29	462	V	582	Shigella sonnei strain CECT 4887 16S ribosomal RNA gene, partial sequence	99%	0.0	99%	NR_104826.1	Enterobacteriaceae
43	467	IX	1049	Clostridium baratii strain IP 2227 16S ribosomal RNA gene, complete sequence	100%	0.0	99%	NR_029229.1	Clostridium baratii

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
72	467	VII	1036	[Clostridium] sordellii strain JCM 3814 16S ribosomal RNA gene, partial sequence	100%	0.0	98%	NR_113140.1	Peptostreptococcaceae
79	399	VII	1095	Romboutsia lituseburensis strain ATCC 25759 16S ribosomal RNA gene, partial sequence	99%	0.0	98%	NR_118728.1	Romboutsia
84	462	VII	455	[Clostridium] bifermentans strain JCM 1386 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	NR_113323.1	Paraclostridium bifermentans
91	501	IX	400	Bacillus anthracis strain SBS1 16S ribosomal RNA gene, partial sequence	87%	2,00E-149	99%	NR_118536.1	Bacillus anthracis
101	519	VII	617	[Clostridium] bifermentans strain JCM 1386 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	NR_113323.1	Paraclostridium bifermentans

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
116	636	II	600	Enterococcus faecalis strain NBRC 100480 16S ribosomal RNA gene, partial sequence	100%	0.0	94%	NR_113901.1	Enterococcaceae
117	636	VII	955	Clostridium tertium strain JCM 6289 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	NR_113325.1	Clostridium tertium
131	516	VII	1018	[Clostridium] bifermentans strain JCM 1386 16S ribosomal RNA gene, partial sequence	99%	0.0	99%	NR_113323.1	Paraclostridium bifermentans
137	516	III	719	Stenotrophomonas maltophilia strain ATCC 13637 16S ribosomal RNA gene, partial sequence	100%	0.0	98%	NR_112030.1	Stenotrophomonas
147	501	VII	958	Bacillus thuringiensis strain NBRC 101235 16S ribosomal RNA gene, partial sequence	99%	0.0	98%	NR_112780.1	Bacillus

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
170	396	IX	1062	Pseudomonas plecoglossicida strain NBRC 103162 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	NR_114226.1	Pseudomonas
184	462	II	629	Pseudomonas alcaligenes strain IAM 12411 16S ribosomal RNA gene, complete sequence	52%	3,00E-85	82%	NR_043419.1	Pseudomonadales
205	467	III	675	Hafnia alvei strain JCM 1666 16S ribosomal RNA gene, partial sequence	99%	0.0	99%	NR_112985.1	Hafniaceae
215	466	IX	966	Pseudomonas nitritireducens strain WZBFD3-5A2 16S ribosomal RNA, partial sequence	99%	0.0	99%	NR_133020.1	Pseudomonas
221	396	IX	400	Pseudomonas nitroreducens strain IAM1439 16S ribosomal RNA gene, complete sequence	96%	0.0	98%	NR_115611.1	Pseudomonas

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
225	467	III	566	Ralstonia pickettii strain NBRC 102503 16S ribosomal RNA gene, partial sequence	100%	0.0	98%	NR_114126.1	Ralstonia
235	466	II	992	Enterococcus faecalis strain NBRC 100480 16S ribosomal RNA gene, partial sequence	99%	0.0	99%	NR_113901.1	Enterococcus faecalis
246	466	IX	474	Lysinibacillus sphaericus strain NBRC 15095 16S ribosomal RNA gene, partial sequence	100%	0.0	98%	NR_112627.1	Lysinibacillus
248	462	IX	1014	Shigella boydii strain P288 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	NR_104901.1	Enterobacteriaceae
260	462	VII	1032	Delftia lacustris strain 332 16S ribosomal RNA gene, partial sequence	99%	0.0	99%	NR_116495.1	Delftia

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
271	396	IX	901	Pseudomonas nitritireducens strain WZBFD3-5A2 16S ribosomal RNA, partial sequence	97%	0.0	97%	NR_133020.1	Pseudomonas
293	505	IX	1043	Pseudomonas plecoglossicida strain NBRC 103162 16S ribosomal RNA gene, partial sequence	99%	0.0	99%	NR_114226.1	Pseudomonas
302	396	IX	1070	Bacillus anthracis strain ATCC 14578 16S ribosomal RNA gene, partial sequence	100%	0.0	98%	NR_041248.1	Bacillus
320	501	VII	1102	Bacillus thuringiensis strain NBRC 101235 16S ribosomal RNA gene, partial sequence	98%	0.0	99%	NR_112780.1	Bacillus
325	501	VII	656	Bacillus marcorestinctum strain LQQ 16S ribosomal RNA gene, partial sequence	93%	0.0	92%	NR_117414.1	Bacillaceae

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
340	516	VII	937	Bacillus toyonensis strain BCT-7112 16S ribosomal RNA gene, complete sequence	100%	0.0	99%	NR_121761.1	Bacillus
344	516	II	248	Enterococcus faecalis strain NBRC 100480 16S ribosomal RNA gene, partial sequence	96%	2E-121	99%	NR_113901.1	Enterococcus faecalis
349	462	VII	1052	Shigella boydii strain P288 16S ribosomal RNA gene, partial sequence	99%	0.0	99%	NR_104901.1	Enterobacteriaceae
366	399	VII	1073	Enterococcus faecium strain DSM 20477 16S ribosomal RNA gene, complete sequence	99%	0.0	99%	NR_114742.1	Enterococcus
388	388	II	1050	Enterococcus hirae strain ATCC 9790 16S ribosomal RNA gene, complete sequence	100%	0.0	99%	NR_075022.1	Enterococcus

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
456	516	OTHER	676	Rummeliibacillus stabekisii strain NBRC 104870 16S ribosomal RNA gene, partial sequence	98%	0.0	99%	NR_114270.1	Rummeliibacillus stabekisii
462	516	VIII	951	Rummeliibacillus stabekisii strain NBRC 104870 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	NR_114270.1	Rummeliibacillus stabekisii
477	501	VIBRIUM	606	Ralstonia pickettii strain NBRC 102503 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	NR_114126.1	Ralstonia pickettii
492	504	II	1094	Enterococcus faecium strain DSM 20477 16S ribosomal RNA gene, complete sequence	99%	0.0	99%	NR_114742.1	Enterococcus

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
505	508	II	950	Pseudomonas nitritireducens strain WZBFD3-5A2 16S ribosomal RNA, partial sequence	97%	0.0	97%	NR_133020.1	Pseudomonas
506	508	XI	1110	Carnobacterium gallinarum strain DSM 4847 16S ribosomal RNA gene, complete sequence	99%	0.0	98%	NR_042093.1	Carnobacterium
509	508	IX	1150	Lysinibacillus fusiformis strain NBRC15717 16S ribosomal RNA gene, partial sequence	100%	0.0	98%	NR_112569.1	Lysinibacillus
531	505	V	122	Lysinibacillus alkaliphilus strain OMN17 16S ribosomal RNA, partial sequence	71%	3,00E-37	98%	NR_136779.1	Lysinibacillus
541	636	II	491	Enterococcus hirae strain LMG 6399 16S ribosomal RNA gene, complete sequence	99%	0.0	99%	NR_114783.2	Enterococcus

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
589	504	VII	401	Bacillus toyonensis strain BCT-7112 16S ribosomal RNA gene, complete sequence	99%	0.0	98%	NR_121761.1	Bacillus
596	508	VIII	500	Bacillus pumilus strain NRRL NRS-272 16S ribosomal RNA gene, partial sequence	100%	0.0	98%	NR_116191.1	Bacillus
611	466	II	974	Enterococcus faecium strain NBRC 100486 16S ribosomal RNA gene, partial sequence	100%	0.0	98%	NR_113904.1	Enterococcus
626	467	II	401	Enterococcus faecium strain DSM 20477 16S ribosomal RNA gene, complete sequence	100%	0.0	100%	NR_114742.1	Enterococcus faecium
627	467	OTHER	1047	Delftia lacustris strain 332 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	NR_116495.1	Delftia lacustris

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
629	467	OTHER	851	Pseudomonas nitroreducens strain NBRC 12694 16S ribosomal RNA gene, partial sequence	96%	0.0	98%	NR_113601.1	Pseudomonas
630	467	OTHER	410	Stenotrophomonas maltophilia strain NBRC 14161 16S ribosomal RNA gene, partial sequence	60%	5,00E-110	95%	NR_113648.1	Xanthomonadaceae
640	462	II	912	Enterococcus durans strain JCM 8725 16S ribosomal RNA gene, partial sequence	100%	0.0	98%	NR_113257.1	Enterococcus
665	383	IX	980	Pseudomonas nitritireducens strain WZBFD3-5A2 16S ribosomal RNA, partial sequence	99%	0.0	98%	NR_133020.1	Pseudomonas

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
680	502	V	224	Bacillus thuringiensis strain NBRC 101235 16S ribosomal RNA gene, partial sequence	99%	2,00E-111	99%	NR_112780.1	Bacillus
687	502	OTHER	408	Stenotrophomonas maltophilia strain ATCC 19861 16S ribosomal RNA gene, complete sequence	99%	0.0	96%	NR_040804.1	Stenotrophomonas
690	502	OTHER	584	Stenotrophomonas maltophilia strain ATCC 13637 16S ribosomal RNA gene, partial sequence	100%	0.0	97%	NR_112030.1	Stenotrophomonas
691	502	OTHER	740	Stenotrophomonas maltophilia strain ATCC 13637 16S ribosomal RNA gene, partial sequence	99%	0.0	98%	NR_112030.1	Stenotrophomonas

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
734	471	VII	1039	Bacillus toyonensis strain BCT-7112 16S ribosomal RNA gene, complete sequence	98%	0.0	99%	NR_121761.1	Bacillus
738	471	IV	1100	Ralstonia insidiosa strain AU2944 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	NR_025242.1	Ralstonia insidiosa
746	471	V	236	Rummeliibacillus stabekisii strain NBRC 104870 16S ribosomal RNA gene, partial sequence	95%	1E-114	99%	NR_114270.1	Rummeliibacillus stabekisii
747	471	V	400	Sporosarcina soli strain I80 16S ribosomal RNA gene, partial sequence	95%	0.0	98%	NR_043527.1	Sporosarcina
756	460	XI	678	Ralstonia pickettii strain NBRC 102503 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	NR_114126.1	Ralstonia pickettii

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
802	463	XI	668	Ralstonia insidiosa strain AU2944 16S ribosomal RNA gene, partial sequence	99%	0.0	99%	NR_025242.1	Ralstonia insidiosa
805	463	VII	360	Bacillus anthracis strain ATCC 14578 16S ribosomal RNA gene, partial sequence	97%	0.0	100%	NR_041248.1	Bacillus anthracis
837	508	V	752	Bacillus cereus ATCC 14579 16S ribosomal RNA (rrnA) gene, complete sequence	100%	0.0	99%	NR_074540.1	Bacillus
852	675	II	477	Pseudomonas nitritireducens 16S ribosomal RNA, partial sequence	84%	2E-160	92%	NR_133020.1	Pseudomonadaceae
864	471	VIII	968	Lysinibacillus sphaericus 16S ribosomal RNA gene, partial sequence	99%	0.0	98%	NR_112627.1	Lysinibacillus

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
879	505	II	571	Lysinibacillus sphaericus strain NBRC 15095 16S ribosomal RNA gene, partial sequence	100%	0.0	98%	NR_112627.1	Lysinibacillus
889	504	VIII	1145	Lysinibacillus sphaericus strain NBRC 15095 16S ribosomal RNA gene, partial sequence	100%	0.0	98%	NR_112627.1	Lysinibacillus
915	516	II	252	Enterococcus faecalis strain NBRC 100480 16S ribosomal RNA gene, partial sequence	94%	1E-113	98%	NR_113901.1	Enterococcus
919	463	VI	220	Rummeliibacillus stabekisii 16S ribosomal RNA gene, partial sequence	98%	3,00E-110	99%	NR_114270.1	Rummeliibacillus stabekisii

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
928	502	VI	139	Geobacillus thermoglucosidasius strain R-35637 16S ribosomal RNA gene, partial sequence	61%	3,00E-28	92%	NR_116983.1	Bacillaceae
942	466	VI	447	Bacillus cereus ATCC 14579 16S ribosomal RNA (rrnA) gene, complete sequence	100%	0.0	100%	NR_074540.1	Bacillus
952	502	VI	412	Romboutsia lituseburensis strain ATCC 25759 16S ribosomal RNA gene, partial sequence	100%	0.0	98%	NR_118728.1	Romboutsia
953G	502	OTHER	801	Pseudomonas nitroreducens strain NBRC 12694 16S ribosomal RNA gene, partial sequence	99%	0.0	98%	NR_113601.1	Pseudomonas

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
956	502	V	312	Bacillus subtilis subsp. inaquosorum strain BGSC 3A28 16S ribosomal RNA gene, partial sequence	97%	4,00E-160	100%	NR_104873.1	Bacillus
963	504	VI	396	Rummeliibacillus stabekisii strain NBRC 104870 16S ribosomal RNA gene, partial sequence	98%	0.0	99%	NR_114270.1	Rummeliibacillus stabekisii
966	463	II	630	Pseudomonas taiwanensis strain BCRC 17751 16S ribosomal RNA gene, partial sequence	61%	6,00E-106	85%	NR_116172.1	Pseudomonadales
978	471	VI	677	Bacillus toyonensis strain BCT-7112 16S ribosomal RNA gene, complete sequence	99%	0.0	99%	NR_121761.1	Bacillus

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
998	516	IX	637	Solibacillus silvestris16S ribosomal RNA gene, partial sequence	99%	0.0	99%	NR_028865.1	Solibacillus
1001	388	VIII	448	Lysinibacillus xylanilyticus 16S ribosomal RNA gene, partial sequence	93%	0.0	98%	NR_116698.1	Lysinibacillus
1004	388	VI	903	Psychrobacillus soli 16S ribosomal RNA, partial sequence	89%	0.0	99%	NR_137244.1	Psychrobacillus soli
1020	383	VII	86	Uncultured bacterium partial 16S rRNA gene	33%	3,00E-04	100%	LT173941.1	Enterobacteriaceae
1023	383	VII	400	Delftia tsuruhatensis strain NBRC 16741 16S ribosomal RNA gene, partial sequence	99%	0.0	99%	NR_113870.1	Delftia

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
1029	467	II	300	Delftia tsuruhatensis 16S ribosomal RNA gene, partial sequence	99%	4,00E-100	89%	NR_113870.1	Comamonadaceae
1040	467	V	655	Bacillus cereus 16S ribosomal RNA gene, complete sequence	100%	0.0	99%	NR_074540.1	Bacillus
1042	462	V	681	Shigella boydii 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	NR_104901.1	Enterobacteriaceae
1047	462	I	945	Enterococcus faecalis 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	NR_113901.1	Enterococcus faecalis

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
1071	466	VII	86	Delftia deserti strain YIM Y792 16S ribosomal RNA, partial sequence	91%	1,00E-35	100%	NR_136837.1	Delftia
1073	466	II	948	Bacillus anthracis strain ATCC 14578 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	NR_041248.1	Bacillus
1110	388	II	356	Clostridium tertium strain JCM 6289 16S ribosomal RNA gene, partial sequence	100%	0.0	100%	NR_113325.1	Clostridium tertium
1120	508	VII	325	Clostridium tertium strain JCM 6289 16S ribosomal RNA gene, partial sequence	98%	5,00E-159	98%	NR_113325.1	Clostridium
1124	508	II	1085	Enterococcus faecium strain DSM 20477 16S ribosomal RNA gene, complete sequence	100%	0.0	99%	NR_114742.1	Enterococcus

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
1153	388	V	400	Propionibacterium acnes strain ATCC 6919 16S ribosomal RNA, complete sequence	100%	0.0	99%	NR_040847.1	Propionibacterium acnes
1162	508	II	1005	Enterococcus faecium strain NBRC 100486 16S ribosomal RNA gene, partial sequence	98%	0.0	99%	NR_113904.1	Enterococcus
1164	508	VII	1026	Robinsoniella peoriensis strain PPC31 16S ribosomal RNA gene, complete sequence	100%	0.0	99%	NR_041882.1	Robinsoniella peoriensis
1185	516	IX	900	Enterococcus faecalis strain NBRC 100480 16S ribosomal RNA gene, partial sequence	100%	0.0	98%	NR_113901.1	Enterococcus
1188	471	IX	774	Pantoea eucrina strain LMG 2781 16S ribosomal RNA gene, partial sequence	97%	0.0	99%	NR_116246.1	Pantoea eucrina

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
1202	504	VII	233	Clostridium tertium strain JCM 6289 16S ribosomal RNA gene, partial sequence	94%	2,00E-111	99%	NR_113325.1	Clostridium tertium
1295	501	II	826	Enterococcus faecium strain NBRC 100486 16S ribosomal RNA gene, partial sequence	99%	0.0	99%	NR_113904.1	Enterococcus
1319	501	II	224	Enterococcus faecium strain NBRC 100486 16S ribosomal RNA gene, partial sequence	98%	2,00E-112	100%	NR_113904.1	Enterococcus
1321	501	IX	1052	Clostridium septicum 16S ribosomal RNA gene, complete sequence	100%	0.0	98%	NR_026020.1	Clostridium

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
1343	519	X	323	Romboutsia sedimentorum strain LAM201 16S ribosomal RNA, partial sequence	90%	2,00E-132	96%	NR_134800.1	Romboutsia
1353	519	IV	220	Paenibacillus borealis 16S ribosomal RNA gene, complete sequence	96%	2,00E-107	99%	NR_025299.1	Paenibacillus borealis
1359	502	IV	998	Enterococcus durans 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	NR_113257.1	Enterococcus
1362	502	X	940	[Eubacterium] tenue 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	NR_115794.1	Paeniclostridium tenue

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
1376	471	IV	1072	Bacillus kokeshiiformis strain MO-04 16S ribosomal RNA, partial sequence	99%	0.0	99%	NR_133975.1	Bacillus kokeshiiformis
1377	516	VII	234	Clostridium tertium strain JCM 6289 16S ribosomal RNA gene, partial sequence	94%	1E-109	99%	NR_113325.1	Clostridium tertium
1381	509	VII	1037	Clostridium septicum strain Pasteur III 16S ribosomal RNA gene, complete sequence	100%	0.0	99%	NR_026020.1	Clostridium septicum
1388	463	VII	214	Clostridium perfringens strain JCM 1290 16S ribosomal RNA gene, partial sequence	98%	4,00E-103	98%	NR_113204.1	Clostridium

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype*
1398	675	II	215	Enterococcus faecium strain NBRC 100486 16S ribosomal RNA gene, partial sequence	99%	1,00E-108	99%	NR_113904.1	Enterococcus
1404	463	VII	1074	Carnobacterium mobile strain DSM 4848 16S ribosomal RNA gene, partial sequence	99%	0.0	98%	NR_040926.1	Carnobacterium
1409	471	II	415	Enterococcus faecium strain NBRC 100486 16S ribosomal RNA gene, partial sequence	100%	0.0	99%	NR_113904.1	Enterococcus
1421	516	VII	774	[Clostridium] sordellii strain JCM 3814 16S ribosomal RNA gene, partial sequence	99%	0.0	98%	NR_113140.1	Paeniclostridium

[1]Based on morphological (cell morphology, Gram, and endospore staining) and biochemical tests (catalase and oxidase tests).
*Phylotype assignment based on information from top three best matches displaying the higher nucleotide pairwise identity, using taxonomic threshold similarity values as discussed in Material and Methods chapter.

Supplementary Table 7. 6 – Information on the ITS nucleotide sequences of a selected group of fungi isolates.

Isolate	Animal	Phenotypic Identification[1]	Nucleotide Sequence Length	Closest Reference Sequence Match	Query cover	E-value	Nucleotide Sequence Identity	Accession Number	Assigned Phylotype[2]
1205	471	XIII	400	Pseudozyma sp. JS1231 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 26S rRNA gene (partial), isolate 1231	80%	5,00E-134	94%	AM176740.1	Pseudozyma
1206	675	XIII	400	Mucor circinelloides strain S032IMR 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence	100%	0.0	99%	KU198340.1	Mucor circinelloides
1212	509	XII	459	Cryptococcus albidus var. kuetzingii culture-collection CBS:6086 large subunit ribosomal RNA gene, partial sequence	98%	0.0	99%	KY106964.1	Cryptococcus albidus/ Naganishia albida
1213	463	XII	400	Cryptococcus albidus var. kuetzingii strain YM26709 26S ribosomal RNA gene, partial sequence	62%	1,00E-105	95%	KY463404.1	Cryptococcus albidus/ Naganishia albida
1331	460	XIII	400	Penicillium citreonigrum strain SFCF20120912-25 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	90%	2,00E-163	96%	KF313080.1	Penicillium
1334	463	XIII	482	Penicillium amaliae strain CV401 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	100%	0.0	99%	JX091440.1	Penicillium amaliae

[1]Based on morphological (hyphal septation and spores color, morphology, and septation, cell morphology, and division) tests.
[2]Phylotype assignment based on information from top three best matches displaying the higher nucleotide pairwise identity, using taxonomic threshold similarity values as discussed in Material and Methods chapter.