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University of the Witwatersrand

Faculty of Health Science

School of Pathology

Characterising the native midgut bacteria from main African malaria vector species, *Anopheles arabiensis* and *An. funestus*

Bianca E Silva

A Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine.

Johannesburg, 2021

DECLARATION



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PUBLICATIONS ARISING FROM THIS STUDY

1. Submitted a manuscript (29 November 2020) entitled “Microbiota identified from preserved *Anopheles*” to *Malaria Journal*.

PRESENTATIONS ARISING FROM THIS STUDY

1. Molecular Biosciences Research Thrust (MBRT) Annual Research Day, September 2019, University of the Witwatersrand, South Africa. Poster presentation.
2. PAMCA-VectorBase Students and Young Investigators Virtual Vector Meeting, December 2020, International, Virtual Conference. Poster presentation.

ABSTRACT

Malaria predominantly affects the African continent. The dominant African malaria vectors include mosquito species from the *Anopheles* (*An.*) *gambiae* complex and the *An. funestus* group. Mosquito midgut microbiota play essential roles in physiology and disease transmission. Thus, if common bacteria exist amongst vector species, this could be exploited for malaria control. Accordingly, this study characterised the native midgut bacteria of *An. arabiensis* (member of *An. gambiae* complex) and *An. funestus* (member of *An. funestus* group). Additionally, field-collected mosquitoes are routinely preserved and transported to a laboratory for analysis and thus far, it is unknown if midgut bacteria can be identified from preserved *Anopheles*. Therefore, this study also investigated if midgut bacteria could be identified after mosquito preservation.

Midgut bacteria were identified using culture-dependent (midgut dissections, culturomics, MALDI-TOF MS) and culture-independent techniques (midgut dissections, bacterial DNA extraction, next generation sequencing). Bacteria were identified from colonised mosquitoes that were fresh (non-preserved) and preserved on silica desiccant or in RNA*later*® solution. Bacteria were also characterised from field-collected *An. arabiensis* from KwaZulu-Natal and Mpumalanga.

Results show that midgut bacteria can be characterised from preserved *Anopheles* samples. *Aeromonas hydrophila*, *Elizabethkingia anophelis*, *Enterobacter cloacae*, *Herbaspirillum huttiense*, *Pseudomonas grimontii*, *Psychrobacter* species, *Raoultella ornithinolytica*, *Serratia oryzae*, and *Staphylococcus epidermidis* were bacteria isolated from both vectors. Of these, *S. epidermidis* was also identified in field-collected *An. arabiensis*, making this a promising bacterium for future studies investigating the use of mosquito midgut bacteria for disease control. Overall, this study characterised the common midgut bacteria between *An. arabiensis* and *An. funestus* and discovered that midgut bacteria could be identified from preserved *Anopheles*.

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TABLE OF CONTENTS

DECLARATION……..	ii
PUBLICATIONS ARISING FROM THIS STUDY……	iii
PRESENTATIONS ARISING FROM THIS STUDY…….	iii
ABSTRACT……..	iv
ACKNOWLEDGEMENTS……..	v
LIST OF FIGURES……..	ix
LIST OF TABLES……..	xi
LIST OF ACRONYMS ABBREVIATIONS.…….	xii
CHAPTER ONE: INTRODUCTION	**1**
1.1. Malaria…….…….……	1
1.1.1. The Vector-Parasite Relationship……	2
1.2. Mosquito Midgut Microorganisms…….…….……..	3
1.2.1. *Anopheles* Midgut Bacteria…….……	4
1.2.1.1. Development and Homeostasis……...	4
1.2.1.2. Digestion and Nutrition……..	5
1.2.1.3. Immunity and Vector Competence…….	5
1.2.1.4. Insecticide Resistance…….	6
1.2.1.5. Reproduction and Behaviour……..	6
1.2.2. Midgut Bacterial Diversity and Composition……..	7
1.2.3. Characterising the Midgut Bacteria…….	8
1.3. Symbiotic Control…….…….……	10
1.4. Mosquito Preservation Methods…….……..	11
1.5. Aims and Objectives…….…….	13
CHAPTER TWO: MATERIALS AND METHODS	**14**
2.1. Biological Material……	14
2.1.1. Colonised *Anopheles*……	14
2.1.2. Field-collected *Anopheles*…….	15
2.2. Mosquito Preservation……...	17
2.2.1. Silica Preservation……	17
2.2.2. RNA*later*® Preservation……	17
2.3. Midgut Dissections……	18
2.3.1. Dissections of Preserved Midgut…….	19
2.4. Culture-dependent Techniques……..	20
2.4.1. Culturomics……..	20
2.4.2. Mass Spectrometry (MALDI-TOF MS)……..……	21
2.5. Culture-independent Techniques……...……	22

2.5.1. Bacterial DNA Extraction……..……....	22
2.5.2. Next Generation Sequencing (NGS)…….……....	24
2.6. Data Analysis, Diversity Indices and Statistical Analysis…….……	24
2.6.1. MALDI-TOF MS MBT 7854 MSP Library……...……..	24
2.6.2. Diversity Indices……...……	26
2.6.3. Statistical Analysis……...	26
CHAPTER THREE: RESULTS	**28**
3.1. Midgut Bacteria from Colonised and Field-collected *Anopheles arabiensis*……	28
3.1.1. Bacteria from *Anopheles arabiensis* and the Larval Rearing Water……	28
3.1.2. *Anopheles arabiensis* Midgut Bacteria Identified After Mosquito Preservation…	32
3.1.3. Midgut Bacteria of *Anopheles arabiensis* from KwaZulu-Natal and Mpumalanga..	36
3.1.3.1. Bacteria from Field-collected *Anopheles arabiensis* from KwaZulu-Natal.	36
3.1.3.2. Bacteria from Field-collected *Anopheles arabiensis* from Mpumalanga…	38
3.1.3.3. Comparison of *Anopheles arabiensis* from KwaZulu-Natal and Mpumalanga……	39
3.2. Midgut Bacteria from Colonised *Anopheles funestus*…….……	40
3.2.1. Bacteria from Fresh *Anopheles funestus* and the Larval Rearing Water……	41
3.2.2. *Anopheles funestus* Midgut Bacteria Identified After Mosquito Preservation……	45
3.3. Comparison of Midgut Bacteria between Colonised *Anopheles arabiensis* and *Anopheles funestus*……	49
3.3.1. Midgut Bacteria between Fresh *Anopheles arabiensis* and *Anopheles funestus*……	50
3.3.2. Midgut Bacteria between Preserved *Anopheles arabiensis* and *Anopheles funestus.*	58
3.3.3. Overview: Midgut Bacteria from *Anopheles arabiensis* and *Anopheles funestus*…	70
CHAPTER FOUR: DISCUSSION	**75**
4.1. Expanding on Existing Literature of the *Anopheles* Midgut Microbiota……	76
4.2. Novel Midgut Bacteria Identified from *Anopheles arabiensis* and *Anopheles funestus*……	79
4.3. Midgut Bacteria can be Identified from Preserved *Anopheles*……	83
4.4. Limitations…….	85
4.5. Future Studies……	87
CONCLUSION	**89**
REFERENCES	**91**

APPENDICES **118**

APPENDIX A: Ethics Waiver…….. 118

APPENDIX B: Bacteria Identified in this Study…….. 120

APPENDIX C: Calculations: Diversity, Relative Abundance and Evenness Indices…… 143

APPENDIX D: Phyla and Genera of *Anopheles arabiensis* Midguts…….. 144

APPENDIX E: Midgut Bacteria Identified from Preserved *Anopheles arabiensis*…….. 146

APPENDIX F: Common bacteria from fresh and preserved *Anopheles arabiensis*…… 152

APPENDIX G: Midgut Bacteria of Field-collected *Anopheles arabiensis*…….. 154

APPENDIX H: Phyla and Genera of *Anopheles funestus* Midguts…….. 158

APPENDIX I: Midgut Bacteria Identified from Preserved *Anopheles funestus*…….. 160

APPENDIX J: Common bacteria from fresh and preserved *Anopheles funestus*……. 167

APPENDIX K: Fresh *Anopheles arabiensis* and *Anopheles funestus*…….. 169

APPENDIX L: Preserved *Anopheles arabiensis* and *Anopheles funestus*……... 175

APPENDIX M: Midgut Bacteria and Diversity at Preservation Time Periods…… 184

APPENDIX N: Fresh and Preserved *Anopheles arabiensis* and *Anopheles funestus*…….. 205

TURNITIN REPORT…… 209

LIST OF FIGURES

CHAPTER ONE

Figure 1.1	Lifecycle of the *Plasmodium* parasite in the *Anopheles* vector……	2

CHAPTER TWO

Figure 2.1	Rainfall (mm) in South Africa in (A) June, (B) July, and (C) August 2019……	16
Figure 2.2	Mosquitoes preserved on (A) silica and in (B) RNA*later*®……	18
Figure 2.3	Dissected mosquito midguts……..	19
Figure 2.4	*Anopheles* anatomy: Abdominal segments I to V were dissected from preserved mosquitoes.	20

CHAPTER THREE

Figure 3.1	Bacteria identified by culture-dependent techniques from female and male *An. arabiensis* and their larval rearing water……...……	29
Figure 3.2	Bacteria identified by culture-independent techniques from female and male *An. arabiensis* and their larval rearing water……	31
Figure 3.3	Bacteria identified by culture-dependent and culture-independent techniques that are common between colonised female and male *An. arabiensis*……	32
Figure 3.4	Common midgut bacteria identified by culture-dependent and culture-independent techniques from fresh and preserved *An. arabiensis*……	36
Figure 3.5	Common midgut bacteria between colonised *An. arabiensis* and field-collected female *An. arabiensis* from Kwa-Zulu Natal, South Africa……	38
Figure 3.6	Common midgut bacteria between colonised *An. arabiensis* and field-collected female *An. arabiensis* from Mpumalanga, South Africa……	38
Figure 3.7	Comparison of diversity indices between colonised *An. arabiensis* and field-collected *An. arabiensis* from KZN and Mpumalanga……	40
Figure 3.8	Bacteria identified by culture-dependent techniques from female and male *An. funestus* and their larval rearing water……	42
Figure 3.9	Bacteria identified by culture-independent techniques from female and male *An. funestus* and their larval rearing water……	44
Figure 3.10	Bacteria identified by culture-dependent and culture-independent techniques that are common between colonised female and male *An. funestus*……	45
Figure 3.11	Common midgut bacteria identified by culture-dependent and culture-independent techniques from fresh and preserved *An. funestus*……	49
Figure 3.12	Comparison of culture-dependent diversity indices between fresh colonised female and male (A, B, C) *An. arabiensis* and (D, E, F) *An. funestus*……	52
Figure 3.13	Comparison of culture-dependent diversity indices between (A, C, E) *An. arabiensis* and *An. funestus* (females and males combined), and (B, D, F) females and males (*An. arabiensis* and *An. funestus* combined)……	53
Figure 3.14	Comparison of culture-independent diversity indices between fresh colonised female and male (A, B, C) *An. arabiensis* and (D, E, F) *An. funestus*……	54

Figure 3.15	Comparison of culture-independent diversity indices between (A, C, E) *An. arabiensis* and *An. funestus* (females and males combined), and (B, D, F) females and males (*An. arabiensis* and *An. funestus* combined)……	55
Figure 3.16	Comparison of diversity indices between (A, C, E) culture-dependent and culture-independent methods (fresh *An. arabiensis* and *An. funestus* combined), and fresh (B, D, F) *An. arabiensis* and *An. funestus* (culture-dependent and culture-independent results combined)……	57
Figure 3.17	Comparison of culture-dependent diversity indices between preserved colonised female and male (A, B, C) *An. arabiensis* and (D, E, F) *An. funestus*……	59
Figure 3.18	Comparison of culture-dependent diversity indices between preserved (A, C, E) *An. arabiensis* and *An. funestus* (females and males combined), and (B, D, F) females and males (*An. arabiensis* and *An. funestus* combined)……	60
Figure 3.19	Comparison of culture-independent diversity indices between preserved colonised female and male (A, B, C) *An. arabiensis* and (D, E, F) *An. funestus*……	62
Figure 3.20	Comparison of culture-independent diversity indices between preserved (A, C, E) *An. arabiensis* and *An. funestus* (females and males combined), and (B, D, F) females and males (*An. arabiensis* and *An. funestus* combined)……	63
Figure 3.21	Comparison of diversity indices between (A, C, E) culture-dependent and culture-independent methods (preserved *An. arabiensis* and *An. funestus* combined), and preserved (B, D, F) *An. arabiensis* and *An. funestus* (culture-dependent and culture-independent results combined)……	64
Figure 3.22	Comparison of diversity indices identified by culture-dependent techniques between fresh and preserved (A, C, E) *An. arabiensis* and (B, D, F) *An. funestus* at different preservation time periods……	67
Figure 3.23	Comparison of diversity indices identified by culture-independent techniques between fresh and preserved (A, C, E) *An. arabiensis* and (B, D, F) *An. funestus* at different preservation time periods.……	69
Figure 3.24	Comparison of culture-dependent diversity indices from fresh and preserved *An. arabiensis* and *An. funestus*……	71
Figure 3.25	Comparison of culture-independent diversity indices from fresh and preserved *An. arabiensis* and *An. funestus*……	72
Figure 3.26	Common bacteria identified by culture-dependent and culture-independent techniques from fresh and preserved colonised *An. arabiensis* and *An. funestus*……	74

LIST OF TABLES

CHAPTER ONE

Table 1.1	Advantages and disadvantages of culture-dependent and culture-independent bacterial identification techniques……	9

CHAPTER TWO

Table 2.1	Selective media used in this study and the types of bacteria they select for……	21
Table 2.2	Bacteria used to represent genetically indistinguishable bacteria……	25

CHAPTER THREE

Table 3.1	Common midgut bacteria between preserved female and male *An. arabiensis*……	33
Table 3.2	One-way ANOVA results of diversity indices from colonised *An. arabiensis* and field-collected *An. arabiensis* from KZN and Mpumalanga……	40
Table 3.3	Common midgut bacteria between preserved female and male *An. funestus*……	46
Table 3.4	Summary of Gram-negative and Gram-positive bacterial proportions from fresh colonised *An. arabiensis* and *An. funestus*……	51
Table 3.5	Diversity indices for fresh colonised female and male *An. arabiensis* and *An. funestus* identified by culture-dependent methods……	52
Table 3.6	Diversity indices for fresh colonised female and male *An. arabiensis* and *An. funestus* identified by culture-independent methods……	54
Table 3.7	Diversity indices for preserved colonised female and male *An. arabiensis* and *An. funestus* identified by culture-dependent techniques……	58
Table 3.8	Diversity indices for preserved colonised female and male *An. arabiensis* and *An. funestus* identified by culture-independent methods……	61
Table 3.9	Diversity indices for *An. arabiensis* midguts across preservation time periods identified by culture-dependent methods……	66
Table 3.10	Diversity indices for colonised *An. funestus* at different preservation time periods identified by culture-dependent methods……	67
Table 3.11	Diversity indices for colonised *An. arabiensis* at different preservation time periods identified by culture-independent methods……	68
Table 3.12	Diversity indices for colonised *An. funestus* at different preservation time periods identified by culture-independent methods ……	69
Table 3.13	Common midgut bacteria between fresh and preserved *An. arabiensis* and *An. funestus*……	73

LIST OF ACRONYMS AND ABBREVIATIONS

ANOVA	Analysis of Variance
BHI	Brain-Heart Infusion
BLASTn	Basic Local Alignment Search Tool for Nucleotide Sequences
$\frac{1}{D}$	Reciprocal of Simpson's Index
df	Degrees of Freedom
E	Pielou's Evenness Index
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
F	F-value
FLASH	Fast Length Adjustment of SHort reads
HCCA	α-Cyano-4-hydroxycinnamic Acid
H	Shannon-Wiener Index
Hz	Hertz
IRS	Indoor Residual Spraying
KZN	KwaZulu-Natal
LLINs	Long-lasting Insecticidal Nets
LSV	Log Score Value
MALDI-TOF MS	Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry
NAP	Nucleic Acid Preservation
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
NICD	National Institute for Communicable Diseases
ns	Not Significant
OTU	Operational Taxonomic Unit
P	Probability/P-value
PBS	Phosphate-buffered Saline
PCR	Polymerase Chain Reaction
PMF	Peptide Mass Fingerprint
QIIME	Quantitative Insights Into Microbial Ecology
rRNA	Ribosomal Ribonucleic Acid
SBS	Sequencing by Synthesis
SD	Standard Deviation
SIT	Sterile Insect Technique
sp.	Species (singular)
spp.	Species (multiple)
VCRL	Vector Control Reference Laboratory
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1. Malaria

Malaria is a vector-borne disease that severely affects people in poverty-stricken regions. In 2019, 94% of the global malaria cases were localised to the World Health Organization (WHO) African Region – one of the most impoverished regions in the world (WHO, 2020). Malaria predominantly affects the youth and pregnant women in underdeveloped countries (WHO, 2020). To illustrate this, 67% of worldwide malaria deaths occurred in children under the age of five. Meanwhile, approximately 12 million pregnant women in sub-Saharan Africa were infected with malaria and, consequently, approximately 820 000 babies were born with low birthweight (WHO, 2020).

Malaria is caused by the protozoan parasite of the *Plasmodium* genus, which has a multifaceted development that alternates between the vector mosquito and vertebrate host (Bannister and Sherman, 2009). Female mosquitoes of the genus *Anopheles* are the protagonists of malaria transmission, where an infected female transmits the parasite to a human host upon ingestion of a blood meal (Vijay *et al.*, 2018). Five *Plasmodium* (*P.*) species are capable of infecting humans, namely *P. falciparum*, *P. knowlesi*, *P. malariae*, *P. ovale*, and *P. vivax* (Antinori *et al.*, 2012; Gilles and Warrell, 1996). Of these, *P. falciparum* is the dominant *Plasmodium* species in Africa as it was responsible for 96% of the malaria cases in sub-Saharan Africa in 2018 (WHO, 2020).

The *Anopheles* (*An.*) *gambiae* complex and the *An. funestus* group consist of dominant vector species that transmit *P. falciparum* (WHO, 2020). Therefore, members of the *An. gambiae* complex and the *An. funestus* group are responsible for a large portion of *P. falciparum* transmission in Africa. The major malaria vectors of the *An. gambiae* complex include *An. arabiensis*, *An. coluzzii*, and *An. gambiae*, while the major malaria vector of the *An. funestus* group is *An. funestus* (Akogbéto *et al.*, 2018; Gillies and Coetzee, 1987; Gillies and De Meillon, 1968; Sinka *et al*., 2012).

To further exacerbate the problem, malaria control strategies face continual challenges (WHO, 2020). For example, because *Plasmodium* can acquire mutations in its genome, it gradually develops resistance to antimalarial drugs (Blasco *et al.*, 2017; Cheeseman *et al.*, 2012). Moreover, conventional vector control methods, such as indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs), have become less efficient at preventing malaria

transmission (Ranson and Lissenden, 2016). This is mainly because of an increase in mosquito resistance to insecticides, as well as changes to mosquito behaviour due to selective pressures (Ranson and Lissenden, 2016). Further, vaccine production has been challenging (reviewed by Duffy and Gorres, 2020; and Ranson and Lissenden, 2016).

Therefore, there is an urgent need to reduce malaria transmission in Africa. Consequently, recent research has explored alternative forms of intervention, such as investigating the properties of the mosquito's native midgut symbionts to understand their role in vector biology and explore using these symbionts to inhibit *Plasmodium* infection. However, to investigate the premise of symbiotic control, it is necessary to understand the vector-parasite relationship.

1.1.1. The Vector-Parasite Relationship

The parasite undergoes a complex lifecycle in the mosquito's midgut, haemolymph, and salivary glands before it can be transmitted to a human host (Ghosh *et al*., 2000). Briefly, *Plasmodium*'s lifecycle in the vector begins when the mosquito ingests gametocytes (sexual precursor cells) from an infected individual (Figure 1.1). Female and male gametocytes transform and fuse to form a zygote. The fertilised zygote then matures into a motile ookinete that penetrates the mosquito's midgut epithelium and forms an oocyst in the haemolymph. Oocysts then mature and produce sporozoites – the transmissible form of the parasite – and upon oocyst rupture, sporozoites invade the salivary glands. The infected mosquito then feeds on a healthy human host and injects sporozoites into that host, where the disease progresses (Clements, 1992; Gillies, 1953).

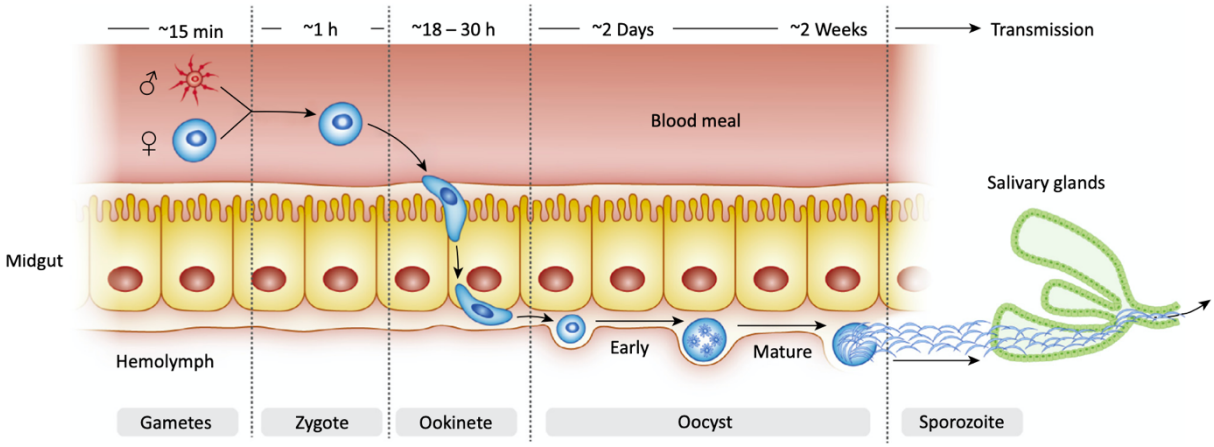

Figure 1.1: Lifecycle of the *Plasmodium* parasite in the *Anopheles* vector. Figure adapted from Smith and Barillas-Mury (2016).

During every stage of parasite development in the mosquito, *Plasmodium* encounters bottlenecks that hamper its development (Smith *et al.*, 2014). One of the major bottlenecks during development occurs when ookinetes penetrate the midgut epithelium (Dimopoulos, 2003). During this stage, the parasite actively evades mosquito immune responses, yet only few ookinetes transverse into the haemolymph (Drexler *et al.*, 2008). The mosquito's immune response is influenced by factors such as ambient environmental temperature, mosquito behaviour, mosquito diet, and mosquito and parasite genetics. However, the major contributor to immune activation, and the reason that few ookinetes thrive past midgut invasion, is the presence of midgut microorganisms (Bahia *et al.*, 2014; Cirimotich *et al.*, 2011a; Cirimotich *et al.*, 2011b; Lefèvre *et al.*, 2013). Thus, mosquito midgut microorganisms have gained considerable attention as targets for intervention.

1.2. Mosquito Midgut Microorganisms

Mosquitoes naturally acquire a variety of microorganisms from their environment (Boissière *et al*., 2012; Chavshin *et al*., 2014; Coon *et al*., 2014; Dennison *et al*., 2014; Dickson *et al*., 2018; Dong and Dimopoulos, 2009; Lindh *et al*., 2005; Merritt *et al.*, 1992; Osei-Poku *et al*., 2012a; Saab *et al*., 2020; Steyn *et al.*, 2016; Wang *et al.*, 2017). Some microorganisms co-evolve with the vector and are transmitted horizontally between females and males, transstadially between developmental stages, and/or vertically through successive generations (Damiani *et al*., 2010; Dennison *et al*., 2014; Favia *et al*., 2007; Lindh *et al*., 2008a; Huang *et al.*, 2020; Hughes *et al*., 2014; O'Neill and Belote, 1992; Villegas and Pimenta, 2014; Wang and Jacobs-Lorena, 2013; Wang *et al*., 2017; Werren *et al*., 1995).

Midgut microorganisms associated with mosquitoes include bacteria, fungi, nematodes, protozoa, and viruses (Bartholomay and Michel, 2018; Chandler *et al.,* 2015; Luis *et al.*, 2019; Lynch *et al.*, 2012; Minard *et al*., 2015; Morales *et al*., 2005; Thongsripong *et al*., 2018). However, only some microorganisms form symbiotic relationships with mosquitoes and are responsible for physiological functioning. For example, fungi are involved in mosquito behaviour (Barnard *et al*., 2007; Eneh *et al*., 2016; Geetha *et al*., 2003; Lindh *et al*., 2015; Liu *et al*., 2010; Reeves, 2004; Xia *et al*., 2008), development (Díaz-Nieto *et al*., 2016; Souza *et al*., 2019; Strand, 2018; Valzania *et al*., 2018a; Valzania *et al*., 2018b), fecundity (Barnard *et al*., 2007; Lorenz and Koella, 2011), nutrition (Souza *et al*., 2019; Stefanini, 2018), survival (Souza *et al*., 2019; Valzania *et al*., 2018b) and immunity (Angleró-Rodríguez *et al*., 2016; Angleró-Rodríguez *et al*., 2017; Bargielowski and Koella, 2009; Cappelli *et al*., 2014; Cappelli

et al., 2019; Da Silva *et al*., 2000; Hernández-Martínez *et al*., 2002; Herren *et al*., 2020; Ricci *et al*., 2011; Tawidian *et al*., 2019; Valzano *et al*., 2016).

Nonetheless, bacteria are the most researched symbionts due to their diverse biological roles, but especially because they influence *Plasmodium* development. Additionally, genetic bacterial manipulation is well established and has been tested for symbiotic control (section 1.4).

1.2.1. *Anopheles* Midgut Bacteria

Generally, there is still knowledge on the *Anopheles* midgut microbiota that remains unknown. Nonetheless, *Anopheles* midgut bacteria have been shown to play essential roles in development, nutrition, immunity, insecticide resistance, and reproduction (described in sections 1.2.1.1–1.2.1.5). Further, while some midgut bacteria have positive effects on mosquito biology, others have been shown to be detrimental.

1.2.1.1. Development and Homeostasis

Some bacteria have no effect on mosquito development while others influence homeostasis and survival (Jadin *et al.*, 1967; Pumpuni *et al.*, 1993; Seitz *et al*., 1987). For example, clearing the midguts of *Anopheles* larvae with antibiotics results in delayed growth and higher mortality rates (Chouaia *et al*., 2012; Wotton *et al*., 1997). This demonstrates the role that the midgut bacteria play in larval development. In addition, Coon *et al.* (2014) discuss that the midgut bacterial community as a whole, as well as the interplay between different bacterial species, is essential for development. However, Mitraka *et al*. (2013) show that introducing *Asaia* in *An. gambiae* promotes larval growth, demonstrating how some midgut bacteria play larger roles in mosquito development than others.

Midgut bacteria have also been shown to affect the synthesis of the peritrophic matrix – the membrane that physically separates the midgut epithelial cells from the midgut contents – that is responsible for midgut homeostasis (Rodgers *et al*., 2017). Wang *et al*. (2011) also describe the ability of bacteria to maintain midgut redox homeostasis. Therefore, *Anopheles* native midgut bacteria play critical roles in development and homeostasis.

1.2.1.2. Digestion and Nutrition

Midgut bacteria influence mosquito digestion either by producing compounds that the mosquito uses directly or by producing enzymes that degrade complex molecules (Minard *et al*., 2013). For example, de O Gaio *et al.* (2011) show that midgut bacteria contribute to mosquito nutrition by supplying vitamins, while Crotti *et al*. (2010) describe that midgut bacteria release lytic enzymes for digestion. Additionally, female and male *Anopheles* have different midgut microbiota because they digest different food sources. Although both genders consume nectar/sugar for energy, females have a more diverse microbiota because they need to digest blood after ingesting a blood meal (de O Gaio *et al*., 2011; Gusmão *et al.*, 2010; Rani *et al*., 2009).

Furthermore, Jadin *et al.* (1967) showed that feeding radioactive *Pseudomonas* species (spp.) to *An. gambiae* and *An. stephensi* resulted in these bacteria localising in the intestinal tract. These mosquitoes also developed radioactive signals throughout their body, suggesting that these bacteria were responsible for host metabolism (Jadin *et al.*, 1967). Moreover, when these anophelines were infected with *Plasmodium*, oocysts and sporozoites developed the same radioactive compounds, suggesting that these bacteria also contributed to parasite nutrition (Jadin *et al*., 1967). Therefore, the *Anopheles* midgut bacteria are specialised for digestion, metabolism, and nutrition.

1.2.1.3. Immunity and Vector Competence

Many studies have shown that the *Anopheles* midgut bacteria influence vector competence, which describes the ability of a vector to transmit a disease (Bahia *et al.*, 2014; Blumberg *et al*., 2013; Boissière *et al*., 2012; Briones *et al.*, 2008; Cirimotich *et al*., 2011a; Dennison *et al*., 2014; Dennison *et al*., 2015; Dennison *et al.*, 2016; Dong and Dimopoulos, 2009; Dong *et al*., 2006; Favia *et al.*, 2007; Favia *et al*., 2008; Garver *et al.*, 2012; Gendrin and Christophides, 2013; Gendrin *et al*., 2017; Gonzalez-Ceron *et al*., 2003; Kalappa *et al*., 2018; Meister *et al*., 2009; Michel *et al.*, 2005; Ngo *et al*., 2015; Ngo *et al*., 2016; Pumpuni *et al.*, 1993; Ramirez *et al*., 2014; Rodrigues *et al*., 2010; Ross, 1911; Saraiva *et al.*, 2016; Smith *et al*., 2014; Stathopoulos *et al.*, 2014; Straif *et al.*, 1998; Tchioffo *et al*., 2013; Tchioffo *et al*., 2016a; Volohonsky *et al*., 2017; Wang and Jacobs-Lorena, 2013; Wang *et al*., 2012; Wang *et al.*, 2019; Zhang *et al*., 2015).

Briefly, treating anophelines with antibiotics to clear their midgut bacteria causes them to become more susceptible to *Plasmodium* infection (Dong and Dimopoulos, 2009; Meister *et al*., 2009). Additionally, when the *Anopheles* immune system is compromised, midgut bacteria proliferate because the immune system cannot control the bacterial load. However, an increased bacterial load compensates for the lack of immunity, where bacteria produce anti-*Plasmodium* molecules to indirectly prime the immune system against *Plasmodium* (Dong and Dimopoulos, 2009; Dong *et al*., 2006; Garver *et al*., 2008).

While some bacteria indirectly influence the immune system, others directly hinder *Plasmodium* infection; for example, *An. albimanus* co-infected with *Serratia marcescens* and *P. vivax* resulted in only 1% of mosquitoes being infected compared with 71% infection for control mosquitoes without *S. marcescens* (Bahia *et al.*, 2014; Cirimotich *et al*., 2011a; Gendrin and Christophides, 2013; Gonzalez-Ceron *et al*., 2003; Ramirez *et al*., 2014). Therefore, the *Anopheles* midgut bacteria play major roles in immune system modulation and vector competence against pathogens.

1.2.1.4. Insecticide Resistance

More recently, research has investigated the role that midgut bacteria play in *Anopheles* insecticide resistance. For example, Soltani *et al.* (2017) demonstrate that bacterial symbionts render *An. stephensi* resistant to temephos, an organophosphate larvicide. Removing these symbionts with antibiotics renders mosquitoes susceptible to temephos. Meanwhile, some midgut bacteria metabolise insecticides and thus, insecticide pressure could be selecting for insecticide-metabolising bacteria (Dada *et al*., 2018; Dada *et al*., 2019; Kwon *et al*., 2005). To illustrate this, insecticide-metabolising bacteria were found in organophosphate-resistant, field-collected *An. albimanus* (Dada *et al*., 2018). Similarly, Barnard *et al.* (2019) found that an insecticide-resistant strain of *An. arabiensis* was predominantly colonised by insecticide-degrading midgut bacteria. Therefore, some midgut bacteria contribute to insecticide resistance, which ultimately poses a challenge to malaria control programmes.

1.2.1.5. Reproduction and Behaviour

Some mosquito midgut bacteria spread through populations by horizontal transmission – that is, between females and males – and are known to influence mosquito reproduction (Lindh *et al.*, 2008b). For example, treating *An. gambiae* with antibiotics removes Enterobacteriacae and

positively affects fecundity (Gendrin *et al*., 2015). Additionally, some bacteria influence *Anopheles* behaviour; for example, bacteria produce messenger molecules in water that female *Anopheles* can sense, which influences their oviposition behaviour (Lindh *et al.*, 2008b). Furthermore, *Anopheles* can also sense bacteria on human skin, which affects their feeding behaviour (Verhulst *et al.*, 2009; Verhulst *et al*., 2011).

Therefore, midgut bacteria are essential to *Anopheles* biological functioning. However, even though multiple midgut bacterial symbionts and their roles have been discovered, many vector-specific bacteria and their functions are yet to be elucidated. Hence, it is necessary to understand the diversity and composition of the *Anopheles* midgut bacteria as these factors ultimately affect physiological functioning.

1.2.2. Midgut Bacterial Diversity and Composition

The diversity and composition of the *Anopheles* midgut bacteria differs according to developmental stage, environment, gender, and species. For example, transstadial transmission of midgut bacteria (that is, between developmental stages) ensures that bacteria acquired by larvae from the aquatic stage are transmitted to adults post-emergence (Briones *et al*., 2008; Buck *et al*., 2016; Damiani *et al*., 2010; Favia *et al*., 2007; Jadin *et al.*, 1967; Lindh *et al*., 2008a; Straif *et al*., 1998). Nonetheless, adult *Anopheles* midguts have low bacterial diversity due to midgut renewal during metamorphosis from pupal to adult stage (Favia *et al*., 2007; Pumpuni *et al*., 1996; Tandina *et al*., 2016).

Nevertheless, studies have revealed that the dominant *Anopheles* midgut phyla are Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Champion and Xu, 2017; Minard *et al*., 2013; Wang *et al*., 2011). Additionally, dominant mosquito midgut genera include *Aeromonas*, *Acinetobacter*, *Asaia*, *Bacillus*, *Chryseobacterium*, *Comamonas*, *Elizabethkingia*, *Enterobacter*, *Klebsiella*, *Mycobacterium*, *Pantoea*, *Pseudomonas*, *Serratia, Sphingomonas*, and *Staphylococcus* (Alvarez *et al*., 2012; Dong and Dimopoulos, 2009; Favia *et al.*, 2007; Gendrin and Christophides, 2013; Garay *et al*., 2016; Hughes *et al.*, 2016a; Hughes *et al*., 2016b; Jiang *et al*., 2012; Kukutla *et al*., 2013; Pei *et al*., 2015; Rani *et al*., 2009).

There are also major differences in the midgut bacteria of colonised and field-collected *Anopheles* because mosquitoes acquire bacteria from their immediate environment (Boissière *et al.*, 2012). Accordingly, field-collected mosquito midguts have been shown to be more diverse than colonised mosquito midguts (Boissière *et al.*, 2012; Osei-Poku *et al*., 2012a).

Similarly, there are major differences in midgut bacteria of field-collected *Anopheles* from different localities (Boissière *et al.*, 2012).

As wild mosquitoes acquire bacteria from natural food sources such as nectar, these mosquito midguts are predominantly colonised with bacteria of the genera *Acetobacter*, *Asaia*, and *Pseudomonas* (Adedara *et al.*, 2015; Yamada and Yukphan, 2008). In terms of midgut bacterial composition, Gram-negative bacteria have been found to predominate the midguts of field-collected *Anopheles* (Boissière *et al.*, 2012; Osei-Poku *et al.*, 2012a). Additionally, as noted previously, the midgut microbiota of females differs from the midgut microbiota of males because females need a blood meal for egg production. However, the female midgut bacteria also change upon ingestion of a blood meal, where bacterial abundance increases but diversity decreases (Boissière *et al*., 2012; Cirimotich *et al.*, 2011a; Wang *et al*., 2007).

Notably, three bacterial species were first discovered and classified from *Anopheles* midguts: *Elizabethkingia anophelis*, *Janibacter anophelis*, and *Thorsellia anophelis* (Kämpfer *et al*., 2006a; Kämpfer *et al.*, 2006b; Kämpfer *et al*., 2011). This demonstrates how some bacteria are specific to the mosquito midgut and could suggest the presence of other mosquito midgut bacteria that are yet to be identified. Hence, many vector-specific bacteria and their functions remain unknown. Therefore, it is necessary to characterise the native midgut bacteria of malaria vectors belonging to the *An. gambiae* complex and *An. funestus* group to determine if there are common bacteria amongst African malaria vectors. This will identify if a native midgut microbiota exists, which could be explored in future studies investigating symbiotic control.

1.2.3. Characterising the Midgut Bacteria

Mosquito midgut symbionts are typically identified by culture-dependent techniques (Chavshin *et al*., 2014; Chouaia *et al*., 2010; Cirimotich *et al*., 2011a; Demaio *et al*., 1996; Djadid *et al.*, 2011; Dong and Dimopoulos, 2009; Favia *et al*., 2007; Gonzalez-Ceron *et al.* 2003; Gusmão *et al*., 2010; Karger *et al*., 2016; Oliveira *et al*., 2011; Pumpuni *et al*., 1993; Pumpuni *et al*., 1996; Straif *et al.*, 1998; Tandina *et al.*, 2016; Terenius *et al*., 2012) or culture-independent techniques (Boissière *et al*., 2012; Chavshin *et al*., 2012; Osei-Poku *et al*., 2012a; Osei-Poku *et al*., 2012b; Wang *et al.*, 2011).

Culturomics is a fast, cost-effective, and reliable technique that is used to identify unknown bacteria (Tandina *et al*., 2016). This culture-dependent technique involves using nutrient media for bacterial growth, where morphologically distinct bacterial colonies are isolated and

subjected to matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS). MALDI-TOF MS identifies bacteria based on their proteome, where proteins are cleaved into peptides and their molecular masses are used to create peptide mass fingerprints (PMFs) (reviewed by Singhal *et al*., 2015). The PMFs of unknown bacteria are compared with the PMFs of known bacteria in a local database for taxonomic identification (Singhal *et al.*, 2015).

However, culturomics has disadvantages and Next Generation Sequencing (NGS) – a culture-independent approach – addresses some of these disadvantages (Table 1.1). NGS identifies bacteria based on their genome: conserved regions of the prokaryotic 16S ribosomal ribonucleic acid (rRNA) gene are used for amplification, and hypervariable regions of the gene are used to identify taxa (Kim *et al*., 2011; Tringe *et al*., 2008; Wang and Qian, 2009). Yet, identifying microbes using NGS has its own disadvantages (Table 1.1).

Table 1.1: Advantages and disadvantages of culture-dependent and culture-independent bacterial identification techniques. Table information acquired from Boers *et al.* (2019), Cobo (2013), Figdor (2011), Lagier *et al.* (2012) and Lagier *et al*. (2015).

Culture-dependent Techniques		**Culture-independent Techniques**	
Advantages	**Disadvantages**	**Advantages**	**Disadvantages**
Identifies culturable microbes	Cannot identify unculturable microbes	Identifies unculturable microbes	Depth bias and detects contaminants
Distinguishes live from dead microbes	Phenotypic biochemistry often needed	High specificity	Cannot distinguish live from dead microbes
Can be quantitative	High skill level required	Modest skill level required	Relies on established sequences
High sensitivity (specific media)	Slow, resource intensive, and risk of contamination	Fast and not many resources needed	Results vary with bioinformatics analysis
Host-interaction studies possible	Identification depends on MALDI-TOF MS library	Samples can be frozen and processed later	Host-interaction studies cannot be performed

Therefore, neither approach is superior (Pandya *et al*., 2017). Rather, these techniques are complementary and using both is advantageous because they identify a large dataset of overlapping bacterial species (Pandya *et al*., 2017). Therefore, some studies investigating the mosquito midgut bacteria have used a combination of culture-dependent and culture-independent techniques (Chavshin *et al*., 2012; Djadid *et al*., 2011; Galeano-Castañeda *et al*.,

2019; Gusmão *et al*., 2010; Kalappa *et al*., 2018; Lindh *et al*., 2005; Ngo *et al*., 2015; Ngo *et al*., 2016; Rani *et al*., 2009; Saab *et al*., 2020; Tandina *et al*., 2016; Tchioffo *et al*., 2013).

Although culture-dependent and culture-independent techniques typically produce qualitative results, they can be used to quantify midgut bacterial diversity. As described by Hill *et al.* (2003), there are three categories that Magurran (1988) highlights that should be used for measuring diversity: species richness, proportional species abundance, and species distribution. Studies on the *Anopheles* midgut bacteria vary in terms of which indices are used to measure bacterial diversity according to these categories.

The most commonly used indices to quantitate mosquito midgut diversity are the Shannon-Wiener (or Shannon-Weaver) index to measure bacterial diversity, Simpson's reciprocal index to measure dominance as an indicator of relative abundance, and Pielou's evenness index to measure relative abundance in terms of bacterial species distribution (Akorli *et al*., 2016; Bando *et al.*, 2013; Boissière *et al.*, 2012; Briones *et al*., 2008; Dada *et al.*, 2018; Galeano-Castañeda *et al*., 2019; Ngo *et al*., 2016; Rani *et al*., 2009; Sharma *et al*., 2020; Tchioffo *et al*., 2016b). Species richness is measured by the number of operational taxonomic units (OTUs), which is defined as the number of genetically distinct bacterial species (Manguin *et al.*, 2013).

Overall, it is essential to characterise the *Anopheles* native midgut bacteria in a species-specific manner using culture-dependent and culture-independent techniques. This will provide a comprehensive overview of bacteria in common between dominant malaria vectors from the *An. gambiae* complex and *An. funestus* group. Thus, if common bacteria exist, they can be used for symbiotic control.

1.3. Symbiotic Control

Symbiotic control involves exploiting a vector's natural symbionts to express effector molecules that prevent the vector from transmitting a disease (Durvasula *et al.*, 1997). The best example of success using this strategy is in the triatomine bug to control Chagas' disease (Durvasula *et al.*, 1997). This involved using paratransgenesis to transform the triatomine's native symbiont, *Rhodococcus rhodnii*, to produce anti-trypanosomal molecules that rendered the vector incapable of transmitting the disease (Durvasula *et al.*, 1997). Thus, paratransgenesis involves genetically transforming symbionts to express anti-pathogen effector molecules (Beard *et al*., 1998; Durvasula *et al*., 1997). This affects vector competence by rendering a host incapable of transmitting a pathogen.

Although paratransgenesis using viruses and fungi has been documented in *Anopheles*, most studies have used bacteria (Fang *et al*., 2011; Ren *et al*., 2008). Early attempts at this involved using exogenous recombinant *Escherichia coli* to express anti-*Plasmodium* proteins (Riehle *et al*., 2007). However, the exogenous bacteria did not survive in the mosquito midgut and recombinant proteins could not be expressed (Riehle *et al*., 2007). Therefore, several requirements for paratransgenesis were constructed. Paratransgenic candidates must: (i) have an existing symbiotic relationship with the vector, (ii) propagate in the vector, (iii) express gene(s) of interest, (iv) not compromise vector fitness, and (v) be easily delivered to wild populations (Riehle *et al*., 2007).

More recent attempts at paratransgenesis in *Anopheles* demonstrate how engineered *Pantoea agglomerans* inhibits *P. falciparum* infection by 98% (Wang *et al.*, 2012). However, this bacterium could not be transmitted horizontally or vertically and thus, did not meet all the requirements to be a practical paratransgenic candidate. Therefore, Wang *et al.* (2017) genetically engineered a *Serratia* strain to inhibit *P. falciparum* development in *An. gambiae*. This engineered bacterium was capable of being transmitted horizontally and vertically, thereby meeting the requirements for paratransgenesis (Wang *et al.*, 2017). Other studies documenting the genetic modification of symbiotic bacteria from anopheline vectors include those by Bongio and Lampe (2015), Capone *et al.* (2013), Corby-Harris *et al.* (2010), Damiani *et al.* (2010), Dong *et al.* (2011), Favia *et al.* (2007), Favia *et al*. (2008), Isaacs *et al.* (2011), Ito *et al.* (2002), Riehle *et al.* (2007) and Shane (2018).

Since few studies have identified common midgut bacteria between African malaria vectors, no studies have documented using paratransgenesis on common symbionts of *Anopheles* vectors. Thus, it is necessary to compare the native midgut bacteria of colonised and field-collected members of the *An. gambiae* complex and *An. funestus* group to identify if common bacteria exist, which is a step towards finding suitable paratransgenic candidates.

1.4. Mosquito Preservation Methods

The success of vector control programmes, as well as the success of routine malaria monitoring programmes, requires the collection of mosquitoes from their natural habitats. However, analysing specimens in field conditions or semi-field conditions is impractical due to the lack of a sterile environment and laboratory equipment. Additionally, field sites are often far from suitably equipped laboratories. Therefore, field-collected mosquitoes are preserved post-collection and transported to a laboratory for analysis. Routine analyses that are conducted on

field-collected mosquitoes include species identification, *Plasmodium*-infection status, and insecticide resistance testing. The type of preservation method chosen is also dependent on the type of downstream analysis being performed because certain preservatives are better suited for identifying certain entomological indicators (Dowell *et al*., 2011).

The most common types of preservation methods include fixation in Allprotect® Tissue Reagent, Carnoy's solution (6:3:1 ethanol: chloroform: glacial acetic acid, with ferric chloride), ethanol (95%), nucleic acid preservation (NAP) buffer (ethylenediaminetetraacetic acid (EDTA), sodium citrate trisodium salt dihydrate, ammonium sulfate), or RNA*later*® (ammonium sulfate, EDTA, sodium citrate); desiccation in drierite (anhydrous calcium sulphate) or silica (silicon dioxide); refrigeration at 4°C or -20°C; and cryopreservation in liquid nitrogen (Dowell *et al*., 2011; Hunt and Coetzee, 1986; Mayagaya *et al*., 2015; Rodríguez-Ruano *et al.*, 2020; Zawada *et al*., 2018). Although refrigeration is cheap and effective, the most cost-effective preservation method is desiccation (Dowell *et al*., 2011; Mayagaya *et al*., 2015).

Mayagaya *et al*. (2015) describe that silica desiccant is the most reliable preservation method for long-term mosquito storage and it is also the most convenient method for transporting large quantities of field-collected specimens (Mayagaya *et al*., 2015). Additionally, silica desiccation is suitable for DNA extraction and is effective for species identification and age grading using near infrared spectroscopy (Dowell *et al*., 2011). However, it is difficult to determine internal mosquito musculature anatomy after using this preservative (Quicke *et al*., 1999). This is overcome when mosquitoes are preserved in RNA*later*® solution, which preserves DNA and RNA in the highest quality (Dowell *et al*., 2011).

While the midgut bacteria of *Aedes vexans* and *Culex pipiens* have been identified post-preservation from AllProtect® Tissue Reagent, ethanol, and NAP buffer, the efficacy of other commonly used, cost-effective preservatives, such as silica and RNA*later*®, has not been investigated (Rodríguez-Ruano *et al.*, 2020). Therefore, there is a gap in the literature regarding whether it is possible to isolate midgut bacteria from silica- and RNA*later*®-preserved *Anopheles*. It is necessary to determine whether this is possible because valuable information is lost as researchers are unaware if the midgut bacteria of preserved *Anopheles* can be characterised. This can be used to provide insight into the midgut bacteria of preserved, field-collected anophelines from different localities, especially those infected with *Plasmodium*. Ultimately, if common bacteria exist between different vector species, these bacteria can be proposed as possible paratransgenic candidates.

1.5. Aims and Objectives

The aim of this study was to characterise the native midgut bacteria of two dominant malaria vectors in Africa, namely *An. arabiensis* (a member of the *An. gambiae* complex) and *An. funestus* (a member of the *An. funestus* group). The objectives were as follows:

1. To characterise the midgut bacteria of laboratory (colonised) *An. arabiensis* and *An. funestus* using culture-dependent and culture-independent techniques.
2. To determine if midgut bacteria could be identified by culture-dependent and culture-independent techniques after mosquito preservation on silica and in RNA*later*®.
3. To characterise the midgut bacteria of silica-preserved, field-collected *An. arabiensis* from two different South African localities – KwaZulu-Natal and Mpumalanga – using culture-dependent techniques.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Biological Material

Two *Anopheles* species were used in this study: *An. arabiensis* (a member of the *An. gambiae* complex) and *An. funestus* (a member of the *An. funestus* group). These mosquitoes were either colonised (see section 2.1.1) or field-collected (see section 2.1.2).

2.1.1. Colonised *Anopheles*

Colonised mosquitoes were obtained from the Botha de Meillon Insectary, located at the Vector Control Reference Laboratory (VCRL), a division of the National Institute for Communicable Diseases (NICD), Sandringham, Johannesburg, South Africa. The insectary provides a controlled environment that mimics the mosquito's natural habit (Hunt *et al.*, 2005). Accordingly, the insectary is maintained at approximately 25ºC with relative humidity around 80% (Hunt *et al*., 2005). Mosquitoes are routinely reared according to a 12-hour day/night period with 45-minute dusk/dawn transition light cycles (Hunt *et al*., 2005). Larvae are also fed a powdered meal (consisting of crushed dog biscuits and brewer's yeast) while adults are maintained on 10% sucrose.

The insectary is separated into rooms that hold different colonised species from various localities. Colonised *An. arabiensis* used in this study were collected from the MBN colony while *An. funestus* were collected from the FUMOZ ('FUnestus from MOZambique') colony. The MBN ('man-baited net': describing the traps used to collect mosquitoes) colony was colonised in 2002 with mosquitoes collected from Mamfene in the northern KwaZulu-Natal (KZN) province of South Africa (Nardini *et al*., 2012). The FUMOZ colony was colonized in 2000 and consists of mosquitoes reared from southern Mozambique (Hunt *et al.*, 2005).

An. arabiensis and *An. funestus* (20 female and 20 male per species per repeat; three biological repeats) were collected from their respective colonies (ethics waiver in Appendix A) between 0- and 24-hours post-emergence. Subsequently, mosquitoes underwent midgut dissections (see section 2.3) prior to bacterial identification (see section 2.4 and section 2.5). Additionally, 1ml of each species' larval rearing water was collected for bacterial characterisation (three biological repeats per species' larval water) to determine if there were bacteria in common between mosquitoes and their immediate environment.

2.1.2. Field-collected *Anopheles*

Mosquitoes are routinely collected from African localities as part of the departmental Sterile Insect Technique (SIT) project. Upon collection from the field, mosquitoes are preserved in microcentrifuge tubes containing blue indicator silica desiccant and transported to a laboratory for analysis. Routine analyses include species identification and *Plasmodium*-infection status using multiplex polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) respectively (Cohuet *et al*., 2003; Koekemoer *et al*., 2002; Scott *et al*., 1993; Wirtz *et al*., 1987). Results are documented in a departmental database and specimens are placed in the departmental archive.

This study retrieved archived *An. arabiensis* that were collected from the field between June and August 2019 from Mamfene, KZN. Mosquitoes were retrieved after 8, 12, and 16 weeks of preservation prior to midgut bacterial characterisation using culture-dependent techniques (see section 2.4). Field-collected mosquitoes were low in numbers during this time because June, July, and August are the winter months in South Africa and Dandalo *et al.* (2017) show that mosquito density varies according to season, where collection numbers are highest in summer and lowest in winter.

Moreover, the low number of samples collected may have also been exacerbated by the extensive drought in South Africa during this time (Figure 2.1) as mosquitoes require water from rainfall to breed and complete their lifecycle (Gillies and De Meillon, 1968; South African Weather Service, 2020). Therefore, only four females were retrieved per time period from this locality. Additionally, culture-dependent bacterial identification was performed due to the low cost of identification for the limited number of samples (culture-independent methods were too costly for the number of samples).

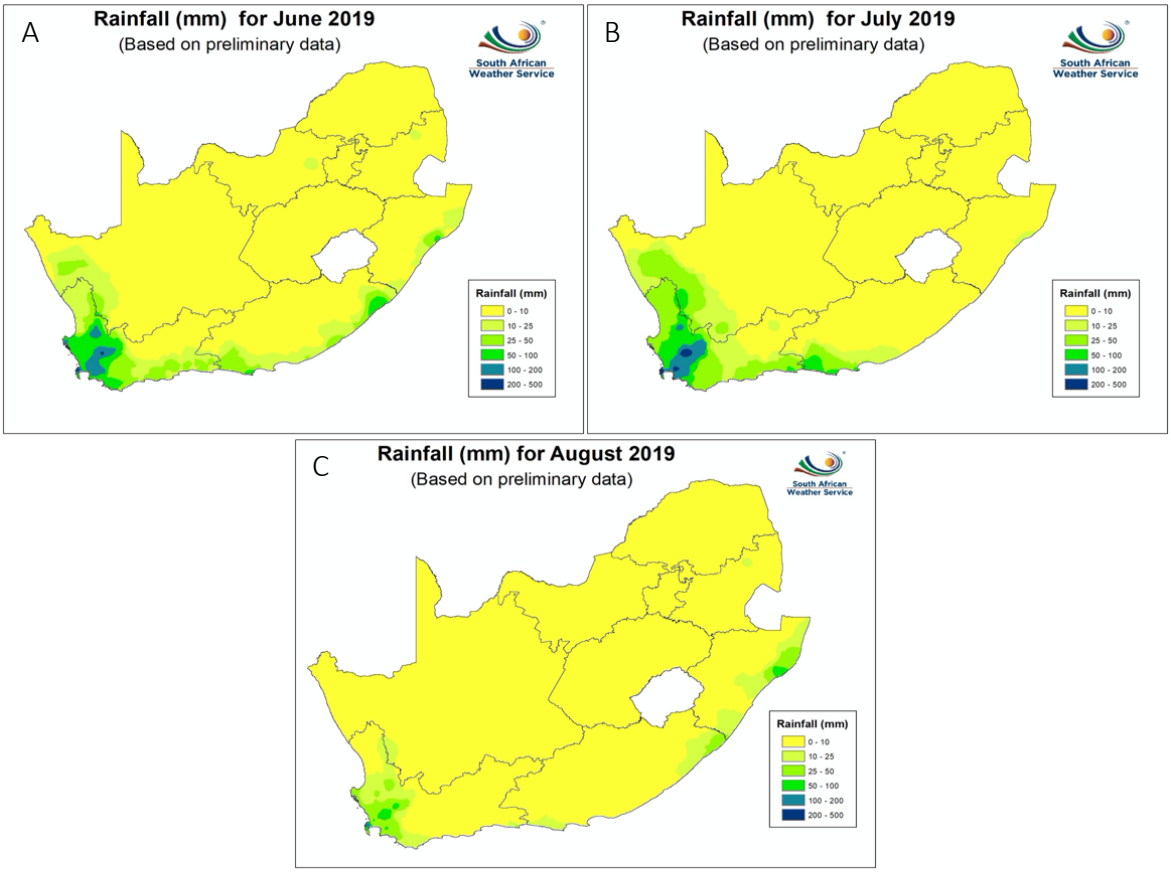

Figure 2.1: Rainfall (mm) in South Africa in (A) June, (B) July, and (C) August 2019. The winter season combined with the lack of rainfall – characteristic of a period of drought – account for the low number of samples collected during this time (images acquired from the South African Weather Service, 2020).

To compare midgut bacteria of field-collected *An. arabiensis* from two South African localities, female *An. arabiensis* from Mpumalanga were also included in this study. These mosquitoes had been collected between June and August 2019 and were retrieved from the departmental archive after 8, 12, and 16 weeks of preservation. However, as noted previously, sample numbers were low. Therefore, only three females were retrieved per time period for this locality. Subsequently, midgut bacteria were characterised using culture-dependent techniques due to the low cost for the limited number of samples.

All field-collected mosquitoes used in this study were confirmed as *P. falciparum*-uninfected. This was necessary because this study aimed to assess the native midgut bacteria of *Anopheles* in the absence of *Plasmodium* infection as infection may alter the native midgut bacteria. Moreover, *An. funestus* from these provinces were older than 16 weeks and could not be used as a direct comparison would not be possible.

2.2. Mosquito Preservation

To determine if mosquito midgut bacteria were conserved during preservation, colonised mosquitoes of each species were preserved either on silica desiccant or in RNA*later*® solution. For each preservation method, mosquitoes were preserved for 1, 4, 8, and 12 weeks (20 female and 20 male per preservative, preservation time, species, and repeat; three biological repeats). For each species, a set of fresh (non-preserved) mosquitoes (20 female and 20 male per species per repeat; three biological repeats) was collected for comparison. Following preservation, midgut dissections (see section 2.3.1) and bacterial identification (see sections 2.4 and 2.5) were performed.

2.2.1. Silica Preservation

Mosquitoes were individually placed (20 females and 20 males per species per repeat; three biological repeats) in 1.5ml microcentrifuge tubes containing approximately five silica beads (silica gel blue self-indicator (copper sulphate-based), B&M Scientific, South Africa; cat no. CSGB0002) and mosquitoes were separated from silica using a piece of paper (Figure 2.2A). Mosquitoes were then preserved for the specified time periods, followed by midgut dissections. All supplies (silica beads, microcentrifuge tubes, separating paper, etc.) were sterilized prior to use and swabs of these supplies were cultured to ensure they were not contaminated.

2.2.2. RNA*later*® Preservation

Mosquitoes preserved in RNA*later*® (Qiagen, Germany; cat no. 76106) were first surface sterilized in 70% (v/v) ethanol, left to dry at room temperature on a sterile piece of Kimwipe® (Kimberly-Clark, TX, USA; cat no. 34155), and individually submerged in 0.5ml of RNA*later*® solution (20 females and 20 males per species per repeat; three biological repeats) in 1.5ml microcentrifuge tubes (Figure 2.2B). Mosquito surface sterilization was necessary prior to RNA*later*®-preservation to ensure that bacteria present on the mosquito surface did not contaminate the RNA*later*® solution because, unlike preservation with silica beads, RNA*later*® is bacteriostatic; that is, the solution stabilises bacterial cells but prevents bacterial growth (Sigma-Aldrich Technical Bulletin, 2016). Mosquitoes preserved in RNA*later*® for up to 4 weeks were stored at 4°C, while mosquitoes preserved for longer than 4 weeks were placed in RNA*later*® overnight at 4°C and subsequently stored at -80°C.

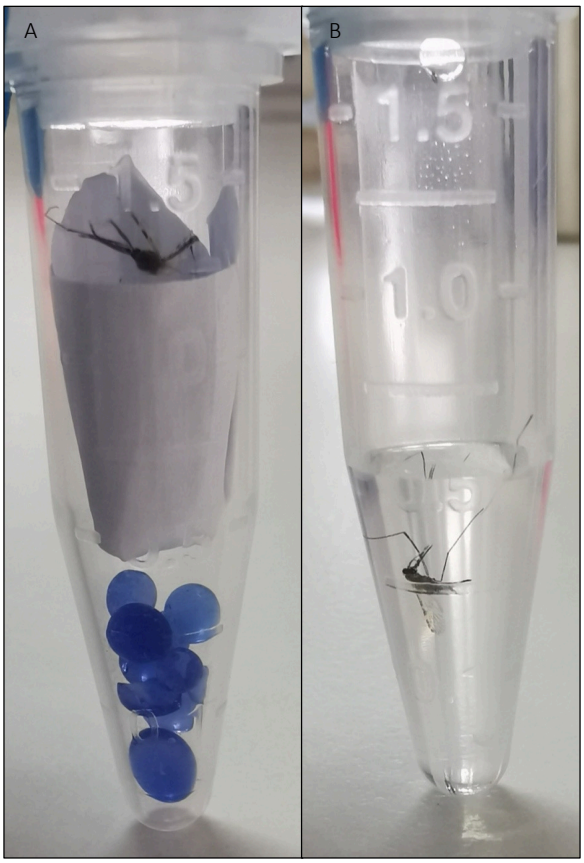

Figure 2.2: Mosquitoes preserved on (A) silica and in (B) RNA*later*®.

2.3. Midgut Dissections

Midgut dissections were performed aseptically as per the protocol by the WHO (1963). Briefly, mosquitoes were immobilised at -20°C for two minutes. Individual mosquitoes were then surface sterilised in 70% (v/v) ethanol, rinsed twice in sterile phosphate-buffered saline (PBS) (137 mM sodium chloride; 10 mM disodium phosphate; 2.7 mM potassium chloride; 1.8 mM monopotassium phosphate; pH 7.4), and transferred onto a microscope slide in a drop of PBS. Each midgut was carefully removed under a stereo microscope (10×magnification) using sterile dissecting forceps (see Figure 2.3 for examples of dissected mosquito midguts). Subsequently, midguts were pooled into 1.5ml microcentrifuge tubes containing sterile PBS, where 4µl of PBS was used per midgut and 20 midguts were pooled per gender, preservative, preservation time period, species, and repeat. Midguts were then homogenised using a TissueLyser II for 4 minutes at a frequency of 24 Hertz (Hz), followed by brief centrifugation. The negative control consisted of PBS alone.

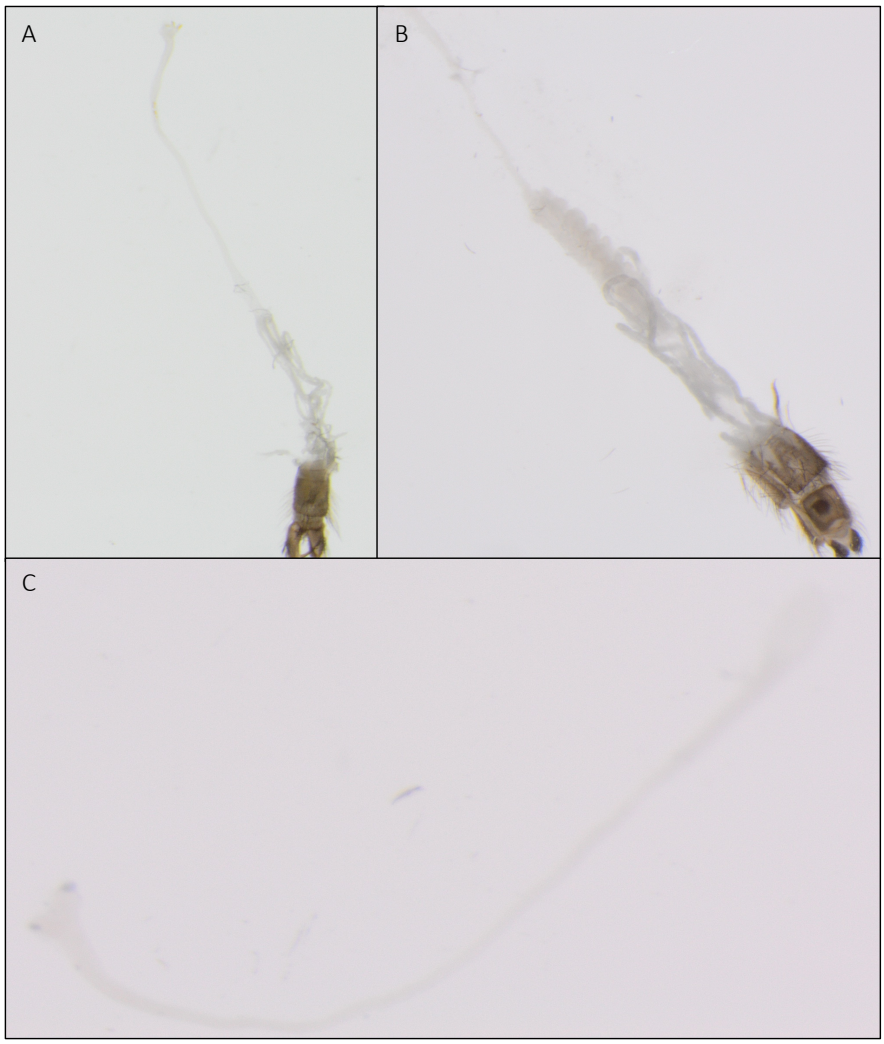

Figure 2.3: Dissected mosquito midguts. *An. funestus* (A) male and (B) female midguts (arrow) still attached to the hindguts prior to excision and (C) midgut isolation.

2.3.1. Dissections of Preserved Midguts

Desiccation caused abdomens to shrivel and become brittle. Therefore, midguts could not be dissected, and an adapted version of the protocol described in section 2.3 was performed. Mosquitoes were surface sterilised in 70% (v/v) ethanol and abdominal segments I to V were dissected (see Figure 2.4 for reference), pooled in sterile PBS, and homogenised as described. Similarly, submerging mosquitoes in RNA*later*® solution caused abdomens to tear apart easily and midguts could not be isolated. Thus, mosquitoes were removed from solution, blotted on tissue paper to remove excess RNA*later*®, and the adapted dissection protocol described for silica-preserved mosquitoes was performed.

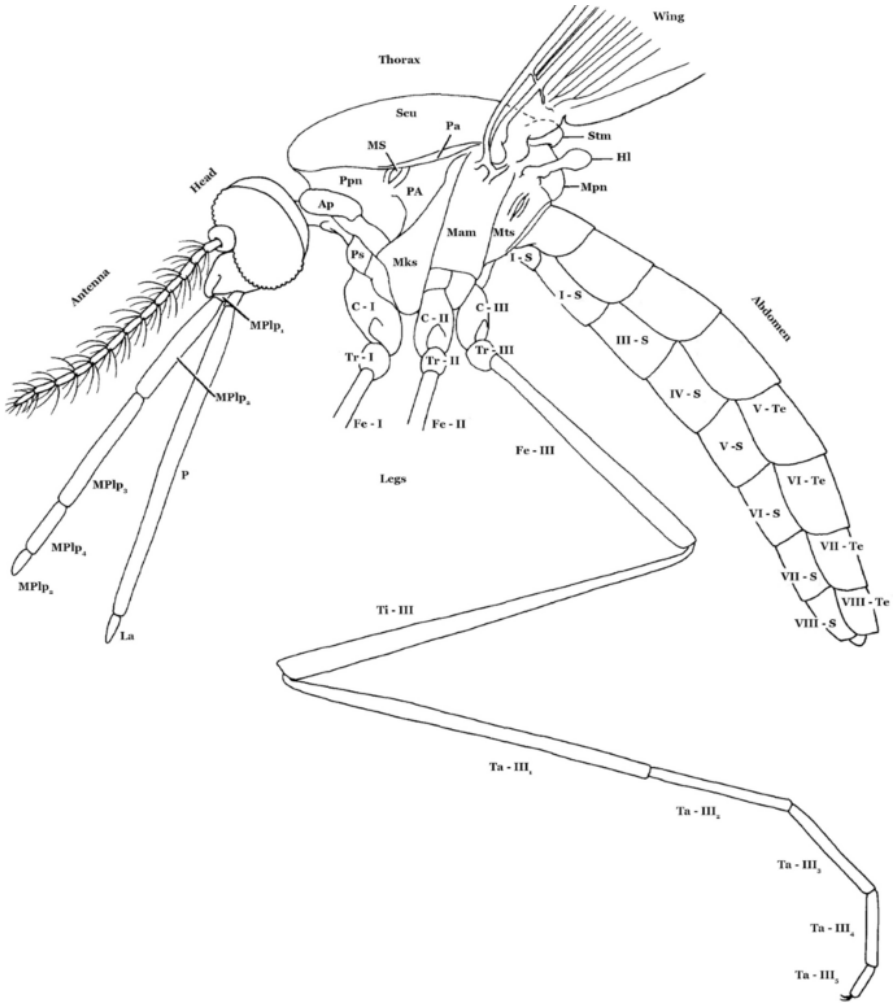

Figure 2.4: *Anopheles* anatomy: Abdominal segments I to V were dissected from preserved mosquitoes. Figure adapted from Sallum *et al.*, 2020.

2.4. Culture-dependent Techniques

Culture-dependent identification involved using culturomics (see section 2.4.1) to grow bacteria from the midgut homogenates of fresh (see section 2.1) and preserved (see section 2.2) mosquitoes, and mass spectrometry was used to identify morphologically distinct bacterial colonies (see section 2.4.2). Additionally, each species' larval rearing water was collected, and bacteria were identified to determine if there were bacteria in common between mosquitoes and their immediate environment.

2.4.1. Culturomics

A volume of 10µl of each homogenate was plated directly onto individual selective media agar plates: MacConkey agar (DMP/NICD, South Africa; cat no. DMPA0315), 10% blood agar

(DMP/NICD, South Africa; cat no. DMPA0115), blood agar with nalidixic acid and colistin (DMP/NICD, South Africa; cat no. DMPA0110), Chapman's agar (or mannitol salt agar) (DMP/NICD, South Africa; cat no. DMPA0316), and Brain-Heart Infusion (BHI) agar (DMP/NICD, South Africa; cat no. DMPB0120); see Table 2.1.

Table 2.1: Selective media used in this study and the types of bacteria they select for.

Type of media agar plate	Selective for
MacConkey agar	non-fastidious Gram-negative enteric bacteria (MacConkey, 1905)
10% blood agar	variety of fastidious bacteria (Murray *et al.*, 1995)
Blood agar with nalidixic acid and colistin	Gram-positive bacteria (Ferrieri and Blair, 1977)
Chapman's agar	Gram-positive bacteria, specifically staphylococci (Chapman *et al.*, 1937)
BHI agar	variety of fastidious bacteria (Rosenow, 1919)

Inoculates were plated using the streak plate method to obtain individual colonies. Subsequently, plates were aerobically incubated for a minimum of 16 hours at 37°C. Plates without observable colonies were re-incubated for 16 hours to account for slow-growing bacteria. Following incubation, bacterial isolates were distinguished morphologically from one another based on colour, elevation, form, margin, opacity, and size (Murray *et al*., 1995).

Morphologically distinct colonies were selected for repeated subculture to ensure single colony isolation until a presumably pure colony was obtained, where isolates were re-inoculated onto fresh primary agar plates and incubated for a minimum of 16 hours at 37°C. Plates without observable colonies were re-incubated for 16 hours to account for slow-growing bacteria. Negative controls were set up for each plate type, and during subsequent incubation periods, to ensure plates were not contaminated during incubation. Each plate type was also inoculated with PBS negative controls from dissections.

2.4.2. Mass Spectrometry (MALDI-TOF MS)

Each morphologically distinct colony was placed directly on an individual spot on a 96-spot reusable polished steel MALDI-TOF target plate (Bruker Daltonics, Wissembourg, France; cat no. 8280800). Each spot on the target plate was then covered with 1µl of portioned α-Cyano-4-

hydroxycinnamic acid (HCCA) matrix (Bruker Daltonics, Wissembourg, France; cat no. 8255344) diluted in standard solvent (50% acetonitrile: 47.5% water: 2.5% trifluoroacetic acid; Sigma-Aldrich, Lyon, France; cat no. 19182). The matrix was then allowed to dry at room temperature.

Subsequently, the target plate was placed in the MALDI Biotyper® with benchtop microflex™ LT/SH mass spectrometer (Bruker Daltonics, Germany). To control for loading on the plate, the quality of the matrix, and the performance of the instrument, a Bacterial Test Standard (Bruker Daltonics, Wissembourg, France; cat no. 8255343) was used according to the manufacturer's instructions to control for loading and matrix. Spectra were compared with the MBT 7854 MSP Library database installed on the computer (Bruker Daltonics, Wissembourg, France; ref no. 182903). An isolate was identified when spectra had a log score value (LSV) ≥ 1.9 (Seng *et al*., 2009). Every unidentified isolate was tested successively, where a portion of the same colony was placed on a new spot on the target plate and identified as described. As MALDI-TOF results were printed on paper, results were manually input into Excel spreadsheets. Subsequently, analyses were manually performed (see section 2.6).

2.5. Culture-independent Techniques

To supplement culture-dependent results, the midguts of fresh (see section 2.1) and preserved mosquitoes (preserved on silica or in RNA*later*® for 4 weeks and 12 weeks) were dissected (see section 2.3). This was followed by bacterial DNA extraction (section 2.5.1) and NGS (section 2.5.2). Additionally, each species' larval rearing water was collected, and bacteria were identified to determine if there were bacteria in common between mosquitoes and their immediate environment.

2.5.1. Bacterial DNA Extraction

Bacterial DNA was extracted from mosquito midguts using the QIAamp® DNA Microbiome Kit (Qiagen, Germany; cat no. 51704). The QIAamp® DNA Microbiome Kit separates microbial DNA from host DNA in mixed samples, resulting in pure bacterial DNA (Qiagen, 2014). The procedure involved adding one volume of Buffer AHL to two volumes of sample homogenate and incubating the mixture for 30 minutes at room temperature with end-over-end rotation. Subsequently, the mixture was centrifuged at 10,000x g for 10 minutes and the supernatant was discarded. Following this, 190µl of Buffer RDD and 2.5µl of Benzonase were

added to the mixture, which was vortexed and incubated at 37°C for 30 minutes. Next, 20µl of Proteinase K was added to the mixture, which was incubated at 56°C for 30 minutes. The tube was briefly centrifuged and 200µl of Buffer ATL (containing Reagent DX) was added to the mixture, followed by brief vortexing.

The mixture was then transferred to a Pathogen Lysis Tube L, which was placed in a TissueLyser II for 10 minutes at 30 Hz to lyse bacterial cells. The tube was centrifuged at 10,000x g for 1 minute and the supernatant was transferred to a fresh microcentrifuge tube. Subsequently, 40µl of Proteinase K was added to the mixture, followed by vortexing and incubation at 56°C for 30 minutes. Next, 200µl of Buffer APL2 was added to the mixture, which was mixed by pulse vortexing for 30 seconds. The mixture was incubated at 70°C for 10 minutes and 200µl of ethanol (100%) was then added to the lysate and mixed by pulse vortexing for 30 seconds.

The mixture (approximately 700µl) was transferred to a QIAamp® UCP Mini Column, which was centrifuged at 6,000x g for 1 minute. The flow-through was then discard, the column was placed back into the collection tube, and centrifugation was repeated. The column was transferred to a fresh collection tube and 500µl of Buffer AW1 was added to the column, followed by centrifugation at 6,000x g for 1 minute. The filtrate was then discarded, and the column was placed into a fresh collection tube. Subsequently, 500µl of Buffer AW2 was added to the column, followed by centrifugation at 13,000x g for 3 minutes. The filtrate was discarded, and the column was placed into a fresh collection tube, followed by centrifugation at 13,000x g for 1 minute. The column was then placed in a fresh collection tube and 50µl of nuclease-free water was added to the column membrane. The tube was then incubated at room temperature for 5 minutes, followed by centrifugation at 6,000x g for 1 minute to elute the bacterial DNA. Negative controls were set up during each stage of DNA extraction, and DNA extraction was also performed on PBS negative controls from dissections. Prior to sequencing, DNA quality and purity were measured using the NanoDrop™ 2000c spectrophotometer (Thermo Scientific™, MA, USA; cat no. ND-2000C). Due to the high cost of NGS and the high number of negative controls, only experimental samples were sequenced. Nonetheless, negative controls were assessed using the NanoDrop™ 2000c spectrophotometer and were cultured to ensure no contaminants were present.

2.5.2. Next Generation Sequencing (NGS)

Following DNA extraction, bacterial DNA was subjected to NGS, which involved library preparation, library construction, sequencing using the Illumina Sequencing by Synthesis (SBS) technology, and conversion of sequencing data to raw data for analysis and taxonomic identification. Specifically, DNA samples were sent to Macrogen Europe (Amsterdam, The Netherlands) for 16S rRNA gene sequencing targeting the V3-V4 regions with universal primers, Bakt 341F and Bakt 805R (Herlemann *et al*., 2011). The Illumina MiSeq system was used to perform paired-end sequencing and the Fast Length Adjustment of SHort reads (FLASH; version 1.2.11) program was used to assemble reads (Magoč and Salzberg, 2011). Pre-processing (denoising) and clustering of sequences were performed with the CD-HIT-OTU and rDnaTools programs (Li *et al*., 2012; Schloss *et al*., 2009). Diversity analyses and taxonomy assignment were performed with the Quantitative Insights Into Microbial Ecology (QIIME) program (Caporaso *et al*., 2010). Analysed bioinformatics results (including, but not limited to, OTU with taxonomy assignment and diversity indices) were received via flash drive.

2.6. Data Analysis, Diversity Indices, and Statistical Analysis

According to the MALDI-TOF MS MBT 7854 MSP Library, several bacteria are phenotypically indistinguishable from one another. Thus, indistinguishable bacteria were grouped according to which bacteria were also identified by NGS (section 2.6.1). Additionally, midgut bacterial species richness was measured, and diversity indices were used to calculate midgut bacterial diversity, relative abundance, and evenness (section 2.6.2). Statistical analyses were performed on the values of these diversity indices to determine if there were any differences amongst midgut bacterial diversity of different mosquito genders, species, preservation conditions, or bacterial identification techniques (section 2.6.3).

2.6.1. MALDI-TOF MS MBT 7854 MSP Library

The library installed on the MALDI-TOF MS database, the MBT Compass 7854 MSP Library, cannot distinguish several closely related bacterial species from one another (refer to the Matching Hints section of the MBT 7854 MSP Library Release Notes for more information). Therefore, MALDI-TOF MS and NGS results were compared (Appendix B, Table B1). Accordingly, indistinguishable bacteria were grouped, where a single bacterium was used to represent indistinguishable bacteria (Table 2.2). Representative bacteria were chosen based on

which one of the indistinguishable bacteria was dominant in NGS results. For example, both *Aeromonas hydrophila* and *Aeromonas veronii* were present in MALDI-TOF MS results. However, according to the Matching Hints Section of the MBT 7854 MSP Library Release Notes, the library cannot distinguish between these two closely related bacteria. Therefore, using culture-independent results, I validated which of these bacteria were 'more likely' to have been identified by MALDI-TOF MS. As *A. veronii* did not appear in NGS results, but *A. hydrophila* was a dominant, frequently recurring bacteria in NGS results, *A. hydrophila* was used to represent both *A. hydrophila* and *A. veronii* identified by MALDI-TOF MS in this study.

Table 2.2: Bacteria used to represent phenotypically indistinguishable bacteria. A single bacterium was used to represent bacteria that were indistinguishable by the MBT 7854 MSP Library.

Indistinguishable bacteria (MBT 7854 MSP Library)	Representative bacterium
A. hydrophila and *A. veronii*	*A. hydrophila*
Corynebacterium propinquum and *Corynebacterium pseudodiphtheriticum*	*C. pseudodiphtheriticum*
Delftia acidovorans and *Delftia tsuruhatensis*	*D. tsuruhatensis*
E. anophelis, *Elizabethkingia meningoseptica*, and *Elizabethkingia miricola*	*E. anophelis**
E. coli and *Escherichia fergusonii*	*E. fergusonii*
Klebsiella oxytoca, *Raoultella ornithinolytica*, *Raoultella planticola*, and *Raoultella terrigena*	*R. ornithinolytica***

**E. anophelis* is often misidentified as *E. meningoseptica* by MALDI-TOF MS (Lin *et al.*, 2018).

***R. ornithinolytica* is often misidentified as *Klebsiella pneumoniae* or *K. oxytoca* (Sitaula *et al*., 2016; Surani *et al*., 2020).

Similarly, *Serratia fonticola* was a dominant bacterium identified by culture-dependent techniques but was unidentified by culture-independent techniques. Since *Serratia oryzae* was undetected by the MBT 7854 MSP Library, it is likely that *S. fonticola* identified by culturomics was *S. oryzae*, especially since *S. oryzae* was identified as a dominant bacterium by NGS. To determine sequence similarity and the likelihood that *S. fonticola* identified by culture-dependent techniques was truly *S. oryzae*, I performed a pairwise alignment using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool for nucleotide sequences (BLASTn) (Zhang *et al*., 2000). Results revealed a 96.75% sequence similarity between *S. fonticola* (accession number: CP011254.1) and *S. oryzae* (accession number: NR_157762.1). Meanwhile, a pairwise alignment between *S. fonticola* and *Serratia*

liquefaciens (accession number: UGYL01000001.1), as well as *S. marcescens* (accession number: CP063354.1) (the other *Serratia* species identified by culture-independent techniques in this study), revealed 94.03% and 85.53% sequence similarity, respectively. Therefore, *S. oryzae* was used to represent *S. fonticola* throughout this study.

2.6.2. Diversity Indices

Midgut bacterial diversity, relative abundance, and species distribution were determined (see Appendix C for calculations used). Additionally, species richness was measured as the number of OTUs per sample. Means and standard deviations (SD) were calculated for diversity, relative abundance, and evenness indices. Therefore, diversity was measured using the Shannon-Wiener diversity index (H) (Barnes *et al.*, 1998; Shannon, 1948). The higher the value of H, the more diverse the bacterial community. If the value of H is low, the community is dominated by one species (Barnes *et al.*, 1998).

The reciprocal of Simpson's index ($\frac{1}{D}$) was calculated to estimate dominance as a measure of relative abundance, which is the probability that a randomly selected species would be the dominant species (Simpson, 1949). One is the lowest value of $\frac{1}{D}$ and indicates that the community is dominated by a single species. Therefore, the higher the value of $\frac{1}{D}$, the greater the diversity (Simpson, 1949).

To estimate evenness as a measure of species distribution, Pielou's evenness index (E) was calculated (Pielou, 1975). Values of E range from zero to one, where zero represents unevenness and one represents complete evenness in a bacterial community. In essence, if there is completeness, there is an even number of species A relative to species B (Pielou, 1975).

2.6.3. Statistical Analysis

Statistical analyses were conducted in GraphPad Prism (San Diego, CA) v8.4.3 with significance set at 95% confidence. Probability/P-values (P) are shown for statistical significance, where no significance (ns) is shown when $P>0.05$. Meanwhile, $P\leq0.05$ is denoted by *, $P\leq0.01$ is denoted by **, $P\leq0.001$ is denoted by ***, and $P\leq0.0001$ is denoted by ****.

Welch's t-tests were used to compare diversity indices individually between females and males of the same species, and between results of fresh and preserved mosquitoes. Additionally, one-way Analysis of Variances (ANOVAs) with multiple comparisons were used to compare:

(i) the diversity indices of preserved colonised *An. arabiensis* with preserved field-collected *An. arabiensis* from KZN and Mpumalanga, and
(ii) diversity indices between fresh and preserved *An. arabiensis* and fresh and preserved *An. funestus* at different preservation time periods.

Moreover, two-way ANOVAs were used to compare diversity indices between:

(i) colonised *An. arabiensis* females and males with colonised *An. funestus* females and males, and females of both species with males of both species,
(ii) colonised fresh mosquitoes of both species with preserved mosquitoes of both species, and fresh and preserved *An. arabiensis* with fresh and preserved *An. funestus*, and
(iii) culture-dependent results with culture-independent results of both species, and *An. arabiensis* with *An. funestus* identified by both techniques.

CHAPTER THREE

RESULTS

3.1. Midgut Bacteria from Colonised and Field-collected *Anopheles arabiensis*

Midgut bacteria of fresh colonised *An. arabiensis* were characterised using culture-dependent and culture-independent techniques (section 3.1.1). This was performed to determine the native midgut bacteria in *An. arabiensis* that may be essential to physiological functioning. Moreover, bacteria were identified from *An. arabiensis* larval rearing water to determine whether mosquitoes acquired bacteria from their environment. Culture-dependent and culture-independent methods were also used to determine if the midgut bacteria of preserved *An. arabiensis* could be identified after mosquito preservation (section 3.1.2). Furthermore, the midgut bacteria of preserved colonised *An. arabiensis* females were compared to preserved field-collected *An. arabiensis* females from KZN and Mpumalanga to determine if there were bacteria in common between colonised and field-collected mosquitoes (section 3.1.3).

3.1.1. Bacteria from *Anopheles arabiensis* and the Larval Rearing Water

Culture-dependent results show that *An. arabiensis* midguts were predominantly colonised by the phylum Proteobacteria, followed by Firmicutes and Bacteroidetes (Appendix D, Figure D1). Additionally, the dominant genus was *Staphylococcus*, followed by *Pseudomonas*, and *An. arabiensis* midguts were colonised by more Gram-negative bacteria (72.84%) than Gram-positive bacteria (27.16%). Specifically, female *An. arabiensis* midguts had a higher proportion of Proteobacteria than males, while males had a higher proportion of Firmicutes and Bacteroidetes (Appendix D, Figure D2). Nonetheless, *Staphylococcus* was the dominant genus in both genders, followed by *Pseudomonas*. Moreover, female and male midguts were predominantly colonised by Gram-negative bacteria (76.92% and 68.75% respectively), while Gram-positive bacteria made up 23.08% of female midguts and 31.25% of male midguts.

Further, culture-dependent results show that female and male *An. arabiensis* had bacteria in common with their larval rearing water: *A. hydrophila*, *E. anophelis*, *S. oryzae*, *Staphylococcus caprae*, and *Staphylococcus epidermidis* (Figure 3.1). These bacteria were likely acquired by mosquitoes from their environment. Additionally, bacteria were common between female and male *An. arabiensis*, but were absent from the larval rearing water: *Klebsiella variicola*, *Pseudomonas libanensis*, *Rahnella aquatilis*, and *R. ornithinolytica*. These bacteria possibly

co-evolved with both genders. Moreover, males had *Sphingobacterium multivorum* and *Staphylococcus hominis* in common with the larval rearing water, which shows that these bacteria were likely acquired from the environment specifically by males.

Additionally, female and male *An. arabiensis* had specific bacteria that belonged exclusively to each gender. *Herbaspirillum huttiense*, *Pseudomonas azotoformans*, a *Psychrobacter* species (sp.), and *Staphylococcus haemolyticus* were exclusive to females, while *Pseudomonas antarctica*, *Pseudomonas extremorientalis*, *Pseudomonas grimontii*, *Staphylococcus aureus*, and *Staphylococcus pasteuri* were exclusive to males. Moreover, a variety of bacteria were isolated from the larval rearing water that were not detected in mosquito midguts, demonstrating that some environmental bacteria could not be acquired by mosquitoes.

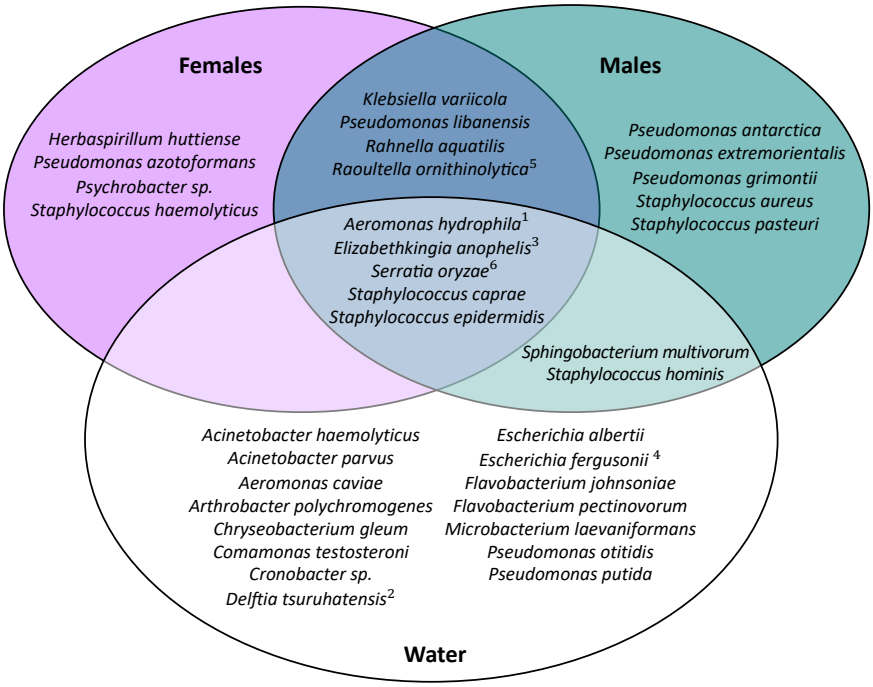

Figure 3.1: Bacteria identified by culture-dependent techniques from female and male *An. arabiensis* and their larval rearing water. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*D. acidovorans* and *D. tsuruhatensis*; [3]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [4]*E. coli* and *E. fergusonii*; [5]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [6]*S. fonticola* and *S. oryzae*.

Like culture-dependent results, culture-independent results show that *An. arabiensis* were predominantly colonised by Proteobacteria (Appendix D, Figure D3). This was followed by Actinobacteria, Bacteroidetes, Firmicutes, and Cyanobacteria (Actinobacteria and Cyanobacteria were not identified by culture-dependent methods). The dominant genus in *An. arabiensis* was *Pseudomonas*, followed by *Yersinia*. Culture-independent results also show that

An. arabiensis midguts were colonised by more Gram-negative bacteria (86.13%) than Gram-positive bacteria (13.87%).

Similar to culture-dependent results, culture-independent results show that female *An. arabiensis* had a higher proportion of Proteobacteria than males (Appendix D, Figure D4). In contrast, males had a higher proportion of Actinobacteria and Firmicutes. Males were also colonised by Cyanobacteria, which was absent in females. Midguts of both genders were predominated by *Pseudomonas*, followed by *Yersinia*. Additionally, culture-independent results show that females were colonised by 92.86% Gram-negative and 7.14% Gram-positive bacteria, while males were colonised by 79.41% Gram-negative and 20.59% Gram-positive bacteria. Thus, culture-independent results reported a larger proportion of Gram-negative bacteria in the midguts of both genders than culture-dependent results.

In terms of bacterial species, culture-independent methods identified a larger number of bacterial species than culture-dependent methods. Nonetheless, culture-independent techniques also show that females and males had bacteria in common with the rearing water: *A. hydrophila*, *E. anophelis*, *Enterobacter cloacae*, *Pseudomonas geniculata*, *Pseudomonas veronii*, *S. oryzae*, *S. multivorum*, *S. epidermidis*, and *Yersinia aldovae* (Figure 3.2). These bacteria were likely acquired by mosquitoes from their environment. Additionally, females and males had bacteria in common that were absent in the larval rearing water: *Aeromonas sanarellii*, *Gluconobacter frateurii*, *Pseudomonas canadensis*, *P. grimontii*, *Pseudomonas savastanoi*, *Yersinia enterocolitica*, *Yersinia entomophaga*, and *Yersinia wautersii*. This suggests that these bacteria possibly co-evolved with both genders of this vector species.

Furthermore, females had *Pedobacter solisilvae* and *Psychrobacter faecalis* in common with the water, suggesting that these bacteria were probably acquired specifically by females. Similarly, *Aerosakkonema funiforme*, *Brevundimonas albigilva*, *Leucobacter iarius*, *Microbacterium maritypicum*, *Paracoccus aerius*, *Phyllobacterium myrsinacearum*, *Reyranella aquatilis*, *Siphonobacter aquaeclarae*, *Streptococcus periodonticum*, and *Streptococcus thermophilus* were common between males and the water, meaning these bacteria were probably acquired exclusively by males.

Females and males also had bacteria isolated exclusively per gender, showing that some bacteria probably co-evolved in a gender-specific manner. *Aeromonas taiwanensis*, *Corynebacterium gottingense*, *Gluconobacter japonicus*, *Hafnia psychrotolerans*, *Microbulbifer maritimus*, *Pseudomonas palleroniana*, *Pseudomonas tolaasii*, and *R. ornithinolytica* were exclusive to females. Meanwhile, *Actinomyces oris*, *Asaia krungthepensis*,

Brucella ovis, *Corynebacterium kroppenstedtii*, *Methylobacterium phyllostachyos*, and *Pseudomonas marginalis* were exclusive to males. Bacteria were also isolated from the larval rearing water that were absent from mosquito midguts, which shows that mosquitoes could not acquire some bacteria from their environment.

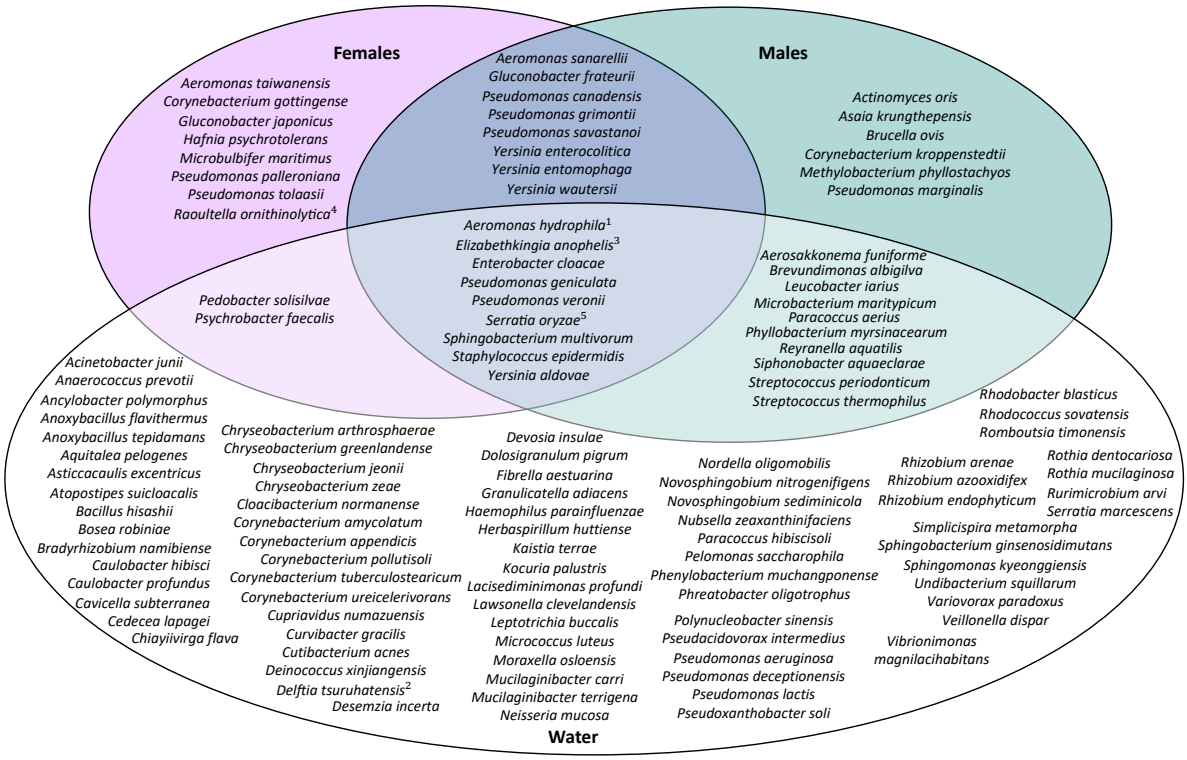

Figure 3.2: Bacteria identified by culture-independent techniques from female and male *An. arabiensis* and their larval rearing water. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*D. acidovorans* and *D. tsuruhatensis*; [3]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [4]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [5]*S. fonticola* and *S. oryzae*.

Collectively, culture-dependent and culture-independent results agree that there were bacteria in common between female and male *An. arabiensis*: *A. hydrophila*, *E. anophelis*, *S. oryzae*, and *S. epidermidis* (Figure 3.3). These bacteria likely form part of the native midgut bacteria of colonised *An. arabiensis* that may be essential to their biological functioning. Additionally, *S. multivorum* was identified in males by culture-dependent techniques and in females and males by culture-independent techniques and *R. ornithinolytica* was identified in females and males by culture-dependent techniques but only in females by culture-independent techniques. Thus, these bacteria likely also contribute to physiological functioning of this vector species despite being variably detected by the identification methods used.

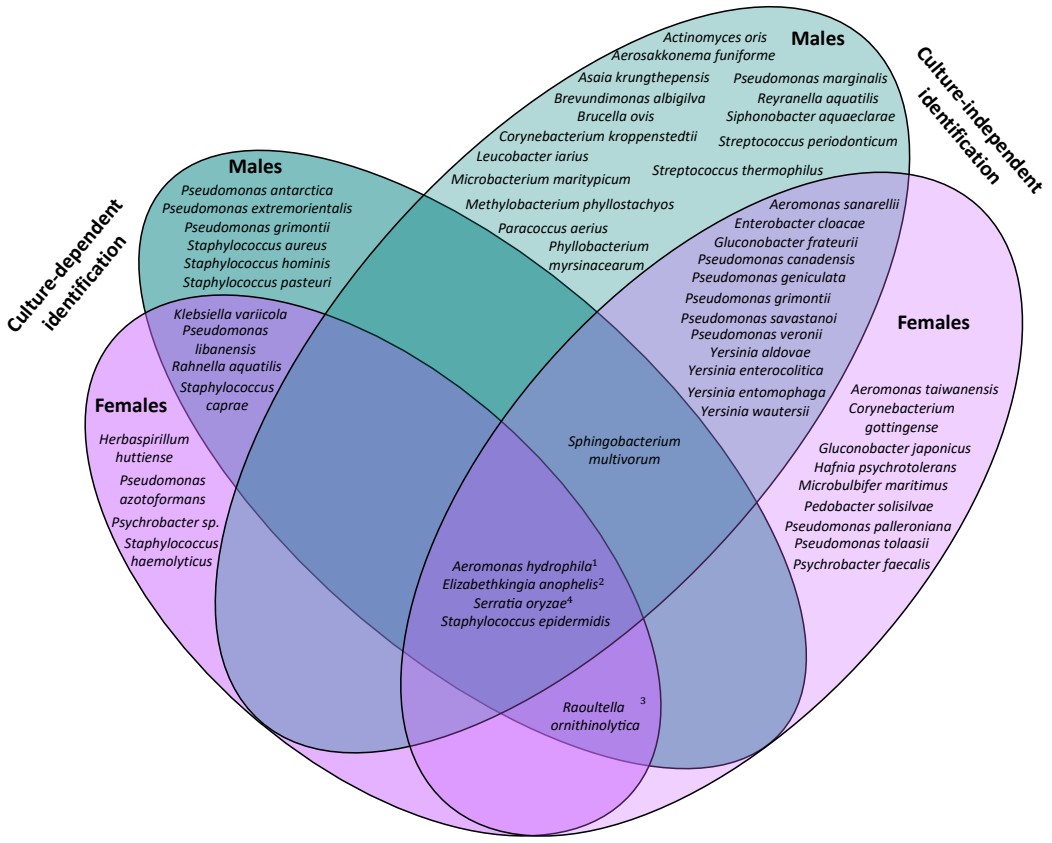

Figure 3.3: Bacteria identified by culture-dependent and culture-independent techniques that are common between colonised female and male *An. arabiensis*. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [3]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [4]*S. fonticola* and *S. oryzae*.

3.1.2. *Anopheles arabiensis* Midgut Bacteria Identified After Mosquito Preservation

Preserved colonised *An. arabiensis* had their midgut bacteria characterised by culture-dependent and culture-independent techniques. Firstly, bacteria grew on selective media from the midguts of preserved mosquitoes, demonstrating that midgut bacteria were conserved during mosquito preservation and could be identified post-preservation. Secondly, culture-dependent results show that mosquitoes preserved on silica and in RNA*later*® had bacteria in common. Thus, both preservatives were capable of conserving mosquitoes and their midgut bacteria (Appendix E, Figure E1).

Specifically, culture-dependent results show that preserved females and males had bacteria in common (Table 3.1). However, bacteria were isolated from silica-preserved females and males that were absent in RNA*later*®-preserved mosquitoes, such as *Cedecea lapagei*, *Cedecea neteri*, and *E. anophelis*. This could imply that silica preservation is better suited than RNA*later*® preservation for conserving specific bacterial species. Further, several bacteria were identified in a gender- and preservative-specific manner. For example, *Arthrobacter woluwensis*,

Ewingella americana, *K. variicola*, *S. oryzae*, and *Staphylococcus capitis* were only identified in silica-preserved males, while a *Psychrobacter* sp. was only identified in RNA*later*®-preserved males.

Culture-independent methods identified more bacteria from preserved *An. arabiensis* than culture-dependent methods. Nonetheless, culture-independent methods also detected common bacteria between preserved female and male *An. arabiensis* (Table 3.1). Many bacteria were also common between females and males preserved either by silica or RNA*later*® in varying combinations per gender (Appendix E, Figure E2). Bacteria were also identified from females or males in a preservative-dependent manner; a detailed analysis of these results can be found in Appendix E (Figures E3–E6). Overall, culture-dependent and culture-independent results agree that *S. epidermidis* was the only bacterium in *An. arabiensis* midguts preserved by both preservatives (Table 3.1 and Appendix E, Figure E7).

Table 3.1: Common midgut bacteria between preserved female and male *An. arabiensis*. Mosquitoes were preserved on silica or in RNA*later*® and midgut bacteria were identified by culture-dependent and culture-independent techniques. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [4]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [5]*S. fonticola* and *S. oryzae*. Green shading represents bacteria identified by both identification methods from mosquitoes conserved by both preservatives.

Bacteria		**Identification Method**	
Genera	**Species**	**Culture-dependent**	**Culture-independent**
Acinetobacter	*johnsonii*		x
	lwoffii		x
Aeromonas	*hydrophila*[1]		x
Aerosakkonema	*funiforme*		x
Asaia	*krungthepensis*		x
Brevundimonas	*albigilva*		x
Cedecea	*lapagei*		x
Corynebacterium	*amycolatum*		x
	aurimucosum		x
	pollutisoli		x
	pseudodiphtheriticum[2]		x
	tuberculostearicum		x
	ureicelerivorans		x
Cutibacterium	*acnes*		x
Deinococcus	*xinjiangensis*		x

Dolosigranulum	*pigrum*		x
Elizabethkingia	*anophelis*[3]		x
Enterobacter	*cloacae*		x
Finegoldia	*magna*		x
Gluconobacter	*frateurii*		x
Herbaspirillum	*huttiense*		x
Lawsonella	*clevelandensis*		x
Methylobacterium	*phyllostachyos*		x
Microbacterium	*maritypicum*		x
Moraxella	*osloensis*		x
Paracoccus	*aerius*		x
Phenylobacterium	*aquaticum*		x
Pseudomonas	*aeruginosa*		x
	geniculata		x
Psychrobacter	*faecalis*		x
Rahnella	*aquatilis*	x	
Raoultella	*ornithinolytica*[4]	x	
Rothia	*dentocariosa*		x
	mucilaginosa		x
Serratia	*marcescens*		x
	oryzae[5]		x
Staphylococcus	*epidermidis*	x	x
	hominis	x	
Streptococcus	*periodonticum*		x
	thermophilus		x
Variovorax	*paradoxus*		x
Yersinia	*aldovae*		x

It should also be noted that *C. lapagei*, *E. anophelis*, and *S. oryzae* were identified by culture-independent methods from mosquitoes preserved on silica and RNA*later*® but only in silica-preserved mosquitoes by culture-dependent methods (Appendix E, Figure E7). Similarly, *R. ornithinolytica* was identified by culture-dependent methods from mosquitoes preserved on silica and in RNA*later*® but only in silica-preserved mosquitoes by culture-independent methods. Also, a *Psychrobacter* sp. was identified by culture-dependent methods from RNA*later*®-preserved mosquitoes and by culture-independent methods from silica-preserved mosquitoes. Hence, although identification methods variably detected these bacteria in specific genders and specific preservatives, these bacteria were preserved in *An. arabiensis* midguts and some of these bacteria also formed part of the native microbiota identified in fresh colonised

An. arabiensis. This means that these bacteria likely also play important physiological functions in *An. arabiensis*.

As an overview, culture-dependent methods show that fresh and preserved female and male *An. arabiensis* had *E. anophelis*, *R. aquatilis*, *R. ornithinolytica*, and *S. epidermidis* in common (Appendix F, Figure F1). Additionally, *K. variicola* and *S. oryzae* were common between fresh females and males but only preserved males. Likewise, a *Psychrobacter* sp. was detected in fresh females but only in preserved males. Further, fresh females and males shared common bacteria, such as *A. hydrophila*, *P. libanensis*, and *S. caprae*, which differed from common bacteria in preserved females and males: *C. lapagei*, *C. neteri*, and *S. hominis*. Nonetheless, these results show that the common native midgut bacteria of fresh colonised *An. arabiensis* can be preserved and identified post-preservation.

In contrast, culture-independent methods identified *A. hydrophila, E. anophelis*, *E. cloacae*, *G. frateurii*, *P. geniculata*, *P. grimontii*, *P. savastanoi*, *P. veronii*, *S. oryzae*, *S. epidermidis*, *Y. enterocolitica*, *Y. entomophaga*, and *Y. aldovae* as common midgut bacteria between fresh and preserved female and male *An. arabiensis* (Appendix F, Figure F2). Additionally, bacteria were common between fresh females and preserved males, fresh males and preserved females, fresh females and preserved females and males, and preserved females and males and fresh males (see Appendix F, Figure F2). Also, *B. ovis*, *P. marginalis*, and *R. aquatilis* were isolated from fresh and preserved males. Hence, these bacteria are likely specific to *An. arabiensis* males.

Overall, both identification methods agree that *E. anophelis*, *Psychrobacter* spp., *R. ornithinolytica*, *S. oryzae*, and *S. epidermidis* were common between fresh and preserved *An. arabiensis* (Figure 3.4). Thus, these bacteria potentially form part of *An. arabiensis* native microbiota that also become conserved upon mosquito preservation.

Additionally, *A. hydrophila* and *P. grimontii* were identified by culture-independent methods in fresh and preserved *An. arabiensis* but only in fresh *An. arabiensis* by culture-dependent methods. Moreover, *H. huttiense* was identified in fresh *An. arabiensis* by culture-dependent methods but only in preserved *An. arabiensis* by culture-independent methods. This means that despite variable detection by both identification techniques, these bacteria likely also form part of the *An. arabiensis* native microbiota and become conserved upon mosquito preservation. In essence, these bacteria were only identified post-preservation in a technique-dependent manner. Further, both identification methods agree that *C. lapagei* was only found in preserved *An. arabiensis*. Therefore, *C. lapagei* is likely a bacterium that only colonises in the midguts during

preservation (i.e., *C. lapagei* is likely present as coloniser in both fresh and preserved mosquitoes, but presumably in numbers that are too low to detect in fresh samples).

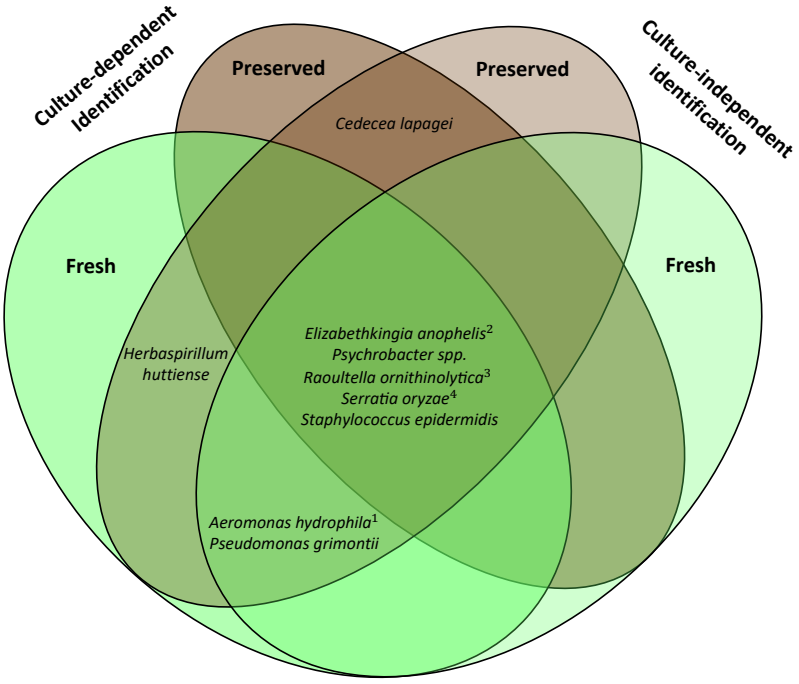

Figure 3.4: Common midgut bacteria identified by culture-dependent and culture-independent techniques from fresh and preserved *An. arabiensis.* Mosquitoes were preserved on silica or in RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [3]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [4]*S. fonticola* and *S. oryzae*.

3.1.3. Midgut Bacteria of *Anopheles arabiensis* from KwaZulu-Natal and Mpumalanga

Field-collected female *An. arabiensis* were preserved on silica for up to 16 weeks and their midgut bacteria were characterised using culture-dependent techniques due to the low cost for the limited number of samples. These mosquitoes were either from KZN (section 3.1.3.1) or from Mpumalanga (section 3.1.3.2). Midgut bacteria of silica-preserved field-collected female *An. arabiensis* from both localities were compared with midgut bacteria of silica-preserved colonised female *An. arabiensis* to determine whether there were significant differences in midgut bacterial diversity and bacterial species composition (section 3.1.3.3).

3.1.3.1. Bacteria from Field-collected Anopheles arabiensis from KwaZulu-Natal

Field-collected *An. arabiensis* from KZN had midguts that were predominantly colonised by the phylum Firmicutes, followed by Proteobacteria and Actinobacteria (Appendix G, Figure G1). This differs from the phyla isolated from colonised *An. arabiensis*, which had

Proteobacteria in highest abundance. However, the dominant genus in field-collected *An. arabiensis* was *Staphylococcus*, which was also the dominant genus in colonised *An. arabiensis* according to culture-dependent results. However, field-collected *An. arabiensis* midguts were colonised by more Gram-positive bacteria than Gram-negative bacteria, while the opposite was true for colonised *An. arabiensis*. Specifically, field-collected *An. arabiensis* were colonised by 75% Gram-positive and 25% Gram-negative bacteria while colonised *An. arabiensis* were colonised by 76.92% Gram-negative and 23.08% Gram-positive bacteria.

Field-collected *An. arabiensis* females differed from colonised *An. arabiensis* females in that the former were predominated by Firmicutes and had a higher proportion of Gram-positive midgut bacteria. In contrast, colonised females were predominated by Proteobacteria and had a higher proportion of Gram-negative midgut bacteria. Since mosquitoes acquire the majority of their midgut bacterial contents from their environment, the difference in midgut bacterial composition is possibly due to the impact of environmentally acquired bacteria, where colonised mosquitoes may have acquired more Gram-negative bacteria from the controlled insectary environment, while field-collected mosquitoes may have acquired their midgut bacteria from an environment that was potentially rich in Gram-positive bacteria.

Furthermore, field-collected *An. arabiensis* (OTUs=12) had higher midgut bacterial species richness than colonised *An. arabiensis* (OTUs=7) (Appendix G, Figure G2). More bacterial species were identified from field-collected *An. arabiensis* females from KZN than from colonised *An. arabiensis* females. Nonetheless, despite differences in phyla, genera, and species richness, field-collected *An. arabiensis* females had *S. epidermidis* and *S. hominis* in common with colonised *An. arabiensis* females (Figure 3.5). See Appendix G for a detailed analysis of common bacteria between fresh colonised female and male and preserved field-collected female *An. arabiensis* (Figure G3A) and colonised and field-collected preserved female *An. arabiensis* (Figure G3B).

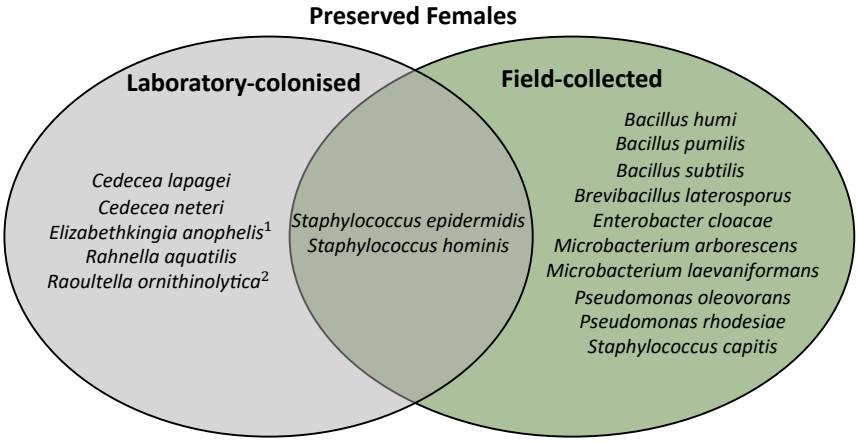

Figure 3.5: Common midgut bacteria between colonised *An. arabiensis* and field-collected female *An. arabiensis* from Kwa-Zulu Natal, South Africa. Field-collected *An. arabiensis* were preserved on silica for up to 16 weeks and bacteria were identified using culture-dependent methods. Bacteria indistinguishable by MALDI-TOF MS include [1]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; and [2]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*.

3.1.3.2. Bacteria from Field-collected Anopheles arabiensis from Mpumalanga

Only two bacteria were identified from the midguts of field-collected *An. arabiensis* from Mpumalanga: *S. aureus and S. epidermidis* (OTUs=2) (Appendix G, Figure G4). Therefore, midguts were only colonised by Firmicutes and were predominated by *Staphylococcus*. Thus, midgut colonisers were composed of 100% Gram-positive bacteria. Moreover, field-collected *An. arabiensis* females had *S. epidermidis* in common with colonised *An. arabiensis* females (Figure 3.6). See Appendix G for a detailed analysis of common bacteria between fresh colonised female and male and preserved field-collected female *An. arabiensis* (Figure G5A) and colonised and field-collected preserved female *An. arabiensis* (Figure G5B).

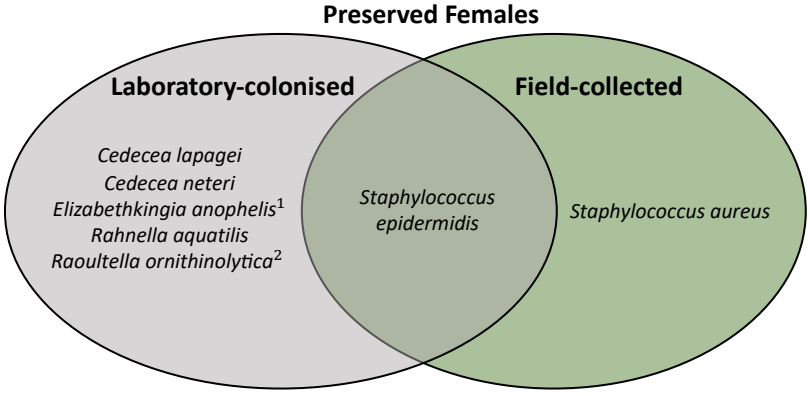

Figure 3.6: Common midgut bacteria between colonised *An. arabiensis* and field-collected female *An. arabiensis* from Mpumalanga, South Africa. Field-collected *An. arabiensis* were preserved on silica for up to 16 weeks and bacteria were identified using culture-dependent methods. Bacteria indistinguishable by MALDI-TOF MS include [1]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; and [2]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*.

3.1.3.3. Comparison of Anopheles arabiensis from KwaZulu-Natal and Mpumalanga

Field-collected *An. arabiensis* from both localities were predominated by Firmicutes and had *Staphylococcus* as their dominant genus. Field-collected *An. arabiensis* from KZN had the highest species richness, while field-collected *An. arabiensis* from Mpumalanga had the lowest species richness (Appendix G, Figure G6). Additionally, field-collected *An. arabiensis* from both localities had more Gram-positive than Gram-negative bacteria. This differs from colonised *An. arabiensis*, which were predominated by Proteobacteria and Gram-negative midgut bacteria. Nevertheless, field-collected and colonised midgut bacteria were predominantly colonised by bacteria of the genus *Staphylococcus*, suggesting that this genus is probably the major midgut bacterial genus of the *An. arabiensis* microbiota.

Field-collected *An. arabiensis* from both localities had *S. epidermidis* in common with one another, as well as with colonised *An. arabiensis* (Appendix G, Figure G7). Hence, this bacterium is a dominant midgut bacterium that is likely essential to *An. arabiensis* physiological functioning and has probably co-evolved with this vector species. Furthermore, *S. hominis* was also common between colonised *An. arabiensis* and field-collected *An. arabiensis* from KZN. Therefore, since *An. arabiensis* of the MBN laboratory colony (the laboratory colony used in this study) had mosquitoes that originated from KZN, this bacterium was likely acquired by mosquitoes from the environment in this locality and has probably co-evolved with mosquitoes in the laboratory colony. Hence, this bacterium was not identified in *An. arabiensis* from Mpumalanga as it may be specific to mosquitoes from KZN.

A one-way ANOVA was performed to determine whether midgut bacterial diversity, relative abundance, and evenness differed between colonised *An. arabiensis* and field-collected *An. arabiensis* (Table 3.2). Results show that no significant differences were found amongst diversity indices between colonised and field-collected *An. arabiensis* (Figure 3.7). Therefore, the midgut bacterial community of colonised *An. arabiensis* was similar to field-collected *An. arabiensis* from both localities, and midgut bacterial communities of field-collected *An. arabiensis* from KZN and Mpumalanga were also similar to each other.

Table 3.2: One-way ANOVA results of diversity indices from colonised *An. arabiensis* and field-collected *An. arabiensis* from KZN and Mpumalanga. Midgut bacteria from silica-preserved colonised females and silica-preserved field-collected females were characterised by culture-dependent methods. Means and standard deviations were calculated for the following indices: Shannon-Wiener (H), Simpson's reciprocal ($\frac{1}{D}$), and Pielou's evenness (E). The one-way ANOVA was performed at 95% confidence and significance was determined using GraphPad Prism v8.4.3. Degrees of freedom (df), F-values (F), and P-values (P) are shown.

Diversity Index		df	F	P
H	Between Groups	2	1.008	0.3847
	Within Groups	18		
	Total	20		
$\frac{1}{D}$	Between Groups	2	2.497	0.1104
	Within Groups	18		
	Total	20		
E	Between Groups	2	1.635	0.2227
	Within Groups	18		
	Total	20		

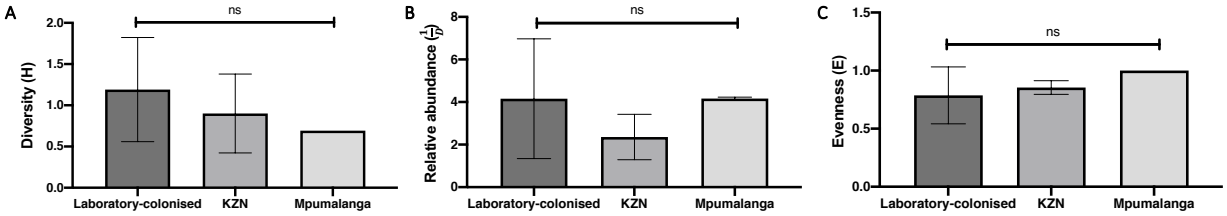

Figure 3.7: Comparison of diversity indices between colonised *An. arabiensis* and field-collected *An. arabiensis* from KZN and Mpumalanga. Midgut bacteria from colonised and field-collected females were characterised by culture-dependent methods. Means and standard deviations were calculated for the following indices: (A) Shannon-Wiener (H), (B) Simpson's reciprocal ($\frac{1}{D}$), and (C) Pielou's evenness (E). GraphPad Prism v8.4.3 was used to determine significance at 95% confidence.

3.2. Midgut Bacteria from Colonised *Anopheles funestus*

Midgut bacteria of fresh colonised *An. funestus* were identified using culture-dependent and culture-independent techniques (section 3.2.1). This was performed to identify the midgut bacteria that may be essential to *An. funestus* physiology. Moreover, bacteria were identified from the larval rearing water to determine whether mosquitoes acquired bacteria from their environment. Both identification techniques were also used to determine if the midgut bacteria of preserved *An. funestus* could be identified after mosquito preservation (section 3.2.2).

3.2.1. Bacteria from Fresh *Anopheles funestus* and the Larval Rearing Water

Culture-dependent results show that colonised *An. funestus* were predominantly colonised by the phylum Proteobacteria, followed by Firmicutes and Bacteroidetes (Appendix H, Figure H1). *Pseudomonas* and *Staphylococcus* were equally dominant genera in *An. funestus* midguts and midguts were colonised by more Gram-negative bacteria (76.74%) than Gram-positive bacteria (23.26%). Specifically, female *An. funestus* had a higher proportion of Proteobacteria than males, while males had higher proportions of Firmicutes and Bacteroidetes (Appendix H, Figure H2). Additionally, the dominant genus in females was *Pseudomonas*, while *Staphylococcus* was the dominant genus in males. Female midguts were also colonised by more Gram-negative bacteria than males (81.25% and 72.22% respectively), while male midguts were colonised by more Gram-positive bacteria than females (18.75% and 27.78% respectively).

In terms of bacterial species, culture-dependent results show that female and male *An. funestus* had bacteria in common with their larval rearing water: *A. hydrophila*, *K. variicola*, *Pseudomonas otitidis*, and *S. epidermidis* (Figure 3.8). These bacteria were likely acquired by mosquitoes from their environment. Additionally, there were bacteria in common between female and male *An. funestus* that were absent from the larval rearing water: *Chryseobacterium gleum*, *E. anophelis*, *P. libanensis*, *Pseudomonas putida*, *R. ornithinolytica*, *S. oryzae*, *S. capitis*, and *S. hominis.* These bacteria likely co-evolved with both genders. Moreover, males had *H. huttiense* in common with the larval rearing water, which shows that this bacterium was probably acquired from the environment in a gender-specific manner.

Also, female and male *An. funestus* had specific bacteria that belonged exclusively to each gender. For example, *E. cloacae*, *P. grimontii*, *R. aquatilis*, and *Stenotrophomonas maltophilia* were isolated exclusively from females while *Bacillus infantis*, *C. lapagei*, *Chryseobacterium arachidis*, *E. americana*, and *S. haemolyticus* were isolated exclusively from males. Some bacteria were also isolated from the larval rearing water that were absent from mosquito midguts. This shows that some environmental bacteria could not be acquired by mosquitoes.

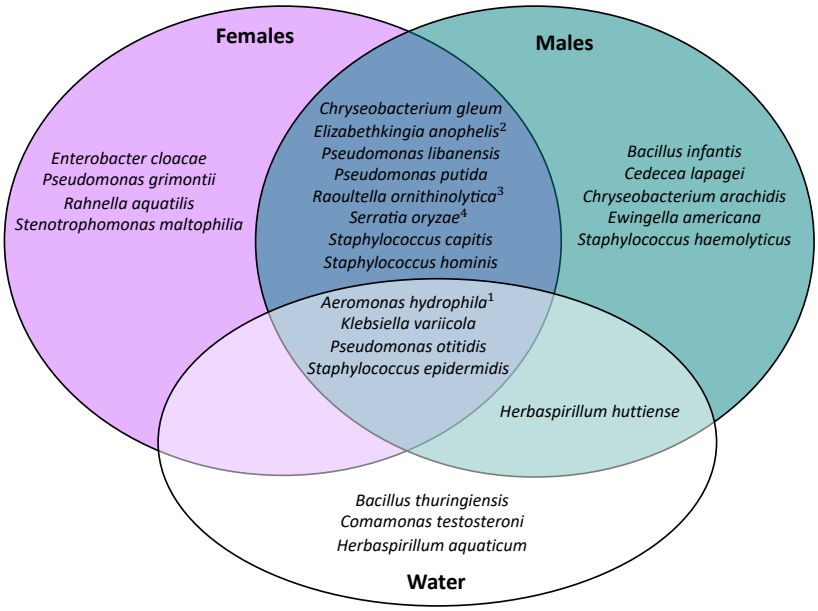

Figure 3.8: Bacteria identified by culture-dependent techniques from female and male *An. funestus* and their larval rearing water. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [3]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [4]*S. fonticola* and *S. oryzae.*

Like culture-dependent results, culture-independent results show that *An. funestus* were predominantly colonised by the phylum Proteobacteria (Appendix H, Figure H3). This was followed by Actinobacteria, Firmicutes, and Bacteroidetes (Actinobacteria were not identified by culture-dependent methods). Culture-independent results also show that the dominant genus in *An. funestus* was *Pseudomonas*, which correlates with culture-dependent results. Additionally, culture-independent results show that *An. funestus* midguts were colonised by more Gram-negative bacteria (77.8%) than Gram-positive bacteria (22.2%).

Specifically, female *An. funestus* had higher proportions of Proteobacteria and Actinobacteria than males, while males had higher proportions of Firmicutes and Bacteroidetes (Appendix H, Figure H4). The dominant genus in females was *Pseudomonas*, while *Acinetobacter* and *Pseudomonas* were equally dominant genera in males. Like culture-dependent methods, culture-independent results show that female and male midguts were colonised by more Gram-negative (80% and 75.61% respectively) than Gram-positive bacteria (20% and 24.39% respectively).

In terms of bacterial species, culture-independent results show that females and males had bacteria in common with the larval rearing water: *A. hydrophila*, *A. krungthepensis*, *Cutibacterium acnes*, *E. anophelis*, *E. cloacae*, *P. myrsinacearum*, *P. geniculata*, *P. veronii*, *S. oryzae*, *S. epidermidis*, and *Y. aldovae* (Figure 3.9). These bacteria were likely acquired by

mosquitoes from their environment. Additionally, females and males had bacteria in common that were not isolated from the larval rearing water: *G. frateurii*, *R. ornithinolytica*, and *Y. enterocolitica*. These bacteria likely co-evolved with both genders of this vector species.

Females also had *Corynebacterium aurimucosum*, *Kaistia terrae*, *M. maritypicum*, and *Rothia dentocariosa* in common with the water, showing that these bacteria were probably acquired specifically by females. Similarly, *Acinetobacter johnsonii*, *Acinetobacter lwoffii*, *C. pseudodiphtheriticum*, *Corynebacterium tuberculostearicum*, *D. tsuruhatensis*, *H. huttiense*, *L. iarius*, *P. aerius*, *P. solisilvae*, *Pseudomonas aeruginosa*, *P. faecalis*, *Rhizobium endophyticum*, and *S. thermophilus* were identified in males and the larval rearing water, showing that these bacteria were likely acquired from the environment specifically by males.

Furthermore, females and males had bacteria isolated exclusively per gender, showing that bacteria potentially co-evolved in a gender-specific manner. For example, *H. psychrotolerans*, *P. grimontii*, and *P. savastanoi* were exclusive to females, while *Acetobacter suratthaniensis*, *Acinetobacter pittii*, *Acinetobacter seohaensis*, *Caenispirillum bisanense*, *Chryseobacterium arthrosphaerae*, *Dolosigranulum pigrum*, *Empedobacter falsenii*, *Gemella taiwanensis*, *Lactobacillus coryniformis*, *Lactobacillus crispatus*, *Massilia suwonensis*, *Pseudomonas lactis*, *Robbsia andropogonis*, *S. marcescens*, and *Stenotrophomonas rhizophila* were exclusive to males. Also, bacteria were isolated from the larval rearing water that were not identified in mosquito midguts, which reiterates that some environmental bacteria cannot be acquired by mosquitoes.

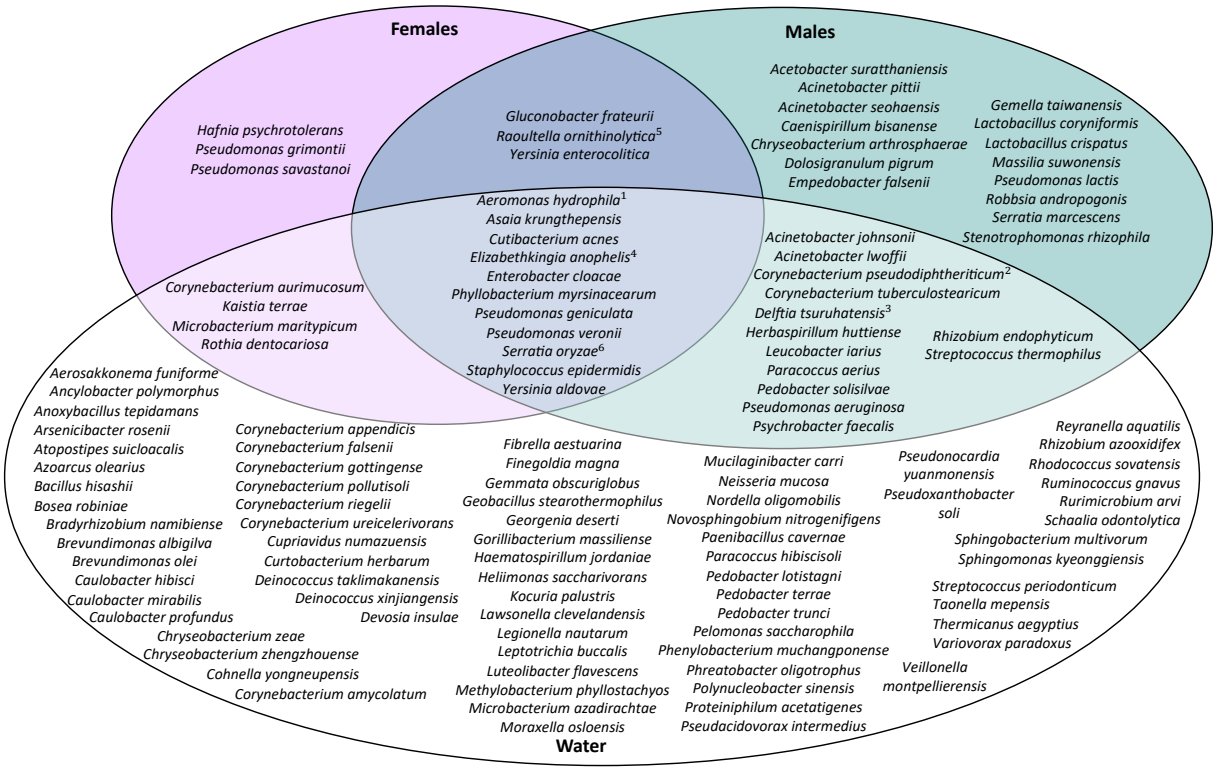

Figure 3.9: Bacteria identified by culture-independent techniques from female and male *An. funestus* and their larval rearing water. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*D. acidovorans* and *D. tsuruhatensis*; [4]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [5]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [6]*S. fonticola* and *S. oryzae.*

Culture-dependent and culture-independent results agree that *A. hydrophila*, *E. anophelis*, *R. ornithinolytica*, *S. oryzae*, and *S. epidermidis* were common between female and male *An. funestus* (Figure 3.10). Thus, these bacteria likely form part of the native microbiota that may be essential to biological functioning of colonised *An. funestus*. Additionally, *E. cloacae* was identified in females and males by culture-independent techniques but only in females by culture-dependent techniques. Although this bacterium was not identified by both identification techniques in both genders, it likely also forms part of the native midgut microbiota of colonised *An. funestus*. Moreover, culture-dependent and culture-independent results agree that *P. grimontii* was found only in females, meaning that this bacterium was probably specific to *An. funestus* females.

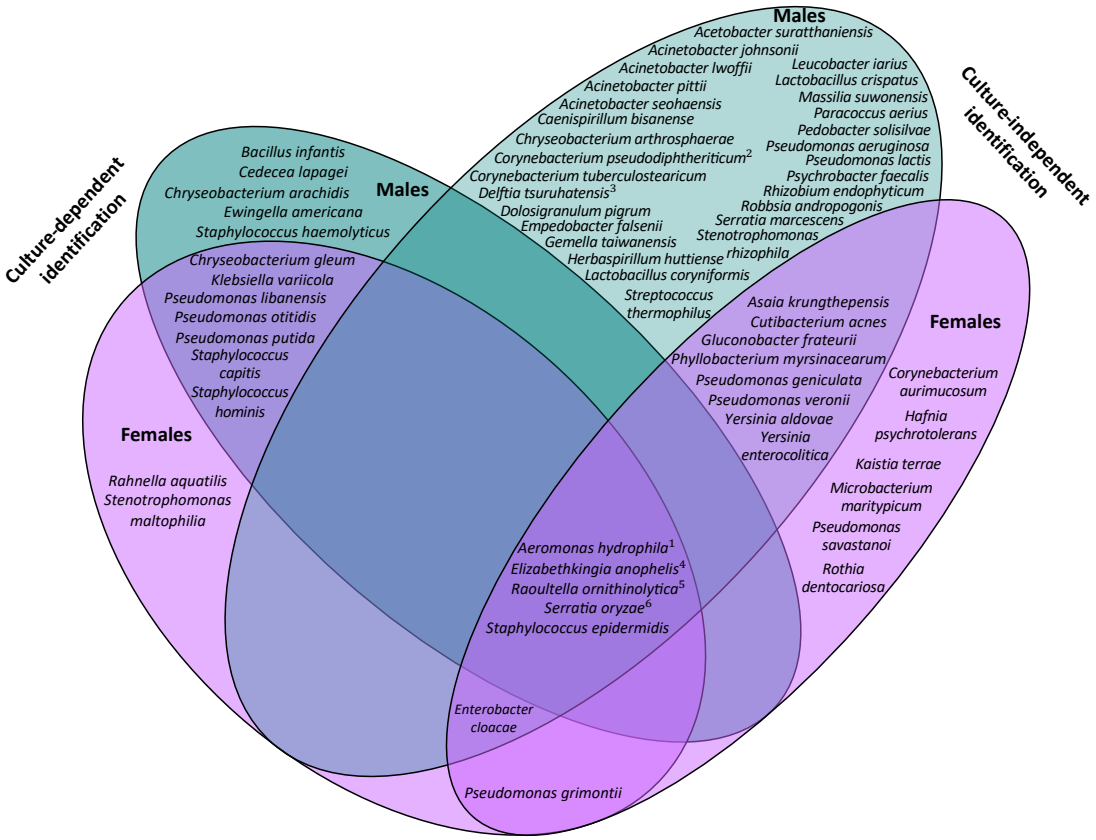

Figure 3.10: Bacteria identified by culture-dependent and culture-independent techniques that are common between colonised female and male *An. funestus*. [1]*A. hydrophila* and *A. veronii*, [2]*C. propinquum* and *C. pseudodiphtheriticum,* [3]*D. acidovorans* and *D. tsuruhatensis*, [4]*E. anophelis*, *E. meningoseptica*, and *E. miricola*, [5]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*, [6]*S. fonticola* and *S. oryzae.*

3.2.2. *Anopheles funestus* Midgut Bacteria Identified After Mosquito Preservation

Preserved colonised *An. funestus* also had their midgut bacteria characterised by culture-dependent and culture-independent techniques. As with *An. arabiensis*, bacteria grew on selective media from the midguts of preserved mosquitoes, demonstrating that midgut bacteria were conserved during mosquito preservation and could be identified post-preservation. Additionally, culture-dependent results echo the notion that mosquitoes preserved on silica and in RNA*later*® have bacteria in common, demonstrating the ability of both preservatives to conserve the midgut bacteria during mosquito preservation (Appendix I, Figure I1).

Specifically, culture-dependent results show that preserved females and males had bacteria in common (see Table 3.3). However, bacteria were isolated from silica-preserved mosquitoes that were absent in mosquitoes preserved in RNA*later*®, such as *E. anophelis* and *E. americana* (Appendix I, Figure I2). These bacteria were likely conserved in a preservative-dependent manner. Moreover, females preserved on silica and in RNA*later*® had *C. lapagei* and *C. neteri* in common, which indicates that these bacteria were likely exclusive to preserved females.

Similarly, males preserved on silica and in RNA*later*® had *S. hominis* in common, which implies that this bacterium may be exclusive to preserved males. Also, *C. pseudodiphtheriticum* and *P. extremorientalis* were only isolated from silica-preserved females, while *C. gleum*, *Pseudomonas gessardii*, and *P. libanensis* were isolated from silica-preserved males. Similarly, *S. capitis* was exclusive to males preserved in RNA*later*®. This means that some bacteria could only be conserved by certain preservatives in specific genders.

In comparison, culture-independent methods identified more bacteria from preserved *An. funestus* than culture-dependent methods (Table 3.3). Many bacteria were also common between females and males preserved either by silica or RNA*later*® in varying combinations per gender. Further, bacteria were identified exclusively from females or males in a preservative-dependent manner; a detailed analysis of these results can be found in Appendix I (Figures I3–I6). This reiterates the notion that some bacteria become conserved in a gender- and/or preservative-dependent manner.

Culture-dependent and culture-independent results agree that *C. lapagei*, *S. oryzae*, and *S. epidermidis* were common in preserved *An. funestus* (Appendix I, Figure I7). These bacteria likely form part of the native midgut bacteria essential to colonised *An. funestus* physiological functioning that can also be conserved during mosquito preservation. Additionally, *C. pseudodiphtheriticum* and *E. anophelis* were common between silica- and RNA*later*®-preserved *An. funestus* identified by culture-independent methods but only silica-preserved *An. funestus* by culture-dependent methods. Thus, although identification of these bacteria differed according to the technique used, these bacteria probably also form part of the native midgut microbiota of colonised *An. funestus* that become conserved during mosquito preservation.

Table 3.3: Common midgut bacteria between preserved female and male *An. funestus*. Mosquitoes were preserved on silica or in RNA*later*® and bacteria were identified by culture-dependent and culture-independent techniques. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [4]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [5]*S. fonticola* and *S. oryzae*. Green shading shows bacteria identified by both identification methods from mosquitoes conserved by both preservatives.

Bacteria		**Identification Method**	
Genera	**Species**	**Culture-dependent**	**Culture-independent**
Acinetobacter	*junii*		x
	lwoffii		x
Aeromonas	*hydrophila*[1]		x

Aerosakkonema	*funiforme*		x
Anaerococcus	*prevotii*		x
Asaia	*krungthepensis*		x
Brevundimonas	*albigilva*		x
Chryseobacterium	*greenlandense*		x
Corynebacterium	*aurimucosum*		x
	pseudodiphtheriticum[2]		x
	tuberculostearicum		x
	ureicelerivorans		x
Cutibacterium	*acnes*		x
Deinococcus	*taklimakanensis*		x
Dolosigranulum	*pigrum*		x
Elizabethkingia	*anophelis*[3]		x
Enterobacter	*cloacae*		x
Gluconobacter	*frateurii*		x
Granulicatella	*adiacens*		x
Haemophilus	*parainfluenzae*		x
Herbaspirillum	*huttiense*		x
Lactobacillus	*crispatus*		x
Lawsonella	*clevelandensis*		x
Leptotrichia	*buccalis*		x
Leucobacter	*iarius*		x
Massilia	*suwonensis*		x
Microbacterium	*maritypicum*		x
Micrococcus	*luteus*		x
Moraxella	*osloensis*		x
Paracoccus	*hibiscisoli*		x
Phyllobacterium	*myrsinacearum*		x
Pseudomonas	*geniculata*		x
	lactis		x
	veronii		x
Psychrobacter	*faecalis*		x
Rahnella	*aquatilis*	x	
Raoultella	*ornithinolytica*[4]	x	
Rhodococcus	*sovatensis*		x
Rothia	*dentocariosa*		x
Serratia	*marcescens*		x
	oryzae[5]	x	x
Staphylococcus	*epidermidis*	x	x
Streptococcus	*periodonticum*		x

	thermophilus		x
Variovorax	*paradoxus*		x
Veillonella	*dispar*		x
Yersinia	*aldovae*		x

As an overview, culture-dependent results show that *E. anophelis*, *R. aquatilis*, *R. ornithinolytica*, and *S. epidermidis* were common between fresh and preserved female and male *An. funestus*. Additionally, *K. variicola* and *S. oryzae* were detected in fresh females and males but only in preserved males, while a *Psychrobacter* sp. was common between fresh females and preserved males (Appendix J, Figure J1).

In comparison, culture-independent results show that *A. hydrophila*, *A. krungthepensis*, *C. acnes*, *E. anophelis*, *E. cloacae*, *G. frateurii*, *P. myrsinacearum*, *P. geniculata*, *P. veronii*, *S. oryzae*, *S. epidermidis*, *Y. aldovae*, and *Y. enterocolitica* were common between fresh and preserved females and males (Appendix J, Figure J2). Many bacteria were also common between preserved females and males and fresh males, or fresh females and preserved males (see Appendix J, Figure J2). Fresh and preserved males also had *A. seohaensis* in common, showing that this bacterium was specific to males. Similarly, females had *H. psychrotolerans* and *P. savastanoi* in common, demonstrating that these bacteria were specific to females.

Altogether, culture-dependent and culture-independent results agree that *E. anophelis* and *S. epidermidis* were the only common bacteria between fresh and preserved *An. funestus* (Figure 3.11). Therefore, these bacteria likely form part of the native midgut microbiota in colonised *An. funestus* that become preserved upon mosquito preservation. Additionally, *A. hydrophila*, *E. cloacae*, *H. huttiense*, *P. grimontii*, and *S. oryzae* were identified from fresh *An. funestus* by culture-dependent methods and fresh and preserved *An. funestus* by culture-independent methods. Similarly, *R. ornithinolytica* was identified in fresh and preserved *An. funestus* by culture-dependent methods but only in fresh *An. funestus* by culture-independent methods.

In addition, *C. pseudodiphtheriticum* was identified by culture-independent methods in fresh and preserved *An. funestus* but only in preserved *An. funestus* by culture-dependent methods. Thus, despite that identification techniques differed in identifying these bacteria, it is likely that these midgut bacteria also form part of the native midgut microbiota essential to *An. funestus* biological functioning and also become conserved during mosquito preservation.

Further, *C. lapagei* was identified in fresh and preserved *An. funestus* by culture-dependent methods but only in preserved *An. funestus* by culture-independent methods. This differs from

An. arabiensis results, which show that *C. lapagei* was only identified from preserved mosquitoes. Hence, it is likely that *C. lapagei* colonises in low quantities in midguts of newly emerged mosquitoes and over time (i.e., during the time it takes for preservation to occur or the time it takes for mosquitoes to age), this bacterium colonises in higher quantities. Therefore, it is difficult to detect in fresh mosquitoes but can be detected in all midguts post-preservation.

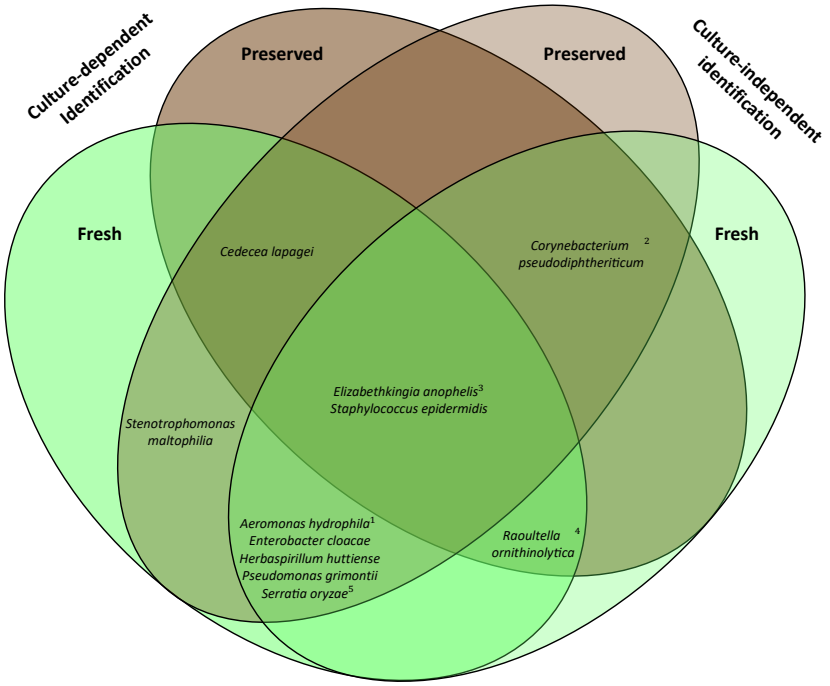

Figure 3.11: Common midgut bacteria identified by culture-dependent and culture-independent techniques from fresh and preserved *An. funestus*. Mosquitoes were preserved on silica or in RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [4]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [5]*S. fonticola* and *S. oryzae.*

3.3. Comparison of Midgut Bacteria between Colonised *Anopheles arabiensis* and *Anopheles funestus*

To provide an overview of the common midgut bacteria between colonised *An. arabiensis* and *An. funestus*, comparisons were made between fresh mosquitoes of both species identified by both identification techniques (section 3.3.1). This depicts the native midgut bacteria common between both colonised species that may be essential to their physiological functioning. Moreover, midgut bacterial diversity was compared between females and males of both vector species to determine whether the midgut bacterial community was different between females and males, as well as between fresh colonised *An. arabiensis* and fresh colonised *An. funestus*.

Similarly, comparisons were made between midgut bacterial species and midgut bacterial diversity from preserved colonised *An. arabiensis* and *An. funestus* identified by culture-dependent and culture-independent methods (section 3.3.2). This outlines which bacteria are capable of being preserved and whether these bacteria form part of the native *Anopheles* microbiota. Additionally, comparing the diversity indices of preserved mosquitoes demonstrates whether the midgut bacteria differed between preserved females and males, as well as between preserved *An. arabiensis* and *An. funestus*.

Finally, comparisons were made between fresh and preserved mosquitoes of both species, as well as between culture-dependent and culture-independent results (section 3.3.3). This provides an overview of the native midgut bacteria in colonised *An. arabiensis* and *An. funestus* that can be preserved and identified by both techniques. This is essential because it can be used in future studies aiming to use *Anopheles* midgut bacteria for malaria intervention strategies or to elucidate the roles that specific bacteria play in *Anopheles*. Moreover, comparing diversity indices also determined whether preservative or preservation time period affected the midgut microbiota and comparing the identification methods determined whether bacterial diversity was over- or under-estimated by specific techniques.

3.3.1. Midgut Bacteria between Fresh *Anopheles arabiensis* and *Anopheles funestus*

Colonised *An. arabiensis* and *An. funestus* were predominantly colonised by the phylum Proteobacteria (Appendix K, Figure K1). However, the proportion of subsequent phyla differed according to the type of identification method used. Culture-dependent methods only isolated three phyla from both species, while culture-independent methods isolated five and four phyla from *An. arabiensis* and *An. funestus* respectively.

Specifically, both identification methods agreed that both species were colonised by Proteobacteria, Firmicutes, and Bacteroidetes, albeit in varying distributions. However, culture-independent results show that both species were also colonised by Actinobacteria (and Cyanobacteria in *An. arabiensis*). This demonstrates that culture-dependent methods can identify dominant phyla, while culture-independent methods are more sensitive at identifying phyla that may be in minority or difficult to identify by culture.

Culture-dependent results also show that *Staphylococcus* was the dominant genus in *An. arabiensis*, while culture-independent results show that the dominant genus in *An. arabiensis* was *Pseudomonas*. Similarly, culture-dependent results show that *Pseudomonas* and

Staphylococcus were equally dominant genera in *An. funestus*, while culture-independent results show that *Pseudomonas* was the dominant genus in *An. funestus*. Collectively, both techniques agree that the midguts of both fresh colonised species were predominantly colonised by *Pseudomonas* and *Staphylococcus*.

An overview of the proportions of Gram-positive and Gram-negative bacteria between females and males of both species can be seen in Table 3.4. Briefly, results of both identification methods were similar, but culture-independent methods reported more Gram-negative bacteria in the midguts of both species. However, the means of Gram-positive and Gram-negative bacteria per species and gender differed according to the technique used, where culture-dependent methods reported a higher proportion of Gram-negative bacteria in *An. funestus*, and culture-independent results reported a higher proportion of Gram-negative bacteria in *An. arabiensis*. Nevertheless, culture-dependent and culture-independent techniques agree that Gram-negative bacteria dominate the midguts of both genders of fresh colonised species used in this study.

Table 3.4: Summary of Gram-negative and Gram-positive bacterial proportions from fresh colonised *An. arabiensis* and *An. funestus*. Bacteria were identified using culture-dependent and culture-independent methods and mean percentages were calculated per gender and species.

Culture-dependent Techniques						
Species	**Gram-negative Bacteria (%)**			**Gram-positive Bacteria (%)**		
	Females	**Males**	**Mean**	**Females**	**Males**	**Mean**
An. arabiensis	76.92	68.75	72.84	23.08	31.25	27.16
An. funestus	81.25	72.22	76.74	18.75	27.78	23.26
Culture-independent Techniques						
Species	**Gram-negative Bacteria (%)**			**Gram-positive Bacteria (%)**		
	Females	**Males**	**Mean**	**Females**	**Males**	**Mean**
An. arabiensis	92.86	79.41	86.13	7.14	20.59	13.87
An. funestus	80	75.61	77.8	20	24.39	22.2

In terms of diversity, culture-dependent results show that males of both species had higher bacterial species richness than females (Appendix K, Figure K2). Collectively, *An. arabiensis* (OTUs=17) had slightly higher species richness than *An. funestus* (OTUs=16). There were no significant differences among diversity indices between colonised female and male *An. arabiensis* identified by culture-dependent techniques (Table 3.5). Similarly, there were no significant differences in midgut bacterial diversity and relative abundance between female and

male *An. funestus*. Yet, there was a significant difference (P=0.0303) between the evenness of female and male *An. funestus* midgut bacteria (Figure 3.12F). This shows that fresh colonised *An. funestus* females had a more evenly distributed midgut bacterial community than males.

Table 3.5: Diversity indices for fresh colonised female and male *An. arabiensis* and *An. funestus* identified by culture-dependent methods. Means and standard deviations (SD) were calculated for the following indices: Shannon-Wiener (H), Simpson's reciprocal ($\frac{1}{D}$), and Pielou's evenness (E).

Fresh colonised		H (± SD)	$\frac{1}{D}$ (± SD)	E (± SD)
An. arabiensis	females	0.913 (± 0.332)	2.207 (± 0.598)	0.726 (± 0.104)
	males	0.879 (± 0.404)	2.090 (± 0.726)	0.723 (± 0.191)
An. funestus	females	0.730 (± 0.189)	1.991 (± 0.386)	0.887 (± 0.099)
	males	0.776 (± 0.708)	2.041 (± 1.105)	0.513 (± 0.454)

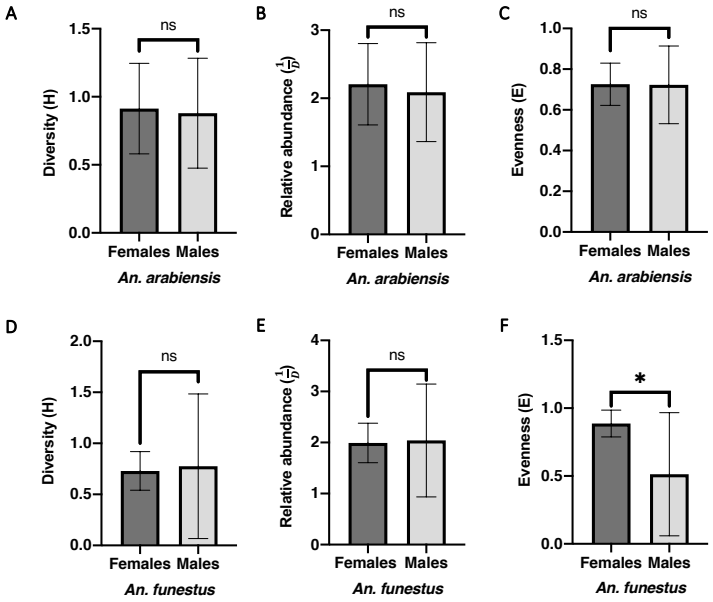

Figure 3.12: Comparison of culture-dependent diversity indices between fresh colonised female and male (A, B, C) *An. arabiensis* and (D, E, F) *An. funestus*. Means and standard deviations were calculated for the following diversity indices: (A, D) Shannon-Wiener (H), (B, E) Simpson's reciprocal ($\frac{1}{D}$), and (C, F) Pielou's evenness (E). GraphPad Prism v8.4.3 was used to determine significance at 95% confidence.

A two-way ANOVA revealed that there were no significant differences among diversity indices between females and males of the same species (Figure 3.13). Fresh colonised *An. arabiensis* (females and males combined) had similar midgut bacterial diversity to *An. funestus*. There

were also no significant differences between females and males of different species. Females (*An. arabiensis* and *An. funestus* combined) and males had similar midgut bacterial diversity. Thus, culture-dependent results show that neither gender nor species influenced the midgut bacterial diversity of fresh colonised mosquitoes.

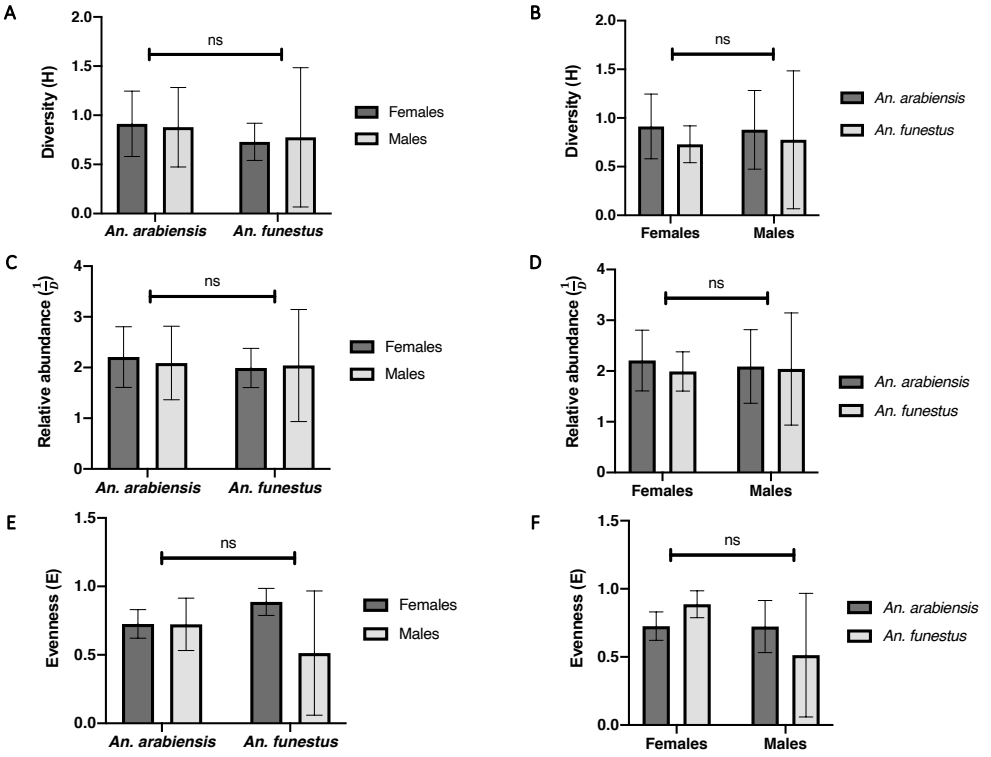

Figure 3.13: Comparison of culture-dependent diversity indices between (A, C, E) *An. arabiensis* and *An. funestus* (females and males combined), and (B, D, F) females and males (*An. arabiensis* and *An. funestus* combined). Means and standard deviations were calculated for the following indices: (A, B) Shannon-Wiener (H), (C, D) Simpson's reciprocal ($\frac{1}{D}$), and (E, F) Pielou's evenness (E). GraphPad Prism v8.4.3 was used to determine significance at 95% confidence.

In terms of midgut bacterial species, culture-dependent results show that female and male *An. arabiensis* and *An. funestus* had *A. hydrophila*, *E. anophelis*, *K. variicola*, *P. libanensis*, *R. ornithinolytica*, *S. oryzae*, and *S. epidermidis* in common (Appendix K, Figure K3). *An. arabiensis* females and males had *R. aquatilis* in common with *An. funestus* females, while *S. hominis* was common between *An. arabiensis* males and both *An. funestus* genders. Further, *P. grimontii* was common between *An. funestus* females and *An. arabiensis* males. Several bacteria were identified in a gender- and/or species-specific manner; see Appendix K for a detailed analysis of midgut bacteria according to species (Figure K4) and gender (Figure K5).

Culture-independent results also show that males of both species had higher bacterial species richness than females (Appendix K, Figure K6). Collectively, *An. funestus* (OTUs=61) had

slightly higher species richness than *An. arabiensis* (OTUs=59). Like culture-dependent results, culture-independent results show no significant differences among diversity indices between colonised female and male *An. arabiensis* (Table 3.6). However, there were significant differences between the diversity (P<0.0001), relative abundance (P<0.0001), and evenness (P=0.0068) of female and male *An. funestus* (Figure 3.14D–F). This demonstrates that culture-independent methods identified a more diverse, abundant, and evenly distributed midgut bacterial community in colonised *An. funestus* males than colonised *An. funestus* females.

Table 3.6: Diversity indices for fresh colonised female and male *An. arabiensis* and *An. funestus* identified by culture-independent methods. Means and standard deviations (SD) were calculated for the following indices: Shannon-Wiener (H), Simpson's reciprocal ($\frac{1}{D}$), and Pielou's evenness (E).

Fresh colonised		H (± SD)	$\frac{1}{D}$ (± SD)	E (± SD)
An. arabiensis	females	1.258 (± 0.69)	2.060 (± 1.065)	0.386 (± 0.212)
	males	1.383 (± 0.242)	2.319 (± 0.462)	0.396 (± 0.069)
An. funestus	females	0.738 (± 0.209)	1.408 (± 0.203)	0.251 (± 0.071)
	males	1.139 (± 0.213)	1.937 (± 0.123)	0.305 (± 0.057)

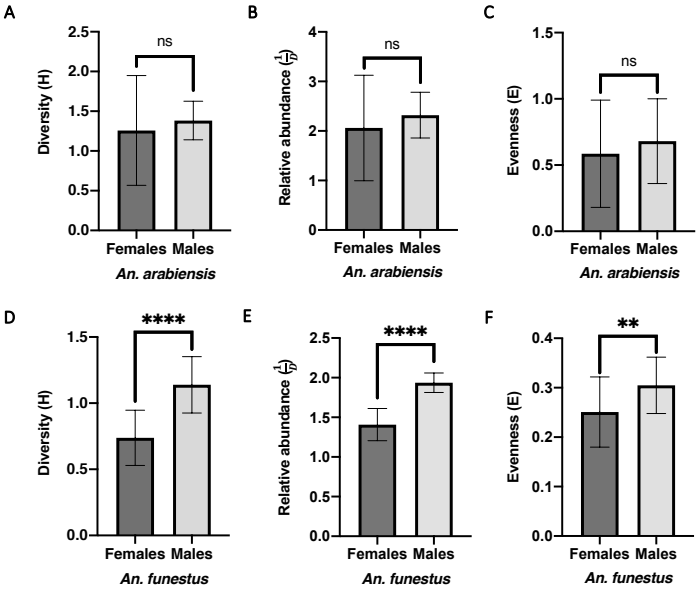

Figure 3.14: Comparison of culture-independent diversity indices between fresh colonised female and male (A, B, C) *An. arabiensis* and (D, E, F) *An. funestus*. Means and standard deviations were calculated for the following indices: (A, D) Shannon-Wiener (H), (B, E) Simpson's reciprocal ($\frac{1}{D}$), and (C, F) Pielou's evenness (E). GraphPad Prism v8.4.3 was used to determine significance at 95% confidence.

Culture-independent results also demonstrated significant differences among diversity (P<0.0001), relative abundance (P<0.0001), and evenness (P<0.0001) between females and males of the same species (Figure 3.15A, C, E), and amongst diversity (P=0.0004) and relative abundance (P=0.0004) between females and males of different species (Figure 3.15B, D). This shows that *An. arabiensis* (females and males combined) had a more diverse, abundant, and evenly distributed midgut bacterial flora than *An. funestus*. Similarly, males (*An. arabiensis* and *An. funestus* combined) had a more diverse and abundant midgut bacterial community than females. Nonetheless, there were no significant differences between females and males of different species. Therefore, in contrast to culture-dependent results, culture-independent results show that both gender and species influenced the midgut bacterial diversity of colonised mosquitoes.

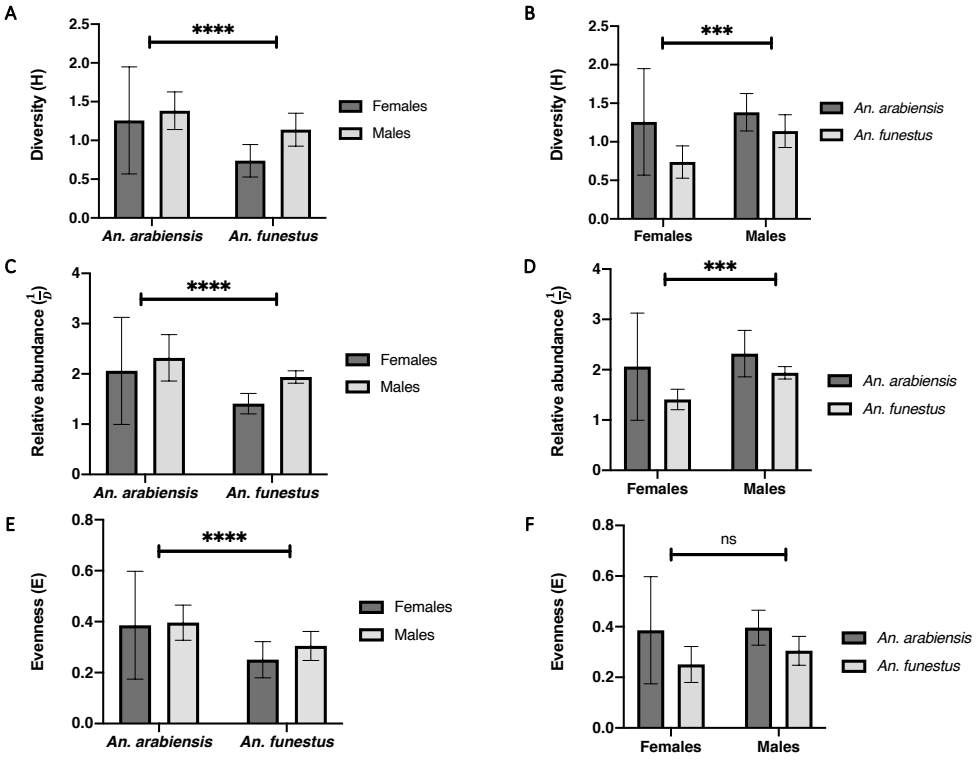

Figure 3.15: Comparison of culture-independent diversity indices between (A, C, E) *An. arabiensis* and *An. funestus* (females and males combined), and (B, D, F) females and males (*An. arabiensis* and *An. funestus* combined). Means and standard deviations were calculated for the following indices: (A, B) Shannon-Wiener (H), (C, D) Simpson's reciprocal ($\frac{1}{D}$), and (E, F) Pielou's evenness (E). GraphPad Prism v8.4.3 was used to determine significance at 95% confidence.

In terms of bacterial species, culture-independent results show that *An. arabiensis* and *An. funestus* had *A. hydrophila*, *E. anophelis*, *E. cloacae*, *G. frateurii*, *P. geniculata*, *P. veronii*, *S. oryzae*, *S. epidermidis*, *Y. aldovae*, and *Y. enterocolitica* in common (Appendix K, Figure K7). Additionally, *An. arabiensis* females and males had *P. grimontii* and *P. savastanoi* in common

with *An. funestus* females, while *R. ornithinolytica* was common between *An. arabiensis* females and *An. funestus* females and males. Further, *A. krungthepensis* and *P. myrsinacearum* were common between *An. arabiensis* males and *An. funestus* females and males.

Meanwhile, *P. solisilvae* and *P. faecalis* were common between *An. arabiensis* females and *An. funestus* males, while *M. maritypicum* was common between *An. arabiensis* males and *An. funestus* females. Moreover, *An. arabiensis* females and *An. funestus* females had *H. psychrotolerans* in common, demonstrating that this bacterium was specific to females. Similarly, *An. arabiensis* males and *An. funestus* males had *A. oris*, *L. iarius*, *P. aerius*, and *S. thermophilus* in common, demonstrating that these bacteria were likely specific to males. Several bacteria were gender- and/or species-specific; see Appendix K for a detailed comparison between species (Figure K8) and between females (Figure K9A) and males (Figure K9B) of each species.

Collectively, there were significant differences among diversity ($P<0.0001$), relative abundance ($P=0.0055$), and evenness ($P<0.0001$) between culture-dependent and culture-independent techniques (Figure 3.16A, C, E). Culture-independent techniques reported higher diversity, relative abundance, and evenness than culture-dependent techniques in both species. This may suggest that culture-dependent techniques underestimate the midgut bacterial community in *Anopheles*, or that culture-independent techniques overestimate the midgut bacterial community. Also, diversity ($P<0.0001$), relative abundance ($P<0.0001$), and evenness ($P=0.0208$) differed significantly between *An. arabiensis* and *An. funestus* when culture-dependent and culture-independent results were combined (Figure 3.16B, D, F). Thus, culture-dependent and culture-independent results show that *An. arabiensis* midguts had higher bacterial diversity, relative abundance, and evenness than *An. funestus*.

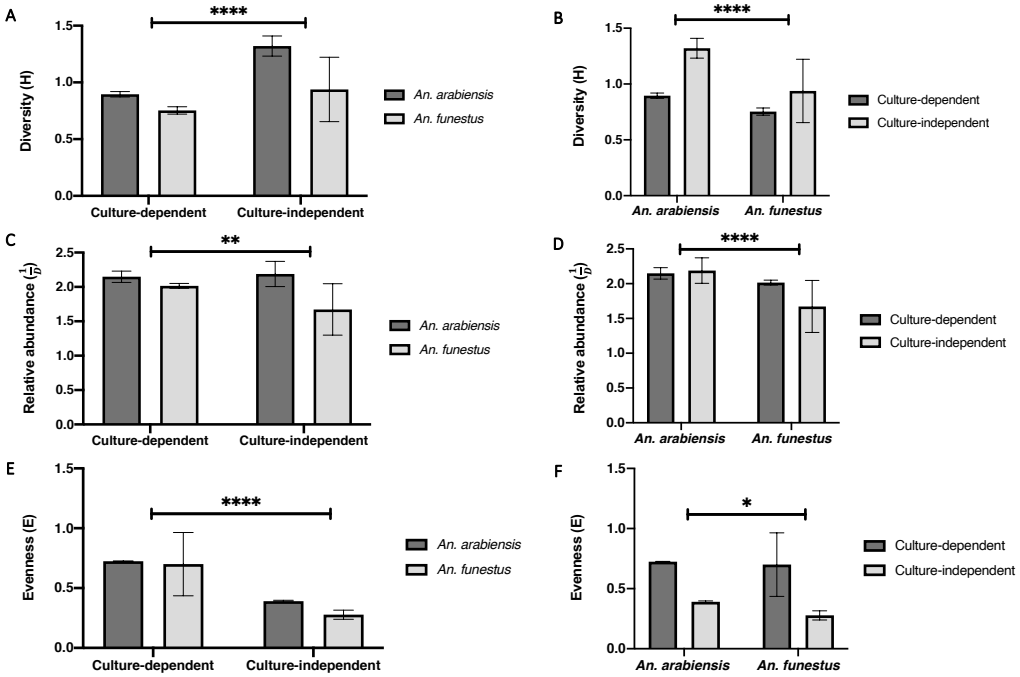

Figure 3.16: Comparison of diversity indices between (A, C, E) culture-dependent and culture-independent methods (fresh *An. arabiensis* and *An. funestus* combined), and fresh (B, D, F) *An. arabiensis* and *An. funestus* (culture-dependent and culture-independent results combined). Means and standard deviations were calculated for the following indices: (A, B) Shannon-Wiener (H), (C, D) Simpson's reciprocal ($\frac{1}{D}$), and (E, F) Pielou's evenness (E). GraphPad Prism v8.4.3 was used to determine significance at 95% confidence.

In summary, both identification techniques show that the midguts of males of both species were richer in bacterial species than females. Culture-dependent results show that *An. arabiensis* midguts had higher species richness than *An. funestus* and both techniques agreed that the midgut bacterial diversity of both of *An. arabiensis* females and males were comparable. However, culture-dependent results show that the midguts of *An. funestus* females were more diverse than males, while culture-independent results show that the midguts of *An. funestus* males were more diverse, abundant, and even than females. Moreover, while culture-dependent results show that neither gender nor species influenced midgut bacterial diversity, culture-independent results reported the opposite. Nonetheless, both identification methods agree that *An. arabiensis* midguts were more diverse, abundant, and even than *An. funestus* midguts.

In terms of bacterial diversity, culture-dependent and culture-independent results agree that *A. hydrophila*, *E. anophelis*, *P. grimontii*, *R. ornithinolytica*, *S. oryzae*, and *S. epidermidis* were common between fresh *An. arabiensis* and *An. funestus* (Appendix K, Figure K10). Therefore, these bacteria potentially form part of the native midgut microbiota common between fresh colonised *An. arabiensis* and *An. funestus* that may be essential to their biological functioning.

Additionally, *Psychrobacter* spp. were common between *An. arabiensis* and *An. funestus* when identified by culture-independent methods but only in *An. arabiensis* when identified by culture-dependent methods. Further, *H. huttiense* was identified by culture-dependent methods in *An. arabiensis* and *An. funestus* but only in *An. funestus* by culture-independent methods. Similarly, *E. cloacae* was found in *An. arabiensis* and *An. funestus* by culture-independent methods but only in *An. funestus* by culture-dependent methods. These bacteria likely also form part of the native midgut microbiota common between both species even though they were identified by different identification techniques. Moreover, culture-dependent and culture-independent results agreed that *S. multivorum* was a bacterium exclusive to *An. arabiensis*.

3.3.2. Midgut Bacteria between Preserved *Anopheles arabiensis* and *Anopheles funestus*

The midgut bacteria of preserved *An. arabiensis* and *An. funestus* were compared from culture-dependent and culture-independent results. Culture-dependent results show that males of both species had higher bacterial species richness than females (Appendix L, Figure L1). Collectively, *An. funestus* (OTUs=21) had slightly higher species richness than *An. arabiensis* (OTUs=20). Nonetheless, there were no significant differences among diversity indices between preserved female and male *An. arabiensis* (Table 3.7). Likewise, there were no significant differences among diversity indices between preserved female and male *An. funestus* (Figure 3.17). Thus, culture-dependent results show that midguts of preserved *An. arabiensis* and *An. funestus* did not differ in terms of diversity, relative abundance, or evenness.

Table 3.7: Diversity indices for preserved colonised female and male *An. arabiensis* and *An. funestus* identified by culture-dependent techniques. Mosquitoes were preserved on silica or in RNA*later*®. Means and standard deviations (SD) were calculated for the following indices: Shannon-Wiener (H), Simpson's reciprocal ($\frac{1}{D}$), and Pielou's evenness (E).

Preserved colonised		H (± SD)	$\frac{1}{D}$ (± SD)	E (± SD)
An. arabiensis	females	0.561 (± 0.513)	1.828 (± 0.902)	0.586 (± 0.405)
	males	0.718 (± 0.545)	2.227 (± 1.244)	0.681 (± 0.32)
An. funestus	females	0.421 (± 0.192)	1.554 (± 0.378)	0.492 (± 0.162)
	males	0.415 (± 0.303)	1.683 (± 0.5)	0.519 (± 0.292)

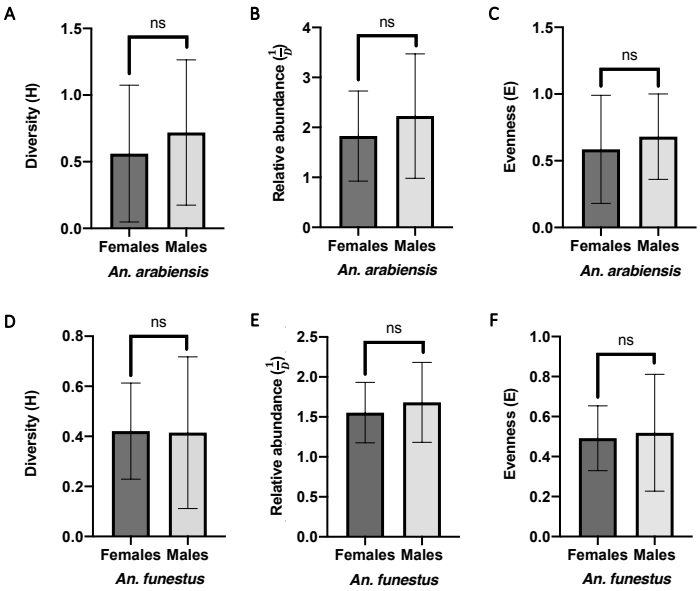

Figure 3.17: Comparison of culture-dependent diversity indices between preserved colonised female and male (A, B, C) *An. arabiensis* and (D, E, F) *An. funestus*. Means and standard deviations were calculated for the following indices: (A, D) Shannon-Wiener (H), (B, E) Simpson's reciprocal ($\frac{1}{D}$), and (C, F) Pielou's evenness (E). GraphPad Prism v8.4.3 was used to determine significance at 95% confidence.

A two-way ANOVA revealed that there were no significant differences among diversity indices between females and males of the same species (Figure 3.18). Preserved colonised *An. arabiensis* and *An. funestus* (females and males combined) had similar midgut bacterial diversity and there were also no significant differences between females and males of different species. Therefore, females (*An. arabiensis* and *An. funestus* combined) and males had similar midgut bacterial diversity. Like the culture-dependent results of fresh *Anopheles*, culture-dependent results show that neither gender nor species influenced the midgut bacterial diversity of preserved colonised mosquitoes.

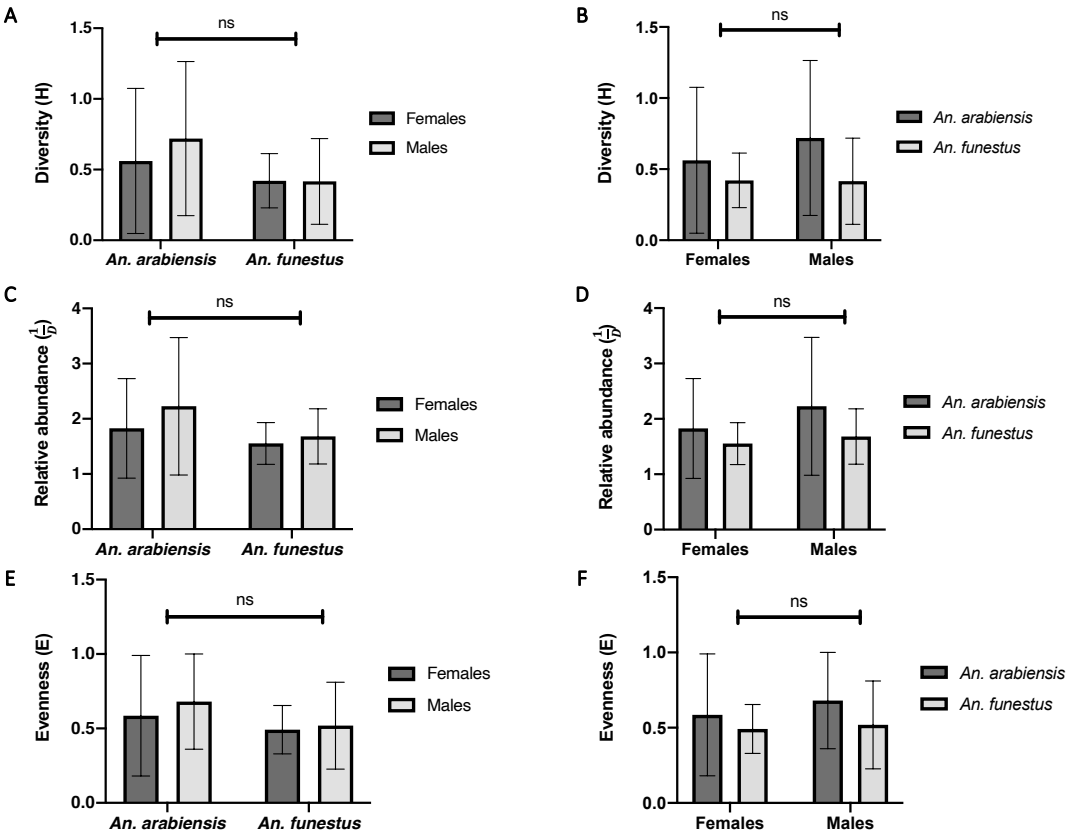

Figure 3.18: Comparison of culture-dependent diversity indices between preserved (A, C, E) *An. arabiensis* and *An. funestus* (females and males combined), and (B, D, F) females and males (*An. arabiensis* and *An. funestus* combined). Means and standard deviations were calculated for the following indices: (A, B) Shannon-Wiener (H), (C, D) Simpson's reciprocal ($\frac{1}{D}$), and (E, F) Pielou's evenness (E). GraphPad Prism v8.4.3 was used to determine significance at 95% confidence.

In terms of bacterial species, culture-dependent results show that preserved *An. arabiensis* and *An. funestus* had *E. anophelis*, *R. aquatilis*, *R. ornithinolytica*, and *S. epidermidis* in common (Appendix L, Figure L2). These bacteria likely form part of the native midgut microbiota of both colonised vector species that can also be conserved upon mosquito preservation. Additionally, *C. lapagei* and *C. neteri* were identified from preserved *An. arabiensis* females and males but only *An. funestus* females. Meanwhile, *S. hominis* was common between *An. arabiensis* females and males and *An. funestus* males, and *E. americana* and *S. oryzae* were common between male *An. arabiensis* and female and male *An. funestus*. *An. arabiensis* and *An. funestus* males also had *S. capitis* in common, showing that this bacterium was preserved in a gender-dependent manner. See Appendix L for a detailed analysis of common bacteria identified by culture-dependent methods between *An. arabiensis* and *An. funestus* females (Figure L3A, B) and *An. arabiensis* and *An. funestus* males (Figure L3C, D) according to preservative.

In comparison, culture-independent results agree with culture-dependent results by showing that preserved males of both species had higher bacterial species richness than females (Appendix L, Figure L4). Collectively, *An. arabiensis* (OTUs=446) had higher species richness than *An. funestus* (OTUs=435). In contrast, culture-independent results show significant differences between preserved *An. arabiensis* females and males in terms of diversity (P=0.0034), relative abundance (P=0.0005), and evenness (P=0.0074) (Figure 3.19A, B, C). Hence, preserved males had more diverse, abundant, and even midgut bacterial communities than preserved females (Table 3.8). Additionally, culture-independent results show no significant differences in diversity and evenness between preserved *An. funestus* females and males. However, there was a significant difference between *An. funestus* female and male midgut relative abundance (P=0.0005) (Figure 3.19E). This means that *An. funestus* males were more diverse than females.

Table 3.8: Diversity indices for preserved colonised female and male *An. arabiensis* and *An. funestus* identified by culture-independent methods. Mosquitoes were preserved on silica or in RNA*later*®. Means and standard deviations (SD) were calculated for the following indices: Shannon-Wiener (H), the Simpson's reciprocal ($\frac{1}{D}$), and Pielou's evenness (E).

Preserved colonised		**H (± SD)**	**$\frac{1}{D}$ (± SD)**	**E (± SD)**
An. arabiensis	females	1.894 (± 1.133)	2.964 (± 1.278)	0.418 (± 0.218)
	males	2.199 (± 1.027)	3.442 (± 1.591)	0.469 (± 0.173)
An. funestus	females	1.805 (± 0.488)	2.886 (± 1.541)	0.413 (± 0.072)
	males	1.867 (± 1.466)	3.526 (± 2.251)	0.392 (± 0.287)

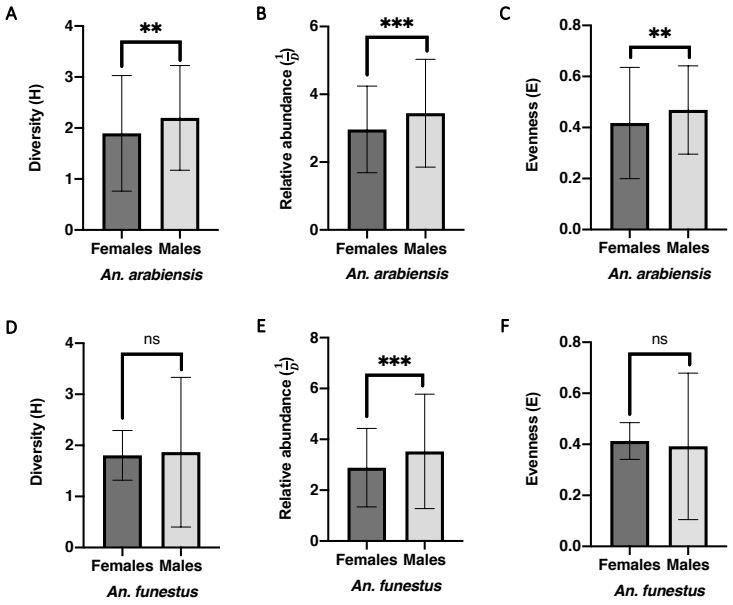

Figure 3.19: Comparison of culture-independent diversity indices between preserved colonised female and male (A, B, C) *An. arabiensis* and (D, E, F) *An. funestus*. Means and standard deviations were calculated for the following indices: (A, D) Shannon-Wiener (H), (B, E) Simpson's reciprocal index ($\frac{1}{D}$), and (C, F) Pielou's evenness (E). GraphPad Prism v8.4.3 was used to determine significance at 95% confidence.

A two-way ANOVA for culture-independent results shows significant differences in diversity (P=0.0048) between preserved *An. arabiensis* and *An. funestus* (females and males combined) (Figure 3.20A), but no significant difference amongst relative abundance and evenness. Therefore, culture-independent results show that the midguts of *An. arabiensis* were more diverse than *An. funestus*. Moreover, *An. arabiensis* and *An. funestus* females differed significantly from *An. arabiensis* and *An. funestus* males in terms of diversity (P=0.0138) and relative abundance (P<0.0001) (Figure 3.20B, D). Thus, culture-independent results show that *An. arabiensis* and *An. funestus* males had more diverse midguts than females and that both gender and species influenced midgut bacterial diversity.

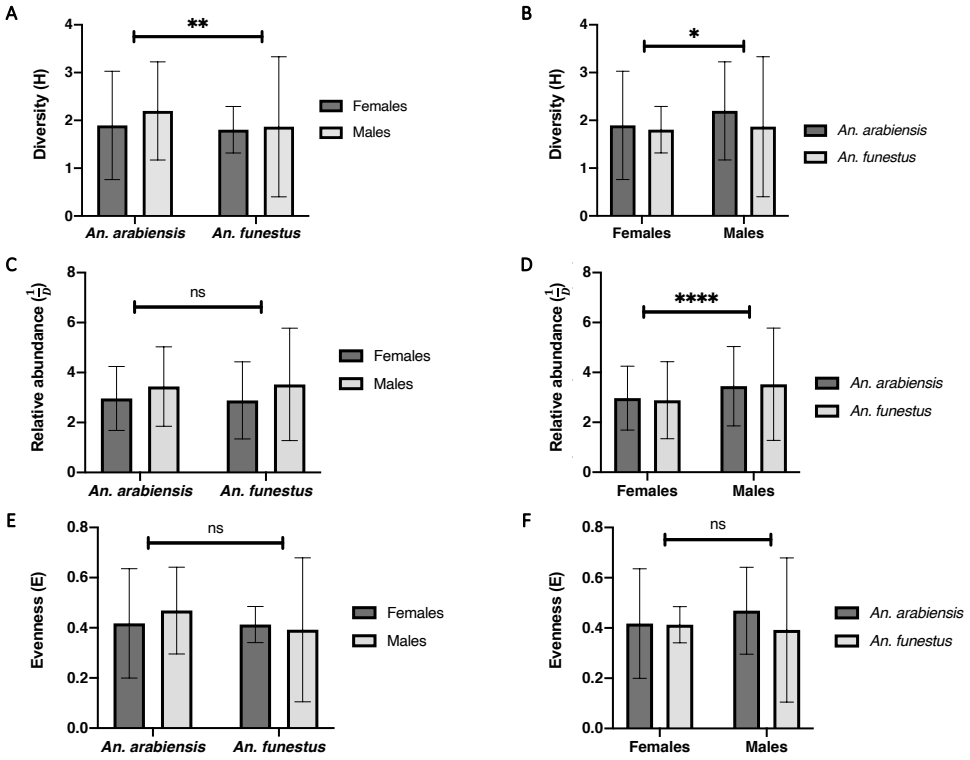

Figure 3.20: Comparison of culture-independent diversity indices between preserved (A, C, E) *An. arabiensis* and *An. funestus* (females and males combined), and (B, D, F) females and males (*An. arabiensis* and *An. funestus* combined). Means and standard deviations were calculated for the following indices: (A, B) Shannon-Wiener (H), (C, D) Simpson's reciprocal ($\frac{1}{D}$), and (E, F) Pielou's evenness (E). GraphPad Prism v8.4.3 was used to determine significance at 95% confidence.

In terms of bacterial species, many bacteria were identified by culture-independent methods that were common between preserved female and male *An. arabiensis* and *An. funestus* (see Appendix L, Figure L5). Bacteria were also identified from both species, but in varying combinations of genders. For example, *Bacillus loiseleuriae*, *Frateuria aurantia*, *Morganella morganii*, *Pedobacter trunci*, and *Ruminococcus gnavus* were identified from females of both species, showing that these bacteria were specific to females. A detailed analysis of common bacteria identified by culture-independent methods between *An. arabiensis* and *An. funestus* females and males according to preservative can be found in Appendix L (Figures L6–L9).

Overall, a two-way ANOVA shows significant differences amongst diversity ($P<0.0001$), relative abundance ($P<0.0001$), and evenness ($P<0.0001$) between culture-dependent and culture-independent results for both preserved species (Figure 3.21A, C, E). This indicates that culture-independent techniques identified a more diverse, abundant, and evenly distributed midgut bacterial community in both species than culture-dependent techniques. Significant differences were also found amongst diversity ($P=0.0211$) and evenness ($P<0.0001$) between

An. arabiensis and *An. funestus* when culture-dependent and culture-independent results were combined (Figure 3.21B, F). Thus, both identification methods agree that preserved *An. arabiensis* have a more diverse and evenly distributed midgut bacterial community than preserved *An. funestus*.

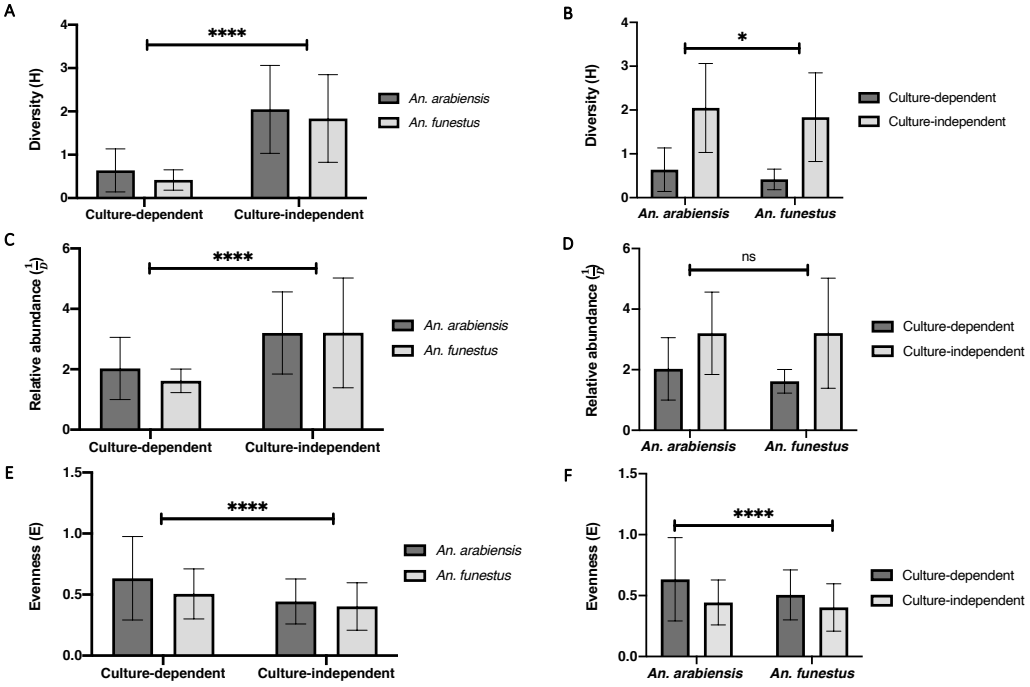

Figure 3.21: Comparison of diversity indices between (A, C, E) culture-dependent and culture-independent methods (preserved *An. arabiensis* and *An. funestus* combined), and preserved (B, D, F) *An. arabiensis* and *An. funestus* (culture-dependent and culture-independent results combined). Means and standard deviations were calculated for the following indices: (A, B) Shannon-Wiener (H), (C, D) Simpson's reciprocal ($\frac{1}{D}$), and (E, F) Pielou's evenness (E). GraphPad Prism v8.4.3 was used to determine significance at 95% confidence.

In summary, both identification techniques show that male midguts of both species had higher midgut bacterial species richness than females. However, culture-dependent results show that *An. funestus* midguts had higher species richness than *An. arabiensis*. Additionally, culture-dependent results show that female and male *An. arabiensis* midgut bacterial diversity was comparable, while culture-independent results show that *An. arabiensis* males had more diverse, abundant, and even midgut bacterial communities than females. Similarly, culture-dependent results show that the midgut bacterial diversity of *An. funestus* females and males were similar, while culture-independent results show that the midguts of *An. funestus* males were more diverse than females. Moreover, while culture-dependent results show that neither gender nor species influenced midgut bacterial diversity, culture-independent results reported the opposite. Nonetheless, both identification methods agree that *An. arabiensis* midguts were

more diverse, abundant and even than *An. funestus* midguts; this was also reported in fresh colonised *An. arabiensis*.

In terms of bacterial species, results from both identification methods show that *C. lapagei*, *E. anophelis*, *S. oryzae*, and *S. epidermidis* were common between preserved *An. arabiensis* and *An. funestus* (Appendix L, Figure L10). Thus, both identification methods agree that these bacteria likely form part of the native midgut bacteria common between both colonised species that can also be conserved upon mosquito preservation. Additionally, *C. pseudodiphtheriticum* was identified by culture-dependent techniques in both species but only in *An. funestus* by culture-dependent techniques.

Similarly, *Psychrobacter* spp. were identified in both species by culture-independent techniques but only in *An. arabiensis* by culture-dependent techniques. *Raoultella ornithinolytica* was also found in both preserved species by culture-dependent methods but only in *An. arabiensis* by culture-independent techniques. Therefore, even though these bacteria were variably identified by both techniques, they likely also form part of the native midgut microbiota common between both colonised species that can be conserved upon mosquito preservation.

Diversity indices were also compared between fresh and preserved mosquitoes at different preservation time periods. Culture-dependent results show significant differences in diversity ($P<0.0001$), relative abundance ($P<0.0001$), and evenness ($P<0.0001$) between fresh *An. arabiensis* and *An. arabiensis* preserved for 4 weeks (Table 3.9; Figure 3.22A, C, E). There were also significant differences in diversity ($P=0.0007$) and relative abundance ($P<0.0001$) between *An. arabiensis* preserved for 4 and 12 weeks (Figure 3.22A, C, E).

This shows that the midgut bacterial community changes during preservation. Nevertheless, culture-dependent results show no significant differences in diversity between fresh *An. arabiensis* and *An. arabiensis* preserved for 12 weeks (Appendix M, Figure M1). Thus, despite changes in midgut bacterial diversity between preservation time periods, the midguts of fresh *An. arabiensis* and *An. arabiensis* preserved for up to 12 weeks were comparable.

Table 3.9: Diversity indices for *An. arabiensis* midguts across preservation time periods identified by culture-dependent methods. Mosquitoes were preserved on silica or in RNA*later*®. Means and standard deviations (SD) were calculated for the following indices: Shannon-Wiener (H), Simpson's reciprocal ($\frac{1}{D}$), and Pielou's evenness (E).

***An. arabiensis* Preservation Time**	**H (± SD)**	$\frac{1}{D}$ **(± SD)**	**E (± SD)**
Fresh	0.896 (± 0.331)	2.148 (± 0.598)	0.725 (± 0.138)
1 week	0 (± 0)	1 (± 0)	0 (± 0)
4 weeks	1.648 (± 0.060)	4.779 (± 0.401)	0.920 (± 0.033)
8 weeks	1.116 (± 0.218)	2.817 (± 0.720)	0.862 (± 0.086)
12 weeks	1.072 (± 0.131)	2.308 (± 0.501)	0.737 (± 0.110)

Similarly, culture-dependent results show significant differences in diversity (P<0.0001), relative abundance (P=0.0002), and evenness (P=0.0021) between fresh *An. funestus* and *An. funestus* preserved for 4 weeks (Table 3.10; Figure 3.22B, D, E). There were also significant differences in diversity (P<0.0001), relative abundance (P<0.0001), and evenness (P<0.0001) between *An. funestus* preserved for 4 and 12 weeks (Figure 3.22B, D, E). This reiterates that the midgut microbiota changes during preservation. However, culture-dependent results show no significant differences in midgut bacterial diversity between fresh *An. funestus* and *An. funestus* preserved for 12 weeks (Appendix M, Figure M1). Yet, there was a significant difference in evenness (P=0.0018) (Appendix M, Figure M1F). Thus, the midgut bacterial diversity of *An. funestus* changes across preservation time periods but the only difference between fresh *An. funestus* and *An. funestus* preserved for 12 weeks was that fresh *An. funestus* had a more evenly distributed midgut bacterial community.

Table 3.10: Diversity indices for colonised *An. funestus* at different preservation time periods identified by culture-dependent methods. Mosquitoes were preserved on silica or in RNA*later*®. Means and standard deviations (SD) were calculated for following indices: Shannon-Wiener (H), Simpson's reciprocal ($\frac{1}{D}$), and Pielou's evenness (E).

An. funestus Preservation Time	H (± SD)	$\frac{1}{D}$ (± SD)	E (± SD)
Fresh	0.753 (± 0.464)	2.016 (± 0.741)	0.700 (± 0.115)
1 week	0 (± 0)	1 (± 0)	0 (± 0)
4 weeks	1.614 (± 0.226)	4.639 (± 1.339)	0.845 (± 0.088)
8 weeks	1.204 (± 0.206)	2.872 (± 0.728)	0.823 (± 0.099)
12 weeks	0.686 (± 0.276)	1.545 (± 0.305)	0.529 (± 0.099)

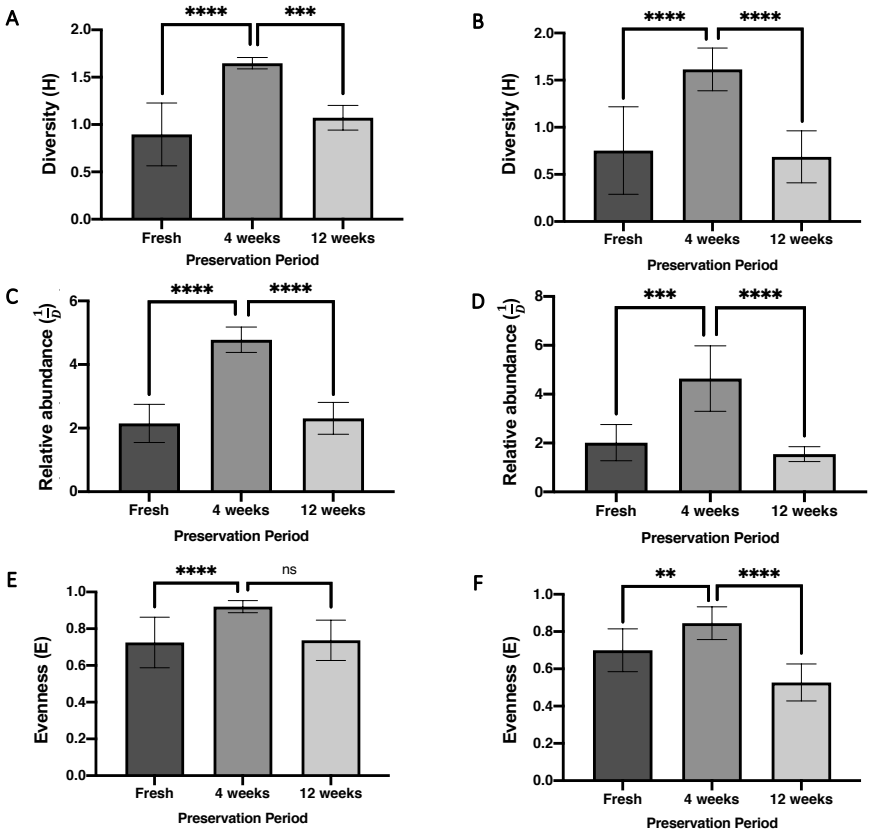

Figure 3.22: Comparison of diversity indices identified by culture-dependent techniques between fresh and preserved (A, C, E) *An. arabiensis* and (B, D, F) *An. funestus* at different preservation time periods. Means and standard deviations were calculated for the following indices: (A, B) Shannon-Wiener (H), (C, D) Simpson's reciprocal ($\frac{1}{D}$), and (E, F) Pielou's evenness (E). GraphPad Prism v8.4.3 was used to determine significance at 95% confidence.

In comparison, culture-independent results show significant differences in midgut bacterial diversity (P=0.0015) and relative abundance (P=0.0056) between fresh *An. arabiensis* and *An. arabiensis* preserved for up to 4 weeks (Table 3.11; Figure 3.23A, C). However, there were no significant differences in midgut bacterial diversity between *An. arabiensis* preserved for 4 and 12 weeks (Figure 3.23). Thus, the midgut bacterial community changes initially after preservation but does not change as the preservation time period is extended. Nevertheless, culture-independent results show that there were significant differences in bacterial diversity (P<0.0001), relative abundance (P<0.0001), and evenness (P<0.0001) between fresh *An. arabiensis* and *An. arabiensis* preserved for 12 weeks (Appendix M, Figure M2A, C, E). Therefore, despite no changes in the bacterial community at different preservation time periods, the midguts of fresh *An. arabiensis* and *An. arabiensis* preserved for up to 12 weeks differed.

Table 3.11: Diversity indices for colonised *An. arabiensis* at different preservation time periods identified by culture-independent methods. Mosquitoes were preserved on silica or in RNA*later*®. Means and standard deviations (SD) were calculated for the following indices: Shannon-Wiener (H), Simpson's reciprocal ($\frac{1}{D}$), and Pielou's evenness (E). In contrast to culture-dependent methods, midgut bacteria were identified by culture-independent methods from mosquitoes preserved for 4 weeks and 12 weeks.

***An. arabiensis* Preservation Time**	**H (± SD)**	**$\frac{1}{D}$ (± SD)**	**E (± SD)**
Fresh	1.320 (± 0.468)	2.189 (± 0.747)	0.391 (± 0.141)
4 weeks	1.723 (± 1.297)	2.662 (± 1.390)	0.370 (± 0.229)
12 weeks	2.370 (± 0.664)	3.744 (± 1.268)	0.517 (± 0.111)

Similarly, culture-independent results show significant differences in midgut bacterial diversity (P<0.0001), relative abundance (P<0.0001), and evenness (P=0.0382) between fresh *An. funestus* and *An. funestus* preserved for 4 weeks (Table 3.12; Figure 3.23B, D, F). Nonetheless, there were no significant differences in midgut bacterial diversity between *An. funestus* preserved for 4 and 12 weeks (Figure 3.23). This reiterates that the midgut bacterial community changes initially after preservation but does not change as the preservation time period is extended. However, culture-independent results show that there were significant differences in midgut bacterial diversity (P<0.0001) and evenness (P<0.0001) between fresh *An. funestus* and *An. funestus* preserved for 12 weeks (Appendix M, Figure M2B, F). Therefore, despite no

changes in the midgut bacterial community between different preservation time periods, the midguts of fresh *An. funestus* differed from *An. funestus* preserved for up to 12 weeks.

Table 3.12: Diversity indices for colonised *An. funestus* at different preservation time periods identified by culture-independent methods. Mosquitoes were preserved on silica or in RNA*later*®. Means and standard deviations (SD) were calculated for the following indices: Shannon-Wiener (H), Simpson's reciprocal ($\frac{1}{D}$), and Pielou's evenness (E). In contrast to culture-dependent methods, midgut bacteria were identified by culture-independent methods from mosquitoes preserved for 4 and 12 weeks.

An. funestus Preservation Time	H (± SD)	$\frac{1}{D}$ (± SD)	E (± SD)
Fresh	0.937 (± 0.290)	1.672 (± 0.326)	0.278 (± 0.065)
4 weeks	1.394 (± 0.972)	3.042 (± 1.847)	0.326 (± 0.207)
12 weeks	2.279 (± 0.961)	3.370 (± 2.058)	0.479 (± 0.172)

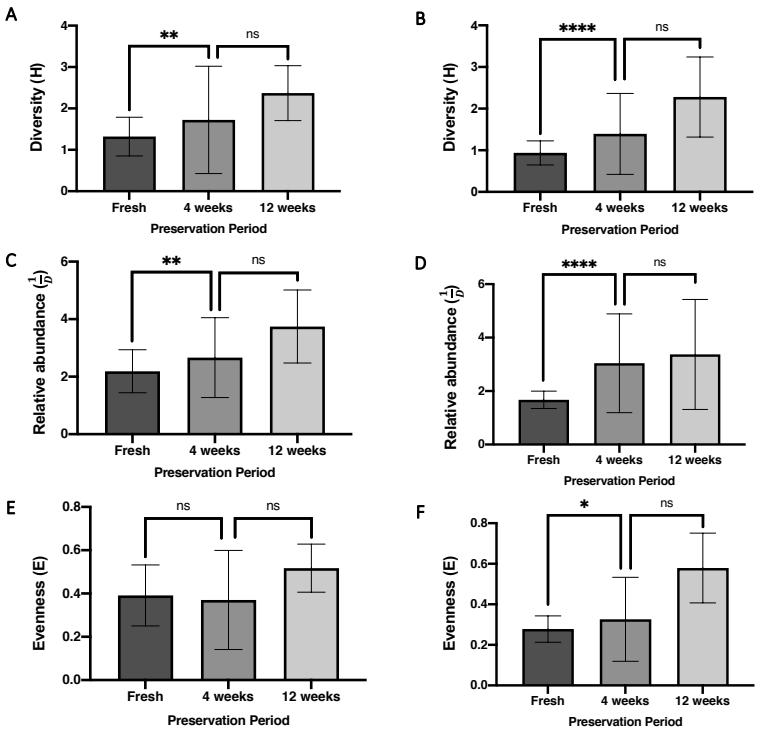

Figure 3.23: Comparison of diversity indices identified by culture-independent techniques between fresh and preserved (A, C, E) *An. arabiensis* and (B, D, F) *An. funestus* at different preservation time periods. Means and standard deviations were calculated for the following indices: (A, B) Shannon-Wiener (H), (C, D) Simpson's reciprocal ($\frac{1}{D}$), and (E, F) Pielou's evenness (E). GraphPad Prism v8.4.3 was used to determine significance at 95% confidence.

In summary, both techniques agree that midgut bacterial diversity differs between fresh mosquitoes and mosquitoes preserved for up to 4 weeks. However, both techniques disagree with whether the midgut bacterial diversity differs between fresh mosquitoes and mosquitoes preserved for up to 12 weeks. In essence, culture-dependent results show that the midgut of fresh mosquitoes was comparable to mosquitoes preserved for up to 12 weeks, but the opposite was true for culture-independent results. Nonetheless, there were common bacteria between fresh and preserved mosquitoes across preservation time periods (Appendix M, Table M1).

There were also bacteria found in fresh mosquitoes that were only identified in mosquitoes preserved after a certain period of time, such as between fresh mosquitoes and mosquitoes preserved for 4 weeks, fresh mosquitoes and mosquitoes preserved for 8 weeks, and fresh mosquitoes and mosquitoes preserved for 12 weeks (see Appendix M, Table M1). Some bacteria were also identified in fresh mosquitoes and mosquitoes preserved at more than one preservation time period, such as between fresh mosquitoes and mosquitoes preserved for 4 and 8 weeks, fresh mosquitoes and mosquitoes preserved for 4 and 12 weeks, fresh mosquitoes and mosquitoes preserved for 8 and 12 weeks, and fresh mosquitoes and mosquitoes preserved for 4, 8, and 12 weeks (Appendix M, Table M1). Overall, however, *E. anophelis*, *R. ornithinolytica*, and *S. epidermidis* were common between fresh mosquitoes and mosquitoes preserved at every preservation time period. Bacteria were also unique to fresh mosquitoes, or were identified only after 4 weeks of preservation, 12 weeks of preservation, both 4 and 12 weeks of preservation, or both 8 and 12 weeks of preservation (see Appendix M, Table M2).

3.3.3. Overview: Midgut Bacteria from *Anopheles arabiensis* and *Anopheles funestus*

A two-way ANOVA was performed from culture-dependent results to compare diversity indices between fresh and preserved *An. arabiensis* and *An. funestus*. Results show significant differences in diversity ($P<0.0001$) and evenness ($P=0.0065$) between both species (Figure 3.24A, E). Thus, fresh *An. arabiensis* and *An. funestus* midgut bacterial communities were more diverse and even than preserved *An. arabiensis* and *An. funestus*. There were also significant differences in diversity ($P=0.0079$) and relative abundance ($P=0.0473$) between *An. arabiensis* and *An. funestus* when fresh and preserved results were combined (Figure 3.24B, D). Thus, culture-dependent results show that fresh and preserved *An. arabiensis* midguts were more diverse than *An. funestus*.

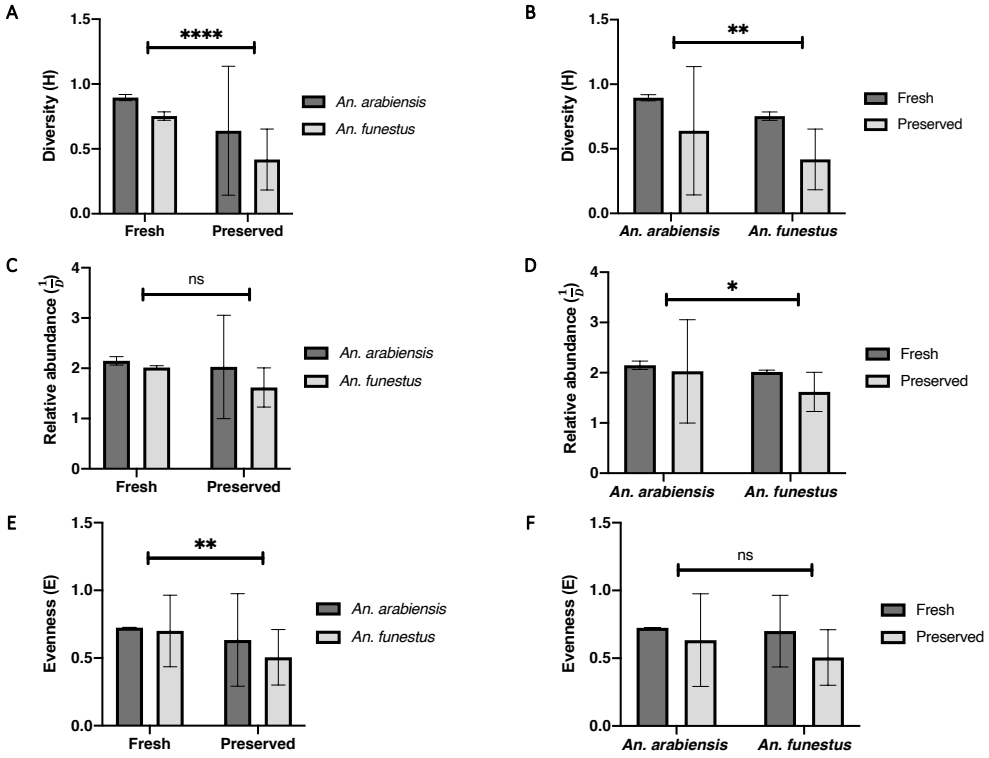

Figure 3.24: Comparison of culture-dependent diversity indices from fresh and preserved *An. arabiensis* and *An. funestus*. Means and standard deviations were calculated for the following indices: (A, B) Shannon-Wiener (H), (C, D) Simpson's reciprocal ($\frac{1}{D}$), and (E, F) Pielou's evenness (E). GraphPad Prism v8.4.3 was used to determine significance at 95% confidence.

In comparison, culture-independent results show a significant difference in diversity (P<0.0001), relative abundance (P<0.0001), and evenness (P=0.0028) between fresh and preserved *An. arabiensis* and *An. funestus* (Figure 3.25A, C, E). In contrast to culture-dependent results, culture-independent results show that preserved mosquitoes had higher diversity, relative abundance, and evenness than fresh mosquitoes. Additionally, *An. arabiensis* differed significantly from *An. funestus* in terms of evenness (P=0.0094) when fresh and preserved results were combined (Figure 3.25F). Thus, *An. arabiensis* had a more evenly distributed midgut bacterial community than *An. funestus*.

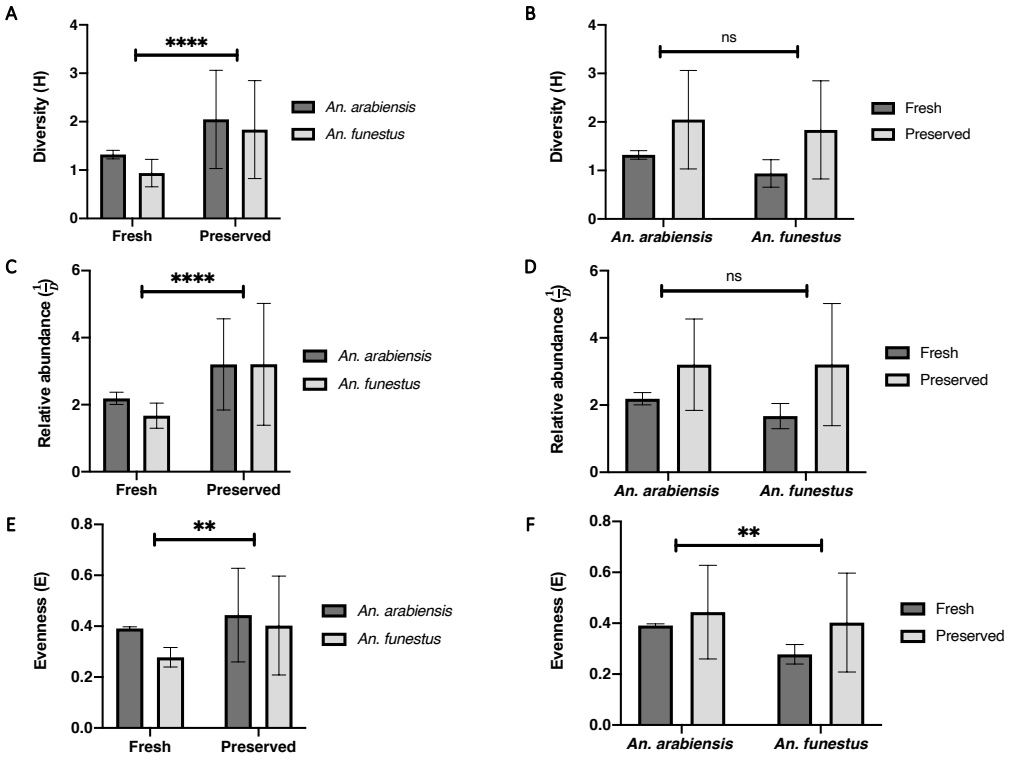

Figure 3.25: Comparison of culture-independent diversity indices from fresh and preserved *An. arabiensis* and *An. funestus*. Means and standard deviations were calculated for the following indices: (A, B) Shannon-Wiener (H), (C, D) Simpson's reciprocal ($\frac{1}{D}$), and (E, F) Pielou's evenness (E). GraphPad Prism v8.4.3 was used to determine significance at 95% confidence.

A comparison of the culture-dependent results from fresh and preserved *An. arabiensis* and *An. funestus* revealed six common bacteria between fresh and preserved mosquitoes of both species (Table 3.13). Culture-dependent results also revealed common bacteria between fresh and/or preserved *An. arabiensis* and/or *An. funestus* (see Appendix N, Figure N1). Nonetheless, *C. gleum* was common between fresh and preserved *An. funestus*, showing that this bacterium was likely specific to *An. funestus*. Similarly, a *Psychrobacter* sp. was common between fresh and preserved *An. arabiensis*. This shows that this bacterium was likely specific to *An. arabiensis*. A detailed analysis of bacteria identified by culture-dependent methods between both genders of both species can be found in Appendix N (Figures N2–N3).

In contrast, a comparison of the culture-independent results from fresh and preserved *An. arabiensis* and *An. funestus* showed that 19 bacteria were common between fresh and preserved mosquitoes of both species (Table 3.13). Meanwhile, many bacteria were identified by culture-independent methods from fresh and/or preserved *An. arabiensis* and/or *An. funestus* in varying combinations (see Appendix N, Figure N4). Additionally, *A. seohaensis* and *R. andropogonis* were found in fresh and preserved *An. funestus*, suggesting that these bacteria were specific to

An. funestus. Similarly, *S. multivorum* and *Y. wautersii* were identified from fresh and preserved *An. arabiensis*, demonstrating that these bacteria were specific to *An. arabiensis*. A detailed analysis of common bacteria identified by culture-independent methods between *An. arabiensis* and *An. funestus* females and males can be found in Appendix N (Figures N5–N6).

Table 3.13: Common midgut bacteria between fresh and preserved *An. arabiensis* and *An. funestus*. Mosquitoes were preserved on silica or in RNA*later*® and midgut bacteria were identified by culture-dependent and culture-independent methods. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [3]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [4]*S. fonticola* and *S. oryzae*. Green shading shows bacteria identified by both identification methods from both species.

Bacteria		Identification Technique	
Genera	**Species**	**Culture-dependent**	**Culture-independent**
Aeromonas	*hydrophila*[1]		x
Asaia	*krungthepensis*		x
Elizabethkingia	*anophelis*[2]	x	x
Enterobacter	*cloacae*		x
Hafnia	*psychrotolerans*		x
Leucobacter	*iarius*		x
Microbacterium	*maritypicum*		x
Paracoccus	*aerius*		x
Pedobacter	*solisilvae*		x
Pseudomonas	*geniculata*		x
	grimontii		x
	savastanoi		x
	veronii		x
Psychrobacter	*faecalis*		x
Rahnella	*aquatilis*	x	
Raoultella	*ornithinolytica*[3]	x	
Serratia	*oryzae*[4]	x	x
Staphylococcus	*epidermidis*	x	x
	hominis	x	
Streptococcus	*thermophilus*		x
Yersinia	*aldovae*		x
	enterocolitica		x

Overall, culture-dependent and culture-independent results show that the common bacteria between fresh and preserved *An. arabiensis* and *An. funestus* are *A. hydrophila*, *C. lapagei*, *E.*

anophelis, *H. huttiense*, *P. grimontii*, *R. ornithinolytica*, *S. oryzae*, and *S. epidermidis* (Figure 3.26). Therefore, these bacteria potentially form part of the native midgut bacteria essential to both colonised species that can also be identified post-preservation.

Additionally, *C. pseudodiphtheriticum* and *E. cloacae* were identified in both species by culture-independent methods but only in *An. funestus* by culture-dependent methods. Similarly, *Psychrobacter* spp. were identified by culture-independent methods in *An. arabiensis* and *An. funestus* but only in *An. arabiensis* by culture-dependent methods. Thus, although these bacteria were identified by varying identification techniques, they likely also form part of the native midgut microbiota of both species. Further, both identification methods agree that *S. maltophilia* was specific to *An. funestus*, while *S. multivorum* was specific to *An. arabiensis*. A detailed analysis of common bacteria identified by both identification methods from specific genders of fresh and preserved *An. arabiensis* and *An. funestus* can be found in Appendix N (Figures N7–N8).

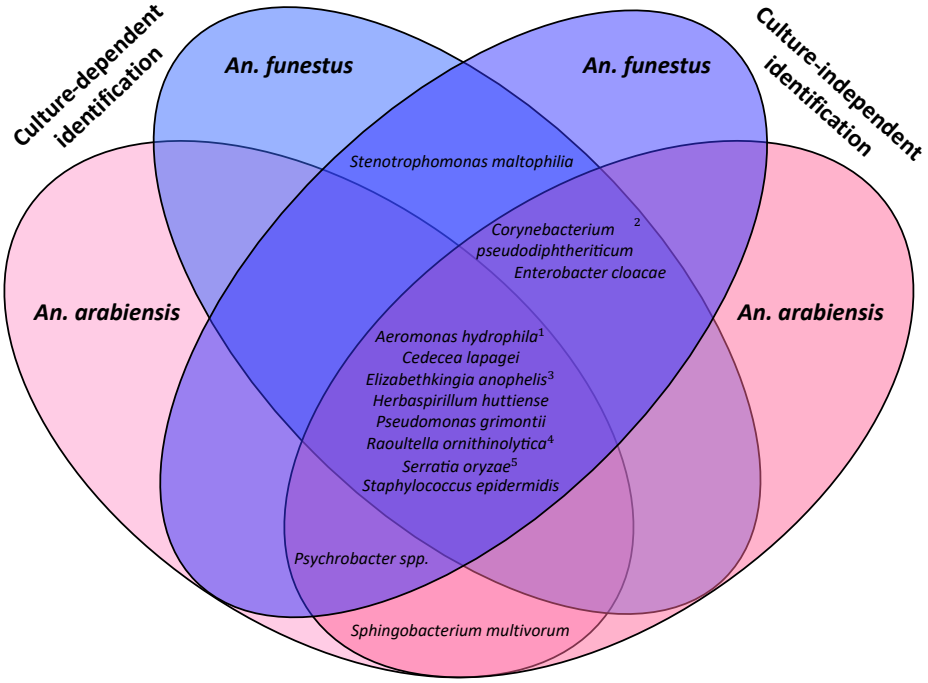

Figure 3.26: Common bacteria identified by culture-dependent and culture-independent techniques from fresh and preserved colonised *An. arabiensis* and *An. funestus*. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [4]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [5]*S. fonticola* and *S. oryzae.*

CHAPTER FOUR

DISCUSSION

Few studies have investigated if common midgut bacteria exist between malaria vectors. Thus, the present study characterised the native midgut bacteria of two dominant African malaria vectors, namely *An. arabiensis* (a member of the *An. gambiae* complex) and *An. funestus* (a member of the *An. funestus* group), using culture-dependent and culture-independent techniques. Furthermore, valuable information is lost when field-collected mosquitoes are preserved as researchers are unaware if the midgut bacteria can be identified post-preservation. Accordingly, this study determined if *Anopheles* midgut bacteria could be identified after mosquitoes were preserved on silica and in RNA*later*®. Moreover, to determine if there were common midgut bacteria between colonised and field-collected mosquitoes, midgut bacteria were characterised from field-collected *An. arabiensis* from two different South African localities, KwaZulu-Natal and Mpumalanga.

As an overview, the results of this study reinforce notions in existing literature on the *Anopheles* midgut microbiota (see section 4.1). For example, there were common bacteria between mosquitoes and their larval rearing water, showing that midgut bacteria are likely acquired from the environment. This study also identified common midgut bacteria between *An. arabiensis* and *An. funestus* that likely form part of the native midgut microbiota essential to the physiological functioning of both these vector species, some of which have been identified in these vectors for the first time (see section 4.2). Also, some bacteria were identified in a gender- and/or species-specific manner.

Several bacteria were common between fresh and preserved *An. arabiensis* and *An. funestus*, while *S. epidermidis* was the only bacterium common between colonised and field-collected mosquitoes. For the first time, this study has shown that midgut bacteria can be identified by culture-dependent or culture-independent techniques from *Anopheles* preserved on silica or in RNA*later*®. Thus, some midgut bacteria become conserved during mosquito preservation and can be identified post-preservation (see section 4.3). Although this study is not without limits (see section 4.4), it has multiple implications for future studies (see section 4.5), many of which could aid current malaria intervention strategies to reduce malaria transmission in Africa.

4.1. Expanding on Existing Literature of the *Anopheles* Midgut Microbiota

The present study expands on the existing knowledge of the *Anopheles* midgut microbiota. For instance, and although not the focus of this study, it was observed that bacteria were acquired from the mosquito's direct environment and colonised in the midgut (Boissière *et al*., 2012; Osei-Poku *et al*., 2012a). Kämpfer *et al*. (2011) and Lindh *et al*. (2008a) describe that mosquitoes ingest water during or shortly after emergence, thereby ingesting bacteria such as *E. anophelis*. Similarly, this study identified *E. anophelis* as a dominant midgut bacterium common between *An. arabiensis* and *An. funestus* that was also common in both species' larval rearing water.

In accordance with previous literature, this study also demonstrates that some bacteria likely co-evolve in a gender- or species-specific manner and must have been transmitted horizontally, transstadially, or vertically as these bacteria were present in female and/or male midguts even when they were not detected in the larval rearing water (Favia *et al*., 2007). However, as Kaufman *et al.* (1999) demonstrates that the abundance of bacteria in the larval rearing water increases in response to larva, it is also possible that the larval rearing water may have transstadially acquired bacteria from the larvae. Further, this study reinforces the notion that culture-dependent and culture-independent techniques are complementary and together provide a large dataset of valuable information (Pandya *et al*., 2017).

In terms of diversity and composition, this study echoes the results of previous studies by showing that colonised *Anopheles* midguts are predominated by Gram-negative bacteria (Berhanu *et al*., 2019; Boissière *et al.*, 2012; Damiani *et al*., 2010; Djadid *et al.*, 2011; Malafronte *et al*., 1999; Osei-Poku *et al.*, 2012a; Rami *et al*., 2018). Additionally, the bacteria identified in this study fall within the common phyla identified by previous studies; that is, Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Champion and Xu, 2017; Minard *et al*., 2013; Wang *et al*., 2011). Of these, this study demonstrated that colonised *Anopheles* midguts were predominated by Proteobacteria, which is also the most common midgut phylum isolated in other *Anopheles* midgut studies (Berhanu *et al*., 2019; Boissière *et al*., 2012; Chavshin *et al*., 2014; Cirimotich *et al*., 2011b; Dennison *et al*., 2014; Djadid *et al*., 2011; Gonçalves *et al*., 2019; Kalappa *et al*., 2018; Ngo *et al*., 2015; Ngo *et al*., 2016; Nilsson *et al*., 2019; Osei-Poku *et al*., 2012a; Rani *et al*., 2009; Tchioffo *et al*., 2016b; Terenius *et al*., 2008; Zoure *et al*., 2020).

Colonised *Anopheles* in this study also had midguts predominated by *Pseudomonas* and *Staphylococcus*, which are dominant genera isolated from colonised and field-collected

Anopheles in existing literature (Berhanu *et al*., 2019; Chavshin *et al.*, 2014; Djadid *et al.*, 2011; Galeano-Castañeda *et al.*, 2019; Ngo *et al.,* 2016; Osei-Poku *et al.,* 2012a; Pumpuni *et al.*, 1996; Rani *et al*., 2009; Terenius *et al*., 2008). Apart from *Pseudomonas* and *Staphylococcus*, *Aeromonas*, *Acinetobacter, Asaia*, *Bacillus*, *Chryseobacterium*, *Comamonas*, *Elizabethkingia*, *Enterobacter*, *Klebsiella*, *Mycobacterium*, *Pantoea*, *Serratia*, and *Sphingomonas* are also common genera associated with the *Anopheles* midgut, all of which were also isolated in the present study (Alvarez *et al*., 2012; Dong and Dimopoulos, 2009; Favia *et al.*, 2007; Garay *et al*., 2016; Gendrin and Christophides, 2013; Hughes *et al.*, 2016a; Hughes *et al*., 2016b; Jiang *et al*., 2012; Kukutla *et al*., 2013; Pei *et al*., 2015; Rani *et al*., 2009).

Few native midgut bacteria have been characterised on a species level from colonised or field-collected *An. arabiensis* or *An. funestus*. For example, *Acidovorax temperans*, *Anaplasma ovis*, *Bacillus silvestris*, *Bacillus simplex*, *Ehrlichia* spp., *Escherichia senegalensis*, *Janibacter limosus*, *Mycoplasma wenyonii*, *Nocardia corynebacterioides*, *Serratia odorifera*, *S. maltophilia*, *Vibrio metschnikovii* (Lindh *et al.*, 2005), *Alloiococcus otitis*, *Enterococcus casseliflavus*, *Kocuria kristinae*, *Kytococcus sedentarius*, *Lactococcus garvieae*, *Pseudomonas luteola*, *Saccharomyces cerevisiae*, *Streptococcus thoraltensis* (Berhanu *et al.*, 2019), *Comamonas* spp., *Pantoea* spp., *P. putida*, *Pseudomonas rhodesiae* (Cirimotich *et al.*, 2011a), *J. anophelis* (Kämpfer *et al.*, 2006a), *T. anophelis* (Briones *et al.*, 2008; Kämpfer *et al*., 2006b), and *Wolbachia* spp. (Baldini *et al.*, 2018) have been isolated from *An. arabiensis* midguts by previous studies.

Similarly, previous studies have only isolated *Bacillus megaterium*, *Brevundimonas diminuta*, *Cedecea davisae*, *Comamonas testosteroni*, *Erwinia chrysanthenum*, *Flavobacterium resinovorum*, *Gluconobacter cerinus*, *K. pneumoniae*, *Klyvera cryosceens*, *P. agglomerans*, *Panteo ananas*, *Pseudomonas mendocina*, *Salmonella choleraesuis* (Straif *et al.*, 1998), *J. anophelis*, and *Spiroplasma* spp. (Lindh *et al.*, 2005) from *An. funestus* midguts.

In accordance with these previous studies, the present study identified *Acinetobacter* spp. (Cirimotich *et al*., 2011a), *Asaia* spp. (Epis *et al.,* 2012), *C. amycolatum*, *E. meningoseptica*/*E. anophelis*, *K. variicola*, *S. hominis* (Barnard *et al.*, 2019), *Paenibacillus* spp. (Lindh *et al.*, 2005), *P. aeruginosa*, *Pseudomonas* spp., and *S. marcescens* (Berhanu *et al.*, 2019) from *An. arabiensis*. Similarly, this study agreed with previous literature that *An. funestus* midguts were colonised by *Aeromonas* spp., *Chryseobacterium* spp., *Pseudomonas* spp. (Osei-Poku *et al.*, 2012a), *E. coli*/*E. fergusonii*, *Pseudomonas stutzeri*, *S. maltophilia* (Straif *et al.*, 1998), and *S. marcescens* (Berhanu *et al*., 2019).

However, only two bacteria have been identified by previous studies as common midgut symbionts between *An. arabiensis* and *An. funestus* species: *S. marcescens* and *S. maltophilia* (Berhanu *et al.*, 2019; Lindh *et al*., 2005; Straif *et al.*, 1998). Therefore, this study added to the existing knowledge on the *An. arabiensis* and *An. funestus* midgut microbiota by expanding on common bacteria associated with both vector species. Moreover, two bacteria were identified in field-collected *An. arabiensis* in this study that had also been identified in *An. arabiensis* midguts in previous studies: *P. rhodesiae* and *S. hominis.*

Overall, this study and previous studies agree that *Pseudomonas* spp. and *S. marcescens* are common between the midguts of *An. arabiensis* and *An. funestus. Pseudomonas* spp. are known anopheline symbionts and have been isolated from *An. albimanus*, *An. culicifacies*, *An. darlingi*, *An. gambiae*, *An. maculipennis*, and *An. stephensi* (Alvarez *et al.*, 2012; Boissière *et al*., 2012; Chavshin *et al*., 2012; Chavshin *et al*., 2014; Djadid *et al*., 2011; Pumpuni *et al*., 1996; Rani *et al*., 2009; Terenius *et al*., 2008; Wang *et al*., 2011). *Pseudomonas* spp. are also known to increase in abundance after females ingest a blood meal and have been implicated in assisting with oxidative stress induced by ingestion of a blood meal (Alvarez *et al*., 2012). Additionally, *Pseudomonas* spp., specifically *P. stutzeri* and *P. putida*, have been shown to inhibit *Plasmodium* development in *Anopheles* (Bahia *et al*., 2014; Tchioffo *et al*., 2013).

Similarly, *S. marcescens* has been isolated as a midgut symbiont from *An. dirus* and *An. gambiae* (Jupatanakul *et al.*, 2020; Tandina *et al*., 2016; Wang *et al*., 2011; Wang *et al*., 2017). Bahia *et al.* (2014) also demonstrated that acute infections with *S. marcescens* reduced mosquito fitness and caused mortality. In addition, Jupatanakul *et al.* (2020) described that *S. marcescens* secretes pathogenic chitinases and proteases but suggested the potential for using these bacteria to increase mosquito susceptibility to entomopathogenic microorganisms as part of vector control strategies.

Also, *S. marcescens* has been shown to inhibit *Plasmodium* infection in *Anopheles* (Bahia *et al*., 2014; Bando *et al*., 2013; Boissière *et al*., 2012; Gendrin and Christophides, 2013; Gonzalez-Ceron *et al*., 2003; Ramirez *et al*., 2014; Seitz *et al*., 1987; Stathopoulos *et al*., 2014; Tchioffo *et al*., 2013; Wang *et al*., 2019). Notably, Wang *et al.* (2017) have shown that a *Serratia* strain, made up of *Serratia marcescens marcescens* and *Serratia marcescens sakuensis*, can be transmitted horizontally and vertically in anophelines, demonstrating that this bacterium meets the requirements for paratransgenesis.

Therefore, these bacteria possibly form part of the native midgut microbiota that may be essential to anopheline physiological functioning and could serve as potential paratransgenic

candidates to aid current malaria intervention strategies and reduce the malaria burden in Africa. Accordingly, it would be worthwhile to determine if these bacteria could serve as paratransgenic candidates in African malaria vectors such as *An. arabiensis* and *An. funestus*, as they are known midgut symbionts of both vector species.

4.2. Novel Midgut Bacteria Identified from *Anopheles arabiensis* and *Anopheles funestus*

Few studies have focused on characterising the native midgut bacteria amongst *Anopheles* vector species. This study added to the existing knowledge of the *Anopheles* microbiota by characterising native midgut bacteria in colonised *An. arabiensis* and *An. funestus* that have not yet been identified in these species. Accordingly, the common native midgut bacteria in *An. arabiensis* and *An. funestus* include *A. hydrophila*, *E. anophelis*, *E. cloacae*, *H. huttiense*, *P. grimontii*, *Psychrobacter* spp., *R. ornithinolytica*, *S. oryzae*, and *S. epidermidis*.

Some of these bacteria have been identified in previous studies investigating the *Anopheles* midgut bacteria. For example, *A. hydrophila* has been identified in the midguts of *An. gambiae*, as well as in the larval rearing water of *An. stephensi* and *An. maculipennis* (Djadid *et al*., 2011; Lindh *et al.*, 2005). Additionally, *A. hydrophila* has been documented in *Culex quinquefasciatus*, however, its role in mosquitoes remains unknown (Chandel *et al*., 2013; Pidiyar *et al.*, 2002). Interestingly, *A. hydrophila* – commonly associated with human infections such as gastroenteritis – has also been identified in dipteran flies such as *Chrysomya megacephala*, *Musca domestica*, and *Musca sorbens*. It is speculated that these flies act as vectors and reservoirs for this bacterium (Gray *et al*., 1990; Gupta *et al*., 2012; Nayduch *et al*., 2001; Rahuma *et al*., 2005; Sulaiman *et al*., 2000). Therefore, perhaps mosquitoes provide a similar role for *A. hydrophila* by acting as reservoirs or vectors for transportation.

Similarly, *E. anophelis* (misidentified as *E. meningoseptica* in most studies) was first discovered in *An. gambiae* midguts, demonstrating the specificity of this bacterium for *Anopheles* midguts (Kämpfer *et al.*, 2011). In addition, *E. anophelis* has been documented in *An. stephensi* and is known to protect *Anopheles* from infection and positively influence their fecundity (Akhouayri *et al.*, 2013; Chen *et al*., 2015; Chen *et al*., 2020; Dong and Dimopoulos, 2009; Kukutla *et al*., 2014). Moreover, *E. anophelis* possesses transstadial transmission abilities and has demonstrated anti-*Plasmodium* activity (Akhouayri *et al.*, 2013; Bahia *et al*., 2014; Chen *et al*., 2015; Ngwa *et al.*, 2013; Wang *et al*., 2011).

Chen *et al*. (2015) have also genetically modified *E. anophelis* to express genes of interest. Thus, the presence of *E. anophelis* in *An. arabiensis* and *An. funestus* likely means that this bacterium has co-evolved with *Anopheles* vectors to play essential roles in immunity and fecundity (Akhouayri *et al.*, 2013; Chen *et al*., 2015; Chen *et al*., 2020; Dong and Dimopoulos, 2009; Kukutla *et al*., 2014). Hence, *E. anophelis* is a favourable candidate for paratransgenesis, especially because it has been identified in the two dominant malaria vectors of the present study, as well as in other anophelines.

Further, *E. cloacae* is a known *Anopheles* midgut symbiont (Berhanu *et al*., 2019; Tandina *et al*., 2016). This bacterium was identified in *An. gambiae* and has been shown to hinder the development of *P. berghei* and *P. falciparum* in *An. stephensi*, and *P. vivax* in *An. albimanus* (Eappen *et al*., 2013; Gonzalez-Ceron *et al*., 2003). This bacterium has also been tested for paratransgenesis in *An. stephensi* and an *Enterobacter* strain (Esp_Z) discovered from field-collected *An. arabiensis* has been shown to inhibit *P. falciparum* in *An. gambiae* (Cirimotich *et al*., 2011a). However, *E. cloacae* has not been previously documented in *An. funestus*. Thus, because this bacterium is known to influence *Plasmodium* development and is found in several *Anopheles* vector species, its potential use in paratransgenesis in *An. arabiensis* and *An. funestus* could aid intervention strategies.

Psychrobacter spp. have also been documented in *Anopheles* midguts, but their role in mosquitoes, as well as in other blood-feeding insects, remains unknown (Malele *et al*., 2018; Manguin *et al*., 2013). Similarly, *S. epidermidis* has been isolated from the midguts of field-collected *An. pharoensis*, from the salivary glands of colonised *An. arabiensis*, and from the midguts of *An. gambiae*, but its role is also yet to be elucidated (Berhanu *et al*., 2019; Tandina *et al*., 2016). Thus, not only can these midgut symbionts be tested for their potential as paratransgenic candidates, but their biological role in *Anopheles* can also be elucidated in future studies.

Likewise, *H. huttiense*, *P. grimontii*, *R. ornithinolytica*, and *S. oryzae* have not been documented in *Anopheles* midguts; therefore, their roles also remain unknown. Moreover, *H. huttiense*, *P. grimontii*, and *S. oryzae* have not been documented in other arthropods or insects. However, *R. ornithinolytica* has been previously documented in *Anopheles* larval rearing water (Tandina *et al.*, 2016). Thus, since the current study shows that *R. ornithinolytica* colonises in *An. arabiensis* and *An. funestus* midguts and is also present in the larval rearing water of both species, *R. ornithinolytica* has potential for paratransgenesis as it can be acquired by mosquitoes from their environment. In addition, *R. ornithinolytica* has been identified in the midgut of the earthworm, *Dendrobaena veneta*, and has shown antimicrobial activity against bacteria and

fungi (Fiołka *et al.*, 2010; Fiołka *et al.*, 2012). Thus, perhaps *R. ornithinolytica* has a similar role in *Anopheles* by potentially protecting them against opportunistic pathogens. This should be investigated further as *R. ornithinolytica* could be a potential symbiont for paratransgenesis.

To my knowledge, this is the first time that *A. hydrophila*, *E. anophelis*, *H. huttiense*, *P. grimontii*, *R. ornithinolytica*, *S. oryzae*, and *S. epidermidis* have been isolated from the midguts of *An. arabiensis* and *An. funestus*. Of these bacteria, *S. epidermidis* was also identified in field-collected *An. arabiensis* from two different localities in South Africa. Therefore, *S. epidermidis* has likely co-evolved with *An. arabiensis* and may be transmitted horizontally, transstadially, and/or vertically. Thus, *S. epidermidis* is the most promising candidate bacterium for future investigations because it was isolated from: (i) colonised *An. arabiensis* and *An. funestus* females and males and their larval rearing water, (ii) colonised *An. arabiensis* and *An. funestus* females and males preserved on silica or in RNA*later*® for up to 12 weeks, and (iii) silica-preserved field-collected female *An. arabiensis* from two different localities in South Africa.

This study also identified gender-specific bacteria from *An. arabiensis* and *An. funestus*. For example, *H. psychrotolerans* was specific to females of both vector species while *A. oris*, *L. iarius*, *P. aerius*, and *S. thermophilus* were specific to males of both vector species. To my knowledge, this is the first time that these bacteria have been identified from the midguts of *Anopheles* – and Diptera in general – in a gender-specific manner. Of these, *H. psychrotolerans*, which was exclusive to females, and *A. oris*, which was exclusive to males, were also absent from the mosquito's larval rearing water. Thus, these bacteria have likely co-evolved with both species in a gender-specific manner and may play vital gender-specific roles in *Anopheles*, possibly for digestion of different food sources (i.e., blood and nectar/sugar by females but only nectar/sugar by males) (de O Gaio *et al*., 2011; Gusmão *et al.*, 2010; Rani *et al*., 2009).

Additionally, some bacteria were identified in a gender-specific manner from specific species. For example, *B. ovis*, *P. marginalis*, and *R. aquatilis* were specific to *An. arabiensis* males, while *P. savastanoi* was specific to *An. funestus* females and *A. seohaensis* was specific to *An. funestus* males. Of these, *B. ovis*, *P. marginalis*, *P. savastanoi*, and *A. seohaensis* were not present in the larval rearing water. Thus, these bacteria co-evolved in a species- and gender-specific manner and are potentially essential for biological functioning of specific genders belonging to specific vector species. These bacteria have also not been previously identified in *Anopheles* or arthropod studies on the midgut bacteria, making these novel discoveries that have potential for paratransgenesis.

Future studies could investigate the role that the aforementioned bacteria play in *Anopheles* to determine whether they have potential to be used in paratransgenesis in a gender-specific manner. For example, genetically engineering *H. psychrotolerans* could be used to hamper *Plasmodium* development in females, while genetically engineering *A. oris* could be used to influence male mating behaviour and prevent males from impregnating females, thereby reducing the propensity for females to bite humans since females only ingest a blood meal to feed their eggs for maturation (Roubaud, 1929).

Thus, *A. oris* has potential to be used in SIT programmes. SIT involves introducing sterile males into a wild population to outcompete wild males, where sterile males copulate with wild females to prevent impregnation (Alphey *et al*., 2010; Alphey *et al.*, 2014; Knipling, 1955; Malcolm *et al*., 2009). The success of this technique lies in the principle that females generally only mate once (Charlwood and Jones, 1979; Tripet *et al*., 2003). Therefore, *A. oris* should be investigated for its potential as a paratransgenic candidate to induce male mosquito sterility, where – if successful – use of this bacterium could form part of SIT programmes to reduce malaria transmission.

The present study also identified midgut bacteria worth mentioning: *S. aureus*, *S. hominis*, *S. multivorum*, and *S. maltophilia*. Although *S. aureus* is a human pathogen responsible for a variety of infections, this study identified *S. aureus* in colonised *An. arabiensis* males and field-collected *An. arabiensis* females from Mpumalanga (Lowy, 1998). Also, *S. aureus* has been isolated from the salivary glands of field-collected *An. gambiae* from Ethiopia (Berhanu *et al*., 2019). Therefore, since this bacterium was identified from colonised and field-collected *An. arabiensis*, *S. aureus* is a potential candidate for paratransgenesis.

This study also identified *S. hominis* as a common bacterium in fresh colonised *An. arabiensis* and *An. funestus* males, preserved colonised females and males of both species, and preserved *An. arabiensis* females from KZN. This bacterium is also a known symbiont of field-collected *An. stephensi* (Rani *et al*., 2009). Additionally, *S. hominis* has been identified in fourth instar *An. arabiensis* larvae from the Botha De Meillon Insectary, albeit from a different colony – the insecticide susceptible SENN colony originating from Sudan (Barnard *et al*., 2019). Therefore, *S. hominis* has potential for paratransgenesis in *An. arabiensis* and *An. funestus*.

Conversely, *S. multivorum*, which was identified as a bacterium specific to *An. arabiensis* midguts in this study, has not previously been identified as a midgut symbiont in *Anopheles*. It was, however, detected in the *An. arabiensis* and *An. funestus* rearing water of the present study and has been previously identified in colonised *An. gambiae* larval rearing water, yet not in

these *An. gambiae* midguts (Tandina *et al*., 2016). Therefore, since the environment plays a role on *Anopheles* midgut bacteria, *S. multivorum* is probably a bacterium common to the larval rearing water in controlled settings (Chavshin *et al.*, 2012). This bacterium likely then becomes acquired by – and colonises in the midguts of – *An. arabiensis* but not *An. funestus*, demonstrating the ability of specific anophelines to acquire specific environmental bacteria. Therefore, *S. multivorum*, should be investigated as a potential candidate for paratransgenesis in *An. arabiensis*.

Similarly, *S. maltophilia* was identified as a bacterium specific to *An. funestus*. This bacterium has been characterised in field-collected *An. funestus*, *An. arabiensis*, and *An. gambiae* (Lindh *et al*., 2005; Straif *et al*., 1998). Since *S. maltophilia* has also been characterised in other anophelines, it is perhaps only specific to colonised *An. funestus* of this study. Nonetheless, because it has been found as a common bacterium amongst *Anopheles* vector species, this bacterium should be explored for use in paratransgenesis.

4.3. Midgut Bacteria can be Identified from Preserved *Anopheles*

For the first time, this study shows that preserving *Anopheles* on silica or in RNA*later*® also conserves their midgut bacteria. This study also demonstrates that it is possible to identify the *Anopheles* midgut bacteria after several weeks of mosquito preservation. Additionally, this study demonstrates that the midgut bacteria of preserved mosquitoes were similar to the midgut bacteria of fresh mosquitoes in terms of diversity and composition, suggesting that preservation does not drastically alter the midgut bacterial community. The dominant phylum in fresh mosquitoes remained Proteobacteria in preserved mosquitoes while *Pseudomonas* and *Staphylococcus* remained the dominant genera, reiterating that the midgut bacterial community did not change severely after preservation. Thus, preserving mosquitoes on silica or RNA*later*® also conserves their midgut bacterial communities.

However, unlike colonised mosquitoes, preserved field-collected *An. arabiensis* in this study were mainly colonised by Firmicutes. Nonetheless, field-collected *An. albimanus* from Colombia and field-collected *Anopheles* spp. from Vietnam were also predominated by Firmicutes (Galeano-Castañeda *et al*., 2019; Ngo *et al*., 2016). These studies also identified *Staphylococcus* and *Bacillus* as dominant genera colonising the midguts of these field-collected *Anopheles*, which is consistent with results from preserved field-collected *An. arabiensis* in this study.

Overall, I hypothesise that silica desiccation compacts the midgut to form a rigid, secure biofilm surrounding the bacteria, providing a suitable environment for preservation. This hypothesis arises from the fact that silica gel is a suitable preservative for long-term bacterial conservation, where encapsulating bacteria in silica gel also preserves bacterial biological activity (Grivell and Jackson, 1969; Mutlu *et al.*, 2015). During preservation, the bacteria then enter a state of dormancy and when they are released into saline solution and placed on selective media, the bacteria exit dormancy and acquire nutrients for active growth. Therefore, using silica preservation in combination with culture-dependent techniques is useful because it distinguishes bacteria capable of entering and exiting dormancy, whereas culture-independent techniques cannot distinguish between live and dead bacteria (Figdor and Gulabivala, 2008). However, silica preservation may only be suitable for conserving specific bacteria at certain preservation time periods.

I also hypothesise that preserving mosquitoes in RNA*later*® causes the midguts to become pulpous. As such, the RNA*later*® solution may come into contact with the midgut bacteria and inhibit bacterial growth when homogenates are placed on nutrient agar as RNA*later*® is bacteriostatic (Sigma-Aldrich Technical Bulletin, 2016). This may account for the few bacteria identified from RNA*later*®-preserved mosquitoes by culture-dependent techniques in comparison to culture-independent techniques. Thus, since RNA*later*® preserves DNA in high quality, this preservation method is suitable when paired with culture-independent techniques. Hence, culture-independent techniques identified a more diverse profile of bacteria than culture-dependent techniques, which was also expected as culture-independent methods are highly sensitive (Figdor and Gulabivala, 2008).

However, a disadvantage of culture-independent identification is possible contamination during bacterial DNA extraction (Figdor and Gulabivala, 2008). This may account for the large number of bacteria identified in silica- and RNA*later*®-preserved mosquitoes that were not identified in fresh mosquitoes. It is possible that during the time it took for preservation to occur, bacterial diversification and growth may have remained active. This could allow the bacterial community to change and may explain why several bacteria appear in the midguts of preserved mosquitoes and not fresh mosquitoes, such as *C. lapagei*, as well as why more bacteria were found in the midguts of preserved mosquitoes after certain preservation time periods.

Cedecea lapagei was identified by both identification methods as a common bacterium in preserved *An. arabiensis* and *An. funestus*. Thus, I hypothesise that *C. lapagei* colonises in low quantities in the midguts of fresh mosquitoes and over time (i.e., during the time it takes for preservation to occur), this bacterium colonises in higher quantities. Therefore, it is likely

difficult to detect this bacterium in fresh mosquitoes, but it can be readily detected in all midguts post-preservation. Similarly, this could be true for many of the bacteria identified post-preservation that were not identified in fresh mosquitoes.

Future studies investigating the midgut bacteria of preserved *Anopheles* need to be cognisant that the composition of the midgut microbiota can change during preservation and that this may be dependent on preservation time as well as the type of preservative used. Future studies investigating the midgut bacteria of preserved *Anopheles* – or arthropods – should compare the midgut bacteria of newly emerged *Anopheles* with aged *Anopheles* prior to preservation to get an overview of the change in composition of the midgut bacteria of fresh mosquitoes over time. This will provide insight into which midgut bacteria colonise in a preservative-dependent manner, and which midgut bacteria are purely age-dependent.

Nevertheless, now that this study has demonstrated that the midgut bacteria of conserved *Anopheles* can be identified after preservation on silica and RNA*later*®, future studies can look at the midgut bacteria of mosquitoes from different localities without the need to work on live, wild mosquitoes (especially mosquitoes potentially infected with *Plasmodium*) or have to travel to various localities to collect these mosquitoes. Not only does this reduce risk of infection and cost of travel for research, it paves the way for future studies aiming to discover common midgut bacteria between *Anopheles* vectors (colonised and field-collected) across different regions to elucidate the relationship between midgut bacteria and *Plasmodium* infection.

4.4. Limitations

As described by Chavshin *et al.* (2012), the microbial diversity of the mosquito midgut is influenced by the origin of specimens as well as the length of time they have been maintained in laboratory or field settings. Thus, selective pressures of laboratory conditions limit bacterial acquisition and composition (Favia *et al.*, 2007; Gusmão *et al.*, 2010; Riehle and Jacobs-Lorena, 2005). Many of the midgut bacteria identified in this study were likely influenced by the laboratory environment, or by the field environment they originated from, making them specific to colonised and field-collected African malaria vectors.

Additionally, the powdered meal fed to colonised larvae likely affected their midgut bacteria, as well as the midgut bacteria identified from the larval rearing water. Further, the sucrose meal that adults were maintained on may have also influenced their midgut bacteria. It is also possible that the human curators may have been a source of water contamination. Another

limitation is that the larval rearing water was not initially sterilized or controlled, and mosquitoes were not raised in sterile conditions. Thus, the conclusions of this study may be limited to the specific insectary they were reared in.

In addition, while adult colonised (and field-collected mosquitoes) used in this study were not fed blood meals, their predecessors were. During rearing, colonised mated female mosquitoes are routinely blood fed using guinea pigs and occasionally supplemented with human blood during unsuccessful feeding (Letinić *et al*., 2020). Thus, it is possible that mosquitoes may have acquired bacteria – such as *Staphylococcus*, which is a bacterium abundant on mammal skin (Kloos and Schleifer, 1986) – from guinea pig or human skin upon ingestion of a blood meal; these bacteria may have colonised in the midguts and likely co-evolved with mosquitoes and may have been transmitted vertically through successive generations.

Another limitation of this study is that the sample size of colonised mosquitoes was larger than the sample size of field-collected mosquitoes since preserved field-collected mosquitoes were scarce. Additionally, the sample size of field-collected *An. arabiensis* from KZN and Mpumalanga differed, where Mpumalanga had a smaller sample size than KZN. This could have accounted for the fewer bacteria identified in *An. arabiensis* from Mpumalanga than KZN, and overall, the fewer bacteria identified from field-collected mosquitoes than colonised mosquitos. Only female field-collected *An. arabiensis* were used in this study, which may have also limited the types of bacteria identified. Moreover, seasonal variation may have affected the midgut bacteria identified in this study as Akorli *et al.* (2016) show that *Anopheles* midgut bacteria change according to season. Thus, because this study used field-collected mosquitoes that were collected during the winter season, this may represent a different *An. arabiensis* midgut microbiota than that of mosquitoes collected in summer.

Additionally, as there is no way of telling mosquito age upon collection, the age of field-collected *Anopheles* is unclear. Since the midgut bacteria change during development, the age of the field-collected mosquitoes used in this study could have influenced the bacteria identified (Gimonneau *et al*., 2014; Wang *et al*., 2011). Further, the conditions that mosquitoes were handled during collection may have affected the types of bacteria that were preserved (i.e., depending on the time it took for mosquitoes to be immobilized and placed on silica after collection, this may have affected the bacterial community).

Moreover, although culture-independent procedures were performed at the same time for all samples, different generations of samples were collected. This may account for the difference in microbial composition between fresh and preserved samples. As culture-dependent

techniques were performed at different times, but with the same generations of mosquitoes, this may have introduced batch effects. Further, pooling does not provide a true representation of the mosquito microbiome because there is high variability between individual mosquitoes, which may have also limited this study (Rodríguez-Ruano *et al*., 2020).

4.5. Future Studies

Apart from aforementioned ideas for future studies, this study provides a broad range of potential paratransgenic candidates that future studies could investigate. Future studies could also determine if these bacteria exist in other *Anopheles* vectors and compare the native midgut bacteria of *P. falciparum*-infected and *P. falciparum*-uninfected vector species. Identifying common symbionts that meet paratransgenic requirements is advantageous for symbiotic control as it would increase the chance of reducing malaria in endemic regions that have more than one dominant malaria vector species. Additionally, it would be interesting to determine if these bacteria exist in other mosquito genera such as *Aedes* or *Culex* to determine if they can potentially be used to control other vector-borne pathogens.

Apart from paratransgenesis, it is also essential to test the physiological role that these bacteria play in *An. arabiensis* and *An. funestus* and determine whether this information is species-specific or can be extended to other *Anopheles*, or even other mosquito genera. Overall, combining the preservatives and identification methods used in this study for future studies is useful for investigating the midgut bacteria of other *Anopheles*. This may further broaden the existing knowledge on the *Anopheles* native midgut microbiota and can aid future investigations elucidate the role that specific midgut bacteria play in mosquito physiology. It could also be used to investigate the midgut bacteria of wild vector populations that have been preserved on silica or in RNA*later*®, where common bacteria can be proposed for paratransgenesis and used in combination with current intervention strategies to reduce malaria transmission.

It would also be worthwhile to determine if mosquito midgut bacterial composition and diversity change over longer preservation time periods (i.e., longer than 12 weeks of preservation). This would determine how far into the past midgut bacteria can be isolated and would be interesting for studies investigating evolutionary changes in mosquito midgut microbiota. It would also be useful to analyse the efficiency of other preservatives to determine if they can also conserve midgut bacteria and if some preservatives are better suited than others for conserving specific midgut bacteria.

Furthermore, it would be interesting to see if the midgut bacteria of other preserved arthropods can also be identified. Since many midgut bacteria are acquired from the environment, identifying preserved midgut bacteria from arthropods in an area can be used to study ecological changes in an environment over time (i.e., if there is a change in a specific environment, it would be worthwhile investigating if there is also a change in the midgut bacteria of the arthropods inhabiting that area). This could aid in understanding the changes in environmental bacteria and the effect that these changes have on the midguts of localised arthropods, and on an ecological system as a whole.

Additionally, this may reveal valuable information about the biological evolution of arthropods and their midgut symbionts, especially when investigating the native midgut bacterial community of preserved arthropods over various time periods and/or various localities. This may also pave the way for studies on arthropods that are disease vectors, where insight into the midgut bacteria of preserved arthropods may aid studies investigating symbiotic control. Additionally, it would be interesting to determine if other midgut symbionts besides bacteria are also preserved during arthropod preservation.

CONCLUSION

Previous studies have shown that there are major differences in the midgut bacteria of different mosquito vector species, as well as between individuals of the same species. However, this study has demonstrated that there are native bacteria that are common between two dominant colonised malaria vectors, *An. arabiensis* (a member of the *An. gambiae* complex) and *An. funestus* (a member of the *An. funestus* group).

These include *Pseudomonas* spp. and *S. marcescens* (in accordance with previous studies), as well as *A. hydrophila*, *E. anophelis*, *E. cloacae*, *H. huttiense*, *P. grimontii*, *Psychrobacter* spp., *R. ornithinolytica*, *S. oryzae*, and *S. epidermidis*. Of these, *A. hydrophila*, *E. anophelis*, *H. huttiense*, *P. grimontii*, *R. ornithinolytica*, *S. oryzae*, and *S. epidermidis* have been identified from the midguts of *An. arabiensis* and *An. funestus* for the first time. Thus, these bacteria show promise for symbiotic control and should be tested for their potential as paratransgenic candidates.

Of these bacteria, *S. epidermidis* is the most promising candidate bacterium for paratransgenesis as it was found in: (i) colonised *An. arabiensis* and *An. funestus* females and males and their larval rearing water, (ii) colonised *An. arabiensis* and *An. funestus* females and males preserved on silica or in RNA*later*® for up to 12 weeks, and (iii) silica-preserved field-collected female *An. arabiensis* from two different localities in South Africa. Other noteworthy bacteria worth investigating include *A. oris*, *A. seohaensis*, *B. ovis*, *H. psychrotolerans*, *P. marginalis*, and *P. savastanoi* because they were gender- and/or species-specific. Similarly, *S. aureus*, *S. hominis*, *S. multivorum*, and *S. maltophilia* are also potential candidates for future paratransgenic studies.

For the first time, this study has discovered that preserving *Anopheles* on silica or in RNA*later*® also conserves their midgut bacteria. These bacteria can then be identified by culture-dependent or culture-independent techniques. This is a crucial discovery because valuable information is lost from field-collected mosquitoes as researchers are unaware that the midgut bacteria can be identified post-preservation. Furthermore, this study reiterated several notions within existing literature: (i) culture-dependent and culture-independent techniques are complementary, (ii) bacteria are acquired from the mosquito's environment and colonise within the midgut, and (iii) some bacteria co-evolve in a gender- or species-specific manner and are likely transmitted horizontally, transstadially, and/or vertically.

In conclusion, this study served the purpose of broadening the existing knowledge on the native midgut bacteria of two dominant African malaria vectors, *An. arabiensis* and *An. funestus*. The uniqueness of this research lies in the discovery that preserving mosquitoes also conserves their

midgut bacteria and that the midgut bacteria can be identified post-preservation. Additionally, many common bacteria were identified for the first time between *An. arabiensis* and *An. funestus*, and *S. epidermidis* was the most promising midgut bacterium as it was found in both genders of both species, both species' larval rearing water, both genders of both preserved species, and field-collected *An. arabiensis* from two different South African localities. Therefore, future studies could investigate the potential for the symbionts discovered in this study to be used as paratransgenic candidates. This could aid current intervention strategies, or introduce novel intervention strategies, to reduce the transmission of malaria, especially in Africa.

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APPENDICES

Ethics Waiver **APPENDIX A**

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

30/05/2018

Ref: W-CBP-180530-04

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: Ms/Professor BE/L Silva/Koekemoer
Student No. (if appropriate): 802873
Staff No. (if appropriate):

Supervisor: Dr Y Dahan-Moss

School: Pathology
Department:
Wits Research Institute for Malaria
NICD
NHLS

Project title: *The relationship between the anopheles midgut and Plasmodium falciparum infection*

Reason: In vitro study.
No human participants will be involved in the study.

Professor CB Penny
Chairperson: Human Research Ethics Committee (Medical)

Copy – HREC (Medical) Secretariat: Zanele Ndlovu and Rhulani Mkansi.

Research Office Secretariat:
Physical address: Phillip Tobias Building, 3rd Floor, Office 302, Corner York Road and Princess of Wales Terrace, Parktown, Johannesburg 2193.
Postal address: Private Bag 3, Wits 2050
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Office E-mail: HREC-Medical.ResearchOffice@wits.ac.za.
Website:http://www.wits.ac.za/research/about-our-research/ethics-and-research-integrity/

Please note that the above project title was changed (see below for approved change of title).

Approved Change of Title

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Private Bag 3 Wits, 2050
Fax: 027117172119
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Reference: Mrs Sandra Benn
E-mail: sandra.benn@wits.ac.za

28 July 2020
Person No: 802873
TAA

Ms BG E Silva
87 Churchill Avenue
Dawnview
Germiston
1401
South Africa

Dear Ms Bianca E Silva

Master of Science in Medicine: Change of title of research

I am pleased to inform you that the following change in the title of your Dissertation for the degree of **Master of Science in Medicine** has been approved:

From:	**The relationship between the anopheles midgut bacteria and plasmodium falciparum infection**
To:	**Characterising the native midgut bacteria from main African malaria vector species, Anopheles arabiensis and An.funestus.**

Yours sincerely

Mrs Sandra Benn
Faculty Registrar
Faculty of Health Sciences

Bacteria Identified in this Study

APPENDIX B

Table B1: Bacteria identified from colonised *An. arabiensis* and *An. funestus*. Bacteria were identified by culture-dependent (*) and culture-independent methods from fresh females (♀) and males (♂) and their larval rearing water (H_2O), as well as from females and males preserved on silica (^{S}) and in RNA*later*® (^{R}) for up to 12 weeks. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*D. acidovorans* and *D. tsuruhatensis*; [4]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [5]*E. coli* and *E. fergusonii*; [6]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [7]*S. fonticola* and *S. oryzae*.

Genera	Species	Gram Stain	Culture-dependent Identification				Culture-independent Identification			
			Fresh		Preserved		Fresh		Preserved	
		Gram-positive (+) or Gram-negative (-)	*An. arabiensis*	*An. funestus*	*An. arabiensis*	*An. funestus*	*An. arabiensis*	*An. funestus*	*An. arabiensis*	*An. funestus*
Abiotrophia	*defectiva**	+							♀^{SR}; ♂^{R}	♀^{SR}; ♂^{R}
Acetatifactor	*muris*	+							♂^{R}	
Acetobacter	*suratthaniensis*	-						♂	♀^{R}; ♂^{SR}	♀^{R}; ♂^{SR}
Acetobacterium	*carbinolicum*	+								♂^{R}
Achromobacter	*animicus*	-							♂^{R}	♂^{R}
Acidibacter	*ferrireducens*	-								♂^{S}
Aciditerrimonas	*ferrireducens*	+							♂^{SR}	
Acinetobacter	*baumannii**	-							♀^{R}	♂^{R}
	dispersus	-								♂^{R}
	*haemolyticus**	-	H_2O							
	*johnsonii**	-						H_2O; ♂	♀^{SR}; ♂^{SR}	♀^{R}; ♂^{SR}
	*junii**	-					H_2O		♀^{R}; ♂^{SR}	♀^{SR}; ♂^{SR}
	*lwoffii**	-						H_2O; ♂	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}

	*parvus**	-	H_2O							
	*pittii**	-						♂	♀^{R}; ♂^{R}	♀^{R}; ♂^{SR}
	*schindleri**	-							♂^{R}	♂^{R}
	seohaensis	-						♂		♂^{R}
	*ursingii**	-								♀^{R}
Actinomyces	*bowdenii**	+								♀^{R}
	*israelii**	+								♂^{R}
	*oris**	+					♂		♀^{R}; ♂^{R}	♀^{SR}; ♂^{R}
	polynesiensis	+								♂^{S}
Aeromonas	*caviae**	-	H_2O							
	*hydrophila**[l]	-	H_2O; ♀; ♂	H_2O; ♀; ♂			H_2O; ♀; ♂	H_2O; ♀; ♂	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
	sanarellii	-					♀; ♂			
	taiwanensis	-					♀			
Aerosakkonema	*funiforme*	-					H_2O; ♂	H_2O	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
Aggregatibacter	*segnis*	-								♀^{R}
Aliterella	*antarctica*	-							♀^{R}	
Alloprevotella	*rava*	-							♀^{R}; ♂^{R}	♀^{R}; ♂^{S}
Amaricoccus	*kaplicensis*	-							♀^{R}; ♂^{R}	♀^{R}; ♂^{R}
	macauensis	-							♀^{R}	
Aminobacter	*niigataensis*	-							♂^{S}	♀^{R}; ♂^{S}
Anaerococcus	*nagyae*	+								♀^{R}
	*octavius**	+							♀^{R}	♀^{R}; ♂^{R}

	*prevotii**	+					H_2O		$♀^{R}$; $♂^{SR}$	$♀^{SR}$; $♂^{SR}$
	provencensis	+							$♀^{R}$; $♂^{R}$	$♀^{R}$
Anaerofilum	*pentosovorans*	+								$♂^{S}$
Anaerostipes	*hadrus*	+								$♂^{S}$
Ancylobacter	*polymorphus*	-					H_2O	H_2O	$♀^{R}$	
Anoxybacillus	*flavithermus*	+					H_2O		$♀^{R}$; $♂^{R}$	$♂^{R}$
	tepidamans	+					H_2O	H_2O	$♀^{R}$; $♂^{S}$	$♀^{S}$
Aquabacter	*spiritensis*	-								$♀^{R}$
Aquabacterium	*parvum*	-								$♀^{R}$; $♂^{R}$
Aquincola	*tertiaricarbonis**	-								$♂^{S}$
Aquisphaera	*giovannonii*	-								$♂^{R}$
Aquitalea	*pelogenes*	-					H_2O			
Arcticibacter	*pallidicorallinus*	-								$♀^{S}$
Arsenicibacter	*rosenii*	-						H_2O		
Arthrobacter	*gandavensis**	+							$♀^{R}$	$♀^{R}$
	*polychromogenes**	+	H_2O							
	*woluwensis**	+			$♂^{S}$					
Asaia	*krungthepensis*	-					$♂$	H_2O; $♀$; $♂$	$♀^{SR}$; $♂^{SR}$	$♀^{SR}$; $♂^{SR}$
Asticcacaulis	*excentricus*	-					H_2O			
Atopostipes	*suicloacalis*	+					H_2O	H_2O	$♀^{R}$; $♂^{SR}$	$♀^{R}$; $♂^{R}$
Azoarcus	*olearius*	-						H_2O	$♂^{R}$	
Bacillus	*cavernae*	+							$♀^{R}$	$♂^{R}$
	*coagulans**	+							$♂^{R}$	$♂^{S}$

	*cohnii**	+							♂^{R}	
	hisashii	+					H_2O	H_2O	♀^{R}; ♂^{R}	♀^{S}
	*infantis**	+		♂						
	infernus	+								♂^{R}
	loiseleuriae	+							♀^{R}	♀^{S}
	*siralis**	+							♂^{R}	
	*thuringiensis**	+		H_2O						
	velezensis	+							♂^{R}	
	wiedmannii	+							♂^{R}	♂^{SR}
Bacteriovorax	*stolpii*	-								♂^{R}
Bacteroides	*coagulans**	+							♀^{R}; ♂^{R}	
	faecichinchillae	-								♂^{R}
	*salyersiae**	-							♂^{R}	
	*uniformis**	-							♂^{R}	
Bdellovibrio	*bacteriovorus*	-								♂^{R}
Bellilinea	*caldifistulae*	-							♂^{R}	
Belnapia	*soli*	-								♂^{S}
Bergeyella	*porcorum*	-							♂^{R}	♂^{R}
Bifidobacterium	*pseudocatenulatum**	+							♂^{SR}	♂^{S}
Blastocatella	*fastidiosa*	-							♂^{R}	♂^{S}
Blastococcus	*aggregatus*	+							♀^{R}; ♂^{SR}	♀^{R}
Bosea	*robiniae*	-					H_2O	H_2O	♀^{R}; ♂^{R}	♀^{S}; ♂^{SR}
Brachybacterium	*faecium**	+							♀^{SR}; ♂^{R}	♀^{S}; ♂^{SR}
Bradyrhizobium	*namibiense*	-					H_2O	H_2O	♀^{R}; ♂^{R}	♀^{S}; ♂^{R}
Brevibacillus	*laterosporus**	+							♂^{SR}	♂^{R}

Brevibacterium	*casei**	+							♂R	
	*paucivorans**	+							♀R; ♂S	♀R; ♂SR
Brevundimonas	*albigilva*	-					H_2O; ♂	H_2O	♀SR; ♂SR	♀SR; ♂SR
	lenta	-							♂S	♂S
	olei	-						H_2O		
Brochothrix	*thermosphacta**	+							♂R	
Brucella	*ovis*	-					♂		♂S	♂S
Burkholderia	*ambifaria**	-							♀R; ♂R	♂SR
Caenispirillum	*bisanense*	-						♂		
Calidifontibacter	*indicus*	+								♀R; ♂SR
Cardiobacterium	*valvarum**	-							♂SR	♂S
Caryophanon	*latum*	+								♀R
Casaltella	*massiliensis*	+							♀R; ♂R	♀S
Caulobacter	*hibisci*	-					H_2O	H_2O		
	mirabilis	-						H_2O	♂R	♀R
	profundus	-					H_2O	H_2O	♀R; ♂R	
Cavicella	*subterranea*	-					H_2O			
Cedecea	*lapagei**	-		♂	♀S; ♂S	♀SR	H_2O		♀SR; ♂SR	♀SR; ♂S
	*neteri**	-			♀S; ♂S	♀SR				
Cellulosilyticum	*lentocellum*	+								♀R
Chelatococcus	*composti*	-								♂R
Chiayiivirga	*flava*	-					H_2O			
Chryseobacterium	*anthropi*	-							♂R	
	aquifrigidense	-							♀R; ♂R	♀R; ♂R

	*arachidis**	-		♂						
	arthrosphaerae	-					H_2O	♂	♀^{R}; ♂^{R}	♀^{R}; ♂^{R}
	*gleum**	-	H_2O	♀; ♂		♂^{S}				
	greenlandense	-					H_2O		♀^{R}; ♂^{SR}	♀^{SR}; ♂^{SR}
	*hominis**	-							♂^{R}	
	jeonii	-					H_2O		♂^{R}	
	profundimaris	-								♂^{S}
	reticulitermitis	-							♂^{R}	♂^{SR}
	shandongense	-							♀^{R}	
	taklimakanense	-							♀^{R}; ♂^{SR}	♀^{R}; ♂^{R}
	zeae	-					H_2O	H_2O	♀^{R}	
	zhengzhouense	-						H_2O		
Chthoniobacter	*flavus*	-								♀^{R}
Cloacibacterium	*normanense*	-					H_2O		♀^{R}; ♂^{R}	♀^{R}; ♂^{R}
Clostridium	*colinum**	+							♂^{R}	
	saccharoperbutylacetonicum	+								♂^{SR}
	saudiense	+								♀^{R}
	swellfunianum	+							♂^{R}	
Cohnella	*yongneupensis*	+						H_2O		
Comamonas	*testosteroni**	-	H_2O	H_2O						
Conexibacter	*arvalis*	+								♂^{R}
Corynebacterium	*amycolatum**	+					H_2O	H_2O	♀^{SR}; ♂^{SR}	♀^{R}; ♂^{SR}
	*appendicis**	+					H_2O	H_2O	♀^{R}; ♂^{SR}	♀^{SR}; ♂^{R}

	*aurimucosum**	+						H_2O; ♀	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
	crudilactis	+								♂^{S}
	*durum**	+							♀^{R}; ♂^{R}	♀^{R}
	*falsenii**	+						H_2O	♂^{R}	♀^{S}; ♂^{SR}
	*glaucum**	+							♂^{R}	♀^{R}
	gottingense	+					♀	H_2O	♀^{SR}; ♂^{R}	♀^{SR}
	*jeikeium**	+							♀^{R}; ♂^{R}	♀^{R}
	*kroppenstedtii**	+					♂		♀^{R}; ♂^{SR}	♀^{R}; ♂^{S}
	otitidis	+								♀^{R}
	pollutisoli	+					H_2O	H_2O	♀^{SR}; ♂^{SR}	♀^{R}; ♂^{R}
	*pseudodiphtheriticum**[2]	+				♀^{S}		H_2O; ♂	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
	*riegelii**	+						H_2O	♀^{R}; ♂^{SR}	♀^{R}; ♂^{S}
	*sundsvallense**	+							♂^{S}	♂^{S}
	*testudinoris**	+							♀^{R}	♀^{R}
	*tuberculostearicum**	+					H_2O	H_2O; ♂	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
	*ureicelerivorans**	+					H_2O	H_2O	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
Cronobacter	*sp.**	-	H_2O							
Cupriavidus	*numazuensis*	-					H_2O	H_2O		
Curtobacterium	*herbarum*	+						H_2O		
Curvibacter	*fontanus*	-							♀^{R}; ♂^{R}	♀^{R}
	gracilis	-					H_2O		♂^{R}	

Cutibacterium	*acnes*	+					H_2O	H_2O; ♀; ♂	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
Deinococcus	*aetherius*	+							♀^{R}	
	taklimakanensis	+						H_2O	♀^{R}; ♂^{SR}	♀^{SR}; ♂^{SR}
	xinjiangensis	+					H_2O	H_2O	♀^{SR}; ♂^{SR}	♀^{R}; ♂^{SR}
Delftia	*tsuruhatensis*[3]	-	H_2O				H_2O	H_2O; ♂	♀^{R}; ♂^{R}	♀^{R}; ♂^{SR}
Desemzia	*incerta*	+					H_2O			♂^{R}
Desulfobulbus	*oligotrophicus*	-							♀^{R}	
Devosia	*insulae*	-					H_2O	H_2O		
Dialister	*micraerophilus**	+							♂^{R}	
	propionicifaciens	+							♀^{R}; ♂^{R}	♂^{S}
Dolosigranulum	*pigrum*	+					H_2O	♂	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
Duganella	*zoogloeoides*	-							♀^{R}	
Dulcicalothrix	*necridiiformans*	-							♂^{R}	
Elizabethkingia	*anophelis*[4]	-	H_2O; ♀; ♂	♀; ♂	♀^{S}; ♂^{S}	♀^{S}; ♂^{S}	H_2O; ♀; ♂	H_2O; ♀; ♂	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
Empedobacter	*falsenii*	-						♂	♀^{R}; ♂^{R}	♀^{R}; ♂^{SR}
Enterobacter	*cloacae**	-		♀			H_2O; ♀; ♂	H_2O; ♀; ♂	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
	mori	-							♀^{S}	
Enterococcus	*cecorum**	+						H_2O	♀^{R}; ♂^{SR}	♀^{SR}; ♂^{R}
Eremococcus	*coleocola*	+							♂^{R}	
Escherichia	*albertii**	-	H_2O							

	fergusonii[5]	-	H_2O						♀^{R}; ♂^{R}	♂^{SR}
Ewingella	*americana**	-		♂	♂^{S}	♀^{S}; ♂^{S}				
Ezakiella	*massiliensis*	+							♀^{R}	
Facklamia	*hominis**	+							♂^{R}	♀^{R}
Faecalibacterium	*prausnitzii*	+							♂^{R}	
Ferruginibacter	*paludis*	-							♂^{R}	
Fibrella	*aestuarina*	-					H_2O	H_2O		
Finegoldia	*magna**	+						H_2O	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{R}
Flavobacterium	*granuli*	-							♀^{R}	
	*johnsoniae**	-	H_2O							
	*pectinovorum**	-	H_2O							
	zhairuonensis	-							♂^{R}	♀^{R}
Flectobacillus	*roseus*	-								♂^{S}
Fontimonas	*thermophila*	-								♀^{R}
Frateuria	*aurantia*	-							♀^{R}	♀^{S}
Fretibacterium	*fastidiosum*	-							♀^{R}	
Fusobacterium	*nucleatum**	-							♀^{R}; ♂^{R}	♂^{S}
Gallicola	*barnesae*	+								♂^{S}
Gardnerella	*vaginalis**	+							♀^{R}	
Gellertiella	*hungarica*	-								♂^{S}
Gemella	*taiwanensis*	+						♂	♀^{R}; ♂^{SR}	♀^{R}; ♂^{S}
Geminicoccus	*roseus*	-							♀^{R}; ♂^{R}	
Gemmata	*obscuriglobus*	-						H_2O		
Gemmatimonas	*aurantiaca*	-							♂^{R}	
Gemmatirosa	*kalamazoonesis*	-								♀^{S}

Geobacillus	*stearothermophilus**	+						H_2O	$♀^{R}$; $♂^{R}$	$♂^{R}$
Georgenia	*deserti*	+						H_2O	$♂^{R}$	$♀^{S}$; $♂^{SR}$
Gilliamella	*apicola*	-							$♂^{S}$	$♂^{R}$
Gluconobacter	*frateurii*	-					♀; ♂	♀; ♂	$♀^{SR}$; $♂^{SR}$	$♀^{SR}$; $♂^{SR}$
	japonicus	-					♀		$♀^{S}$; $♂^{S}$	$♀^{S}$
Gordonia	*otitidis*	+							$♀^{R}$; $♂^{R}$	$♀^{R}$
Gorillibacterium	*massiliense*	+						H_2O		
Granulicatella	*adiacens**	+					H_2O		$♀^{R}$; $♂^{SR}$	$♀^{SR}$; $♂^{SR}$
	*elegans**	+							$♀^{SR}$; $♂^{R}$	$♀^{R}$
Haematobacter	*massiliensis*	-							$♀^{R}$	
Haematospirillum	*jordaniae*	-						H_2O		
Haemophilus	*haemolyticus**	-							$♀^{R}$; $♂^{SR}$	$♀^{SR}$; $♂^{R}$
	*parahaemolyticus**	-							$♀^{R}$	$♂^{R}$
	*parainfluenzae**	-					H_2O		$♀^{R}$; $♂^{SR}$	$♀^{SR}$; $♂^{SR}$
Hafnia	*psychrotolerans*	-					♀	♀	$♀^{S}$; $♂^{S}$	$♀^{S}$
Hartmannibacter	*diazotrophicus*	-							$♂^{R}$	
Helicobacter	*pylori**	-							$♂^{R}$	
Heliimonas	*saccharivorans*	-						H_2O		
Herbaspirillum	*aquaticum**	-		H_2O						
	*huttiense**	-	♀	H_2O; ♂			H_2O	H_2O; ♂	$♀^{SR}$; $♂^{SR}$	$♀^{SR}$; $♂^{SR}$
Hydrogenophaga	*carboriunda*	-								$♀^{R}$

Iamia	*majanohamensis*	+							♂^{R}	
Ilumatobacter	*fluminis*	+							♂^{R}	♀^{R}
Ilyomonas	*limi*	-								♀^{S}
Intestinibacter	*bartlettii*	+							♂^{S}	
Intestinimonas	*butyriciproducens*	+								♂^{R}
Isoptericola	*cucumis*	+							♀^{R}	♂^{S}
Jeotgalicoccus	*schoeneichii*	+							♂^{R}	
Kaistia	*terrae*	-					H_2O	H_2O; ♀	♀^{S}; ♂^{SR}	♀^{R}; ♂^{S}
Kineococcus	*xinjiangensis*	+								♀^{R}
Kineosporia	*rhizophila*	+								♀^{S}
Kineothrix	*alysoides*	+							♂^{R}	♀^{R}
Kingella	*oralis**	-							♂^{R}	
Klebsiella	*variicola**	-	♀; ♂	H_2O; ♀; ♂	♂^{S}					
Kocuria	*palustris**	+					H_2O	H_2O	♀^{R}; ♂^{S}	♀^{SR}
Krasilnikovia	*cinnamomea*	+							♀^{R}	
Krasilnikoviella	*muralis*	+							♂^{R}	♂^{S}
Kytococcus	*sedentarius**	+								♀^{R}
Laceyella	*sacchari*	+							♂^{S}	
Lachnoclostridium	*pacaense*	+							♂^{R}	♀^{R}
Lacibacterium	*aquatile*	-							♀^{R}	♀^{R}
Lacisediminimonas	*profundi*	-					H_2O			♀^{R}
Lactobacillus	*acetotolerans*	+								♂^{R}
	aquaticus	+							♀^{R}; ♂^{R}	♂^{R}
	*coryniformis**	+						♂		

	*crispatus**	+						♂	♀^{R}; ♂^{SR}	♀^{SR}; ♂^{SR}
	*iners**	+								♀^{S}
	*ingluviei**	+								♀^{R}
	mellis	+								♂^{R}
	*panis**	+							♀^{R}	♂^{R}
	rodentium	+								♂^{R}
	silagincola	+							♀^{R}	♂^{R}
Lactococcus	*lactis**	+							♀^{R}; ♂^{R}	♀^{R}; ♂^{S}
Lapillicoccus	*jejuensis*	+							♀^{R}	♀^{R}; ♂^{S}
Larkinella	*arboricola*	-							♀^{R}	
Lautropia	*mirabilis**	-							♂^{R}	
Lawsonella	*clevelandensis*	+					H_2O	H_2O	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
Legionella	*cardiaca*	-							♂^{R}	
	nautarum	-						H_2O		
Leifsonia	*rubra*	+							♀^{S}; ♂^{SR}	
Leminorella	*grimontii**	-								♂^{R}
Leptotrichia	*buccalis*	-					H_2O	H_2O	♀^{R}; ♂^{SR}	♀^{SR}; ♂^{SR}
	*goodfellowii**	-								♂^{S}
	shahii	-							♂^{SR}	♀^{R}
Leucobacter	*iarius*	+					H_2O; ♂	H_2O; ♂	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
Leuconostoc	*mesenteroides**	+							♂^{R}	♀^{S}

Levyella	*massiliensis*	+								♂^{R}
Longimicrobium	*terrae*	-								♀^{R}
Luteolibacter	*flavescens*	-						H_2O		
Lysobacter	*oculi*	-							♀^{R}; ♂^{R}	♂^{SR}
Lyticum	*flagellatum*	-							♂^{R}	
Marinoscillum	*luteum*	-							♂^{R}	
Marmoricola	*scoriae*	+								♂^{S}
Massilia	*aurea*	-							♂^{R}	
	suwonensis	-						♂	♀^{R}; ♂^{R}	♀^{SR}; ♂^{SR}
Mediterranea	*massiliensis*	-								♀^{R}
Megamonas	*funiformis*	+								♀^{S}; ♂^{S}
Methylobacterium	*bullatum*	-							♂^{R}	♀^{R}; ♂^{S}
	phyllostachyos	-					♂	H_2O	♀^{SR}; ♂^{SR}	♀^{R}; ♂^{R}
Methyloceanibacter	*caenitepidi*	-								♂^{R}
Methylorubrum	*populi*	-							♂^{SR}	♀^{S}
Methyloversatilis	*discipulorum*	-							♂^{R}	
Microbacter	*margulisiae*	-							♂^{R}	
Microbacterium	*azadirachtae*	+						H_2O		
	*laevaniformans**	+	H_2O							
	*maritypicum**	+					H_2O; ♂	H_2O; ♀	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
Microbulbifer	*maritimus*	-					♀			
Micrococcus	*luteus**	+					H_2O		♀^{R}; ♂^{SR}	♀^{SR}; ♂^{SR}

Microlunatus	*lacustris*	+							♀^{R}	
Microvirga	*brassicacearum*	-							♀^{S}; ♂^{S}	♀^{S}; ♂^{SR}
	subterranea	-							♀^{R}	
Mixta	*calida*	-							♂^{R}	
Moheibacter	*stercoris*	-							♀^{R}	
Moraxella	*nonliquefaciens**	-								♀^{R}
	*osloensis**	-					H_2O	H_2O	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
	porci	-								♂^{R}
Morganella	*morganii**	-							♀^{R}	♀^{S}
Mucilaginibacter	*carri*	-					H_2O	H_2O	♂^{R}	
	terrigena	-					H_2O			
Murdochiella	*massiliensis*	+								♂^{S}
Muribaculum	*intestinale*	-								♂^{R}
Mycobacterium	*florentinum*	+							♀^{R}	♂^{S}
	*gordonae**	+							♀^{R}	
Mycolicibacterium	*doricum*	+							♂^{R}	
	iranicum	+							♀^{R}; ♂^{R}	♀^{R}
Nakamurella	*flavida*	+								♀^{R}
Neisseria	*elongata**	-							♂^{R}	
	*mucosa**	-					H_2O	H_2O	♀^{R}; ♂^{SR}	♀^{SR}; ♂^{S}
Nitratireductor	*aestuarii*	-							♀^{R}	
Nocardia	*coeliaca*	+							♀^{R}; ♂^{SR}	♂^{R}
Nocardioides	*caricicola*	+							♂^{R}	
	glacieisoli	+							♂^{S}	♀^{R}; ♂^{R}

	halotolerans	+								♂^{R}
	iriomotensis	+							♀^{R}; ♂^{R}	♀^{R}; ♂^{SR}
	islandensis	+								♂^{R}
	solisilvae	+								♀^{S}
Nordella	*oligomobilis*	-					H_2O	H_2O		♂^{R}
Novosphingobium	*arabidopsis*	-							♂^{R}	
	*nitrogenifigens**	-					H_2O	H_2O	♀^{R}	
	sediminicola	-					H_2O			
Nubsella	*zeaxanthinifaciens*	-					H_2O			
Oblitimonas	*alkaliphila*	-								♂^{R}
Olegusella	*massiliensis*	+								♂^{S}
Oribacterium	*sinus*	+							♀^{R}	♀^{R}
Paenibacillus	*cavernae*	+						H_2O		
	psychroresistens	+							♀^{R}	
	silvae	+							♀^{R}; ♂^{SR}	♀^{S}; ♂^{S}
Paludibaculum	*fermentans*	-							♀^{R}	♂^{S}
Pantoea	*vagans*	-								♂^{R}
Paracoccus	*aerius*	-					H_2O; ♂	H_2O; ♂	♀^{SR}; ♂^{SR}	♀^{R}; ♂^{SR}
	hibiscisoli	-					H_2O	H_2O	♀^{SR}; ♂^{R}	♀^{SR}; ♂^{SR}
Parvimonas	*micra**	+								♀^{R}
Parviterribacter	*multiflagellatus*	+								♀^{R}
Pedobacter	*lotistagni*	-						H_2O		
	solisilvae	-					H_2O; ♀	H_2O; ♂	♂^{SR}	♀^{R}; ♂^{R}
	terrae	-						H_2O		

	trunci	-						H_2O	$♀^{R}$	$♀^{S}$
Pedomicrobium	*ferrugineum*	-								$♀^{R}$
Pelomonas	*saccharophila**	-					H_2O	H_2O	$♀^{R}$; $♂^{S}$	$♀^{S}$; $♂^{S}$
Peptococcus	*niger**	+								$♂^{R}$
Peptoniphilus	*asaccharolyticus**	+							$♂^{R}$	$♂^{R}$
	*coxii**	+								$♀^{R}$; $♂^{SR}$
	lacydonensis	+							$♀^{R}$; $♂^{SR}$	$♀^{R}$; $♂^{SR}$
Peptostreptococcus	*stomatis*	+							$♀^{R}$	$♀^{R}$
Peredibacter	*starrii*	-								$♂^{S}$
Phascolarctobacterium	*succinatutens*	+							$♂^{R}$	
Phenylobacterium	*aquaticum*	-							$♀^{SR}$; $♂^{SR}$	$♀^{R}$; $♂^{S}$
	muchangponense	-					H_2O	H_2O		$♂^{R}$
Phreatobacter	*oligotrophus*	-					H_2O	H_2O	$♂^{R}$	$♀^{R}$
Phyllobacterium	*endophyticum*	-							$♂^{S}$	$♀^{R}$; $♂^{S}$
	myrsinacearum	-					H_2O; $♂$	H_2O; $♀$; $♂$	$♀^{SR}$; $♂^{SR}$	$♀^{SR}$; $♂^{SR}$
Phytobacter	*ursingii*	-							$♀^{S}$	
Pleomorphomonas	*carboxyditropha*	-							$♂^{R}$	
Polynucleobacter	*sinensis*	-					H_2O	H_2O	$♂^{R}$	
Pontibacter	*humi*	-							$♂^{R}$	
Porphyromonas	*bennonis**	-							$♀^{R}$	
	*catoniae**	-								$♀^{R}$
	pasteri	-								$♂^{S}$
Prevotella	*bivia**	-							$♀^{SR}$; $♂^{R}$	$♀^{S}$
	*buccalis**	-							$♀^{R}$; $♂^{R}$	

	colorans	-							♀^{R}	
	*corporis**	-								♂^{R}
	jejuni	-							♀^{R}; ♂^{R}	
	*melaninogenica**	-							♀^{R}	♂^{S}
	*nanceiensis**	-							♀^{R}	♂^{S}
	*nigrescens**	-							♀^{R}	
	*shahii**	-								♂^{S}
	*stercorea**	-								♂^{S}
	*timonensis**	-							♀^{R}; ♂^{S}	♀^{R}
Proteiniphilum	*acetatigenes*	-						H_2O	♀^{R}; ♂^{R}	
Providencia	*vermicola**	-								♀^{R}; ♂^{R}
Pseudacidovorax	*intermedius**	-					H_2O	H_2O		
Pseudomonas	*aeruginosa**	-					H_2O	H_2O; ♂	♀^{SR}; ♂^{SR}	♀^{R}; ♂^{SR}
	*antarctica**	-	♂							
	*azotoformans**	-	♀							
	canadensis	-					♀; ♂			
	chengduensis	-							♂^{S}	♀^{S}
	deceptionensis	-					H_2O		♀^{R}; ♂^{R}	♂^{R}
	*extremorientalis**	-	♂			♀^{S}				
	geniculata	-					H_2O; ♀; ♂	H_2O; ♀; ♂	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
	*gessardii**	-				♂^{S}				
	*grimontii**	-	♂	♀			♀; ♂	♀	♀^{S}; ♂^{S}	♀^{S}; ♂^{S}
	lactis	-					H_2O	♂	♀^{R}; ♂^{SR}	♀^{SR}; ♂^{SR}

	*libanensis**	-	♀; ♂	♀; ♂		♂^{S}				
	*marginalis**	-					♂		♂^{S}	♀^{S}
	*orientalis**	-								♀^{S}
	*otitidis**	-	H_2O	H_2O; ♀; ♂						
	palleroniana	-					♀			
	*putida**	-	H_2O	♀; ♂						
	*savastanoi**	-					♀; ♂	♀	♀^{S}; ♂^{S}	♀^{S}
	soli	-								♂^{S}
	*stutzeri**	-							♀^{R}; ♂^{R}	♂^{SR}
	tolaasii	-					♀			
	*veronii**	-					H_2O; ♀; ♂	H_2O; ♀; ♂	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
	xiamenensis	-								♂^{R}
Pseudonocardia	*oroxyli*	+								♂^{S}
	yuanmonensis	+						H_2O	♂^{R}	♀^{R}; ♂^{SR}
Pseudoxanthobacter	*soli*	-					H_2O	H_2O		
Pseudoxanthomonas	*taiwanensis*	-							♂^{R}	♂^{R}
Psychrobacter	*aquimaris*	-							♀^{R}	
	cibarius	-							♀^{R}	♀^{R}; ♂^{R}
	faecalis	-					H_2O; ♀	H_2O; ♂	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
	*sp.**	-	♀		♂^{R}					
Rahnella	*aquatilis**	-	♀; ♂	♀	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}				

Ralstonia	*insidiosa**	-							♀^{R}	
Raoultella	*ornithinolytica*[6]*	-	♀; ♂	♀; ♂	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}	♀	♀; ♂	♂^{S}	
Reyranella	*aquatilis*	-					H_2O; ♂	H_2O	♂^{R}	
Rhizobium	*arenae*	-					H_2O		♀^{R}; ♂^{R}	
	azooxidifex	-					H_2O	H_2O	♂^{R}	♂^{R}
	daejeonense	-								♂^{S}
	endophyticum	-					H_2O	H_2O; ♂	♀^{R}; ♂^{R}	♀^{R}; ♂^{S}
	zeae	-							♂^{R}	♂^{S}
Rhodobacter	*blasticus*	-					H_2O			
Rhodococcus	*pedocola*	+								♀^{R}
	sovatensis	+					H_2O	H_2O	♀^{R}; ♂^{SR}	♀^{SR}; ♂^{SR}
Rhodoferax	*antarcticus*	-							♂^{S}	♂^{S}
Rhodovulum	*kholense*	-							♂^{R}	♂^{S}
Robbsia	*andropogonis*	-						♂		♀^{S}; ♂^{S}
Romboutsia	*timonensis*	+					H_2O		♀^{R}; ♂^{R}	♀^{R}; ♂^{S}
Rosenbergiella	*epipactidis*	-								♂^{S}
Roseomonas	*aquatica*	-								♂^{R}
	gilardii	-							♀^{R}; ♂^{R}	♀^{R}; ♂^{S}
Rothia	*dentocariosa**	+					H_2O	H_2O; ♀	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
	*mucilaginosa**	+					H_2O		♀^{SR}; ♂^{SR}	♀^{R}; ♂^{SR}
Rubellimicrobium	*rubrum*	-								♂^{R}
Rubrobacter	*spartanus*	+								♀^{R}

Ruminococcus	*champanellensis*	+								♂^{R}
	*gnavus**	+						H_2O	♀^{R}	♀^{S}
	torques	+							♂^{R}	
Rurimicrobium	*arvi*	-					H_2O	H_2O		
Saccharothrix	*ghardaiensis*	+							♀^{R}	♀^{R}
Sandaracinus	*amylolyticus*	-								♂^{R}
Schaalia	*odontolytica*	+						H_2O	♀^{R}; ♂^{R}	♂^{S}
Schlegelella	*aquatica*	-								♂^{R}
Serratia	*liquefaciens**	-							♀^{S}; ♂^{S}	♂^{S}
	*marcescens**	-					H_2O	♂	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
	oryzae [7]	-	H_2O; ♀; ♂	♀; ♂	♂^{S}	♀^{SR}; ♂^{SR}	H_2O; ♀; ♂	H_2O; ♀; ♂	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
Shinella	*curvata*	-							♀^{R}; ♂^{R}	♀^{R}; ♂^{SR}
Simplicispira	*metamorpha*	-					H_2O			
Singulisphaera	*acidiphila*	-							♀^{R}	
Siphonobacter	*aquaeclarae*	-					H_2O; ♂			
Snodgrassella	*alvi*	-							♀^{R}; ♂^{SR}	♀^{R}; ♂^{R}
Solobacterium	*moorei**	+								♀^{R}
Sphaerobacter	*thermophilus*	-							♂^{R}	♀^{SR}; ♂^{R}
Sphingobacterium	*ginsenosidimutans*	-					H_2O			
	*multivorum**	-	H_2O; ♂				H_2O; ♀; ♂	H_2O	♀^{R}	
Sphingobium	*rhizovicinum*	-							♂^{S}	
Sphingomonas	*fennica*	-								♂^{S}
	kyeonggiensis	-					H_2O	H_2O	♀^{R}; ♂^{R}	♀^{R}; ♂^{S}

	olei	-							♀^{R}; ♂^{R}	♀^{S}; ♂^{S}
	palustris	-							♀^{R}; ♂^{S}	♂^{R}
	rhizophila	-								♂^{R}
Sphingorhabdus	*rigui*	-								♂^{R}
Spirosoma	*horti*	-								♀^{R}
Staphylococcus	*aureus**	+	♂							
	*capitis**	+		♀; ♂	♂^{S}	♂^{R}				
	*caprae**	+	H_2O; ♀; ♂							
	*epidermidis**	+	H_2O; ♀; ♂	H_2O; ♀; ♂	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}	H_2O; ♀; ♂	H_2O; ♀; ♂	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
	*haemolyticus**	+	♀	♂						
	*hominis**	+	H_2O; ♂	♀; ♂	♀^{SR}; ♂^{SR}	♂^{SR}				
	*pasteuri**	+	♂							
	*sciuri**	+							♂^{R}	
Stenotrophobacter	*terrae*	-								♂^{S}
Stenotrophomonas	*maltophilia**	-		♀						♀^{S}
	pavanii	-								♀^{S}
	*rhizophila**	-						♂	♀^{R}; ♂^{R}	♀^{R}; ♂^{SR}
Streptacidiphilus	*anmyonensis*	+							♂^{R}	
Streptococcus	*agalactiae**	+							♂^{R}	♀^{S}; ♂^{R}
	periodonticum	+					H_2O; ♂	H_2O	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
	*sinensis**	+								♂^{R}
	thermophilus	+					H_2O; ♂	H_2O; ♂	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}

Streptomyces	*asenjonii*	+							♀^{R}	
	xanthophaeus	+							♀^{R}; ♂^{R}	♂^{S}
Streptosporangium	*jiaoheense*	+								♀^{R}
Taonella	*mepensis*	-						H_2O		
Tepidimonas	*fonticaldi*	-							♂^{R}	♀^{R}; ♂^{R}
Tetragenococcus	*halophilus*	+							♂^{R}	
Thermicanus	*aegyptius*	+						H_2O	♀^{R}; ♂^{S}	♂^{R}
Thermoanaerobaculum	*aquaticum*	-								♂^{S}
Thermomicrobium	*carboxidum*	-								♀^{R}
Thiohalocapsa	*halophila*	-							♂^{R}	
Tidjanibacter	*massiliensis*	-							♂^{R}	
Undibacterium	*squillarum*	-						H_2O	♀^{R}; ♂^{R}	
Variovorax	*boronicumulans*	-							♀^{R}	♂^{R}
	*paradoxus**	-						H_2O	H_2O	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
Veillonella	*dispar**	+						H_2O		♀^{R}; ♂^{SR}	♀^{SR}; ♂^{SR}
	*montpellierensis**	+							H_2O	♀^{SR}; ♂^{R}	♀^{R}
Vibrionimonas	*magnilacihabitans*	-						H_2O		♂^{R}	
Vicinamibacter	*silvestris*	-									♀^{R}
Vogesella	*mureinivorans*	-									♂^{S}
Yersinia	*aldovae**	-						H_2O; ♀; ♂	H_2O; ♀; ♂	♀^{SR}; ♂^{SR}	♀^{SR}; ♂^{SR}
	*enterocolitica**	-						♀; ♂	♀; ♂	♀^{SR}; ♂^{S}	♀^{SR}; ♂^{S}
	entomophaga	-						♀; ♂		♀^{S}; ♂^{S}	♀^{S}

	wautersii	-					♀; ♂		$♀^{R}$	
Zhizhongheella	*caldifontis*	-							$♂^{R}$	$♂^{S}$
Zoogloea	*resiniphila*	-								$♀^{R}$; $♂^{R}$

Calculations: Diversity, Relative Abundance and Evenness Indices **APPENDIX C**

The Shannon-Wiener diversity index (H) is calculated as follows:

$$H = \sum_{i=1}^{s} -(p_i . \ln p_i)$$

where p_i is the fraction of the entire population made up of species i, s is the number of species encountered, and $\sum$ is the sum of the calculations from species 1 to species s.

The reciprocal of Simpson's index ($\frac{1}{D}$) is calculated as follows:

$$\frac{1}{D} = \frac{1}{\sum_{i=1}^{s} {p_i}^2}$$

where p_i is the fraction of the entire population made up of species i, s is the number of species encountered, and $\sum$ is the sum of the calculations from species 1 to species s.

Pielou's evenness index (E) is calculated as follows:

$$E = \frac{H'}{H_{max}}$$

Here, H' is the Shannon-Wiener diversity value and H_{max} is $\ln(s)$, where s is the total number of species encountered.

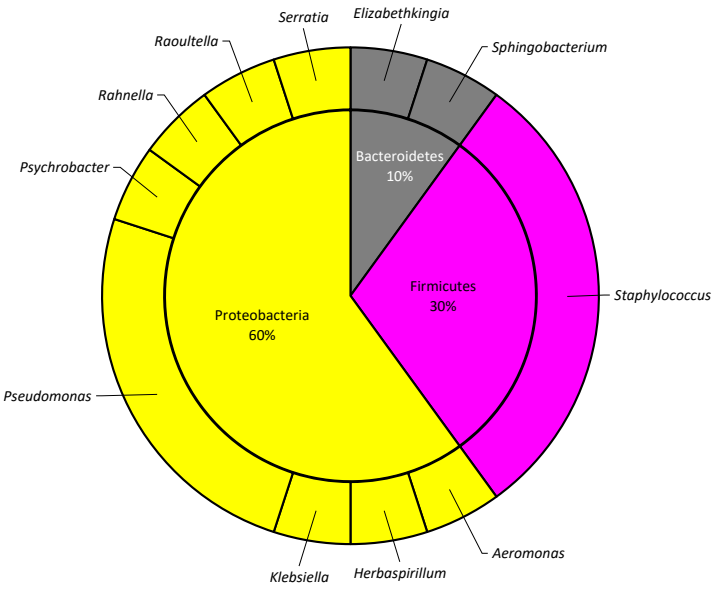

Figure D1: Bacterial phyla and genera identified by culture-dependent techniques from the midguts of colonised *An. arabiensis*.

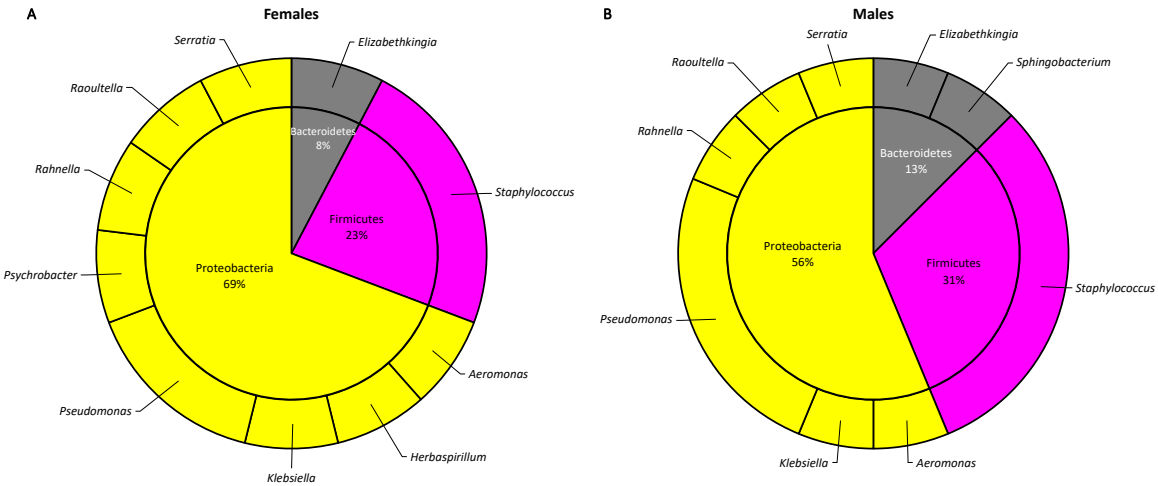

Figure D2: Phyla and genera identified by culture-dependent techniques from colonised (A) female and (B) male *An. arabiensis*.

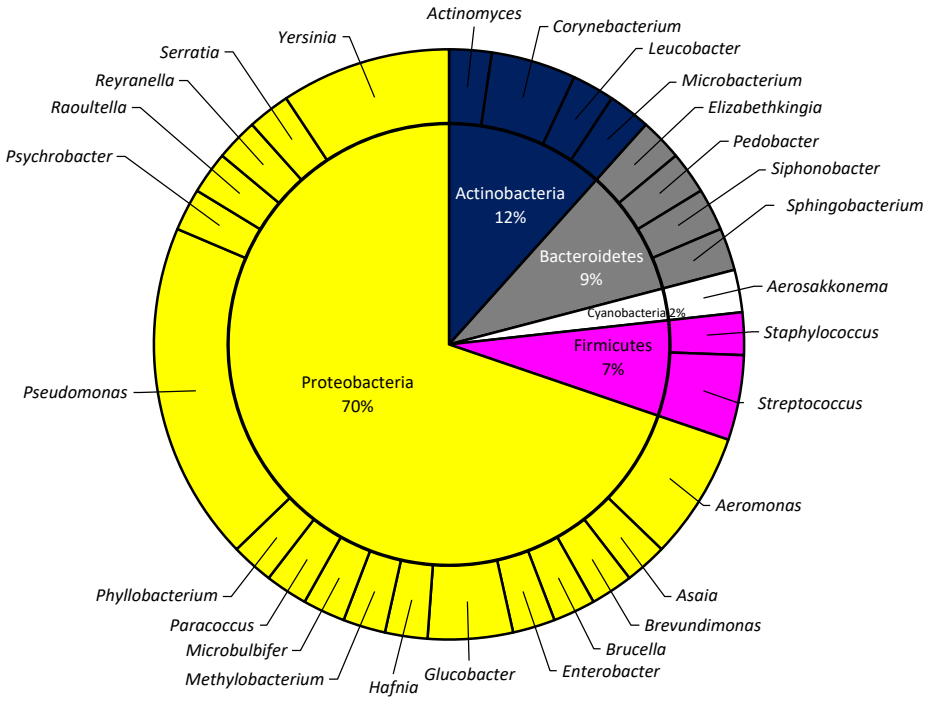

Figure D3: Bacterial phyla and genera identified by culture-independent techniques from midguts of colonised *An. arabiensis*.

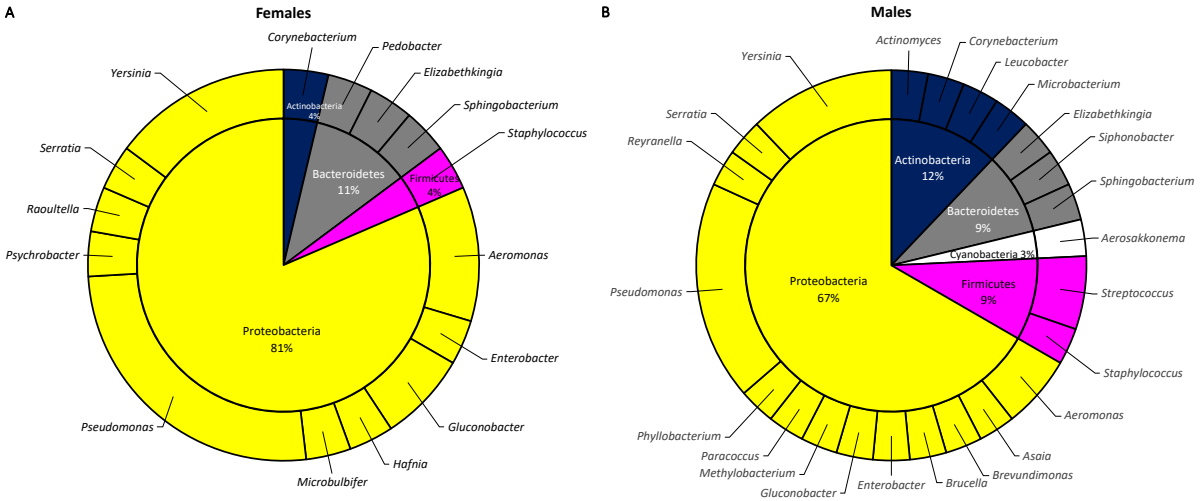

Figure D4: Bacterial phyla and genera identified by culture-independent techniques from colonised (A) female and (B) male *An. arabiensis*.

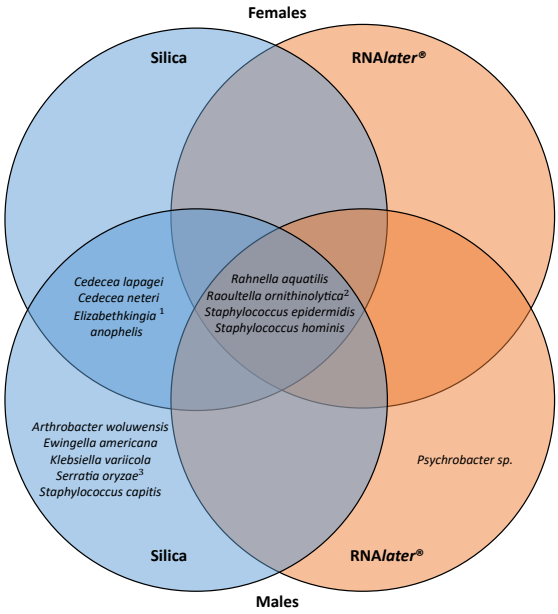

Figure E1: Bacteria identified by culture-dependent techniques from preserved female and male *An. arabiensis*. Mosquitoes were preserved on silica or in RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*E. anophelis*, *E. meningoseptica* and *E. miricola*; [2]*K. oxytoca*, *R. ornithinolytica*, *R. planticola* and *R. terrigena*; and [3]*S. fonticola* and *S. oryzae*.

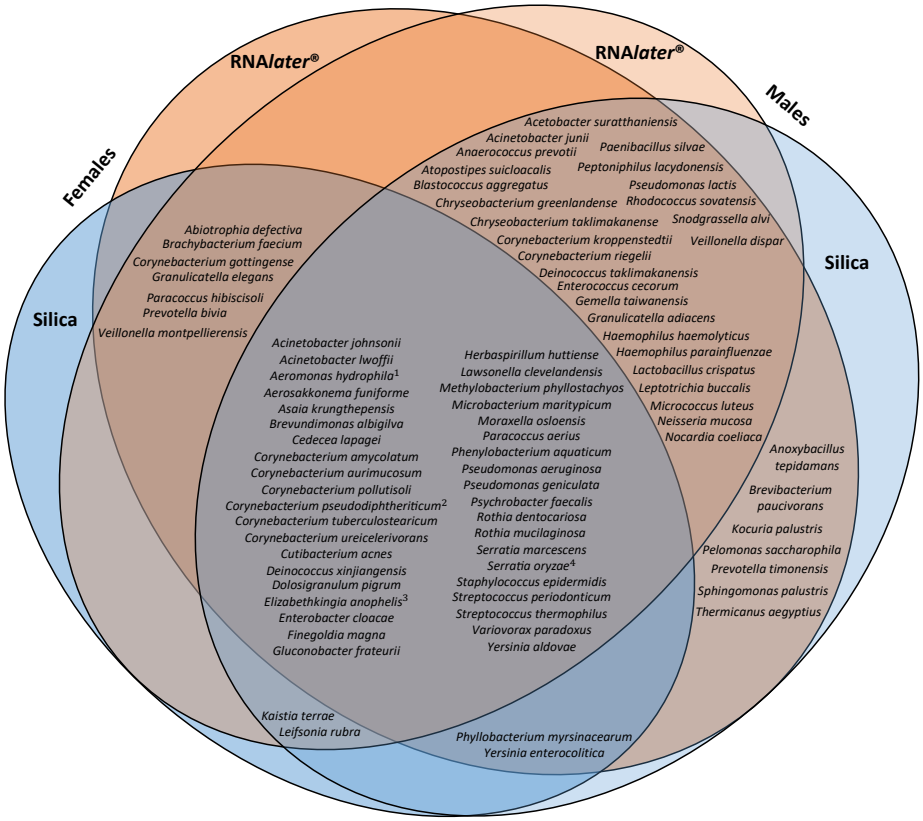

Figure E2: Bacteria identified by culture-independent techniques from preserved female and male *An. arabiensis*. Mosquitoes were preserved on silica or in RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; and [4]*S. fonticola* and *S. oryzae.*

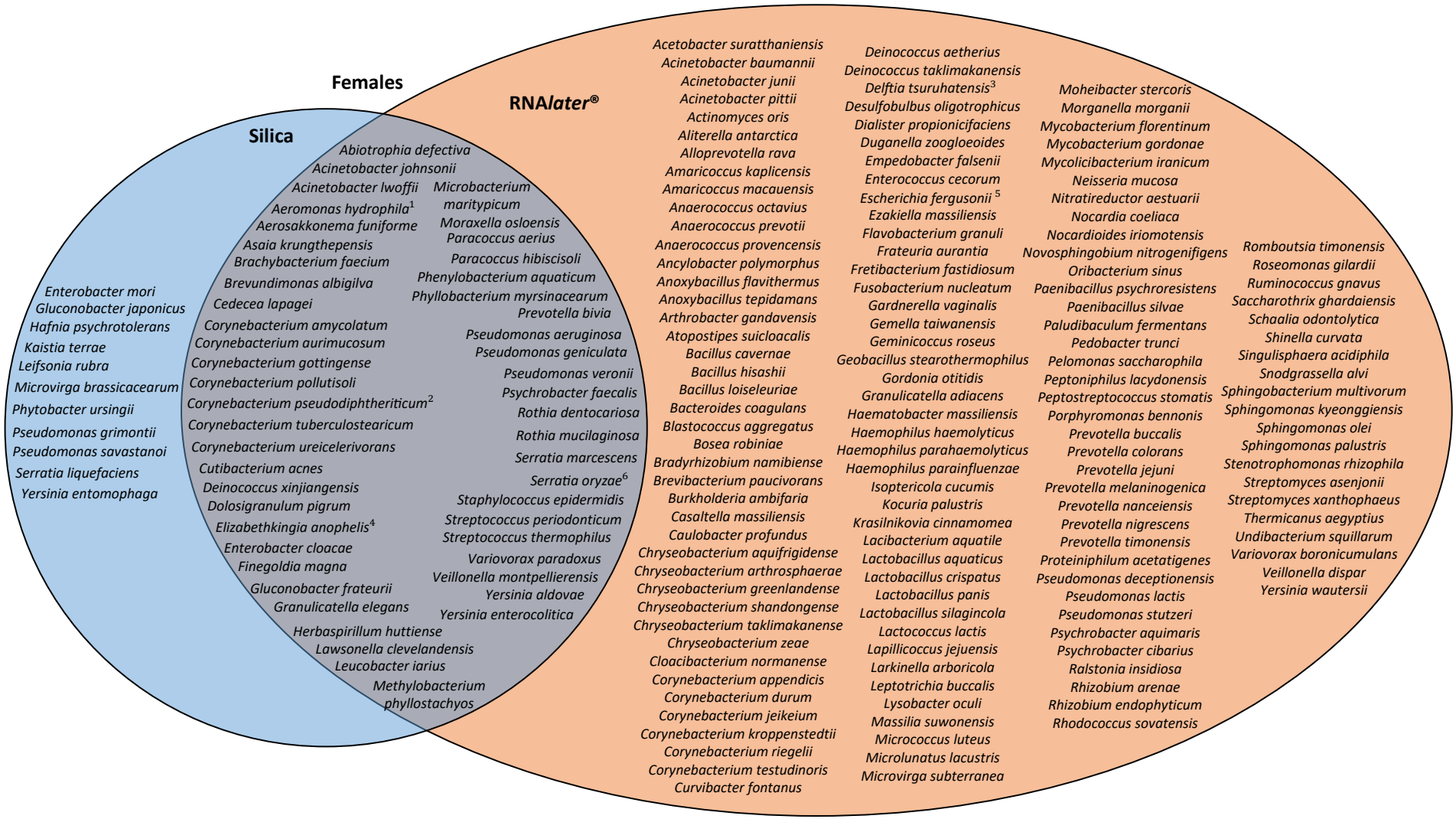

Figure E3: Bacteria identified by culture-independent techniques from preserved female *An. arabiensis*. Mosquitoes were preserved on silica or in RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*D. acidovorans* and *D. tsuruhatensis*; [4]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [5]*E. coli* and *E. fergusonii*; and [6]*S. fonticola* and *S. oryzae*.

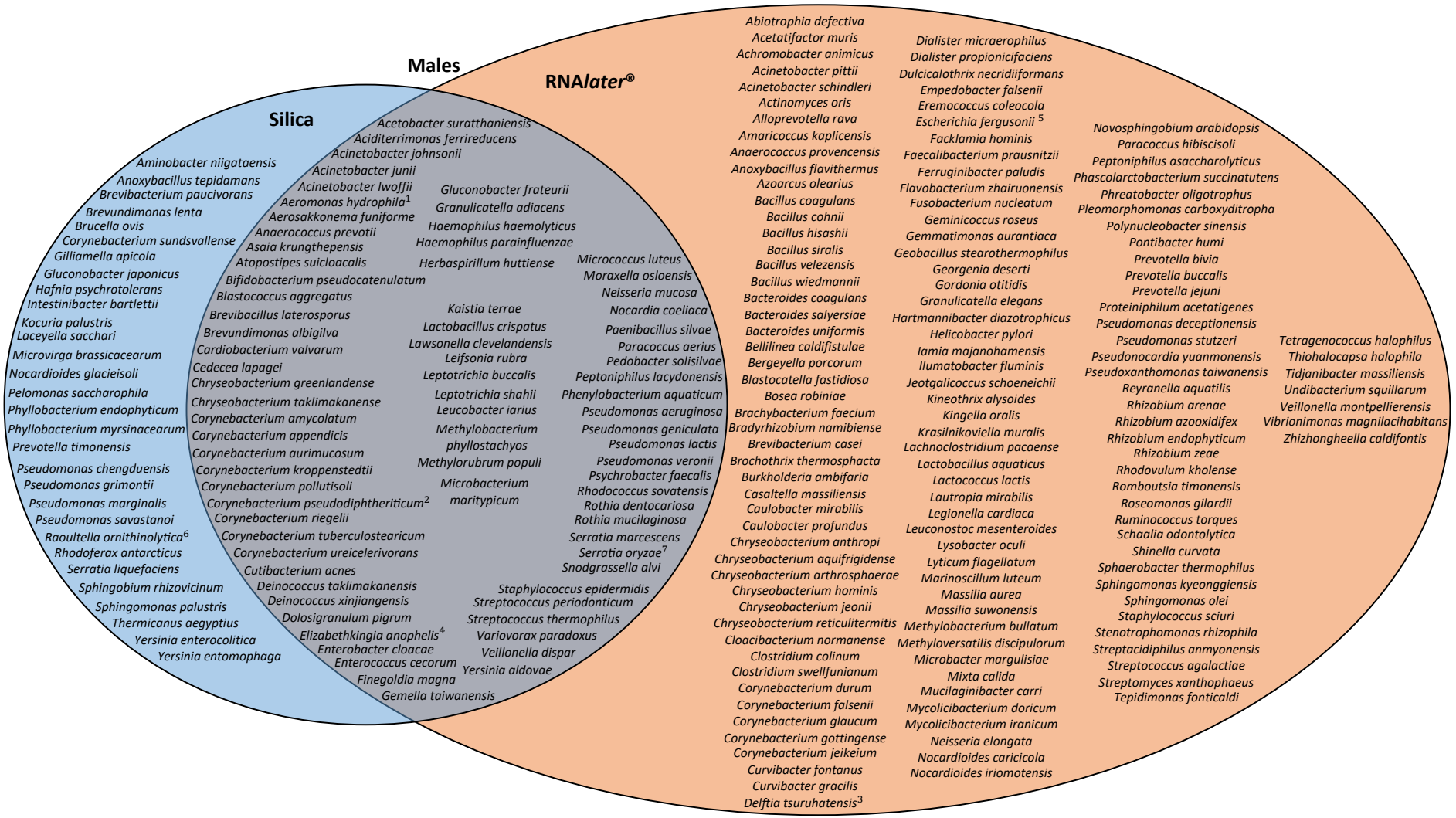

Figure E4: Bacteria identified by culture-independent techniques from preserved male *An. arabiensis*. Mosquitoes were preserved on silica or in RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*D. acidovorans* and *D. tsuruhatensis*; [4]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [5]*E. coli* and *E. fergusonii*; [6]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [7]*S. fonticola* and *S. oryzae*.

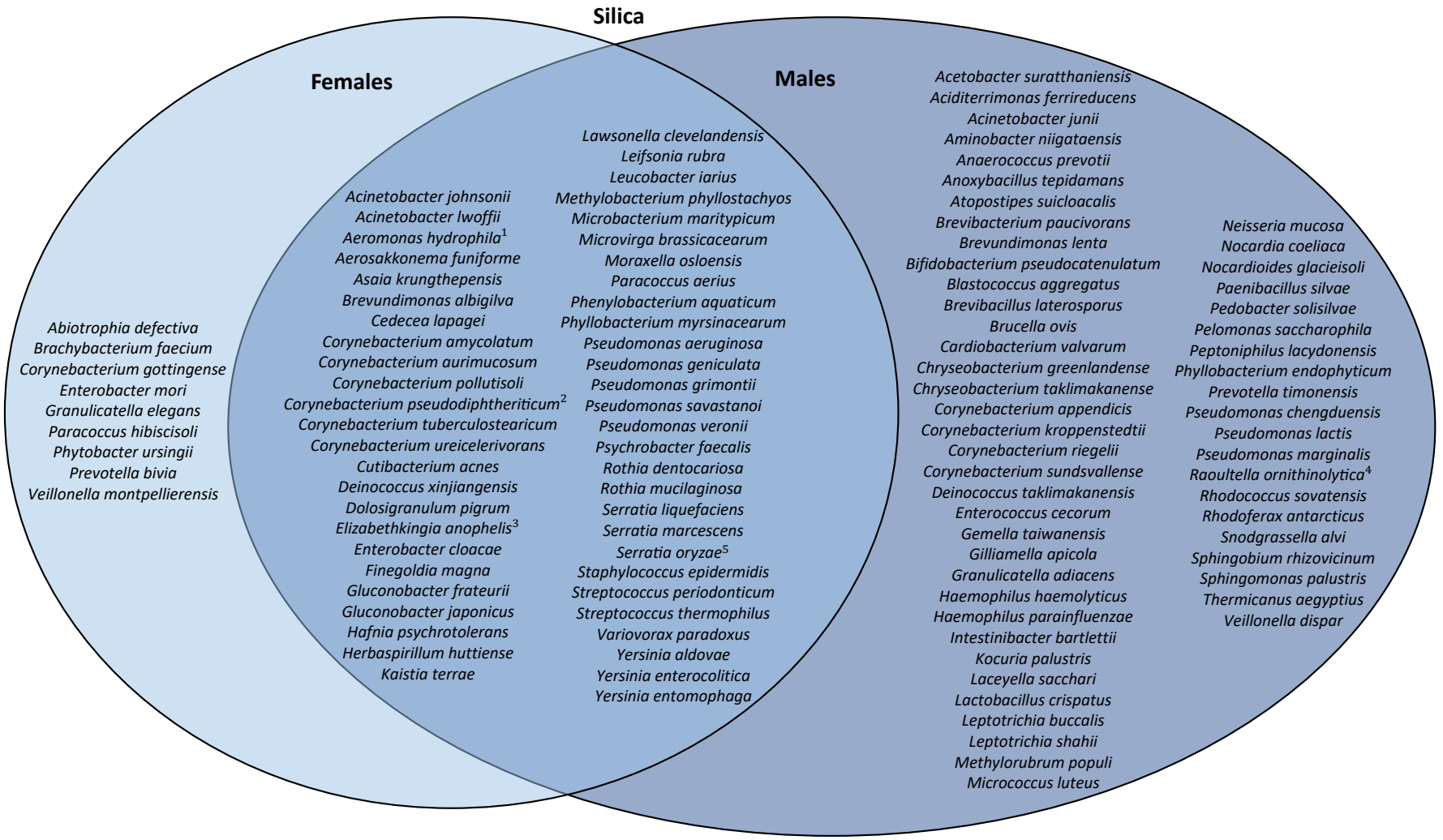

Figure E5: Bacteria identified by culture-independent techniques from silica-preserved *An. arabiensis*. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [4]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [5]*S. fonticola* and *S. oryzae*.

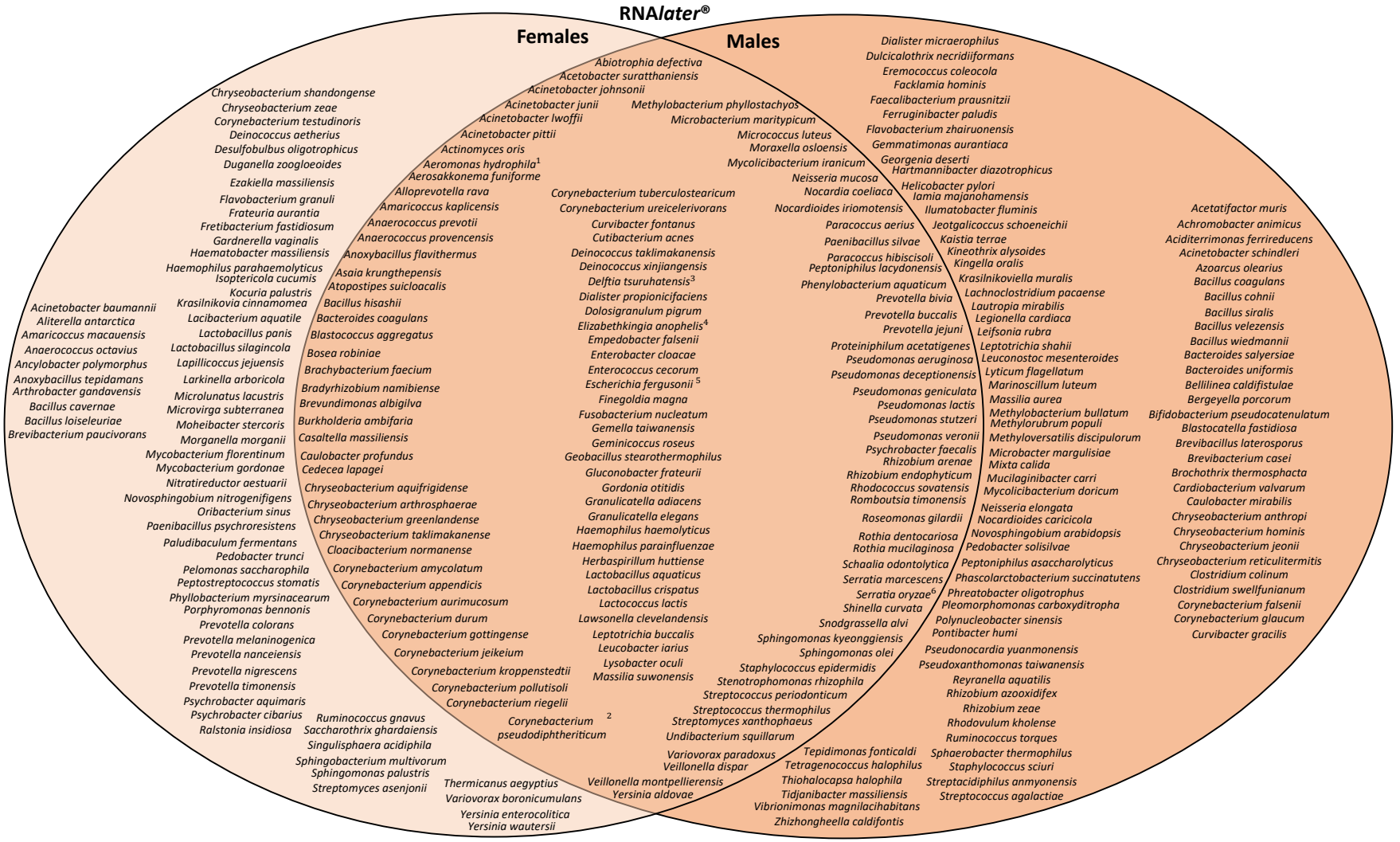

Figure E6: Bacteria identified by culture-independent techniques from RNA*later*®-preserved *An. arabiensis.* Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*D. acidovorans* and *D. tsuruhatensis*; [4]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [5]*E. coli* and *E. fergusonii*; and [6]*S. fonticola* and *S. oryzae*.

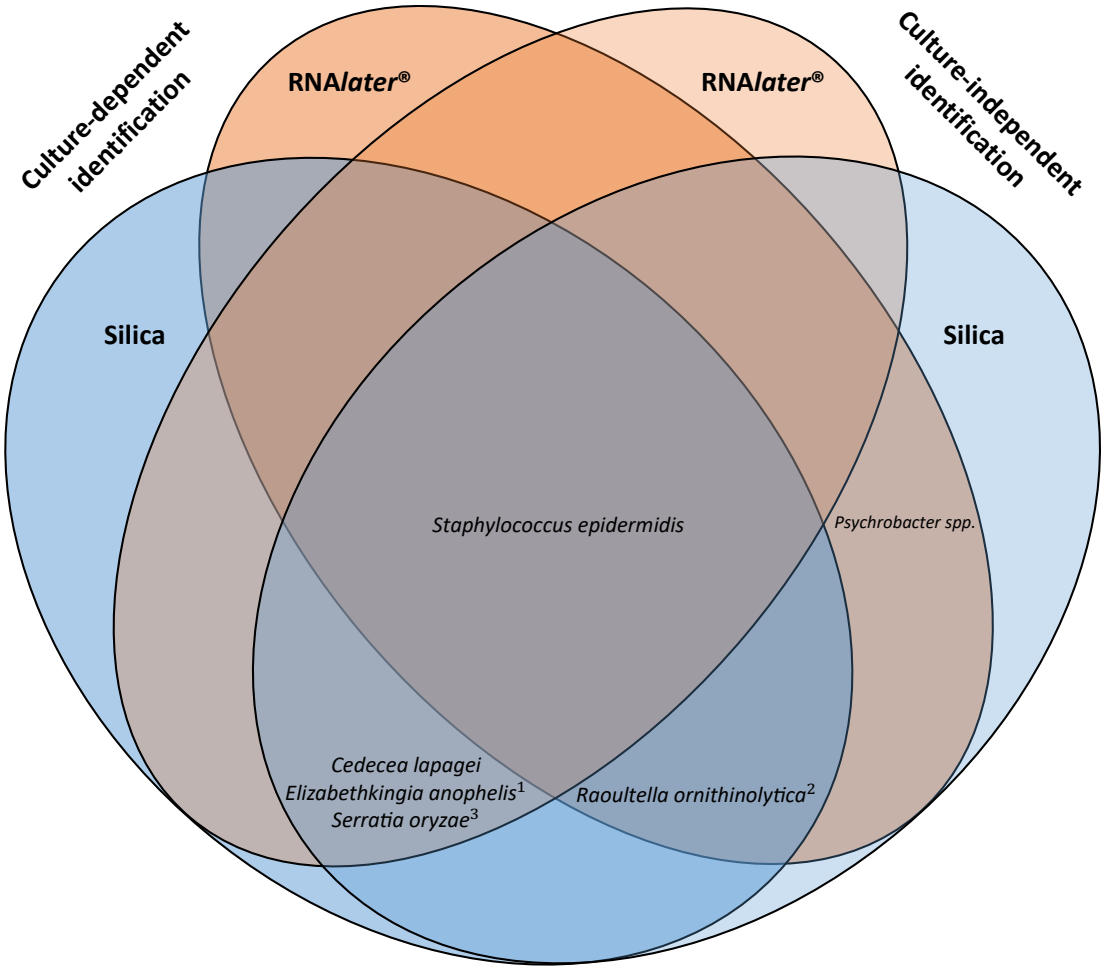

Figure E7: Common bacteria identified by culture-dependent and culture-independent techniques from preserved *An. arabiensis*. Mosquitoes were preserved on silica or in RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [2]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [3]*S. fonticola* and *S. oryzae*.

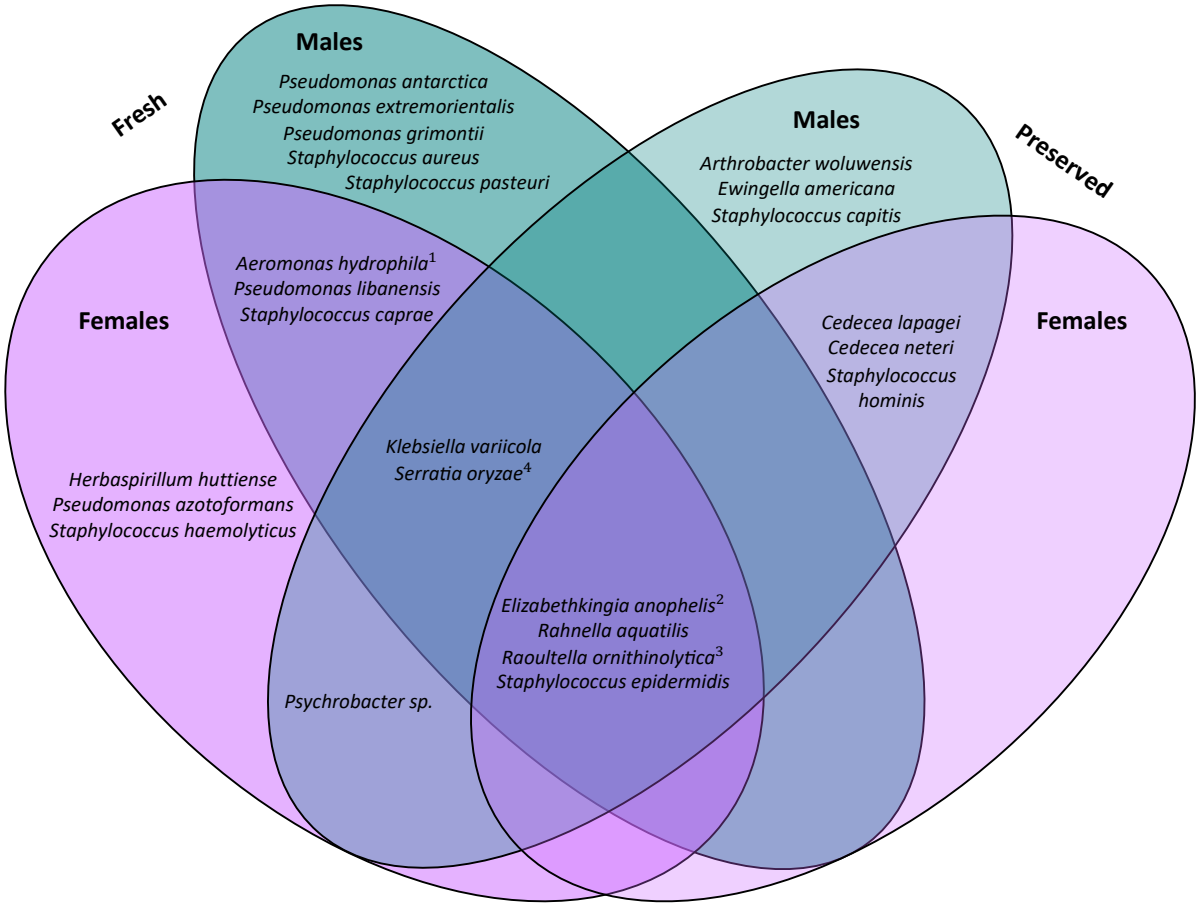

Figure F1: Common bacteria identified by culture-dependent techniques between fresh and preserved female and male *An. arabiensis.* Mosquitoes were preserved on silica or in RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [3]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [4]*S. fonticola* and *S. oryzae*.

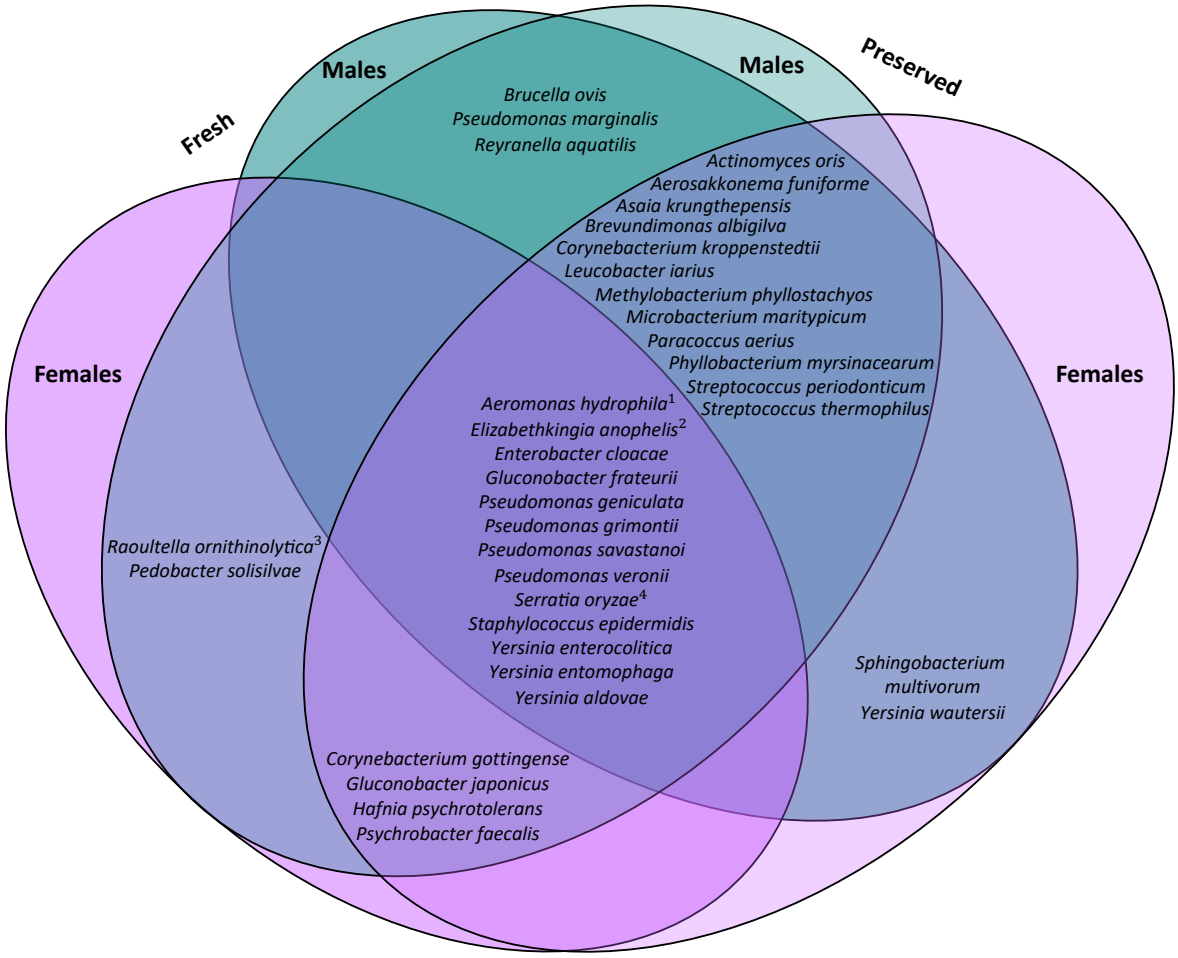

Figure F2: Common bacteria identified by culture-independent techniques between fresh and preserved female and male *An. arabiensis.* Mosquitoes were preserved on silica or in RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [3]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [4]*S. fonticola* and *S. oryzae*.

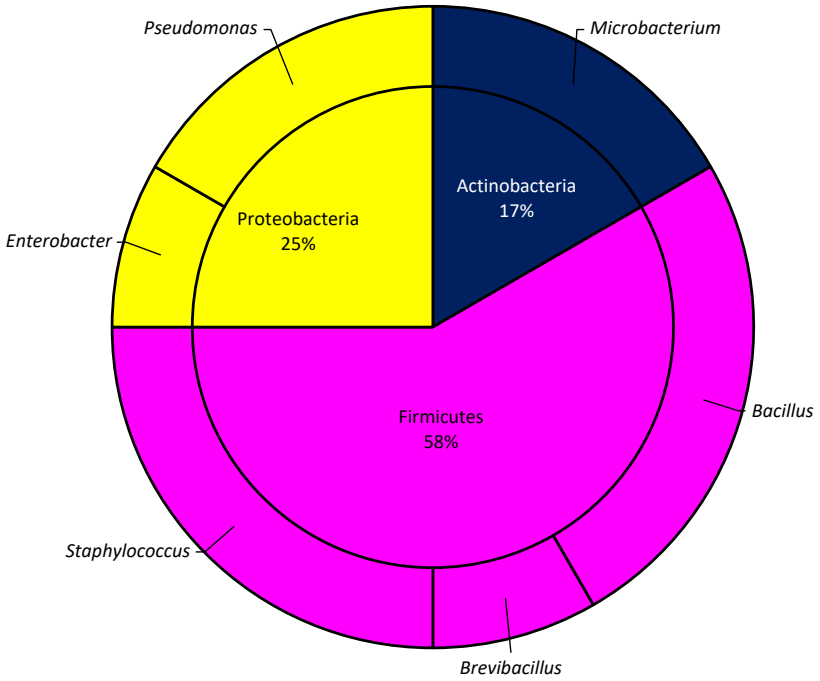

Figure G1: Bacterial phyla and genera identified from the midguts of field-collected female *An. arabiensis* from Mamfene, Kwa-Zulu Natal, South Africa. *An. arabiensis* were preserved on silica for up to 16 weeks and bacteria were identified using culture-dependent methods.

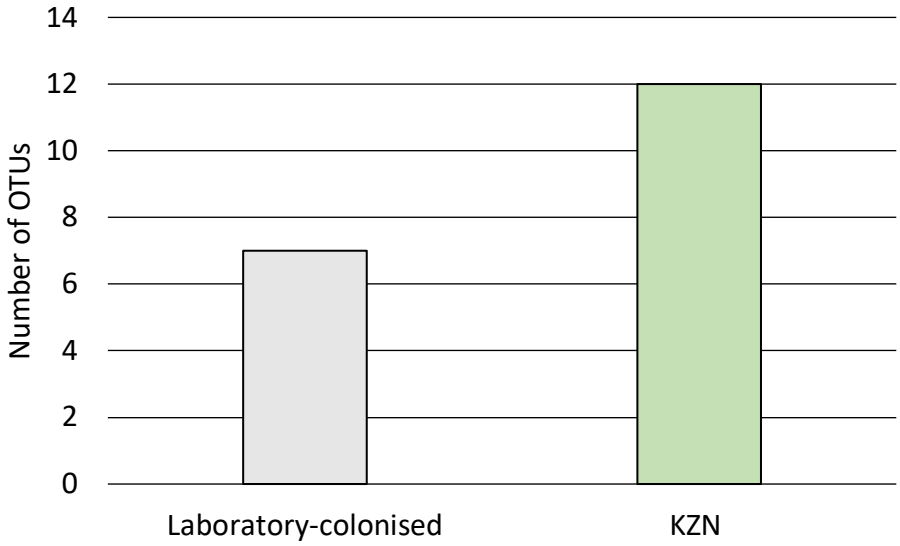

Figure G2: Comparison of species richness between colonised and field-collected *An. arabiensis* females from KZN. Bacteria were identified by culture-dependent methods from silica-preserved mosquitoes and species richness was measured by the number of OTUs.

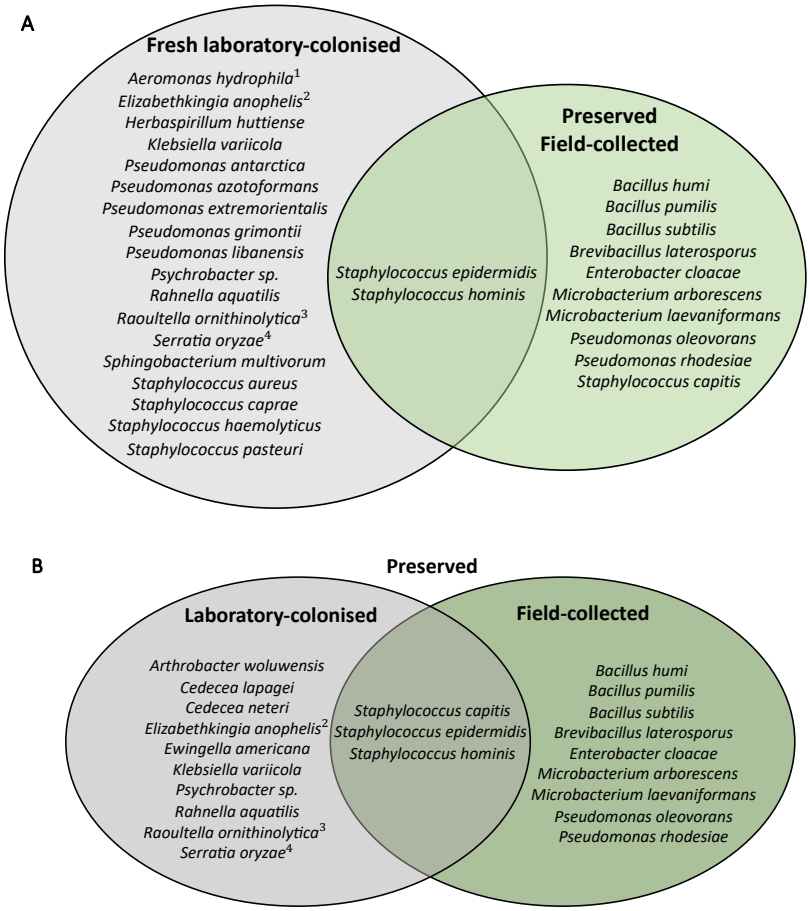

Figure G3: Common bacteria between (A) fresh, colonised female and male *An. arabiensis* and preserved, field-collected female *An. arabiensis* from KwaZulu-Natal, South Africa, and (B) preserved colonised female and male *An. arabiensis* and field-collected female *An. arabiensis* from KwaZulu-Natal, South Africa. Bacteria were identified from field-collected females preserved on silica for up to 16 weeks. Midgut bacteria from colonised and field-collected mosquitoes were identified by culture-dependent techniques. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [3]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [4]*S. fonticola* and *S. oryzae*.

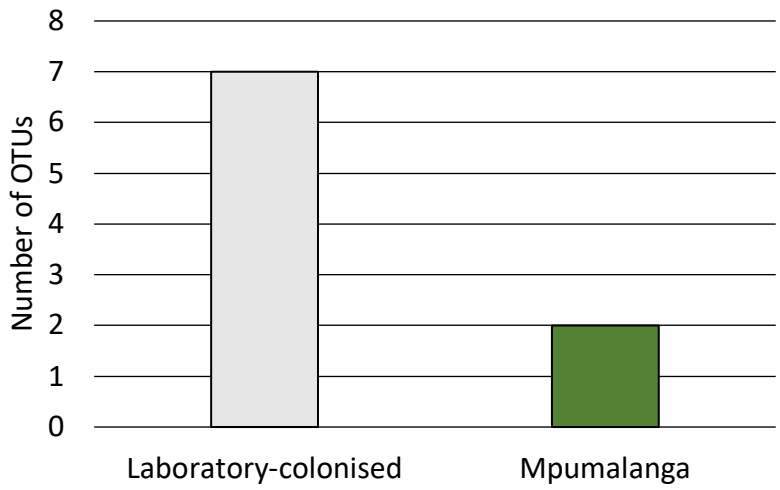

Figure G4: Comparison of species richness between colonised and field-collected *An. arabiensis* females from Mpumalanga. Bacteria were identified by culture-dependent methods from silica-preserved mosquitoes and species richness was measured by the number of OTUs.

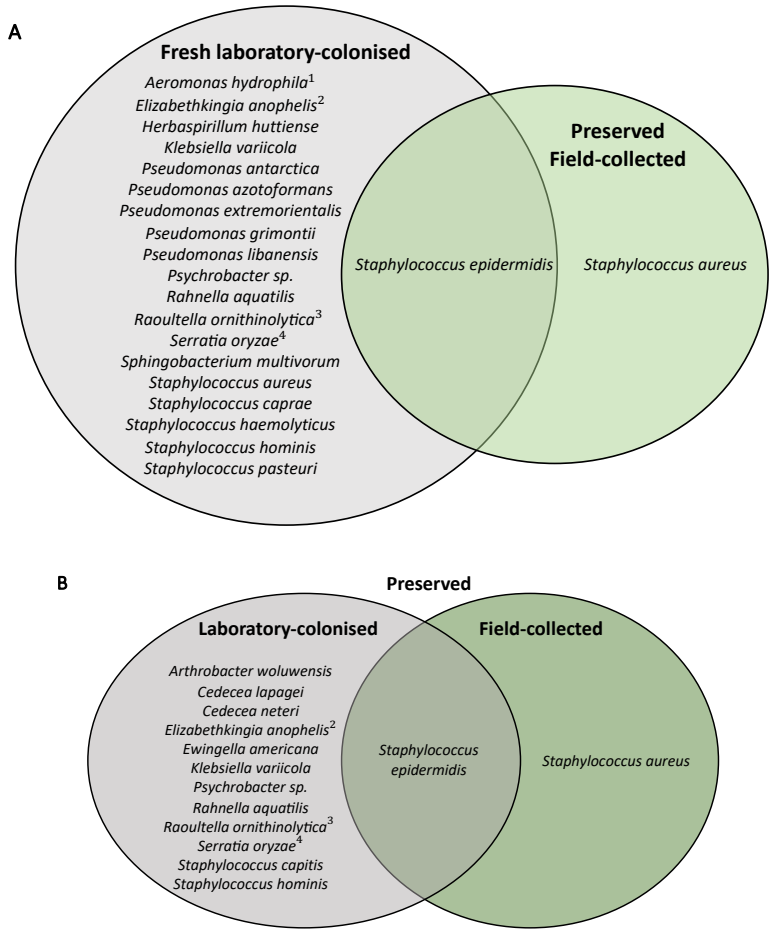

Figure G5: Common bacteria between (A) fresh, colonised female and male *An. arabiensis* and preserved, field-collected female *An. arabiensis* from Mpumalanga, South Africa, and (B) preserved colonised female and male *An. arabiensis* and field-collected female *An. arabiensis* from Mpumalanga, South Africa. Bacteria were identified from field-collected females preserved on silica for up to 16 weeks. Midgut bacteria from colonised and field-collected mosquitoes were identified by culture-dependent techniques. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [3]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [4]*S. fonticola* and *S. oryzae*.

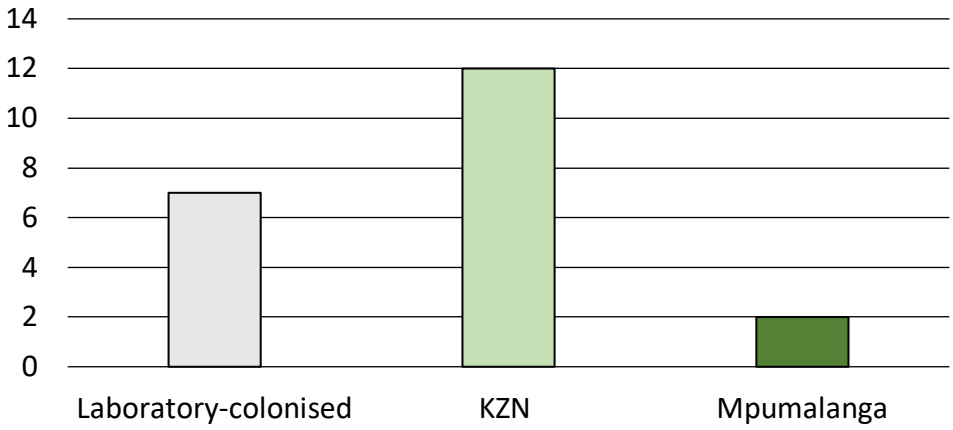

Figure G6: Comparison of species richness between colonised *An. arabiensis* and field-collected *An. arabiensis* females from KZN and Mpumalanga. Bacteria were identified by culture-dependent methods from silica-preserved mosquitoes and species richness was measured by the number of OTUs.

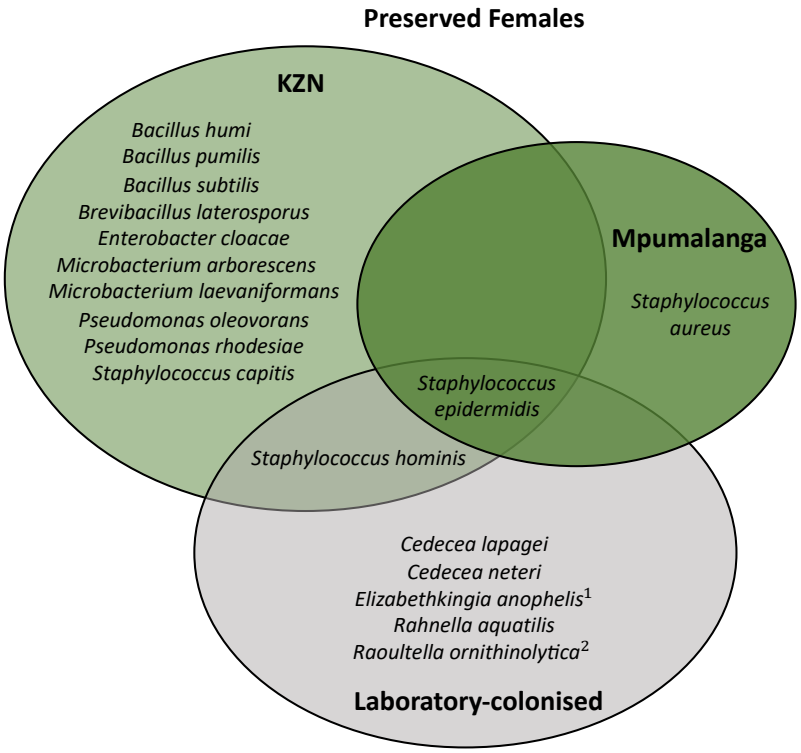

Figure G7: Common midgut bacteria between colonised *An. arabiensis* and field-collected *An. arabiensis* from KZN and Mpumalanga. Field-collected females were preserved on silica for up to 16 weeks and midgut bacteria were identified by culture-dependent methods. Midgut bacteria from silica-preserved colonised females were also identified by culture-dependent techniques. Bacteria indistinguishable by MALDI-TOF MS include [1]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; and [2]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*.

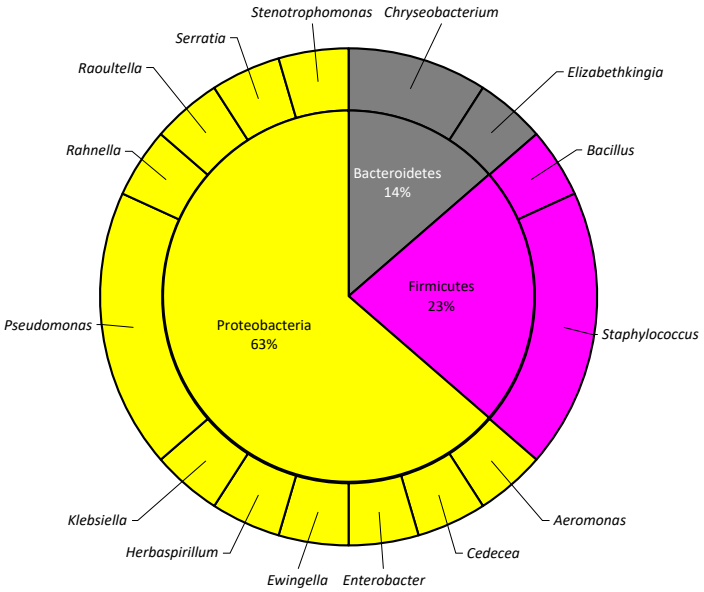

Figure H1: Bacterial phyla and genera identified by culture-dependent techniques from midguts of colonised *An. funestus*.

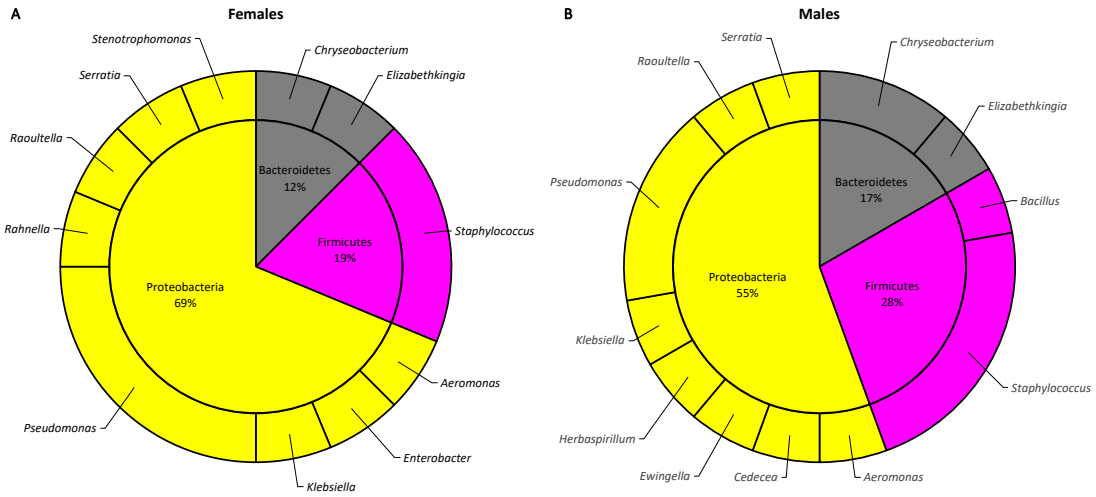

Figure H2: Bacterial phyla and genera identified by culture-dependent techniques from colonised (A) female and (B) male *An. funestus*.

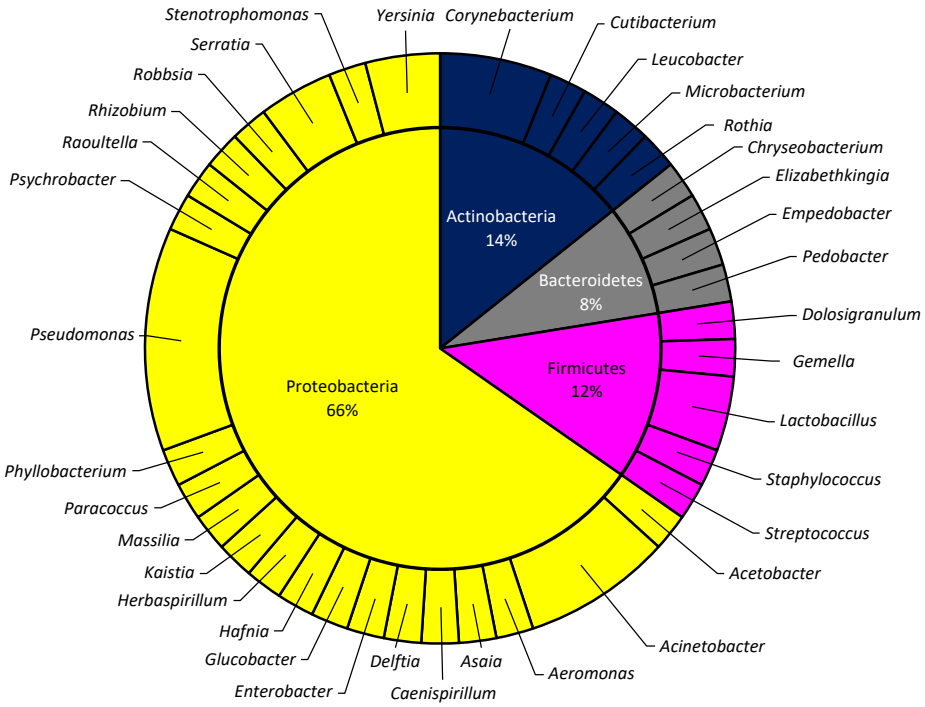

Figure H3: Bacterial phyla and genera identified by culture-independent techniques from the midguts of colonised *An. funestus*.

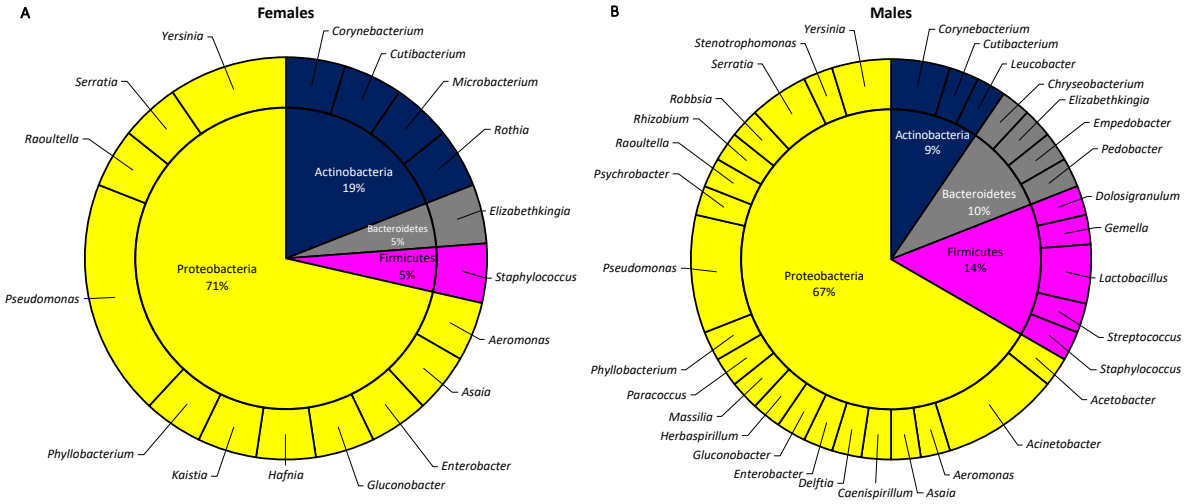

Figure H4 Bacterial phyla and genera identified by culture-independent techniques from colonised (A) female and (B) male *An. funestus*.

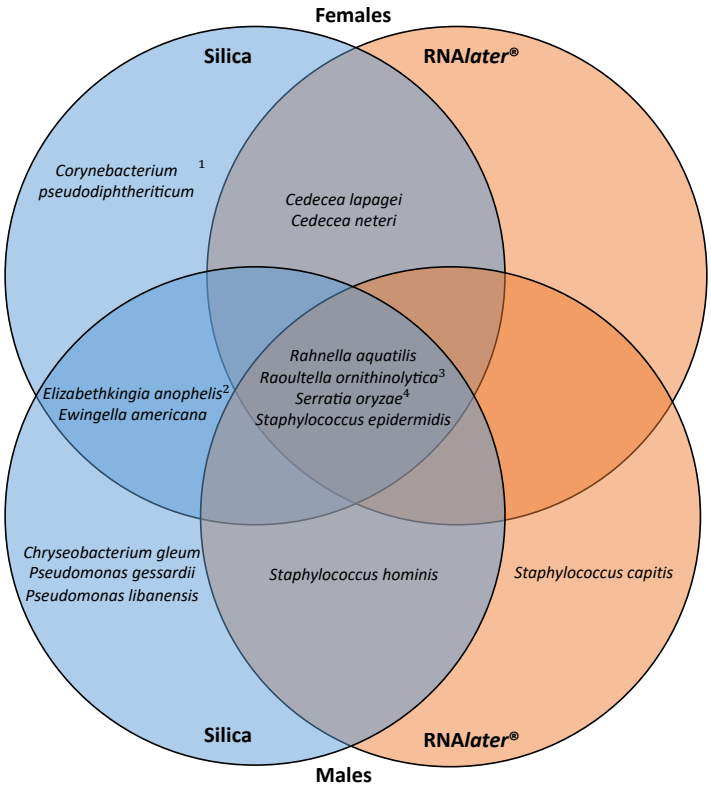

Figure I1: Bacteria identified by culture-dependent techniques from preserved female and male *An. funestus.* Mosquitoes were preserved on silica or in RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*C. propinquum* and *C. pseudodiphtheriticum*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [3]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [4]*S. fonticola* and *S. oryzae.*

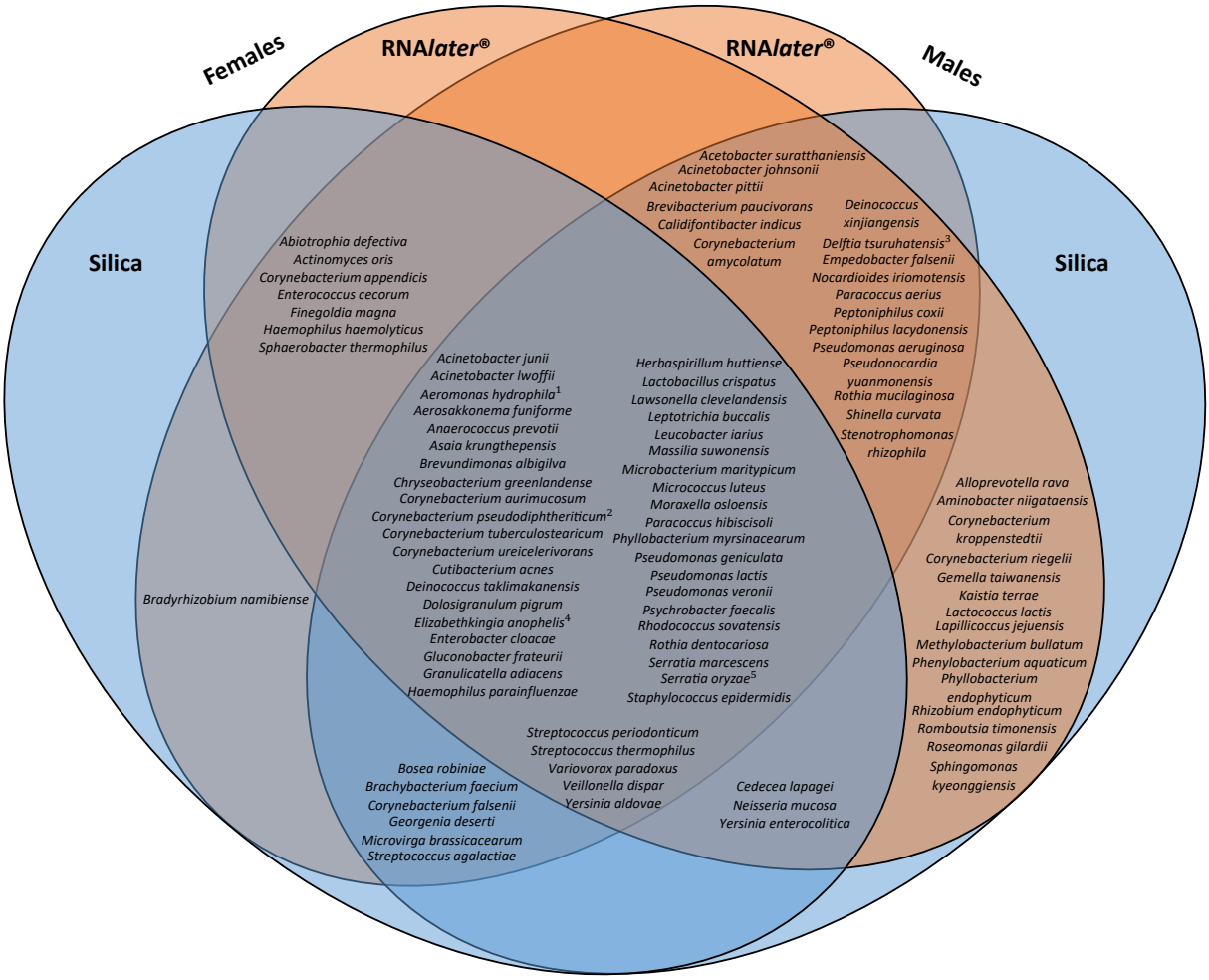

Figure I2: Common bacteria identified by culture-independent techniques from preserved female and male *An. funestus.* Mosquitoes were preserved on silica or in RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*D. acidovorans* and *D. tsuruhatensis*; [4]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; and [5]*S. fonticola* and *S. oryzae.*

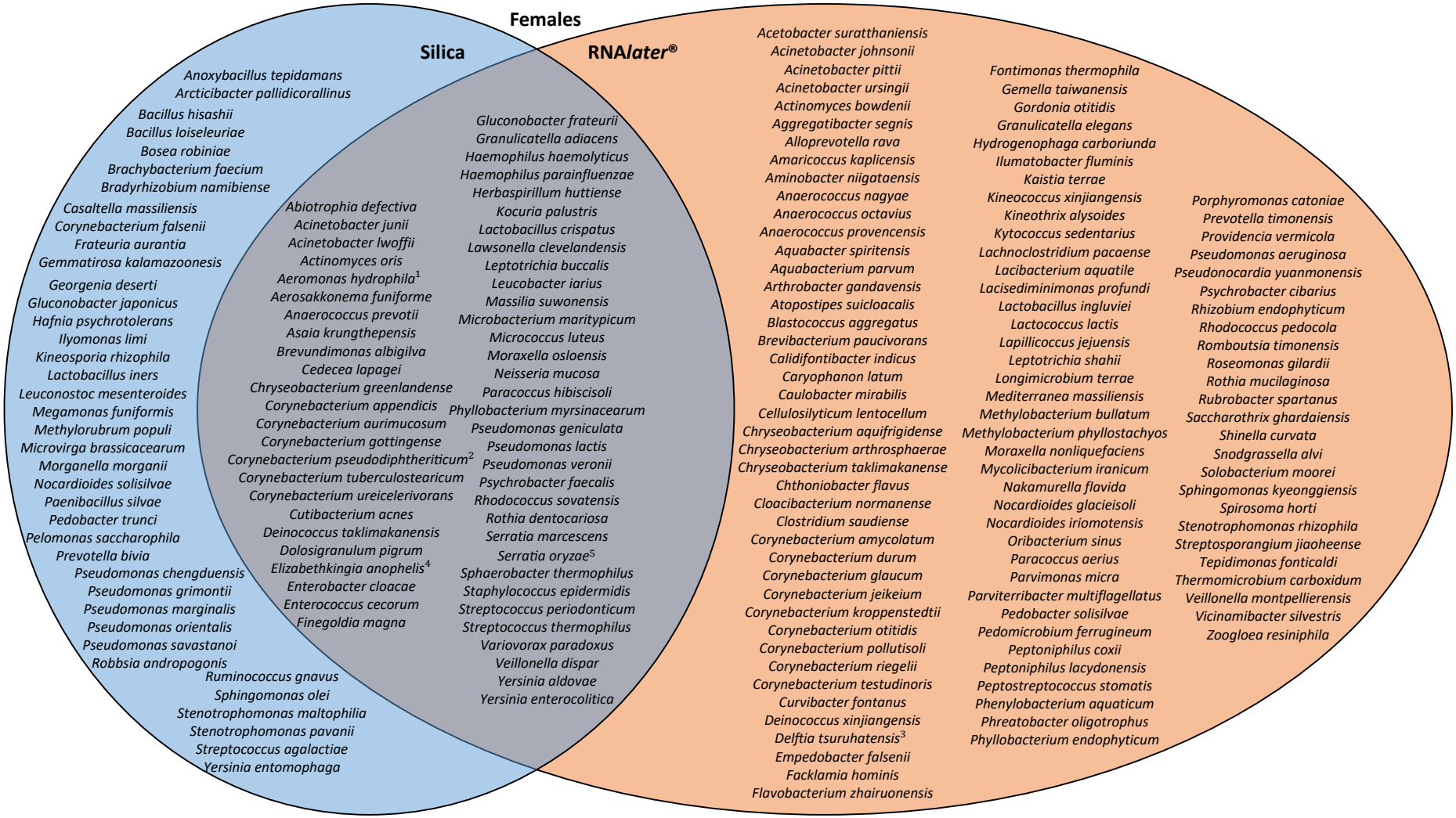

Figure I3: Bacteria identified by culture-independent techniques from preserved female *An. funestus*. Mosquitoes were preserved on silica or in RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*D. acidovorans* and *D. tsuruhatensis*; [4]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; and [5]*S. fonticola* and *S. oryzae*.

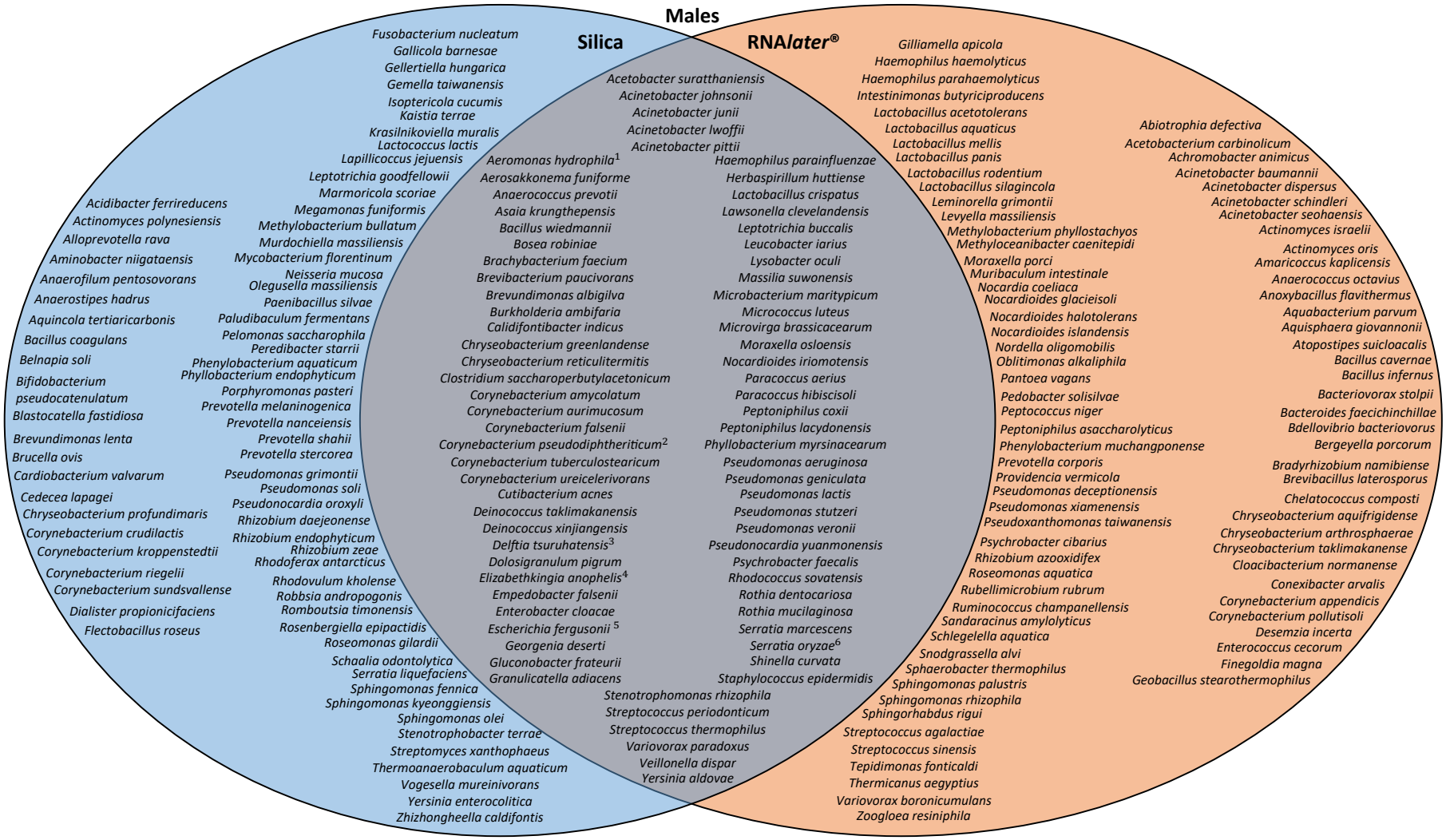

Figure I4: Bacteria identified by culture-independent techniques from preserved male *An. funestus*. Mosquitoes were preserved on silica or in RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*D. acidovorans* and *D. tsuruhatensis*; [4]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [5]*E. coli* and *E. fergusonii*; and [6]*S. fonticola* and *S. oryzae*.

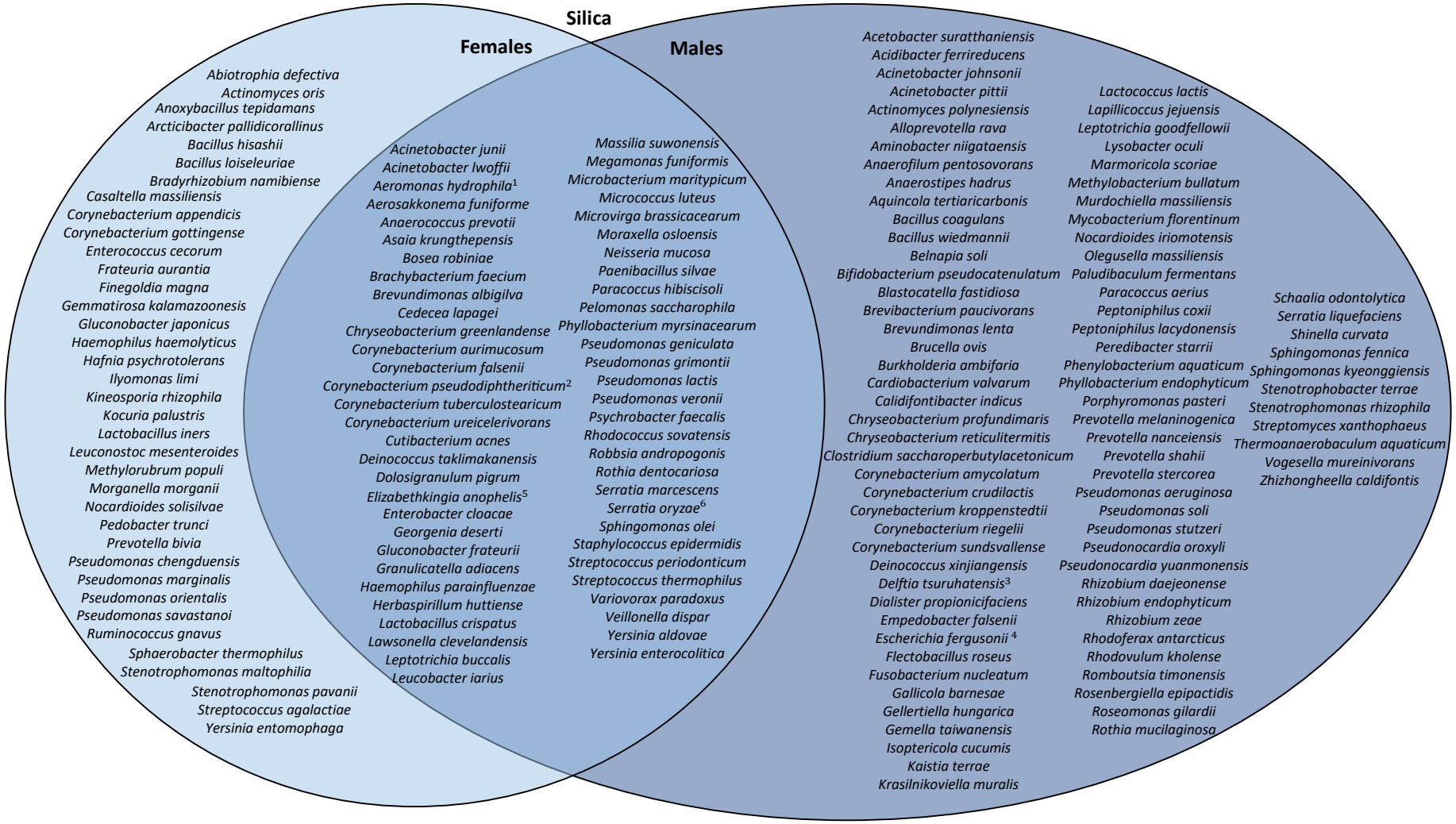

Figure I5: Bacteria identified by culture-independent techniques from silica-preserved *An. funestus*. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*D. acidovorans* and *D. tsuruhatensis*; [4]*E. coli* and *E. fergusonii*; [5]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; and [6]*S. fonticola* and *S. oryzae*.

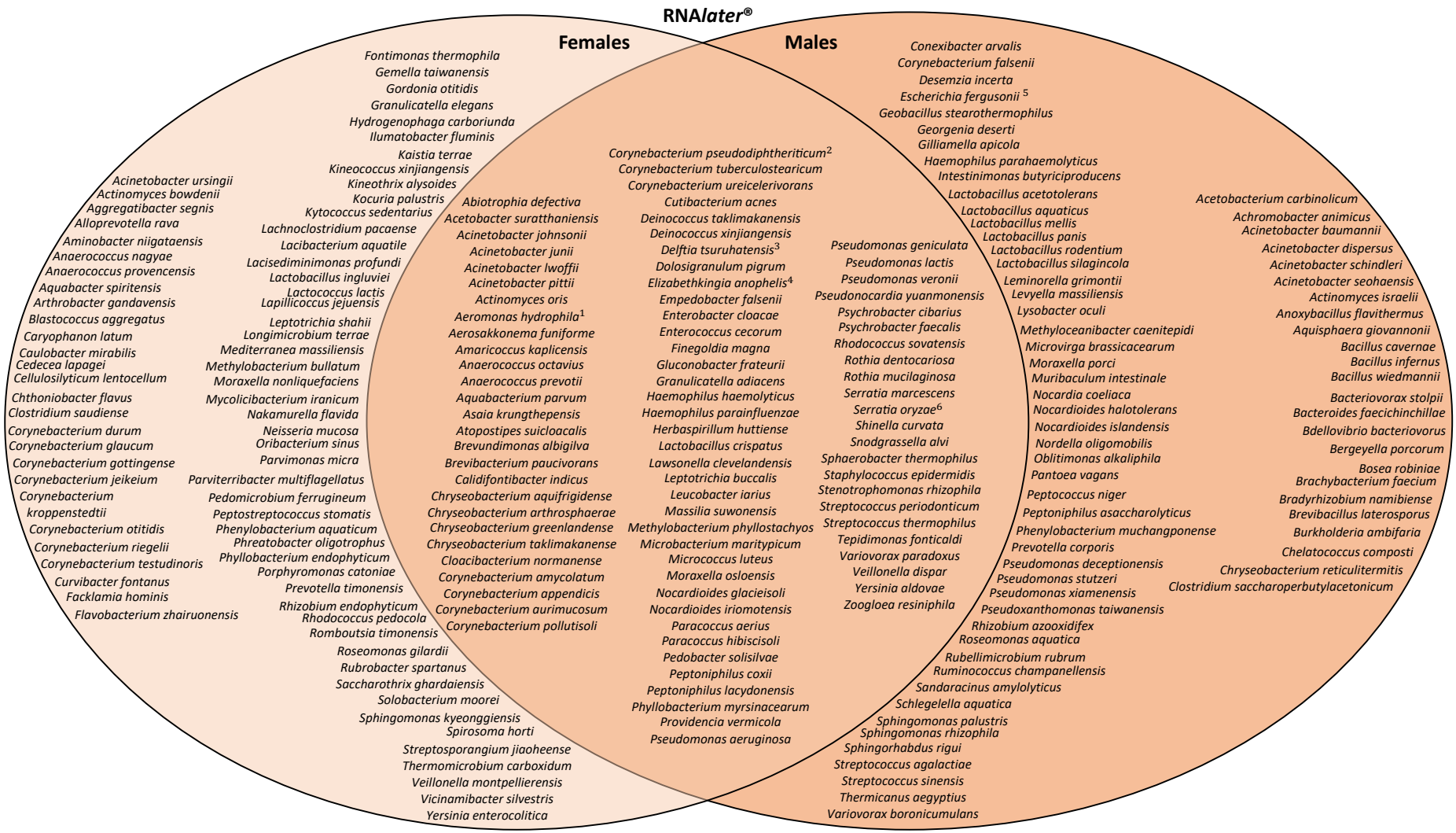

Figure I6: Bacteria identified by culture-independent techniques from RNA*later*®-preserved *An. funestus*. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*D. acidovorans* and *D. tsuruhatensis*; [4]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [5]*E. coli* and *E. fergusonii*; and [6]*S. fonticola* and *S. oryzae*.

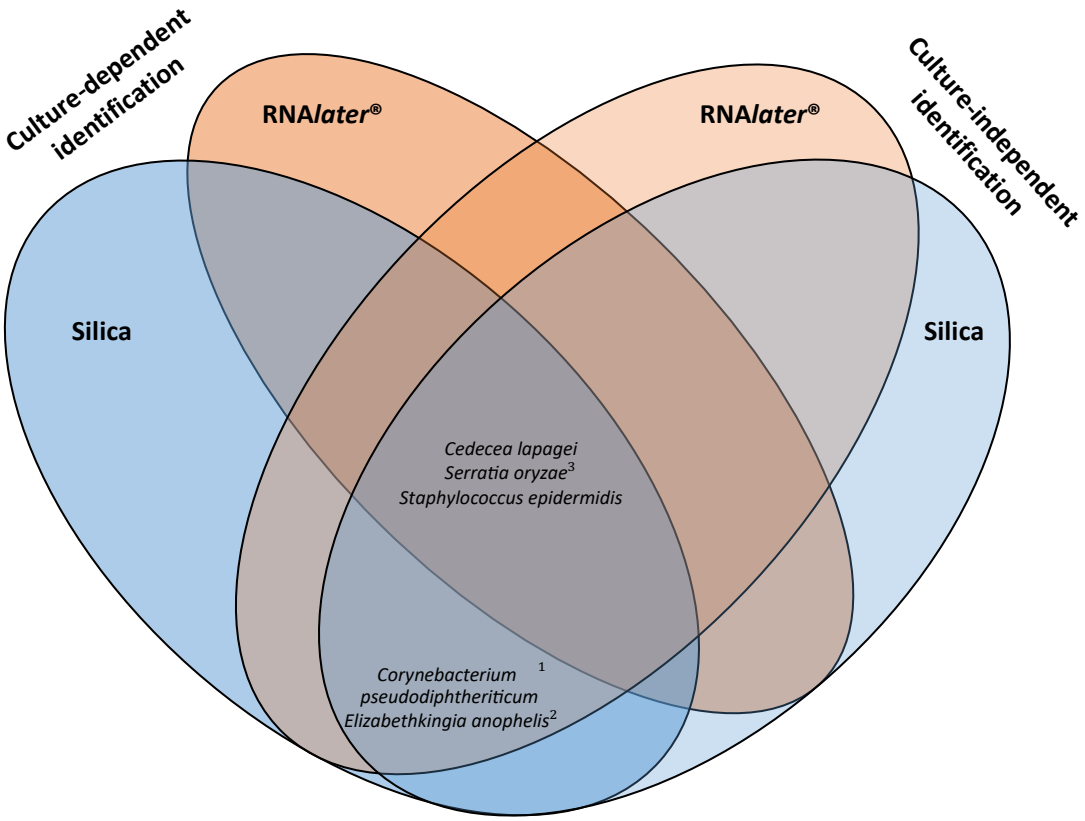

Figure I7: Common bacteria identified by culture-dependent and culture-independent techniques from preserved *An. funestus.* Mosquitoes were preserved on silica or in RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*C. propinquum* and *C. pseudodiphtheriticum*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; and [3]*S. fonticola* and *S. oryzae.*

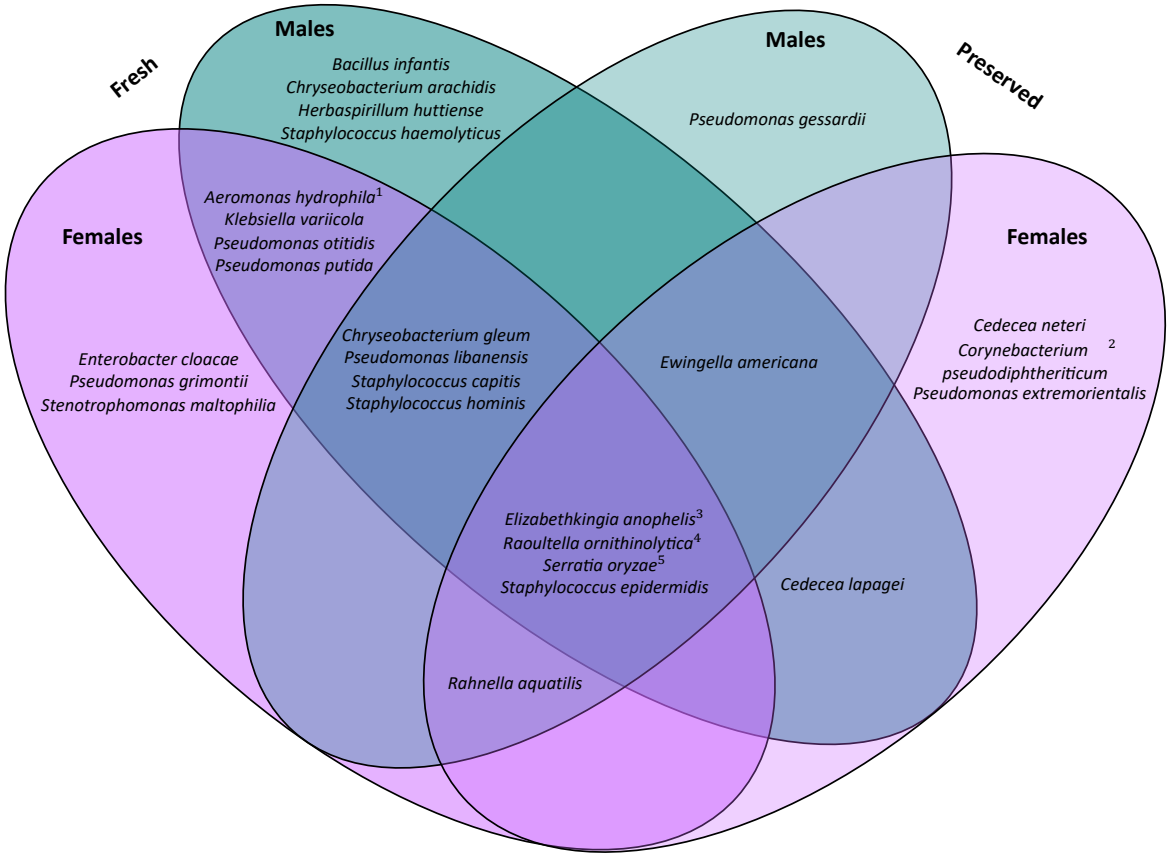

Figure J1: Common bacteria identified by culture-dependent techniques from fresh and preserved female and male *An. funestus.* Mosquitoes were preserved on silica or in RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [3]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [4]*S. fonticola* and *S. oryzae.*

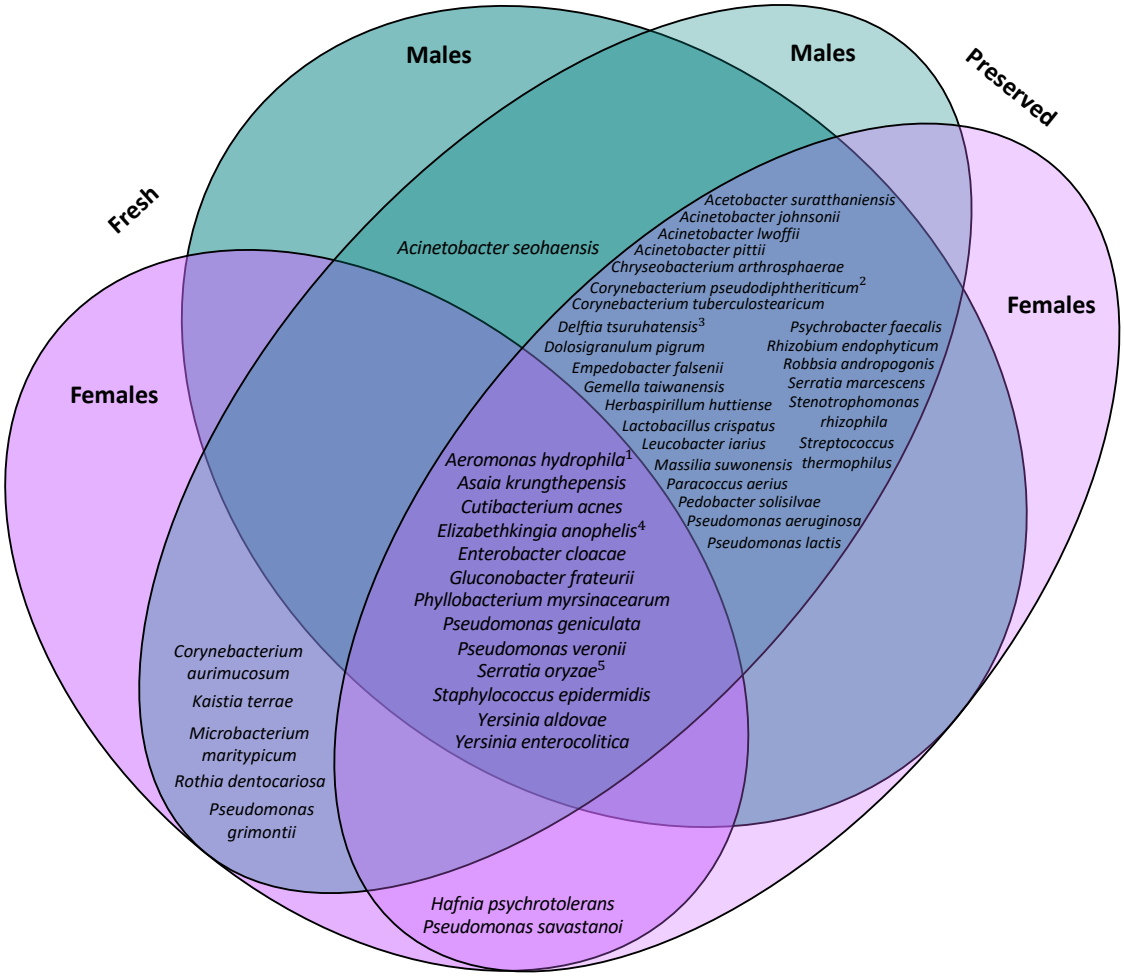

Figure J2: Common bacteria identified by culture-independent techniques from fresh and preserved female and male *An. funestus.* Mosquitoes were preserved on silica or in RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*D. acidovorans* and *D. tsuruhatensis*; [4]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; and [5]*S. fonticola* and *S. oryzae.*

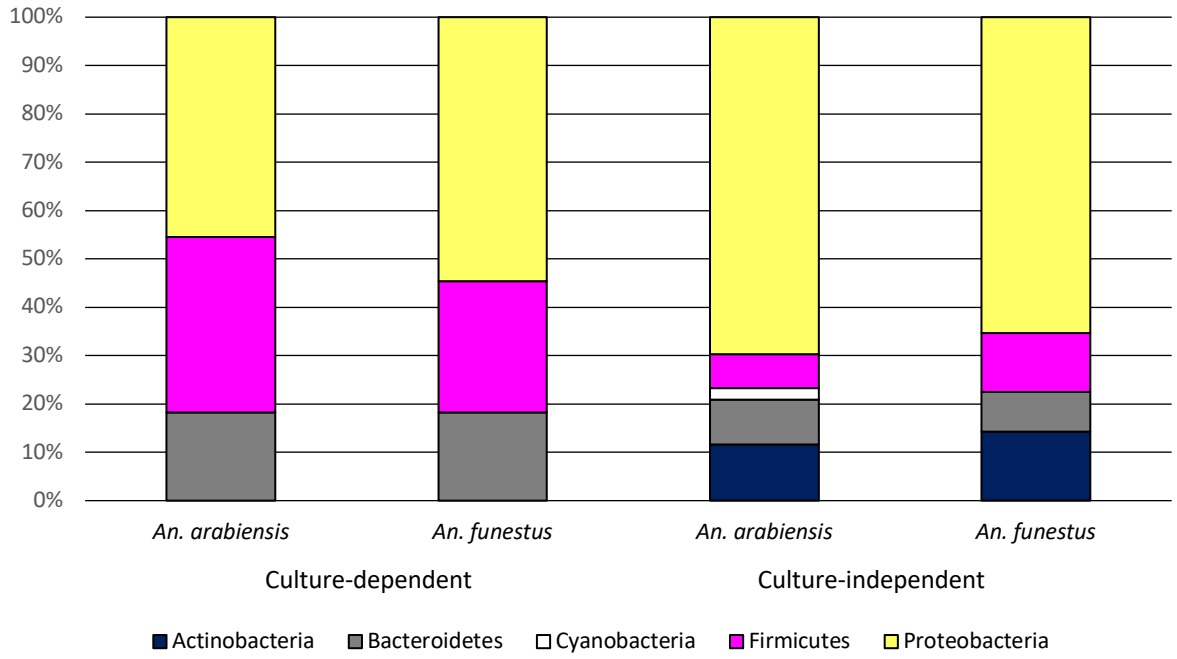

Figure K1: Bacterial phyla identified by culture-dependent and culture-independent techniques from the midguts of fresh colonised *An. arabiensis* and *An. funestus*.

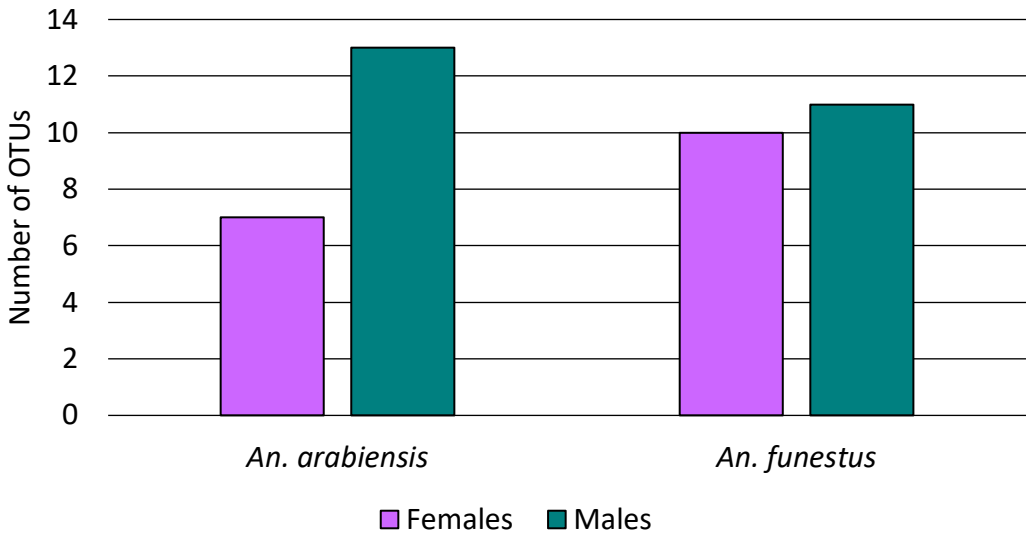

Figure K2: Species richness of fresh colonised *An. arabiensis* and *An. funestus* identified by culture-dependent methods. Species richness was measured by the number of OTUs.

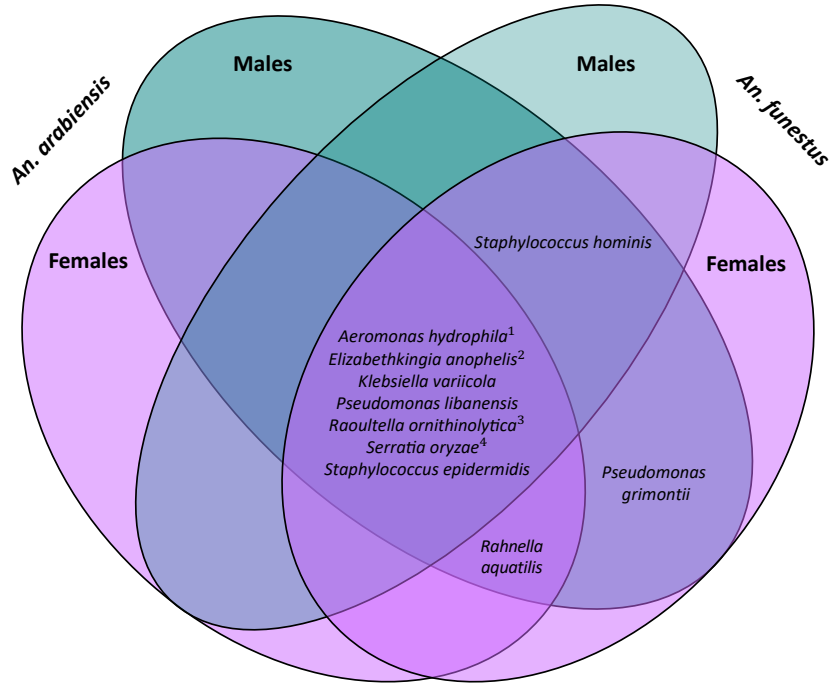

Figure K3: Bacteria identified by culture-dependent techniques from colonised *An. arabiensis* and *An. funestus*. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [3]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [4]*S. fonticola* and *S. oryzae.*

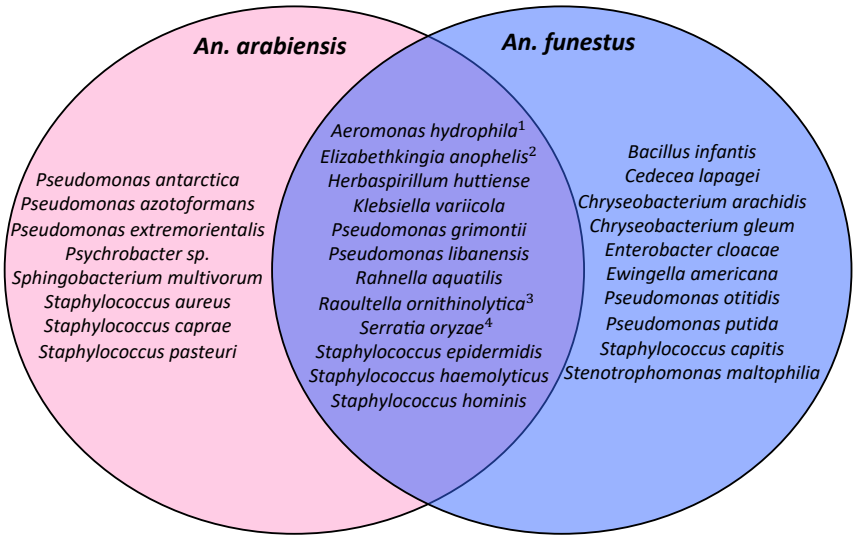

Figure K4: Common bacteria identified by culture-dependent techniques from *An. arabiensis* and *An. funestus*. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [3]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [4]*S. fonticola* and *S. oryzae*.

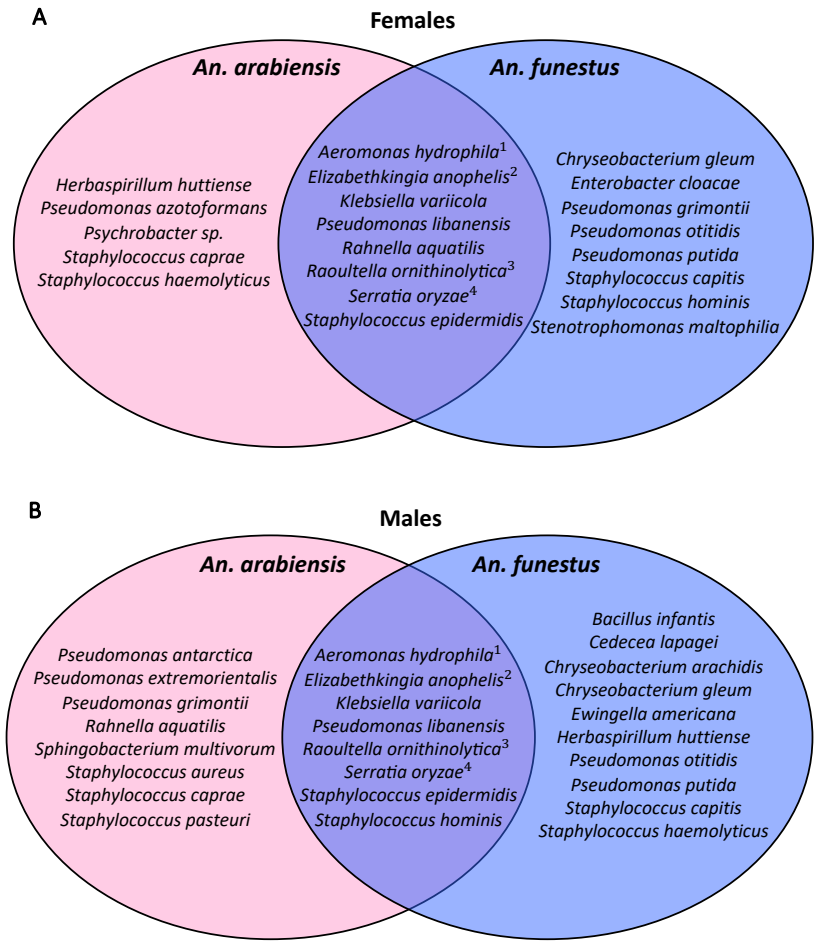

Figure K5: Comparison of bacteria identified by culture-dependent techniques from female and male *An. arabiensis* and *An. funestus*. Bacteria are compared between (A) females of both species and (B) males of both species. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [3]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [4]*S. fonticola* and *S. oryzae*.

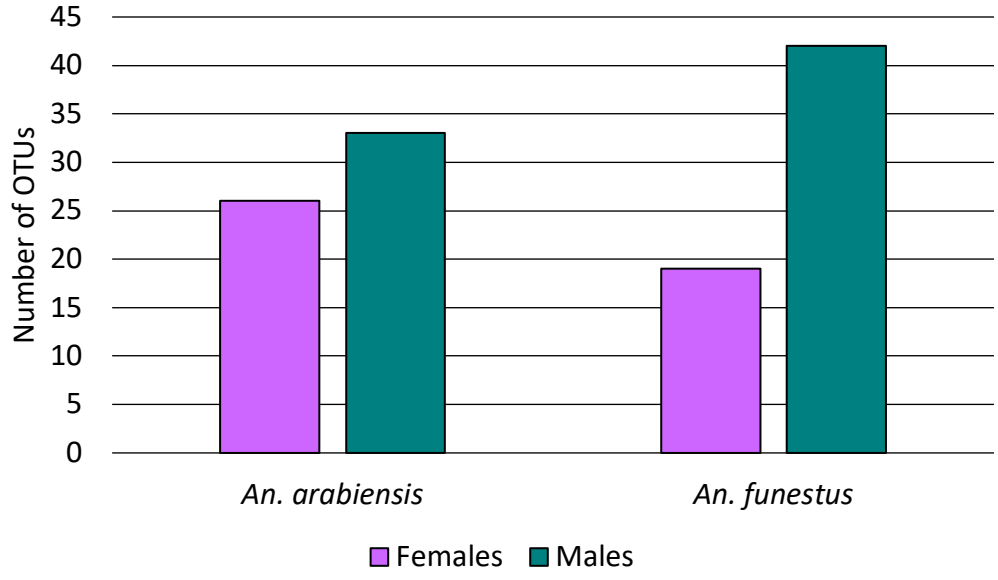

Figure K6: Species richness of fresh colonised *An. arabiensis* and *An. funestus* identified by culture-independent methods. Species richness was measured by the number of OTUs.

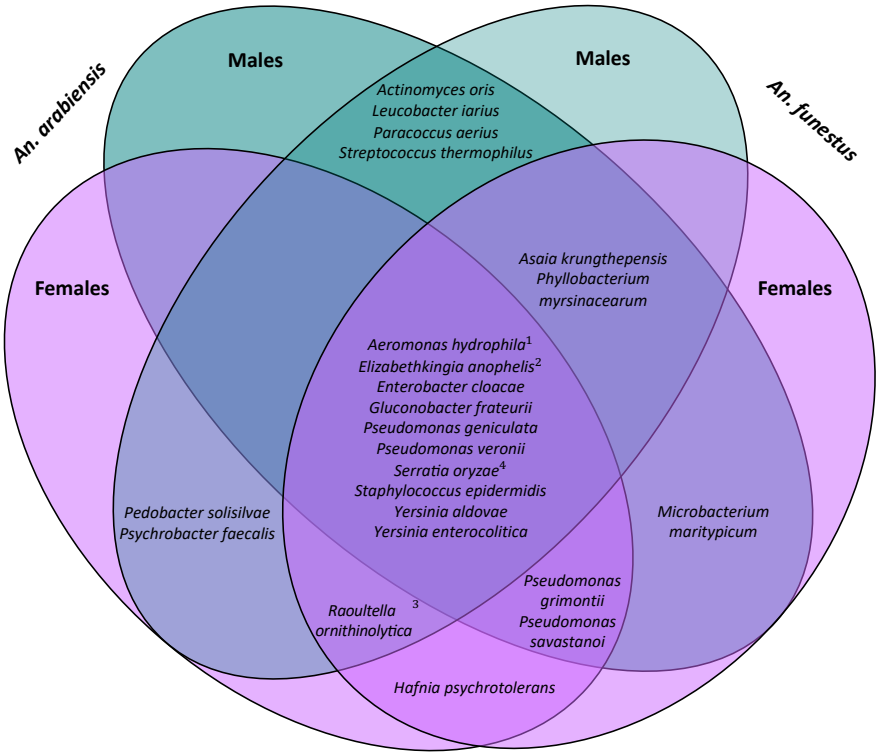

Figure K7: Bacteria identified by culture-independent techniques from colonised *An. arabiensis* and *An. funestus*. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [3]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [4]*S. fonticola* and *S. oryzae.*

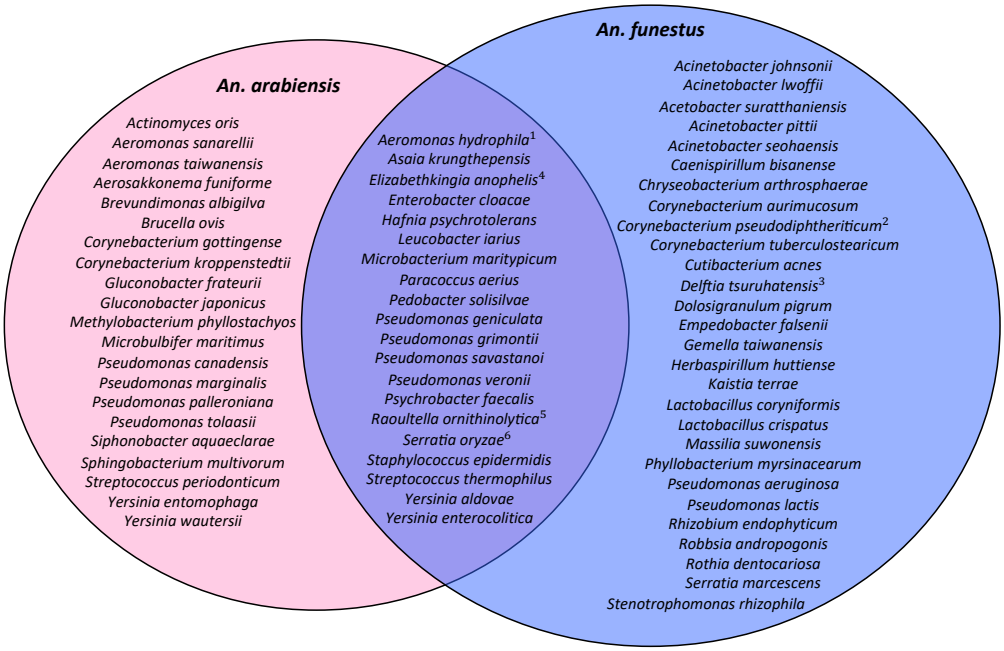

Figure K8: Common bacteria identified by culture-independent techniques from *An. arabiensis* and *An. funestus*. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [3]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [4]*S. fonticola* and *S. oryzae*.

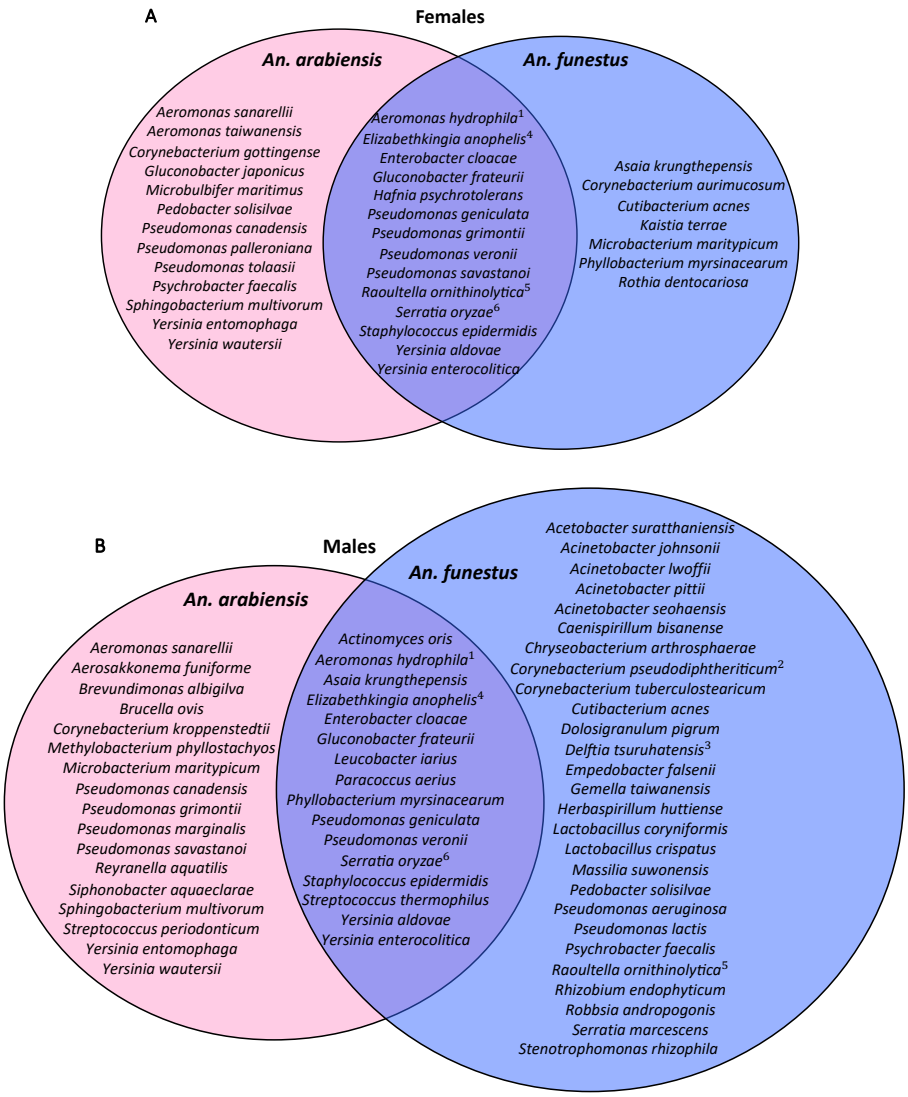

Figure K9: Comparison of bacteria identified by culture-independent techniques from female and male *An. arabiensis* and *An. funestus*. Bacteria are compared between (A) females of both species and (B) males of both species. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [3]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [4]*S. fonticola* and *S. oryzae*.

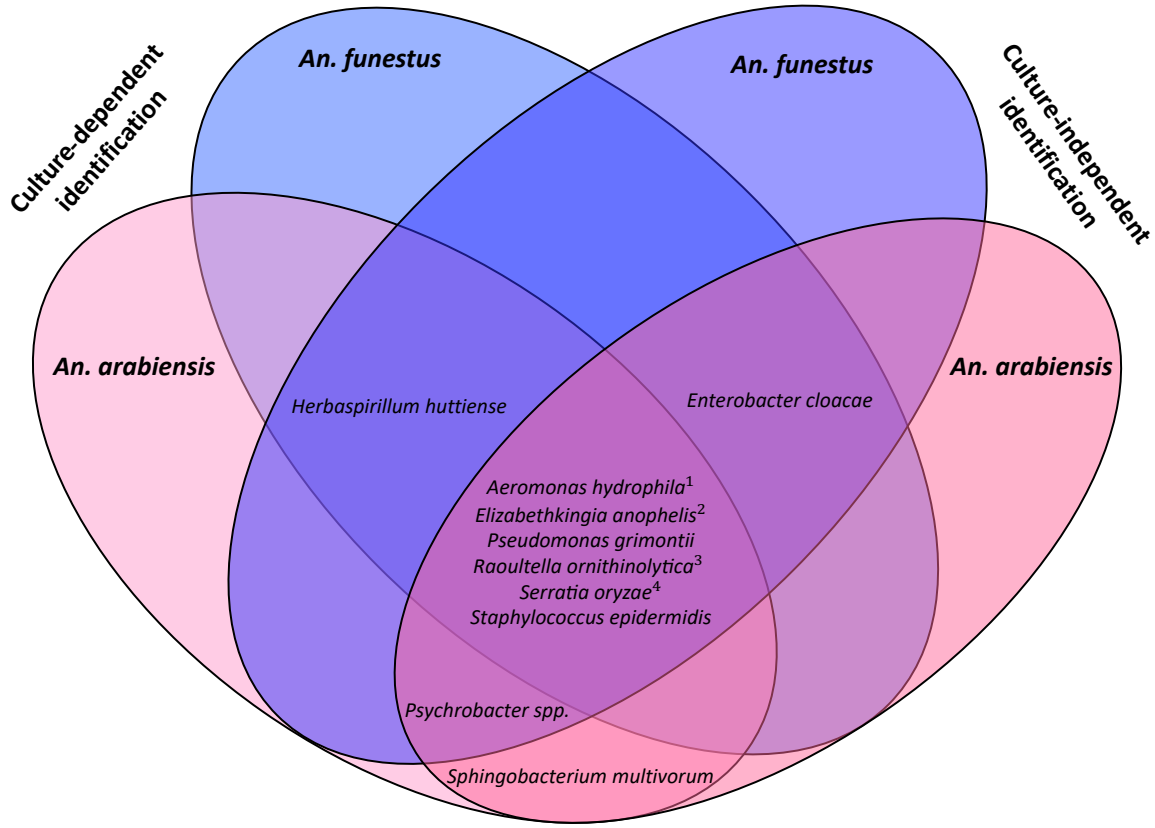

Figure K10: Bacteria identified by culture-dependent and culture-independent techniques from colonised *An. arabiensis* and *An. funestus*. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [3]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [4]*S. fonticola* and *S. oryzae.*

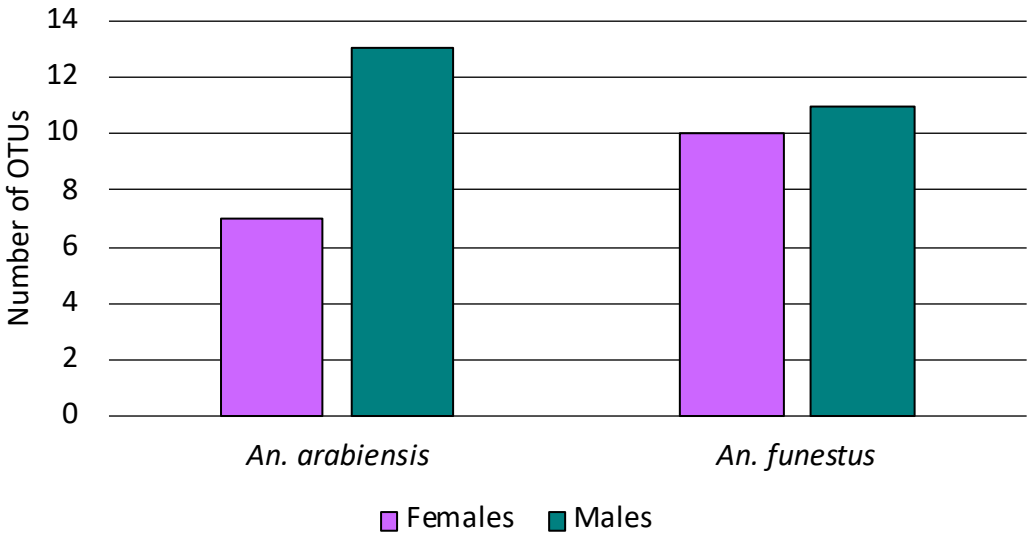

Figure L1: Species richness of preserved colonised *An. arabiensis* and *An. funestus* identified by culture-dependent methods. Species richness was measured by the number of OTUs.

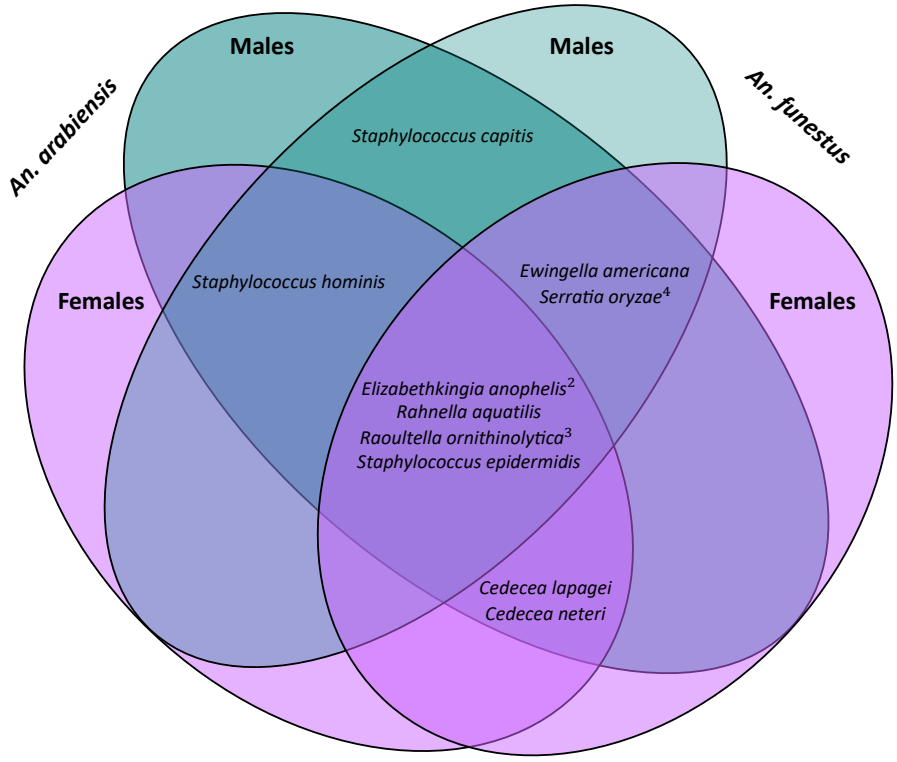

Figure L2: Bacteria identified by culture-dependent techniques from preserved *An. arabiensis* and *An. funestus*. Mosquitoes were preserved on silica or in RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [3]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [4]*S. fonticola* and *S. oryzae.*



Figure L3: Comparison of bacteria identified by culture-dependent techniques from preserved female and male *An. arabiensis* and *An. funestus*. Bacteria are compared between females preserved on (A) silica and (B) RNA*later*®, and males preserved on (C) silica and (D) RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [3]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [4]*S. fonticola* and *S. oryzae*.

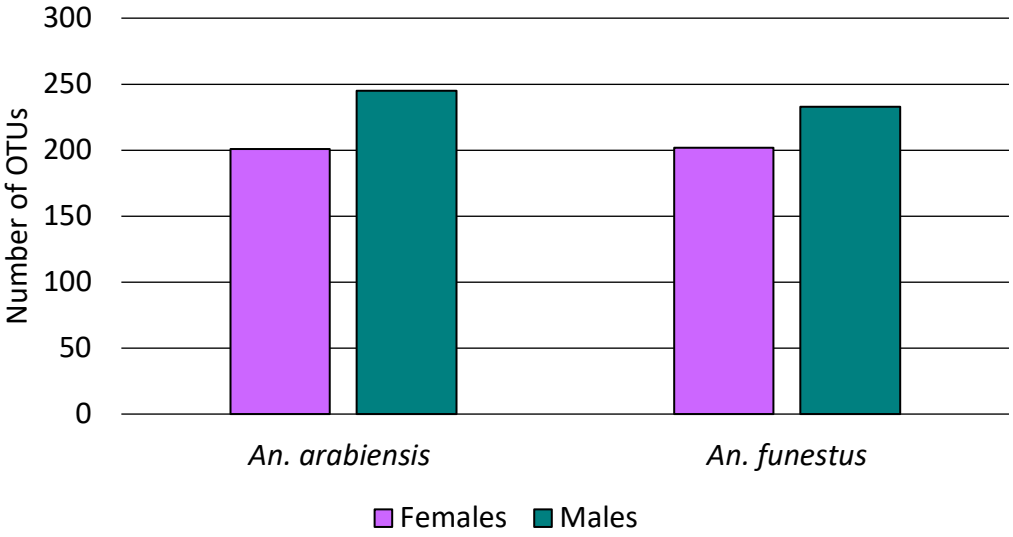

Figure L4: Species richness of preserved colonised *An. arabiensis* and *An. funestus* identified by culture-independent methods. Species richness was measured by the number of OTUs.

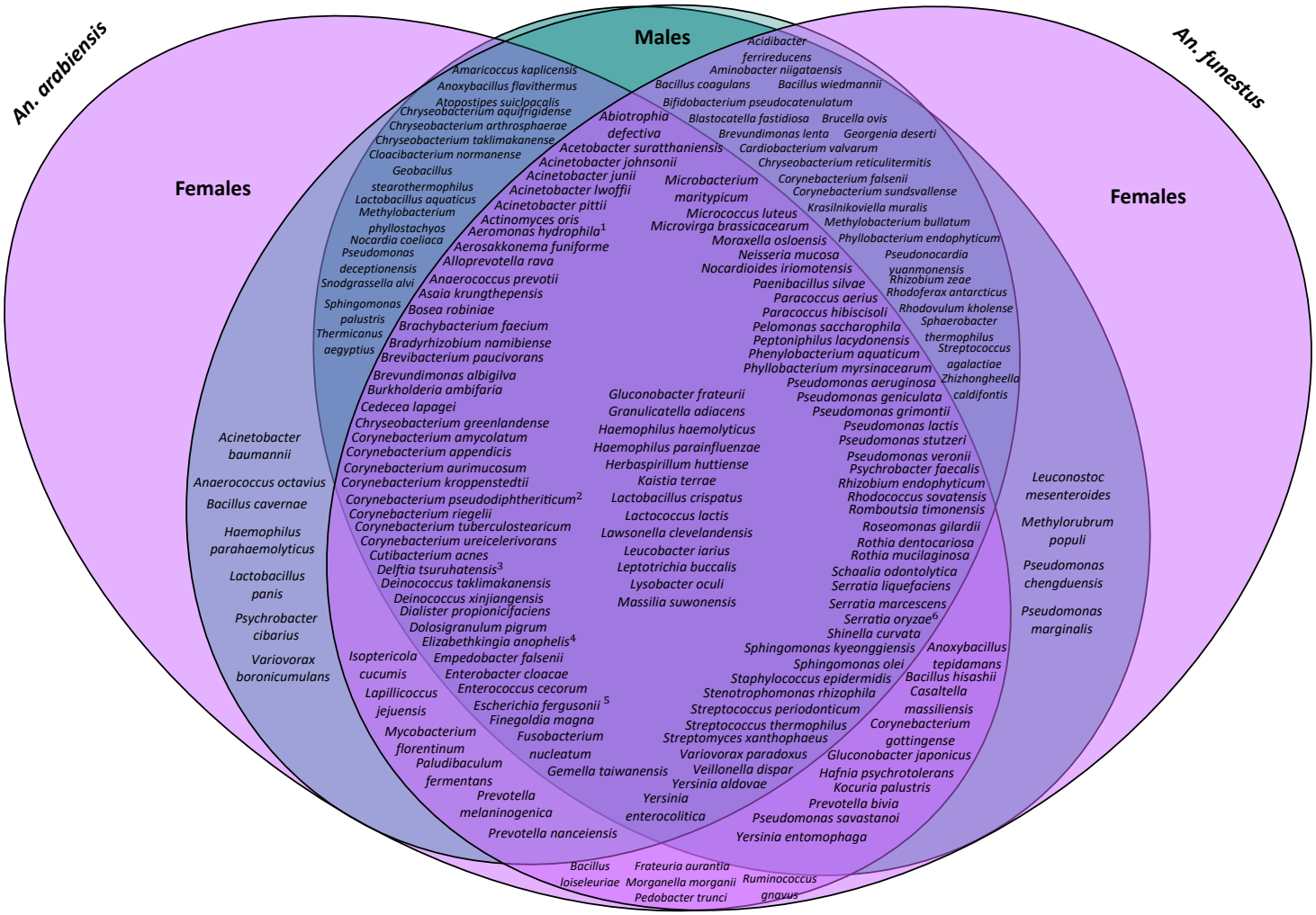

Figure L5: Bacteria identified by culture-independent techniques from preserved *An. arabiensis* and *An. funestus*. Mosquitoes were preserved on silica or in RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*D. acidovorans* and *D. tsuruhatensis*; [4]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [5]*E. coli* and *E. fergusonii*; and [6]*S. fonticola* and *S. oryzae.*

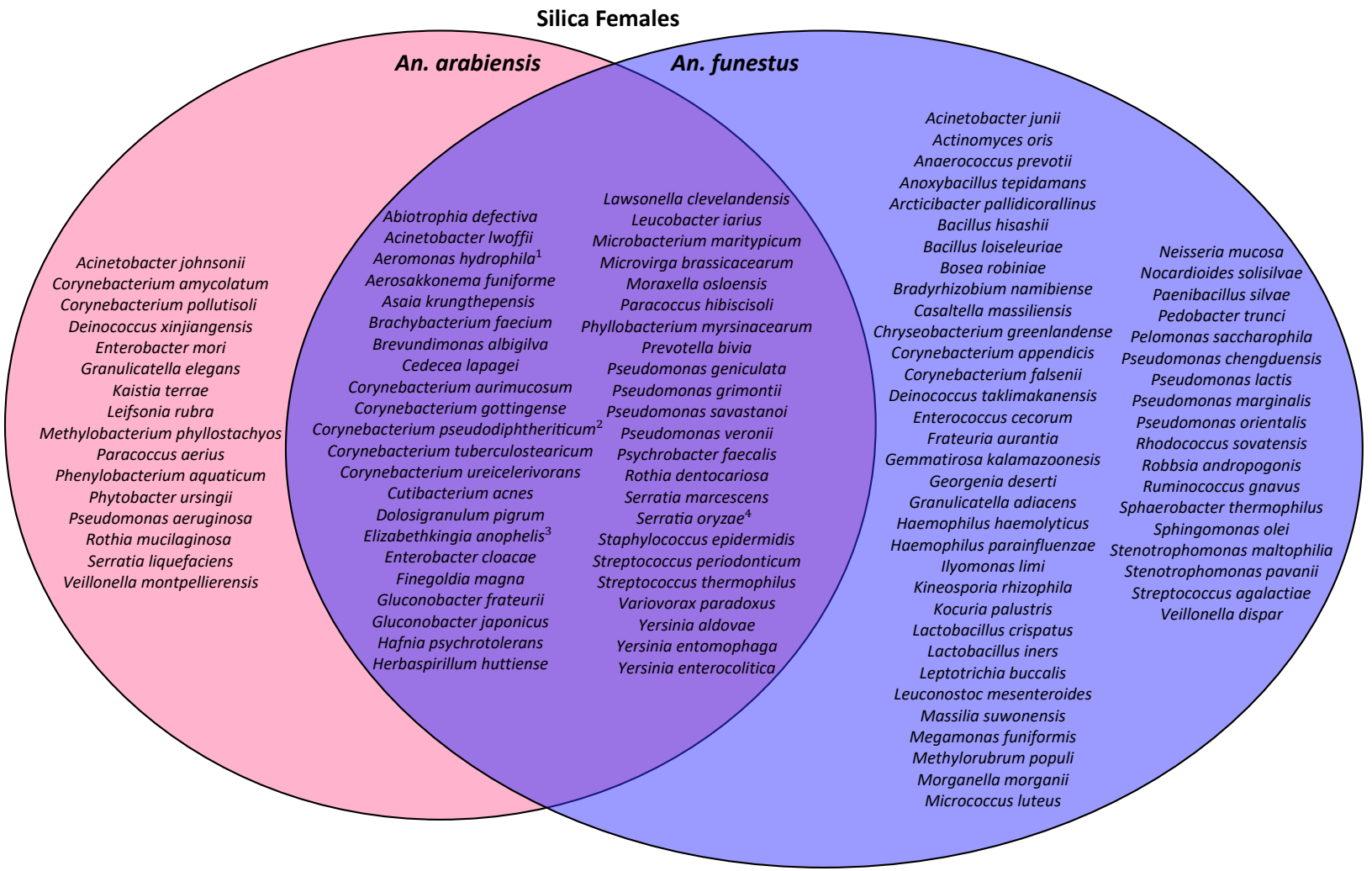

Figure L6: Bacteria identified by culture-independent techniques from silica-preserved *An. arabiensis* and *An. funestus* females. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; and [4]*S. fonticola* and *S. oryzae.*

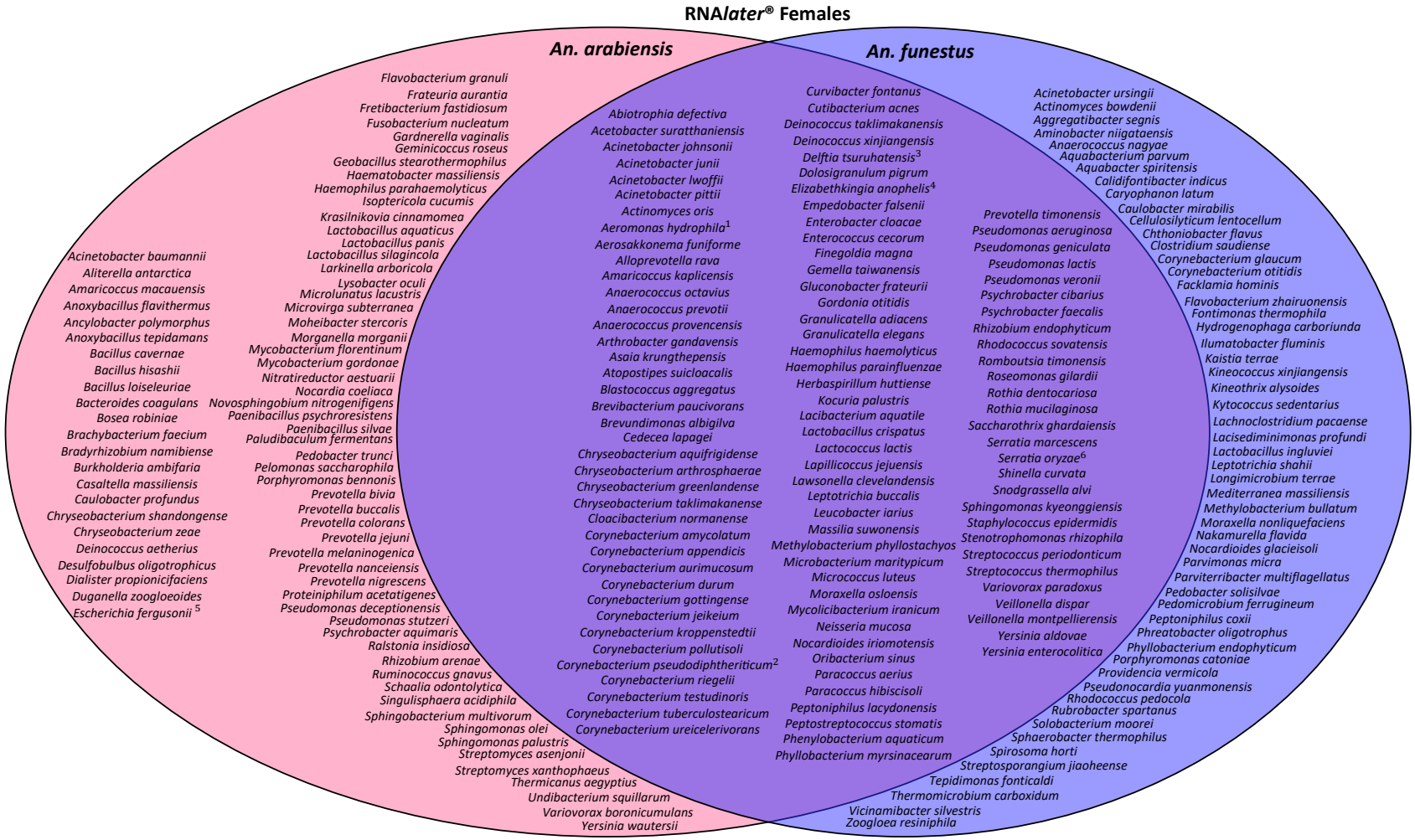

Figure L7: Bacteria identified by culture-independent techniques from RNA*later*®-preserved *An. arabiensis* and *An. funestus* females. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*D. acidovorans* and *D. tsuruhatensis*; [4]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [5]*E. coli* and *E. fergusonii*; [6]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [7]*S. fonticola* and *S. oryzae*.

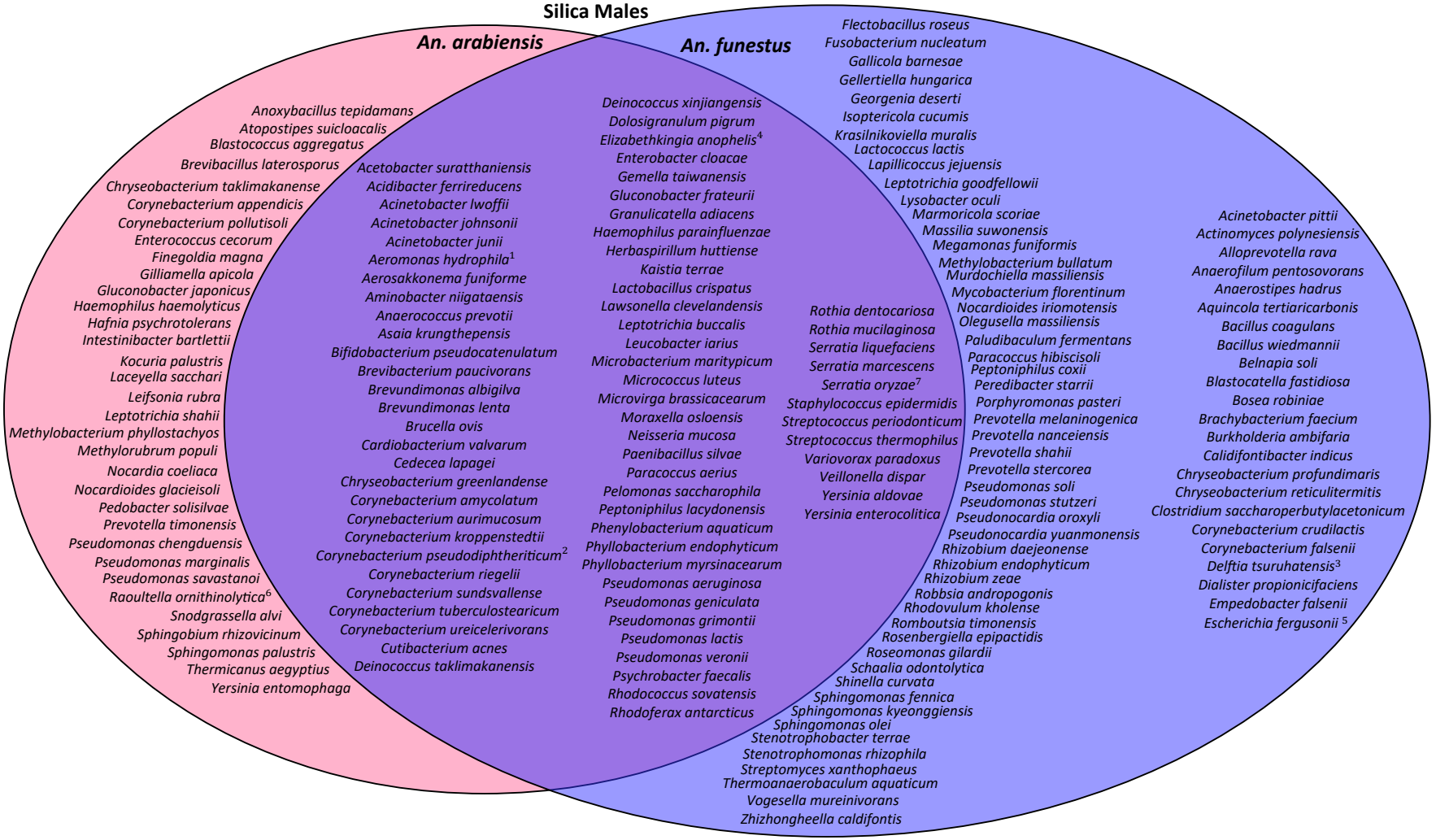

Figure L8: Bacteria identified by culture-independent techniques from silica-preserved *An. arabiensis* and *An. funestus* males. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*D. acidovorans* and *D. tsuruhatensis*; [4]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [5]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [6]*S. fonticola* and *S. oryzae*.

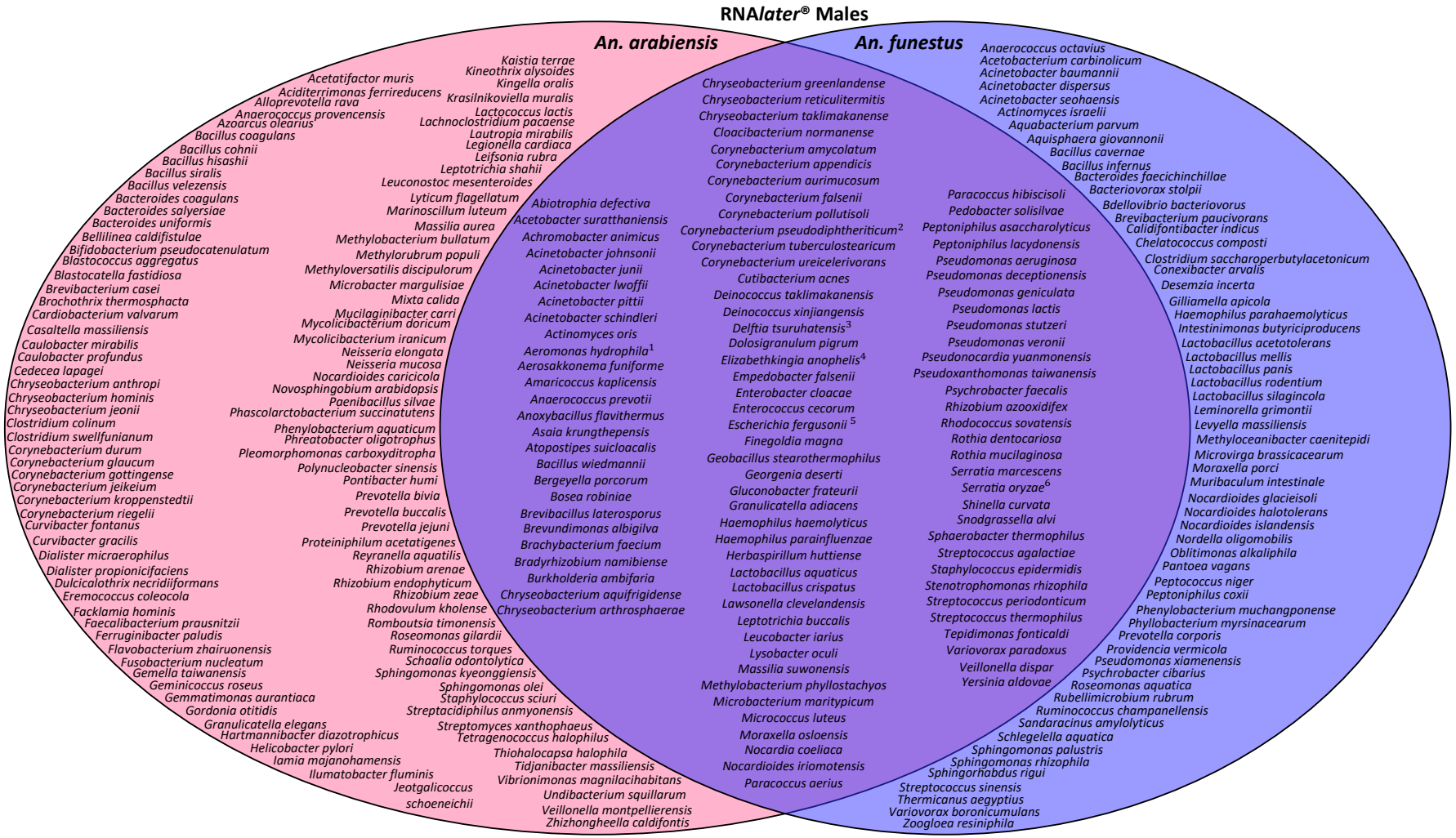

Figure L9: Bacteria identified by culture-independent techniques from RNA*later*®-preserved *An. arabiensis* and *An. funestus* males. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*D. acidovorans* and *D. tsuruhatensis*; [4]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [5]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [6]*S. fonticola* and *S. oryzae*.

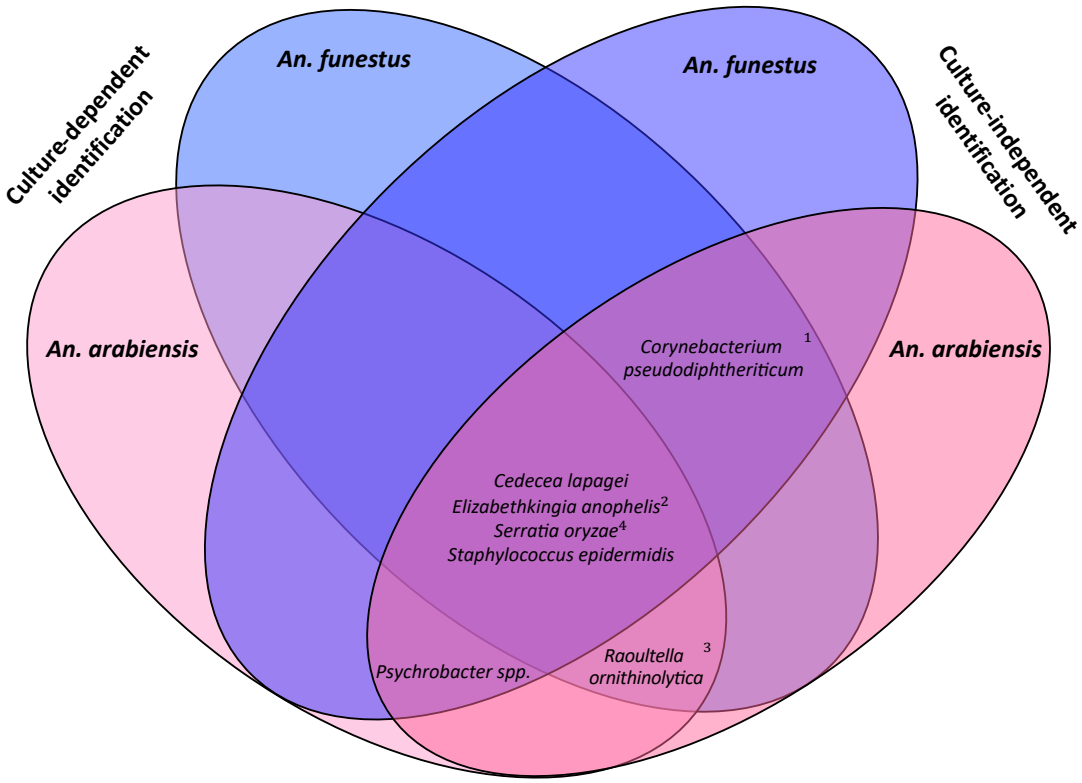

Figure L10: Bacteria identified by culture-dependent and culture-independent techniques from preserved colonised *An. arabiensis* and *An. funestus*. Mosquitoes were preserved on silica or in RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*C. propinquum* and *C. pseudodiphtheriticum*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [3]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [4]*S. fonticola* and *S. oryzae.*

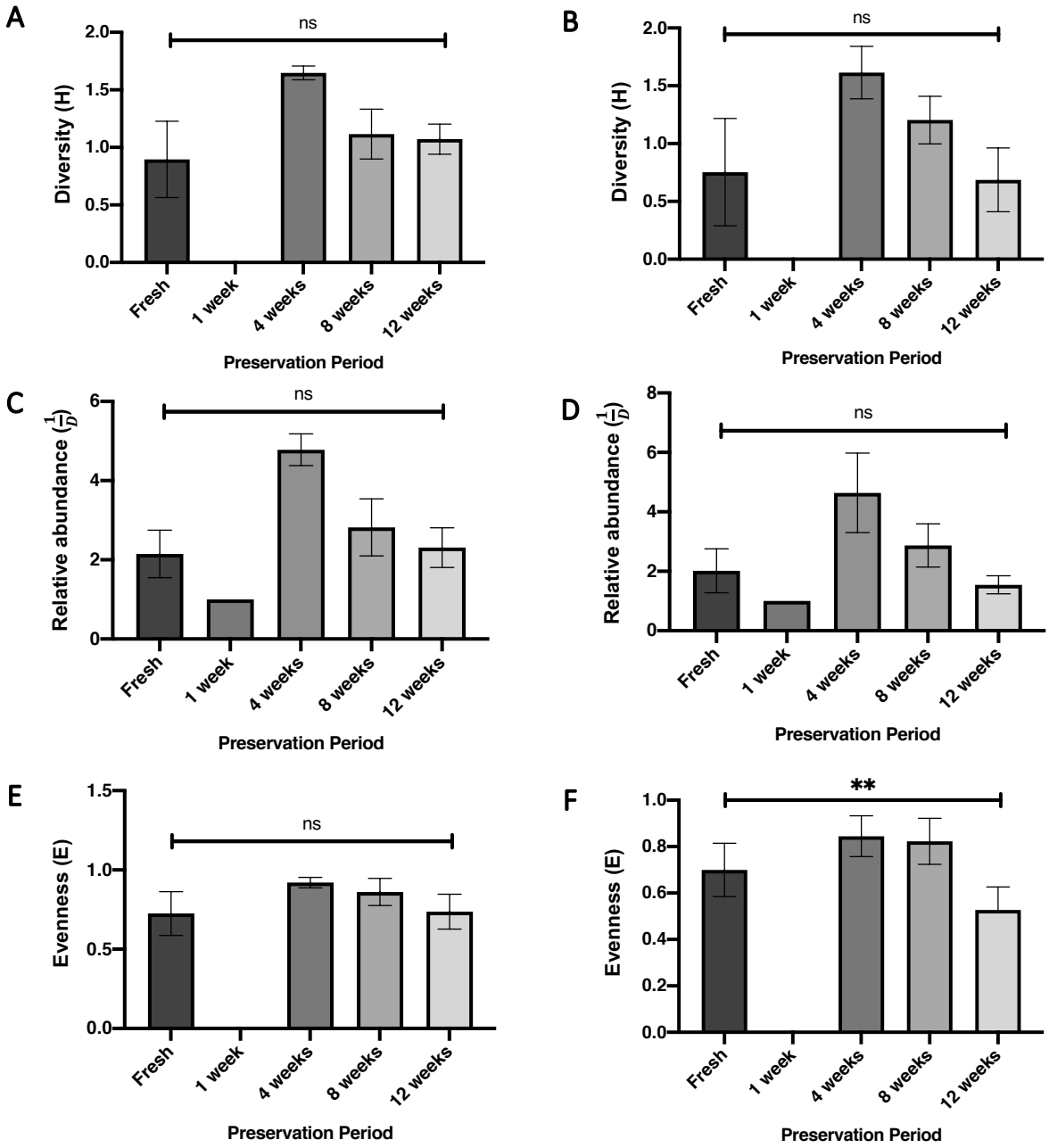

Figure M1: Comparison of diversity indices from fresh and preserved (A, C, E) *An. arabiensis* and (B, D, F) *An. funestus* identified by culture-dependent techniques. Means and standard deviations were calculated for the following indices: (A, B) Shannon-Wiener (H), (C, D) Simpson's reciprocal ($\frac{1}{D}$), and (E, F) Pielou's evenness (E). GraphPad Prism v8.4.3 was used to determine significance at 95% confidence.

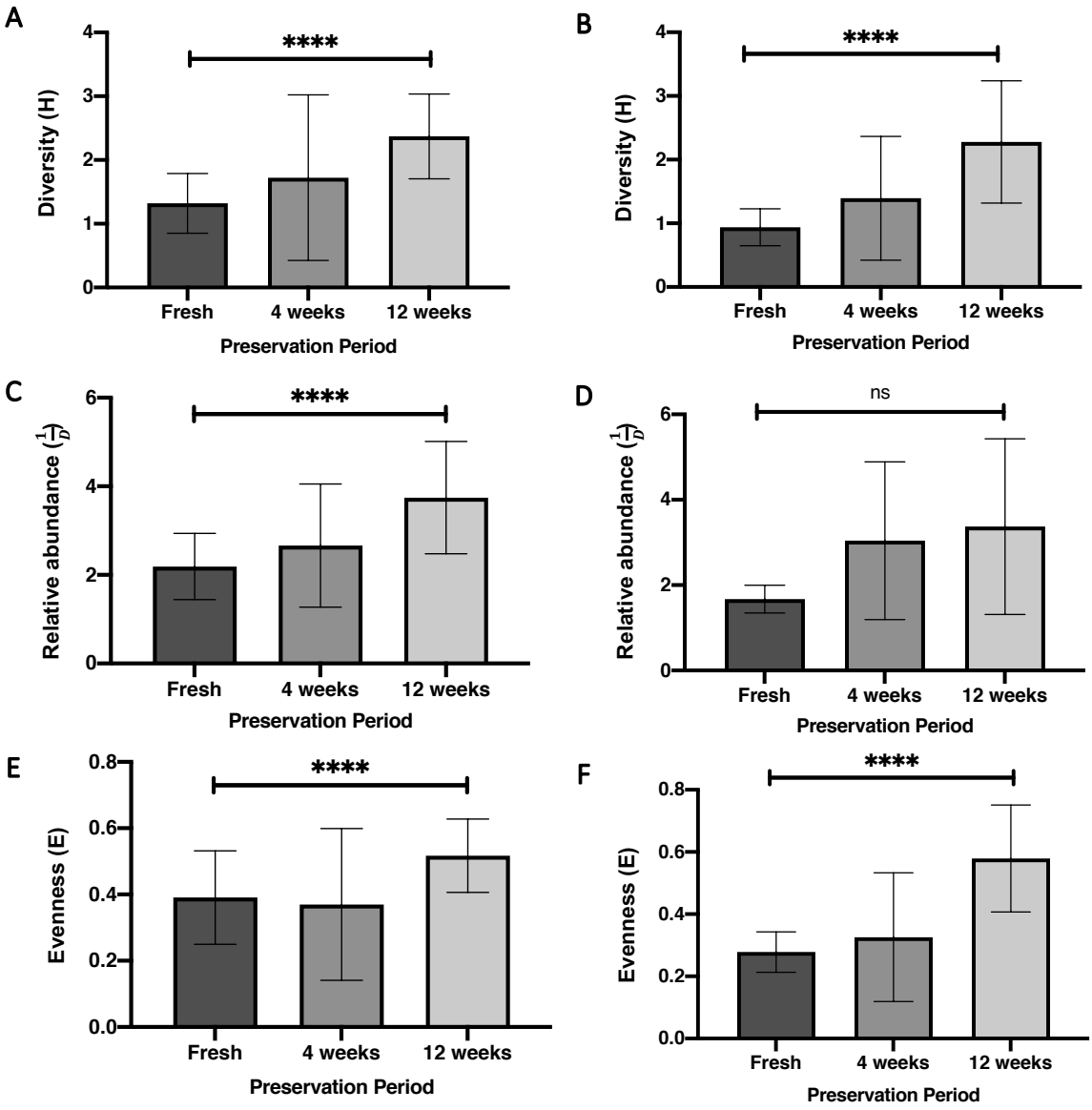

Figure M2: Comparison of diversity indices from fresh and preserved (A, C, E) *An. arabiensis* and (B, D, F) *An. funestus* identified by culture-independent techniques. Means and standard deviations were calculated for the following diversity indices: (A, B) Shannon-Wiener (H), (C, D) Simpson's reciprocal ($\frac{1}{D}$), and (E, F) Pielou's evenness (E). GraphPad Prism v8.4.3 was used to determine significance at 95% confidence.

Table M1: Common bacteria identified from fresh and preserved *An. arabiensis* and *An. funestus*. Bacteria from fresh mosquitoes were compared with bacteria from preserved mosquitoes at different preservation time periods. Bacteria were identified by culture-dependent and culture-independent methods from fresh and preserved mosquitoes. Colours represent common bacteria between certain preservation time periods. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*D. acidovorans* and *D. tsuruhatensis*; [4]*S. fonticola* and *S. oryzae*; [5]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; and [6]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*.

Common between	Bacterial Species	Fresh				1 week of Preservation		4 weeks of Preservation				8 weeks of Preservation		12 weeks of Preservation			
		An. arabiensis		*An. funestus*		*An. arabiensis*	*An. funestus*	*An. arabiensis*		*An. funestus*		*An. arabiensis*	*An. funestus*	*An. arabiensis*		*An. funestus*	
		Culture-dependent	Culture-independent	Culture-dependent	Culture-independent	Culture-dependent	Culture-dependent	Culture-dependent	Culture-independent	Culture-dependent	Culture-independent	Culture-dependent	Culture-dependent	Culture-dependent	Culture-independent	Culture-dependent	Culture-independent
Fresh and 4 weeks	*Brucella ovis*		x								x						
	Hafnia psychrotolerans		x		x				x								
	*Pseudomonas grimontii**	x	x	x	x				x		x						
	*Pseudomonas marginalis**		x								x						
	Pseudomonas soli		x		x				x		x						
	Reyranella aquatilis		x						x								
	*Sphingobacterium multivorum**	x	x						x								
	*Staphylococcus hominis**	x		x				x		x		x					
	*Stenotrophomonas maltophilia**				x						x						
Fresh and 8 weeks	*Pseudomonas extremorientalis**	x											x				
	*Pseudomonas libanensis**	x		x									x				
	*Psychrobacter sp**	x										x					

Fresh and 12 weeks	*Acinetobacter seohaensis*				x												x
	Gluconobacter japonicus		x												x		
	Microbulbifer maritimus		x												x		
Fresh, 4 weeks and 8 weeks	*Klebsiella variicola**	x		x					x			x					
	*Staphylococcus capitis**			x						x		x					
Fresh, 4 weeks and 12 weeks	*Acetobacter suratthaniensis*				x				x		x						x
	*Acinetobacter johnsonii**				x				x		x				x		x
	*Acinetobacter lwoffii**				x				x		x				x		x
	*Acinetobacter pittii**				x				x		x						x
	*Actinomyces oris**		x						x								x
	*Aeromonas hydrophila** [1]	x	x	x	x				x		x				x		x
	Aerosakkonema funiforme		x						x		x				x		x
	Asaia krungthepensis		x		x				x		x				x		x
	Brevundimonas albigilva		x						x		x				x		x
	*Cedecea lapagei**			x				x	x	x	x			x	x	x	x
	Chryseobacterium arthrosphaerae				x				x								x
	*Chryseobacterium gleum**			x						x							
	*Corynebacterium aurimucosum**				x				x		x				x		x
	Corynebacterium gottingense		x						x		x				x		x
	*Corynebacterium kroppenstedtii**		x								x				x		x
	*Corynebacterium pseudodiphtheriticum** [2]				x				x		x				x		x
	*Corynebacterium tuberculostearicum**				x				x		x				x		x

	Cutibacterium acnes				x				x		x				x		x
	Delftia tsuruhatensis [3]				x				x		x				x		x
	Dolosigranulum pigrum				x				x		x				x		x
	Empedobacter falsenii				x				x		x				x		x
	*Enterobacter cloacae**		x	x	x				x		x				x		x
	Gemella taiwanensis				x				x		x				x		
	Gluconobacter frateurii		x		x				x		x				x		x
	*Herbaspirillum huttiense**	x		x	x				x		x				x		x
	Kaistia terrae				x						x				x		x
	*Lactobacillus crispatus**				x				x		x				x		x
	Leucobacter iarius		x		x				x		x				x		x
	Massilia suwonensis				x				x		x				x		x
	Methylobacterium phyllostachyos		x						x						x		x
	*Microbacterium maritypicum**		x		x				x		x				x		x
	Paracoccus aerius		x		x				x		x				x		x
	Pedobacter solisilvae		x		x						x				x		x
	Phyllobacterium myrsinacearum		x		x				x		x				x		x
	*Pseudomonas aeruginosa**				x				x		x				x		x
	Pseudomonas geniculata		x		x				x		x				x		x
	Pseudomonas lactis				x				x		x				x		x
	*Pseudomonas veronii**		x		x				x		x				x		x
	Psychrobacter faecalis		x		x				x		x				x		x
	Rhizobium endophyticum				x				x		x				x		x
	Robbsia andropogonis				x						x						x

	*Rothia dentocariosa**				x				x		x				x		x
	*Serratia marcescens**				x				x		x				x		x
	Streptococcus periodonticum		x						x		x				x		x
	Streptococcus thermophilus		x		x				x		x				x		x
	*Yersinia aldovae**		x		x				x		x				x		x
	*Yersinia enterocolitica**		x		x				x		x				x		
Fresh, 8 weeks and 12 weeks	*Ewingella americana**			x									x	x		x	
Fresh, 4 weeks, 8 weeks and 12 weeks	*Rahnella aquatilis**	x		x						x		x	x	x		x	
	Serratia oryzae [4]	x	x	x	x				x	x	x			x	x	x	x
Fresh and all weeks	*Elizabethkingia anophelis* [5]	x	x	x	x	x	x	x	x	x	x		x		x	x	x
	*Raoultella ornithinolytica** [6]	x	x	x	x	x		x		x		x	x	x		x	
	*Staphylococcus epidermidis**	x	x	x	x	x		x	x	x	x	x	x		x		x

Table M2: Bacteria unique to fresh or preserved *An. arabiensis* and *An. funestus* at different preservation periods. Bacteria were identified by culture-dependent and culture-independent methods from fresh and preserved mosquitoes. Colours represent bacteria unique to certain preservation time periods. Bacteria indistinguishable by MALDI-TOF MS include [1]*E. coli* and *E. fergusonii*.

Unique to	Bacterial Species	Fresh		4 weeks of Preservation				8 weeks of Preservation	12 weeks of Preservation		
		An. arabiensis	*An. funestus*	*An. arabiensis*		*An. funestus*		*An. funestus*	*An. arabiensis*	*An. funestus*	
		Culture-dependent	Culture-dependent	Culture-dependent	Culture-independent	Culture-dependent	Culture-independent	Culture-dependent	Culture-independent	Culture-dependent	Culture-independent
Fresh only	*Aeromonas sanarellii*	x									
	Aeromonas taiwanensis	x									
	*Bacillus infantis**		x								
	Caenispirillum bisanense		x								
	*Chryseobacterium arachidis**		x								
	*Lactobacillus coryniformis**		x								
	*Pseudomonas antarctica**	x									
	*Pseudomonas azotoformans**	x									
	Pseudomonas canadensis	x									
	*Pseudomonas otitidis**		x								
	Pseudomonas palleroniana		x								
	*Pseudomonas putida**	x									
	Pseudomonas tolaasii	x									
	Siphonobacter aquaeclarae	x									
	*Staphylococcus aureus**	x									
	*Staphylococcus caprae**	x									
	*Staphylococcus haemolyticus**	x	x								

	*Staphylococcus pasteuri**	x									
	Yersinia entomophaga	x									
	Yersinia wautersii	x									
4 weeks only	*Aciditerrimonas ferrireducens*				x						
	*Acinetobacter baumannii**				x						
	Actinomyces polynesiensis						x				
	Aggregatibacter segnis						x				
	Alloprevotella rava				x		x				
	Amaricoccus macauensis				x						
	Aminobacter niigataensis				x		x				
	Anaerococcus nagyae						x				
	Anaerofilum pentosovorans						x				
	Anaerostipes hadrus						x				
	Ancylobacter polymorphus				x						
	*Aquincola tertiaricarbonis**						x				
	*Arthrobacter woluwensis**			x							
	Azoarcus olearius				x						
	*Bacillus cohnii**				x						
	Bacillus infernus						x				
	Bacillus velezensis				x						
	*Bacteroides coagulans**				x						
	Blastocatella fastidiosa				x		x				
	*Brochothrix thermosphacta**				x						
	*Cedecea neteri**			x		x					
	Cellulosilyticum lentocellum						x				
	Chryseobacterium anthropi				x						
	*Chryseobacterium hominis**				x						

	Chryseobacterium profundimaris						x				
	Chryseobacterium shandongense				x						
	Chryseobacterium zeae				x						
	Chthoniobacter flavus						x				
	*Clostridium colinum**				x						
	Clostridium swellfunianum				x						
	Curvibacter fontanus				x						
	Deinococcus aetherius				x						
	Desulfobulbus oligotrophicus				x						
	*Dialister micraerophilus**				x						
	Duganella zoogloeoides				x						
	Ferruginibacter paludis				x						
	Flavobacterium granuli				x						
	Fontimonas thermophila						x				
	Fretibacterium fastidiosum				x						
	*Gardnerella vaginalis**				x						
	Geminicoccus roseus				x						
	Gemmatimonas aurantiaca				x						
	Hartmannibacter diazotrophicus				x						
	Hydrogenophaga carboriunda						x				
	Isoptericola cucumis				x		x				
	Jeotgalicoccus schoeneichii				x						
	*Kingella oralis**				x						
	Lactobacillus silagincola				x						
	*Lactococcus lactis**				x		x				
	Larkinella arboricola				x						
	*Lautropia mirabilis**				x						

	*Leuconostoc mesenteroides**				x						
	Lyticum flagellatum				x						
	Marinoscillum luteum				x						
	Marmoricola scoriae						x				
	Mediterranea massiliensis						x				
	Microbacter margulisiae				x						
	Microlunatus lacustris				x						
	Microvirga subterranea				x						
	Mixta calida				x						
	Moheibacter stercoris				x						
	*Moraxella nonliquefaciens**						x				
	Mucilaginibacter carri				x						
	*Mycobacterium gordonae**				x						
	Mycolicibacterium doricum				x						
	Nitratireductor aestuarii				x						
	Nocardioides caricicola				x						
	Novosphingobium arabidopsis				x						
	*Novosphingobium nitrogenifigens**				x						
	Olegusella massiliensis						x				
	Paenibacillus psychroresistens				x						
	*Parvimonas micra**				x						
	Parviterribacter multiflagellatus						x				
	Phyllobacterium endophyticum				x		x				
	*Porphyromonas catoniae**						x				
	Porphyromonas pasteri						x				
	Prevotella colorans				x						
	*Prevotella melaninogenica**				x		x				

	*Prevotella nanceiensis**				x		x				
	*Prevotella nigrescens**				x						
	*Prevotella stercorea**						x				
	Proteiniphilum acetatigenes				x						
	Pseudomonas chengduensis						x				
	*Pseudomonas orientalis**						x				
	Pseudoxanthomonas taiwanensis				x						
	Psychrobacter cibarius				x		x				
	*Ralstonia insidiosa**				x						
	Rhodoferax antarcticus						x				
	Rosenbergiella epipactidis						x				
	Roseomonas aquatica						x				
	*Ruminococcus gnavus**				x						
	*Solobacterium moorei**						x				
	Sphingomonas fennica						x				
	Spirosoma horti						x				
	*Staphylococcus sciuri**				x						
	Stenotrophomonas pavanii						x				
	Streptacidiphilus anmyonensis				x						
	Tetragenococcus halophilus				x						
	Thermomicrobium carboxidum						x				
	Thiohalocapsa halophila				x						
	Vicinamibacter silvestris						x				
12 weeks only	*Acetatifactor muris*								x		
	Acetobacterium carbinolicum										x
	Acinetobacter dispersus										x
	*Acinetobacter ursingii**										x

	*Actinomyces bowdenii**										x
	*Actinomyces israelii**										x
	Aliterella antarctica								x		
	Anoxybacillus tepidamans								x		x
	Aquabacter spiritensis										x
	Aquisphaera giovannonii										x
	Arcticibacter pallidicorallinus										x
	*Arthrobacter gandavensis**								x		x
	*Bacillus siralis**								x		
	Bacillus wiedmannii								x		x
	Bacteriovorax stolpii										x
	Bacteroides faecichinchillae										x
	*Bacteroides salyersiae**								x		
	*Bacteroides uniformis**								x		
	Bdellovibrio bacteriovorus										x
	Belnapia soli										x
	Bergeyella porcorum										x
	*Brevibacillus laterosporus**								x		x
	Brevundimonas lenta								x		
	Calidifontibacter indicus										x
	*Cardiobacterium valvarum**								x		
	Caryophanon latum										x
	Casaltella massiliensis								x		
	Caulobacter mirabilis										x
	Chelatococcus composti										x
	Clostridium saudiense										x
	Conexibacter arvalis										x

	*Corynebacterium appendicis**								x		x
	Corynebacterium crudilactis										x
	Corynebacterium otitidis										x
	Corynebacterium pollutisoli								x		x
	*Corynebacterium propinquum**									x	
	Curvibacter gracilis								x		
	Desemzia incerta										x
	Dulcicalothrix necridiiformans								x		
	Enterobacter mori								x		
	Eremococcus coleocola								x		
	Ezakiella massiliensis								x		
	Faecalibacterium prausnitzii								x		
	Flectobacillus roseus										x
	Gallicola barnesae										x
	Gellertiella hungarica										x
	Gemmatirosa kalamazoonesis										x
	Gilliamella apicola										x
	Haematobacter massiliensis								x		
	*Helicobacter pylori**								x		
	Iamia majanohamensis								x		
	Ilyomonas limi										x
	Intestinibacter bartlettii								x		
	Intestinimonas butyriciproducens										x
	Kineococcus xinjiangensis										x
	Kineosporia rhizophila										x
	Krasilnikovia cinnamomea								x		
	*Kytococcus sedentarius**										x

	Laceyella sacchari								x		
	Lacisediminimonas profundi										x
	Lactobacillus acetotolerans										x
	*Lactobacillus iners**										x
	*Lactobacillus ingluviei**										x
	Lactobacillus mellis										x
	*Lactobacillus panis**								x		
	Lactobacillus rodentium										x
	Legionella cardiaca								x		
	Leifsonia rubra								x		
	*Leminorella grimontii**										x
	*Leptotrichia goodfellowii**										x
	Levyella massiliensis										x
	Longimicrobium terrae										x
	Lysobacter oculi										x
	Massilia aurea								x		
	Megamonas funiformis										x
	Methyloceanibacter caenitepidi										x
	Methyloversatilis discipulorum								x		
	Moraxella porci										x
	*Morganella morganii**								x		x
	Murdochiella massiliensis										x
	Muribaculum intestinale										x
	Nakamurella flavida										x
	*Neisseria elongata**								x		
	Nocardioides glacieisoli								x		x
	Nocardioides halotolerans										x

	Nocardioides islandensis										x
	Nocardioides solisilvae										x
	Nordella oligomobilis										x
	Oblitimonas alkaliphila										x
	Oribacterium sinus								x		x
	Pantoea vagans										x
	Pedobacter trunci									x	
	Pedomicrobium ferrugineum										x
	*Pelomonas saccharophila**								x		x
	*Peptococcus niger**										x
	*Peptoniphilus coxii**										x
	Peredibacter starrii										x
	Phascolarctobacterium succinatutens								x		
	Phenylobacterium aquaticum								x		x
	Phenylobacterium muchangponense										x
	Phytobacter ursingii								x		
	Pleomorphomonas carboxyditropha								x		
	Polynucleobacter sinensis								x		
	Pontibacter humi								x		
	*Prevotella bivia**								x		x
	*Prevotella buccalis**								x		
	*Prevotella corporis**										x
	Prevotella jejuni								x		
	*Prevotella shahii**										x
	*Providencia vermicola**										x
	*Pseudomonas savastanoi**										x
	Pseudomonas xiamenensis										x

	Pseudonocardia oroxyli										x
	Rhizobium arenae								x		
	Rhizobium daejeonense										x
	Rhizobium zeae								x		x
	Rhodococcus pedocola										x
	Rubellimicrobium rubrum										x
	Rubrobacter spartanus										x
	Ruminococcus champanellensis										x
	Ruminococcus torques								x		
	Saccharothrix ghardaiensis								x		x
	Sandaracinus amylolyticus										x
	Schlegelella aquatica										x
	Singulisphaera acidiphila								x		
	Snodgrassella alvi								x		x
	Sphingobium rhizovicinum								x		
	Sphingomonas rhizophila										x
	Sphingorhabdus rigui										x
	Stenotrophobacter terrae										x
	*Streptococcus agalactiae**								x		x
	*Streptococcus sinensis**										x
	Streptomyces xanthophaeus								x		
	Streptosporangium jiaoheense										x
	Thermoanaerobaculum aquaticum										x
	Tidjanibacter massiliensis								x		
	Vibrionimonas magnilacihabitans								x		
	Vogesella mureinivorans										x
	Zoogloea resiniphila										x

4 and 12 weeks	*Abiotrophia defectiva**			x	x				x		x
	Achromobacter animicus				x						x
	Acidibacter ferrireducens						x		x		
	*Acinetobacter junii**				x		x		x		x
	*Acinetobacter schindleri**				x						x
	Amaricoccus kaplicensis				x		x		x		x
	*Anaerococcus octavius**						x				x
	*Anaerococcus prevotii**				x		x		x		x
	Anaerococcus provencensis				x		x		x		x
	Anoxybacillus flavithermus				x				x		x
	Aquabacterium parvum						x				x
	Atopostipes suicloacalis						x		x		x
	Bacillus cavernae				x						x
	*Bacillus coagulans**				x						x
	Bacillus hisashii				x				x		x
	Bacillus loiseleuriae				x						x
	Bellilinea caldifistulae				x						
	*Bifidobacterium pseudocatenulatum**				x				x		x
	Blastococcus aggregatus				x				x		x
	Bosea robiniae				x		x		x		x
	*Brachybacterium faecium**				x		x				x
	Bradyrhizobium namibiense				x		x		x		
	*Brevibacterium casei**				x				x		
	*Brevibacterium paucivorans**						x		x		x
	*Burkholderia ambifaria**				x		x				x
	Caulobacter profundus				x				x		
	Chryseobacterium aquifrigidense				x		x				x

	Chryseobacterium greenlandense				x		x		x		x
	Chryseobacterium jeonii				x				x		
	Chryseobacterium reticulitermitis				x						x
	Chryseobacterium taklimakanense						x		x		x
	Cloacibacterium normanense				x		x		x		x
	Clostridium saccharoperbutylacetonicum						x				x
	*Corynebacterium amycolatum**				x		x		x		x
	*Corynebacterium durum**				x		x				x
	*Corynebacterium falsenii**						x		x		x
	*Corynebacterium glaucum**						x		x		
	*Corynebacterium jeikeium**				x				x		x
	*Corynebacterium riegelii**				x		x		x		x
	*Corynebacterium sundsvallense**						x		x		
	*Corynebacterium testudinoris**				x						x
	*Corynebacterium ureicelerivorans**				x		x		x		x
	Deinococcus taklimakanensis				x		x		x		x
	Deinococcus xinjiangensis				x		x		x		x
	Dialister propionicifaciens				x						x
	*Enterococcus cecorum**				x		x		x		x
	*Escherichia fergusonii** [1]				x		x		x		x
	*Facklamia hominis**						x		x		
	*Finegoldia magna**				x				x		x
	Flavobacterium zhairuonensis				x						x
	Frateuria aurantia				x				x		x
	*Fusobacterium nucleatum**				x		x				x
	*Geobacillus stearothermophilus**				x						x

	Georgenia deserti						x				x
	Gordonia otitidis				x		x		x		
	*Granulicatella adiacens**				x				x		x
	*Granulicatella elegans**				x				x		
	*Haemophilus haemolyticus**				x		x		x		x
	*Haemophilus parahaemolyticus**				x				x		x
	*Haemophilus parainfluenzae**				x		x		x		x
	Ilumatobacter fluminis				x				x		x
	Kineothrix alysoides						x		x		
	*Kocuria palustris**						x				x
	Krasilnikoviella muralis				x						x
	Lachnoclostridium pacaense						x		x		
	Lacibacterium aquatile				x						x
	Lactobacillus aquaticus				x						x
	Lapillicoccus jejuensis						x		x		
	Lawsonella clevelandensis				x		x		x		x
	Leptotrichia buccalis				x		x		x		x
	Leptotrichia shahii				x						x
	Methylobacterium bullatum				x				x		x
	Methylorubrum populi				x				x		x
	*Micrococcus luteus**				x		x		x		x
	Microvirga brassicacearum						x		x		
	*Moraxella osloensis**				x		x		x		x
	Mycobacterium florentinum				x						x
	Mycolicibacterium iranicum				x				x		x
	*Neisseria mucosa**				x		x		x		x
	Nocardia coeliaca				x				x		x

	Nocardioides iriomotensis				x		x		x		x
	Paenibacillus silvae				x				x		x
	Paludibaculum fermentans				x						x
	Paracoccus hibiscisoli				x		x		x		x
	*Peptoniphilus asaccharolyticus**				x						x
	Peptoniphilus lacydonensis						x		x		x
	Peptostreptococcus stomatis				x		x				
	Phreatobacter oligotrophus				x				x		x
	*Porphyromonas bennonis**						x		x		
	*Prevotella timonensis**				x				x		x
	Pseudomonas deceptionensis				x				x		x
	*Pseudomonas stutzeri**				x				x		x
	Pseudonocardia yuanmonensis				x		x				x
	Psychrobacter aquimaris				x						x
	Rhizobium azooxidifex				x				x		x
	Rhodococcus sovatensis				x		x		x		x
	Rhodovulum kholense				x						x
	Romboutsia timonensis				x				x		x
	Roseomonas gilardii				x		x		x		x
	*Rothia mucilaginosa**				x		x		x		x
	Schaalia odontolytica				x				x		x
	*Serratia liquefaciens**				x				x		
	Shinella curvata						x		x		x
	Sphaerobacter thermophilus				x						x
	Sphingomonas kyeonggiensis				x		x		x		x
	Sphingomonas olei				x						x
	Sphingomonas palustris				x						x

	*Stenotrophomonas rhizophila**				x		x		x		x
	Streptomyces asenjonii				x						x
	Tepidimonas fonticaldi				x		x				x
	Thermicanus aegyptius				x				x		x
	Undibacterium squillarum				x				x		
	Variovorax boronicumulans						x		x		
	*Variovorax paradoxus**				x		x		x		x
	*Veillonella dispar**				x		x		x		x
	*Veillonella montpellierensis**						x		x		
	Zhizhongheella caldifontis				x				x		x
8 and 12 weeks	*Pseudomonas gessardii**							x		x	

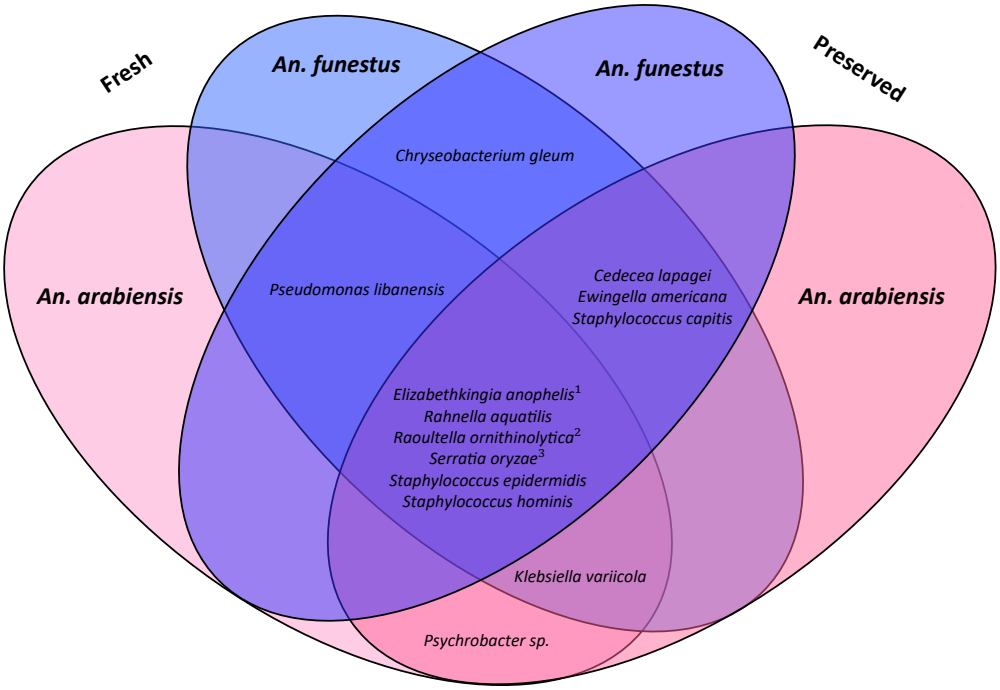

Figure N1: Common bacteria between fresh and preserved colonised *An. arabiensis* and *An. funestus* identified by culture-dependent techniques. Mosquitoes were preserved on silica or in RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [2]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [3]*S. fonticola* and *S. oryzae.*

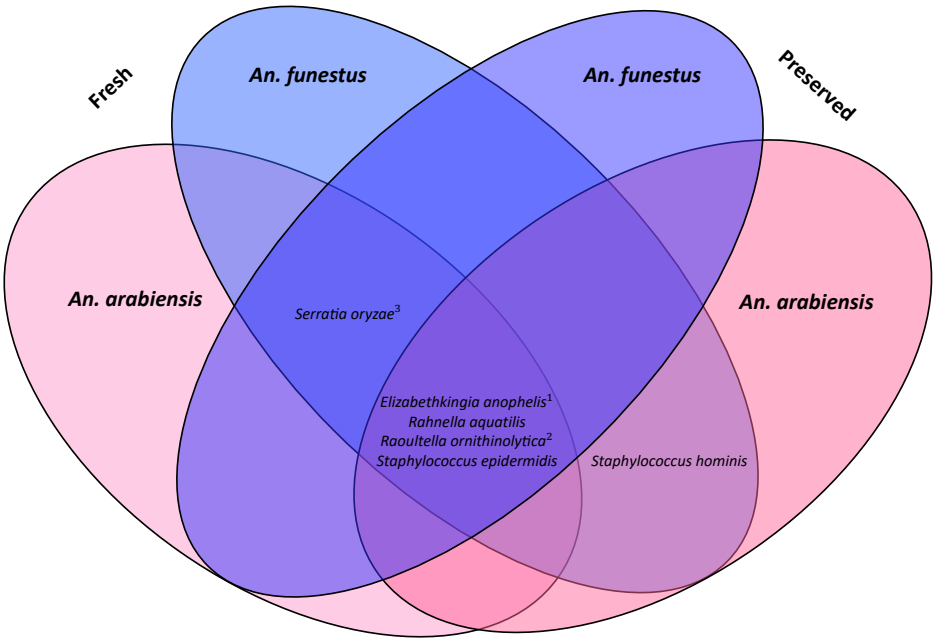

Figure N2: Bacteria identified by culture-dependent techniques from fresh and preserved *An. arabiensis* and *An. funestus* females. Bacteria indistinguishable by MALDI-TOF MS include [1]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [2]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [3]*S. fonticola* and *S. oryzae*.

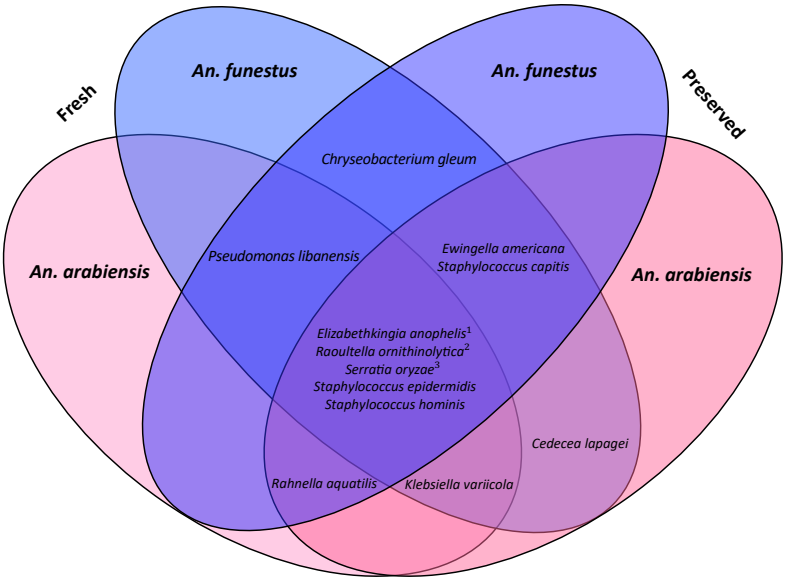

Figure N3: Bacteria identified by culture-dependent techniques from fresh and preserved *An. arabiensis* and *An. funestus* males. Bacteria indistinguishable by MALDI-TOF MS include [1]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [2]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [3]*S. fonticola* and *S. oryzae*.

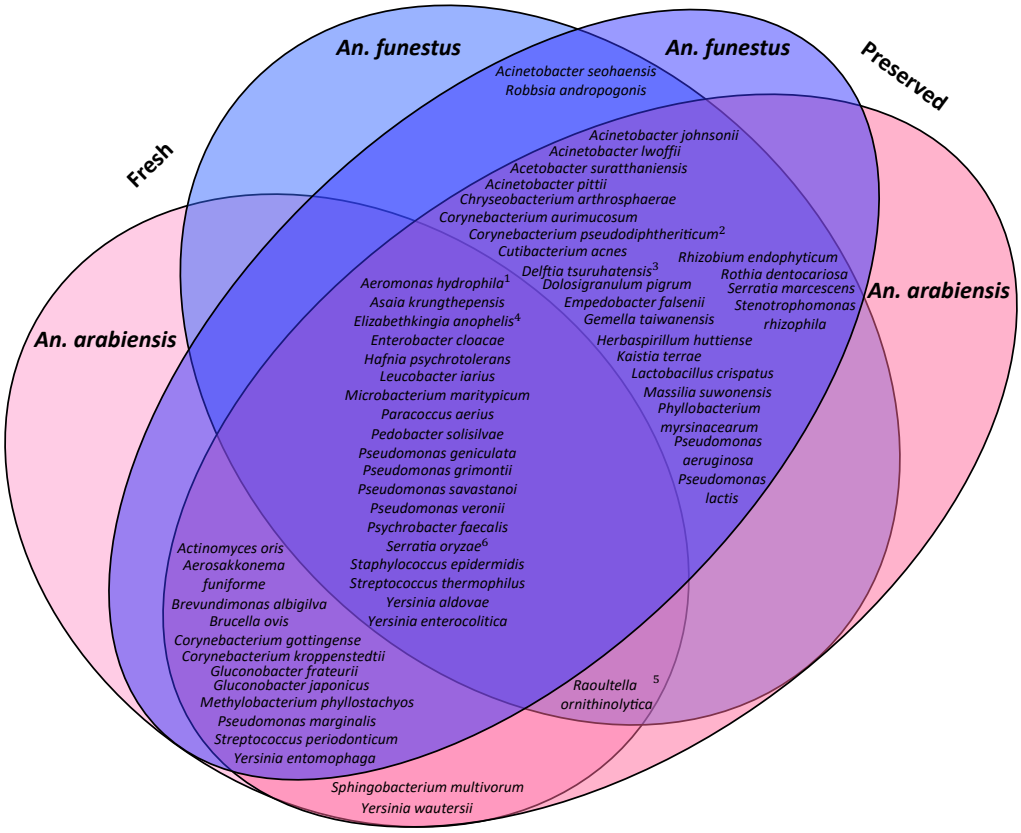

Figure N4: Common bacteria identified by culture-independent techniques between fresh and preserved colonised *An. arabiensis* and *An. funestus*. Mosquitoes were preserved on silica or in RNA*later*®. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*D. acidovorans* and *D. tsuruhatensis*; [4]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [5]*E. coli* and *E. fergusonii*; and [6]*S. fonticola* and *S. oryzae.*

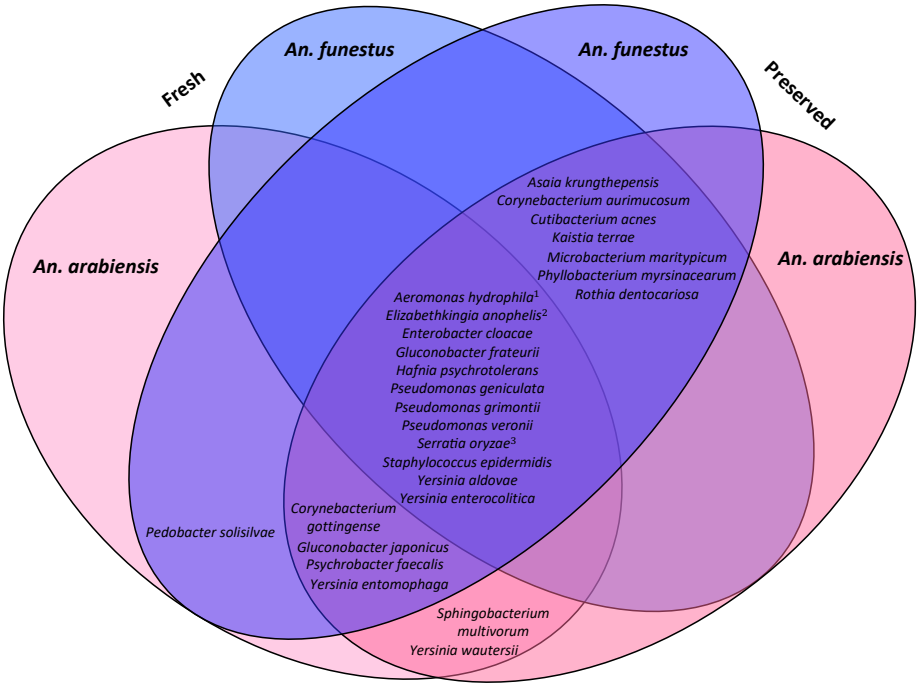

Figure N5: Bacteria identified by culture-independent techniques from fresh and preserved *An. arabiensis* and *An. funestus* females. Bacteria indistinguishable by MALDI-TOF MS include [1]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [2]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [3]*S. fonticola* and *S. oryzae*.

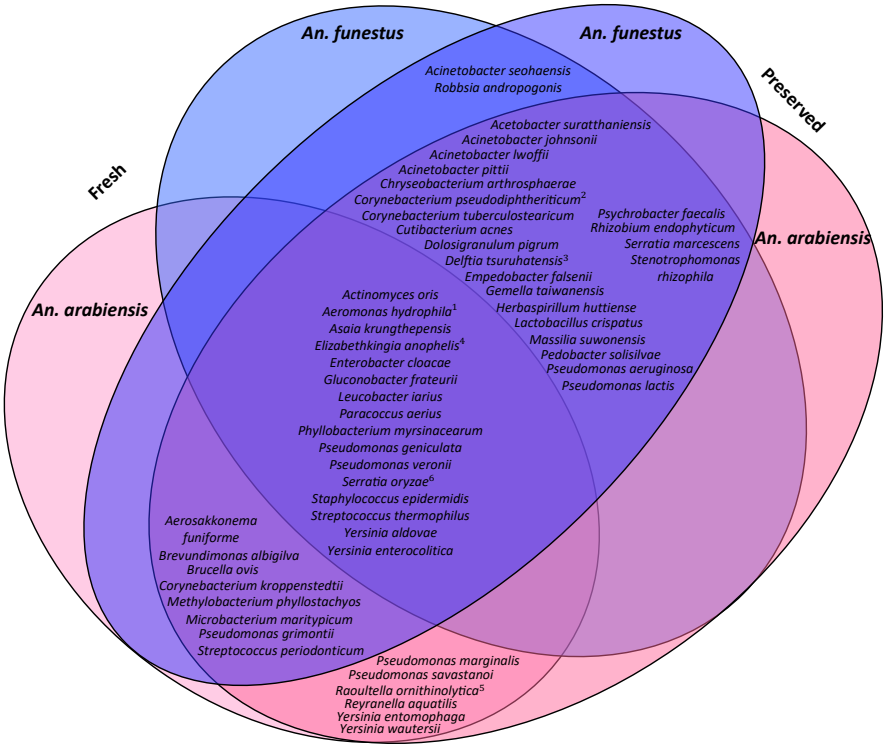

Figure N6: Bacteria identified by culture-independent techniques from fresh and preserved *An. arabiensis* and *An. funestus* males. [1]*A. hydrophila* and *A. veronii*, [2]*C. propinquum* and *C. pseudodiphtheriticum*, [3]*D. acidovorans* and *D. tsuruhatensis*, [4]*E. anophelis*, *E. meningoseptica*, and *E. miricola*, [5]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*, [6]*S. fonticola* and *S. oryzae*.

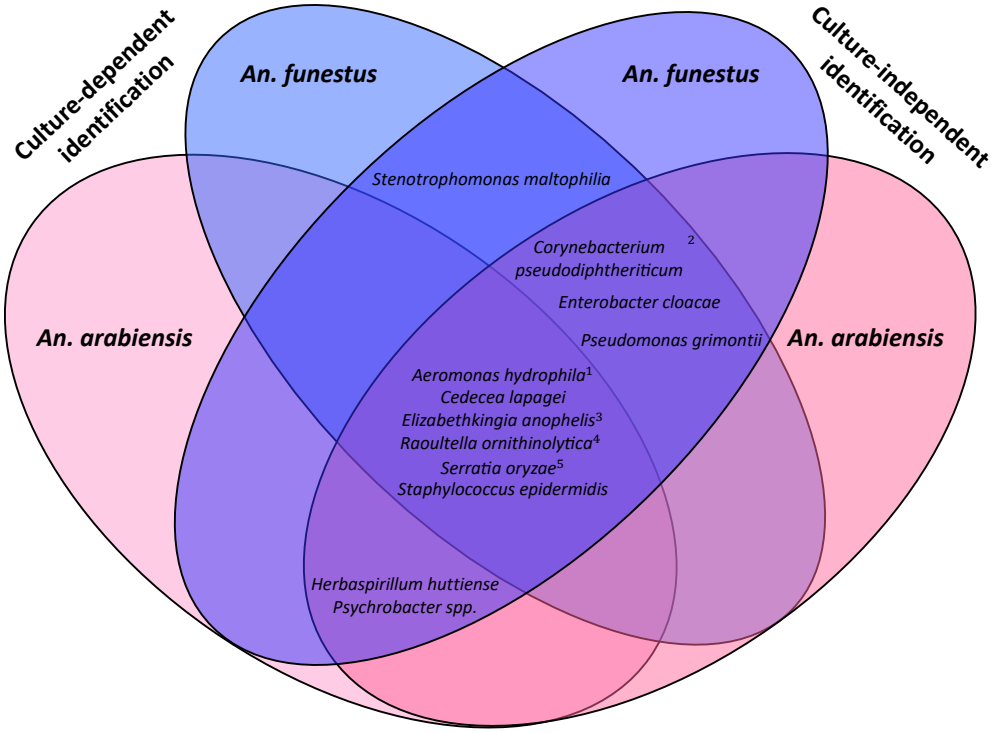

Figure N7: Bacteria identified by culture-dependent and culture-independent techniques from fresh and preserved colonised *An. arabiensis* and *An. funestus* females. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*C. propinquum* and *C. pseudodiphtheriticum*; [3]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [4]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [5]*S. fonticola* and *S. oryzae*.

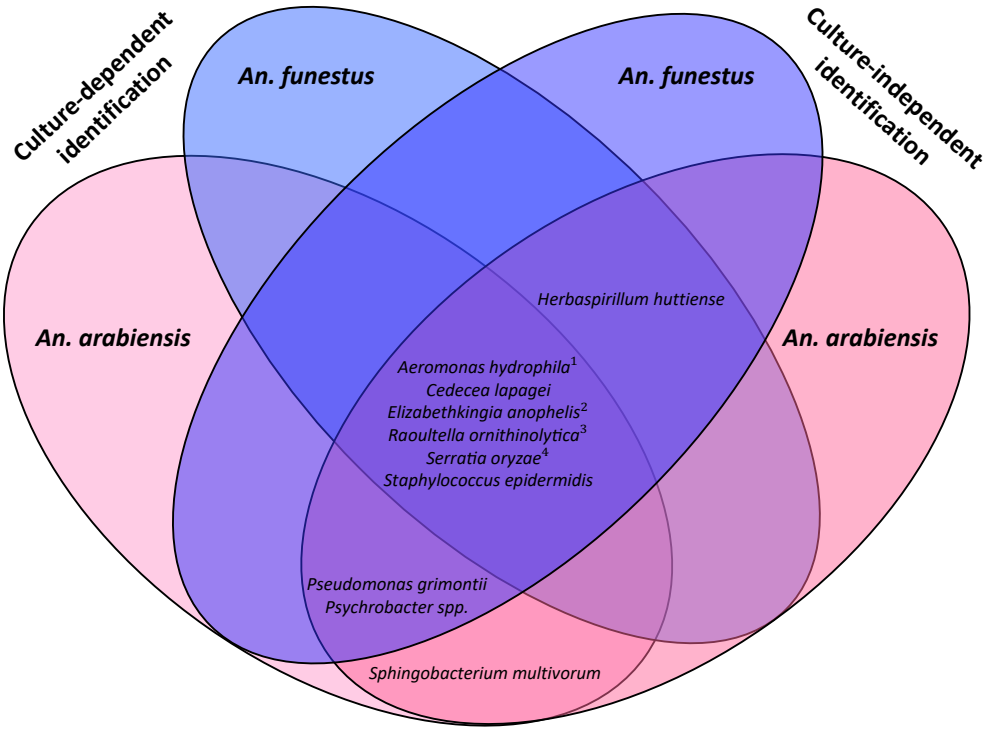

Figure N8: Bacteria identified by culture-dependent and culture-independent techniques from fresh and preserved colonised *An. arabiensis* and *An. funestus* males. Bacteria indistinguishable by MALDI-TOF MS include [1]*A. hydrophila* and *A. veronii*; [2]*E. anophelis*, *E. meningoseptica*, and *E. miricola*; [3]*K. oxytoca*, *R. ornithinolytica*, *R. planticola*, and *R. terrigena*; and [4]*S. fonticola* and *S. oryzae*.

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