ISOLATION AND IDENTIFICATION OF MICROFLORA FROM THE MIDGUT AND SALIVARY GLANDS OF LABORATORY REARED AND FIELD COLLECTED *ANOPHELES* SPECIES FROM SOME MALARIA ENDEMIC AREAS OF ETHIOPIA.

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A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfilment of the Requirement for the Degree of Master of Science in Zoological Sciences (Insect Sciences Stream)

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Isolation and identification of microflora from the midgut and salivary glands of laboratory reared and field collected *Anopheles* species from some malaria endemic areas of Ethiopia.

By

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A Thesis Presented to the College of Graduate Studies of the Addis Ababa University in Partial Fulfillment of the requirements for the Degree of Master of Science in Zoological Sciences (Insect Sciences Stream)

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ABSTRACT

Anopheles mosquitoes are of great importance to human health. They transmit pathogens including malaria parasites, filarial worms, and O'nyong-nyong and rift valley viruses. The numbers of studies have shown that, midgut and salivary gland microflora have an impact on malaria parasite burden through colonization mechanisms, involving either direct *Plasmodium* microbiota interaction or bacterial mediated induction of mosquito immune response. The objective of this study was to isolate and identify the microflora from the midgut and salivary gland of laboratory reared and field collected *Anopheles* species in some malaria endemic areas of Ethiopia. A total of twenty pools of mosquitoes, ten per pool (insectary), and ten pools, thirty per pool (field collected) were anesthetized by chloroform and dissected. 70% of ethanol was used for surface sterilization of mosquitoes followed by washing of each pool four times by 1x PBS and the environment. Each pool of dissected midgut and salivary gland samples was transferred in 3ml phosphate buffer saline, squashed and incubated in water bath until enriched in tryptic soya broth for 24hrs at 35±2^{o}c. After enrichment, a loopful of each sample was taken and inoculated on Blood, Chocolate, Mac Conkey and Sabouraud dextrose agar for 24 hrs. at 35±2^{o}c. Finally, the microbiota was isolated based on their colony characteristics and identified by conventional biochemical tests and automated VITEK 2 Compact Analyzer. All identified microbiota was stored in 20% tryptic soya broth with glycerol at -80 ^{o}c. From all field collected and insectaries, *Pseudomonas* 38 (29%) was found to be the dominant microbiota from all species. *Anopheles gambiae s.l* 77 (50.33%) had got the largest number of microbiota identified and *An. arabiensis* had identified diversified types of microbiota from the rest species. From this report, 40 genera of microbiota were identified and can be a milestone for studying relationship between microbiota and mosquitoes and for the development of a new malaria control strategy.

Keywords: *Anopheles* species, identification, malaria, microflora, midgut, salivary glands

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LIST OF ABBREVIATIONS AND ACRONYMS

ACE	Abundance-based Coverage Estimator
AhpC	Alkyl hydroperoxide reductase
ATTC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
CDC	Center for Disease Control and Prevention
CLSI	Clinical and Laboratory Standard Institute
EPHI	Ethiopian Public Health Institute
GltA	Glutamate synthase
HLC	Human Landing Catch
IRB	Institutional Review Board
KIA	Kligler Iron Agar
LB agar	Luria Bertani agar
LDC	Lysine Decarboxylase
Mac	MacConkey agar
MAB	Midgut associated bacteria
NCBI	National Center for Biotechnology Information
Omp A	Outer Membrane Protein A
OTUs	Operational Taxonomic Units
PBS	Phosphate Buffer Saline
RDP	Remote Desktop Protocol
RNA	Ribonucleic acid
rRNA	ribosomal Ribonucleic acid
Sca4	Spinocerebellar ataxia 4
SDA	Sabouraud Dextrose Agar
SIM	Sulfide Indole Motility
TIDRC	Tropical and Infectious Disease Research Center
TSIA	Triple Sugar Iron Agar
VP	Voges-Proskauer test

INTRODUCTION

1.1 Background

Malaria is a mosquito borne infectious disease of humans and other animals caused by parasitic protozoans (a type of single cell microorganism) of the *Plasmodium* type. Commonly, the disease is transmitted by the bite of an infected female *Anopheles* mosquito. This bite introduces the parasites from the mosquito's saliva into a person's blood (Caraballo and King, 2014). According to the most recent classification of mosquitoes, the family culicidae (Diptera) includes two sub families, 11 tribes, 113 genera and 3531 species in the world fauna and the genus *Anopheles meigen* includes seven subgenera and at least 465 species (Harbach, 2007).

Certain species of *Anopheles* are involved in the transmission of various arboviral and filarial diseases to humans and domestic animals and/or are important for their biting in different parts of the world, but the most important disease transmitted by them is malaria. About 70 *Anopheles* species are malaria vectors in which about 40 are important vectors (Service, 1993). Malaria parasite transmission depends on the availability of competent mosquito vectors (Omumbo *et al*., 1998). However, commensal bacteria in the mid gut can reduce the ability of mosquitoes to transmit disease, either by having direct anti-parasite effects or by stimulating basal immune responses of the insect host. As different bacteria have different effects on parasite development, the composition of bacterial community in the mosquito gut is likely to affect the probability of disease transmission (Osei-Poku *et al*., 2012). Though, these studies have not identified the causal mechanisms through which the presence of bacteria negatively impacts on malaria parasite development (Dong *et al*., 2009).

A few studies have been performed to identify bacterial species in field collected *Anopheles* mosquitoes, using microbe culturing techniques. As a result of this, the wide range of bacteria like *Pseudomonas cepacia*, *Entrobacter agglomerans*, and *Flavobacterium* species were found in high abundance in laboratory reared *An. stephensi*, *An. gambiae* and *An. albimanus* mosquitoes (Pumpuni *et al*., 1996). Further, the gut microflora varied depending up on the ecological niche or geographical location of mosquitoes. Straif and his colleagues (1998) identified *Enterobacter agglomerans* and *Escherichercia coli* as the most frequently isolated bacteria from mid gut of *An. gambiae* and *An. funestus* mosquitoes caught in Kenya and Mali.

In addition to this, another study was done in Kenya showed that; the mosquito ecosystem harbors a complex microbiota with a dynamic taxonomic composition at different stages of the insect's life. Taxa from the family *Entrobacteriaceae* were selectively enriched by the blood meals in adult mosquitoes (Wang *et al*., 2011). Numerous surveys of mosquito midgut-associated bacteria (MAB) in laboratory and wild *Anopheline* mosquitoes have been performed, and common bacterial genera (*Enterobacter, Pseudomonas, Pantoea*, and others) have been identified (Terenius *et al*., 2008), with some of these bacteria closely associated with *Anopheles* mosquitoes (Dong *et al*., 2006). Even though, the bacteria are the symbiont in the mid gut of *Anopheles* mosquitoes, they are important source for the food for mosquito's larvae (Yee *et al*., 2004). However, the larvae survived on the sterile fish food added for several weeks (Lindh *et al*., 2005).

The midgut flora of field caught (Abalain-Colloc *et al*., 1998) and laboratory reared (Dieng *et al*., 2010) mosquitoes has been screened. Many and diverse bacterial species were identified in these studies. However, bacterial species from the Gamma- Proteobacteria class were most frequently identified; in addition, the majority of these belong to the *Entrobacteriacea* family. Bacterial species from this class and family have frequently been found and classified as symbionts in insects (Pontes and Dale, 2006). Similarly, a study done by Djadid and his colleagues for the identification of the midgut microbiota of *An. stephensi* and *An. maculipennis* were investigated. The majority of bacteria belong to the *Gammaproteobactria* class (Djadid *et al*., 2011).

Beyond these few studies, not much is known about the source of the adult bacterial flora, the quantitative and qualitative changes in this flora during mosquito development, and the impact of midgut bacteria on the plasmodium sporogenic development. In *P. falciparum*-infected *An. stephensi*, it showed that gram-negative bacteria in high concentrations block oocyst formation. Thus, the potential exists to use mosquito midgut bacteria to modulate vector competence (Pumpuni *et al*., 1993). Gram-negative bacteria seem to have a stronger effect on plasmodium development than gram-positive, possibly due to the different immune responses they elicit from the mosquito (Aguilar *et al*., 2005). In agreement, several of the immune responses affecting *Plasmodium* development also control bacterial infection in mosquitoes and it was suggested that ookinete invasion of the midgut epithelium facilitates exposure to bacteria and hence increase the immune response mediated by bacteria (Dong *et al*., 2006). In contrast to the studies on

inhibition of parasite development by presence of bacteria, on study reported a correlation between the presence of a gram-negative midgut bacteria (*Pseudomonas* sp.) and enhanced development of *Plasmodium* (Jadin *et al*., 1996). Therefore, the differences in the gut microbiota observed between individuals with in a host species could be causing variation in vector competence.

In Ethiopia, to our knowledge, no research in the isolation and identification of microflora in the midgut and saliva of *Aanopheles* mosquitoes has been done so far. Consequently, the transmission of the parasite which is influenced by resident microbiota in the midgut of the vector could not to be controlled. Thus, the microbiota which resides in the malaria vector can enhance or suppress the development of the parasites that to be transmitted to the mammalian host. Therefore, isolation and identification of microflora in the midgut and salivary gland of *Anpheles* mosquitos' species help to generate data for further analysis of microbiota interaction with the development of plasmodium parasite which is part of integrated malaria control in endemic areas. Therefore, the aim of this study is to assess, isolate and identify bacterial microflora from the midgut and salivary gland of laboratory reared and filed collected *Anopheles* mosquitoes in some malaria endemic areas of Ethiopia.

1.2 Statement of the problem and justification

The globally malaria cases were 216 million in 2016 of which 90% occurred in Africa region, followed by South East Asia region (3%) and Eastern Mediterranean region (2%). The number of deaths due to malaria was 445,000 globally in 2016. Most of the deaths were in WHO African region (91%), followed by the WHO South East Asia region (6%) and least in Eastern Mediterranean region. Moreover, the number of malaria deaths in children aged less than 5 years estimated 285, 000 in 2016. Most of the deaths occurred in Africa especially in sub-Saharan Africa. As a result of these, malaria was the fourth highest cause of death, accounting for 10% of child death in Sub-Saharan African countries.

Mosquito-borne diseases such as malaria, dengue fever and filariasis cause an enormous health burden to people living in tropical and subtropical regions of the world. Despite years of intense effort to control them, many of these diseases are increasing in prevalence, geographical distribution and severity, and options to control them are limited. The use of chemical mosquito control means is considered a fundamental part of early launched malaria eradication program. However, it has its own limitation on the development of resistance of the vector, changing the vector behavior as avoidance of contact with chemical, adversely affect the environment, co-use of chemicals like in agriculture causes resistance and the less potent and effective of the chemical against mosquito vector. Moreover, application of insecticides for controlling the mosquito vector has a negative and devastating effect on the non-target and the natural ecosystem of the environment. Thus, the aim of this study is to assess, isolate and identify the microflora from the midgut and saliva of lab-reared and field collected *Anopheles* species in some malaria endemic areas of Ethiopia.

As a result of this, isolation and identification of microflora in the midgut and salivary gland of mosquitoes will provide us potential isolates that could be used for further molecular analysis and expression of novel genetic products in vector species towards novel biological vector control strategy in malaria endemic areas of Ethiopia. Isolation and identification of microflora from the midgut and salivary gland of *Anopheles* mosquitoes and their association with the development of malaria parasites to the infective sporozoites stage in the mosquito will potentially explain the difference in the vectorial capacity of vector and non vector species in the transmission of malaria. These results would be exploited to suppress parasite development and block malaria transmission as part of integrated malaria control in endemic areas of Ethiopia.

1.3 Objectives

1.3.1. General objective

To isolate and identify the microflora from the midgut and salivary gland of laboratory reared and field collected *Anopheles* species from some malaria endemic areas of Ethiopia.

1.3.2. Specific objectives

- ✓ To isolate microflora from the midgut and salivary gland of laboratory reared and field collected *Anopheles* species.
- ✓ To identify microflora from the midgut and salivary gland of laboratory reared and field collected *Anopheles* species.
- ✓ To compare the microflora identified from laboratory reared and field collected *Anopheles* species.

2. LITRATURE REVIEW

2.1 Bacteria as mosquito food

Microorganisms and particulate organic detritus are generally constituting the major food sources for mosquito larvae and adults (Merritt *et al*., 1992). This interaction was observed in nature between mosquito species and microflora commonly found in their breeding environment. This was investigated in *Culex pipiens* survival rate of larval stages, pupae and adults exposed to a variety of microorganisms in laboratory conditions and assessed its transmission to the offspring by those organisms that secured development up to adult hood. Three complementary experiments were designed: explore the nutritional value of yeasts and other microorganisms during *Cx. pipiens* development; the transstadial transmission of yeast to the host offspring and relevance of all these microorganisms in female choice for oviposition substratum. The yeast *Saccharomyces cerevisiae* was proved to be the most nutritional diet but despite the highest survival rate, vertical transmission to offspring was never confirmed (Diaz-Nieto *et al*., 2016).

In addition, during oviposition trials, none of the gravid females was attracted to the yeast substratum. However, two bacterial strains *Klebsiella* species and *Aeromonas* were preferred oviposition media and managed to feed neonates until molting the 2nd instar larvae. Yeast was considered to be the most supportive diet for completing the mosquito development (Diaz-Nieto *et al*., 2016). Besides, *Aedes aegypti*, *An. gambiae*, *An. quadrimaculatus*, and *Cx. quinquefasciatus* larvae were developed in the presence of algae in water bodies of their habitat (Lindh, 2007).

The diversity and abundance bacteria of *Anopheles* mosquito larvae in breeding habitat of Asendabo, South western Ethiopia was analyzed. This study was investigated that, the bacteria population in this habitat was dominated by the species of *Bacillus*, *Pseudomonas*, *Micrococcus* and *Serratia*. Besides, the mosquito breeding places have particulate organic matter including microorganisms has a nutriment quality support proliferation of large array of bacteria which results in increased larval nourishment. As a result, increased malaria transmission in this area could thus be caused as the bacteria serves as a source of nutrients for mosquito larvae for the continuation of mosquito life cycle (Chukalo and Abate, 2017). This source of food for mosquito supported by the different quantities of abundant taxonomic groups of bacteria and their

exposure to nutriment source can alter abundance in mosquito aquatic habitats and its contribution to malaria transmission.

2.2 Transstadial transfer of bacteria

Paternal transmission of symbiotic bacteria in the male reproductive organs makes this additional transmission route worth being investigated. Bacteria of the genus *Asaia* are associated with different species of malaria vectors and are located in the midgut, salivary glands and reproductive organs of female and male mosquitoes. These bacteria can be transmitted from male reproductive organ during the mating of *Anopheles stephensi* mosquito. Subsequently, the bacteria acquired by the female are vertically transmitted to the progeny so that it is possible to use male mosquitoes, which do not bite, to spread *Asaia* strains interfering with malaria transmission (Damiani *et al.*, 2008).

Mosquitoes are holometabola that undergo four gradual stages of metamorphosis: egg, larvae and pupae are aquatic, whereas adult mosquitoes live in terrestrial environments. The microflora that is acquired from the surrounding environment is thus likely to differ during the insect life cycle. At the larval stage, individuals consume bacteria and plankton as a nutritive resource but not found in adult stages. Larval and pupal stages have cyanobacteria were abundant but modified during molting. This phenomenon could explain why the proportions of different bacteria classes or phyla alter drastically between immature and adult stages. For instance, the number of bacteria in larvae and pupae was three fold higher than adults (Wang *et al*., 2011).

The comparative studies of bacteria composition between stages have been done in *Anopheles* mosquitoes, in which transstadial maintenance of some bacterial genera such as *Acinetobacter*, *Bacillus*, *Enterobacter*, *Staphylococcus*, *Pseudomonas* and *Serratia* species has been observed (Chavshin *et al*., 2012). Evidence of a transstadial transfer of the symbiotic bacterium *Asaia* from larvae to pupae and from pupae to adults of *An. stephensi* also been obtained. For long time, such bacterial transfers in mosquitoes have been controversial issues (Pumpuni *et al*., 1996). The mechanism by which the bacteria in the genus *Asaia*, are transmitted to the offspring is through egg-smearing, according to which the symbiotic bacteria are smeared by the mother on the surface of eggs. This transmission process has been recently described for *Asaia* symbionts of *An. gambiae* (Damiani *et al*., 2010).

2.3 Bacteria in immature and adult mosquito midguts

A study conducted by Rani *et al*. (2009) on bacterial diversity analysis of larvae and adult midgut microflora using culture-dependent and culture-independent methods in lab-reared and field-collected *Anopheles stephensi*-an Asian malaria vector of male, female and larvae were screened. Five 16S rRNA gene library were constructed from laboratory and field-caught *An. stephensi* mosquitoes and a total of 115 culturable isolates from both samples were analyzed further. Altogether, 68 genera were identified from midgut of adult and larval *An. stephensi*, 53 from field-caught and 15 from laboratory reared mosquitoes.

In recent years, it has been well documented that, the gut flora not only influence mosquito physiology, but also significantly alter vector competency. Although, salivary gland and gut constitute key partners of the digestive system, it is still believed that salivary gland may harbor fewer floras than gut (Minard *et al*., 2013). However, a study conducted by Sharma *et al*. (2014) showed that, the salivary glands harbor more diverse microbial communities than gut in *Anopheles culicifacies*. *An. culcifacies* sibling species A were reared and maintained at 28 ± 2 ^{o}C/ Relative Humidity in the insectary fitted with a stimulated dawn and dusk machine for proper mating and pupal stages of mosquitoes were used for meta genomic analysis.

Moreover, salivary glands and gut from 3-4 day old sugar fed adult female mosquitoes were collected for DNA isolation. Interestingly, the tag distribution frequency of the overlapped microbial community was dominated by salivary gland (70%) over the gut (50%). Furthermore, the comparative rarefaction analysis showed the large variability in the bacterial community between the two tissues, covering more taxa counts (OTUs) in the salivary gland. Subsequently, Chao/ ACE estimator and multiple α-diversity indices analysis indicated that salivary gland harbors more diverse bacterial flora as compared to the gut in the laboratory reared adult female mosquitoes (Sharma *et al*., 2014).

The midgut flora of field caught Abalain-Colloc *et al*. (1998) and laboratory reared (Dieng *et al*., 2010) mosquitoes has been screened. Many and diverse bacterial species were identified in this study. However, bacterial species from the *Gamma- Proteobacteria* class were most frequently identified; in addition, the majority of these belong to the *Enterobacteriaceae* family. Bacterial species from this class and family have frequently been found and classified as symbionts in insects (Pontes and Dale, 2006). Similarly, a study done by Djadid and his colleagues for the

identification of the midgut microbiota of *An. stephensi* and *An. maculipennis* were investigated. The majority of bacteria belonging to the *Gammaproteobactria* class (Djadid *et al*., 2011).

Bacterial biodiversity in midguts of *Anopheles* mosquitoes, malaria vectors in South East Asia was assessed. Among the 175 specimens of *Anopheles* mosquitoes was investigated by 16S rRNA gene PCR-TTGE anchored in the V3 hypervariable region. A representative gel is given and TTGE profiles were obtained for 144 samples, 31 samples (17.7%) giving no amplification in PCR or a faint PCR signals leading to non-detectable TTGE profiles. Negative results suggested a low bacterial inoculum rather than a total absence of bacteria in the corresponding samples. Most negative samples came from Vietnam mosquitoes (n=26), compared to Thailand (n=5), and seemed to be unrelated to the *Anopheles* species.

Finally, V3 16S PCR-TTGE approach led to the description of microbial community, members of the genus *Acinetobacter* as well as most members of the genera affiliated to the family *Enterobacteriaceae* displayed a high level of 16S rRNA gene heterogeneity leading to complex banding patterns in V3 16S PCR-TTGE. However, probable species affiliation will be proposed for several genera when the phylogenetic signal of the V3 region was significant. Contrasting with the low diversity per specimen, OUT (Operational Taxonomic Unit) diversity in the whole population was high with the detection of 31 different bacterial genera distributed in four phyla, *Proteobacteria*, *Bacteroidetes*/Chlorobi, *Firmicutes* and *Actinobacteria* largely dominated the midgut microbiota of *Anopheles* mosquitoes with 232 OTUs in the population studied. Their diversity encompassed Alpha, Beta- and Gamma super classes of *Proteobacteria* (Manguin *et al*., 2013).

Another study done by Alvarez *et al*. (2012) showed that, blood meals drastically alter mosquito gut microbial composition; *Pseudomonas* spp. is among the taxa that are enriched in blood-fed guts. Besides, the genetic adaptations that mosquitoes have developed throughout their evolution, the associated microbes in the blood-fed gut may provide the additional genetic capacity to cope with oxidative stress: catalase, manganese superoxidase dismutase, superoxide dismutase (Fe), heme oxygenase, alkyl hydroperoxide reductase (AhpC), and glutathione peroxidase are present as well. This supports the proposed correlation between a genetic tolerance and fitness in the stressful gut environment induced by a blood meal.

Similarly, an isolate of *Enterobacter* sp. was obtained from the microbial community with in the gut of the *Anopheles gambiae*. Draft genome was sequenced and annotated. According to the genome sequences, several colonies were identified as *Enterobacter* sp. based on the 16S rRNA gene sequences. The genome was annotated using NCBI Prokaryotic Genome Automatic Annotation Pipeline which predicted 4,433 protein-coding features and 83 RNA genes. One thousand nine hundred seventy one (54.5%) which belongs to different subsystems and *Enterobacter* is among the bacteria that expand the blood fed gut (Jiang *et al*., 2012).

The deep sequencing reveals extensive variation in the gut microbiota. The result was classified on the level of genus by Operational Taxonomic Units. The filtered sequence was compared to 16S rRNA sequences in the ribosomal Database Project library using Bayesian approach which implemented by the Remote Desktop Protocol (RDP) classifier. This classification was resulted in 144 unique bacterial genera that were mainly composed of four abundant classes of bacteria. The Gram-negative *Gammaproteobacteria, Alphaproteobacteria* and *Flavobacteria* represented 62.3%, 18.3% and 11.6% of these classified bacteria, respectively. The Gram-positive *Bacilli* constituted 3.8% of the bacteria. 17.5% of the bacteria could not be classified below the level of class (Poku *et al*., 2012).

Anopheles gambiae mosquitoes were sampled in their aquatic habitats and immature stages of mosquitoes were collected in four localities in the vicinity of Yaound'e: Mvan Cameroon. This *Anopheles* mosquito was cultured and gram negative bacteria were selectively isolated especially the family of *Enterobacteriaceae*. The pure bacterial isolates were sub-cultured and subjected to genomic DNA extraction kit analysis. The result showed that, culturable bacterial isolates were recovered from 227 individual midguts of mosquitoes collected in four localities and 14 water samples from larval habitats. A total of 597 bacterial colonies were isolated from the MacConkey agar plates and 562 (94%) were successfully sub-cultured into Luria-Bertani (LB) agar. After sequencing analysis, 464 (84%) high quality sequences of the 16S rRNA gene were obtained, 437 were from midgut samples and 27 from aquatic breeding sites. *Bacillus, Serratia* and *Pseudomonas,* the 16S rRNA sequences from the isolates showed higher relatedness with the bacteria isolated from *Anopheles mosquitoes* (Tchioffo *et al*., 2013).

Seasonal abundance, diversity, and Arbovirus associations with Mosquitoes in Western China were investigated. Mosquitoes were collected in Mangshi and Ruili cities of China and Yunnan province. Abundance of mosquito species were monitored through ultraviolet light, carbon

dioxide baited CDC light and gravid traps. The presence of virus was tested to evaluate mosquito-virus associations in agricultural settings in the area. A total of 43 species of mosquitoes from seven genera were collected, including 15 *Culex* species, 15 *Anopheles* spp., four *Aedes* spp., three *Armigeres* spp., one *Mimomyia* spp., two *Uranotaenia* spp. and three *Mansonia* spp. Species richness and diversity varied between Mangshi and Ruili. *Culex tritaeniorhynchus*, *Culexquinquefasciatus*, *Anopheles sinensis* and *Anopheles peditaeniatus* were the most abundant species in both sampling sites.

Ultraviolet light traps collected more specimens than CDC light traps baited with dry ice, though both collected the same variety of mosquito species. The CDC gravid trap was the most effective trap for capture of *Culex quinquefasciatus*, a species underrepresented in light trap collections. A total of 26 virus strains were isolated, which included 13 strains of Japanese encephalitis virus, four strains of Getah virus, one strain of Ova virus, one strain from the arbovirus genus, and seven strains of *Culex pipien* pallens densovirus (Zhang *et al*., 2013).

A study done by Socolovschi and his research team on *Rickettsia* Species in Africa *Anopheles* Mosquitoes; quantitative and traditional PCR assay specific for *Rickettsia* genes detected rickettsial DNA in 13 of 848 (1.5%) *Anopheles* mosquitoes collected from Cote d'lvoire, Gabon and Senegal. *Rickettsia felis* was detected in one *An. gambiae* molecular form S mosquitoes collected from Kahin, Cote d'lvoire (1/77, 1.3%). Additionally, a new *Rickettsia* genotype was detected in five *An. gambiae* molecular form S mosquitoes collected from Cote d'lvoire (5/77, 6.5%) and one mosquito from Libreville, Gabon (1/88, 1.1%) as well as six *An. melas* (6/67, 9%) mosquitoes collected from port Gentil, Gabon.

A sequence analysis of the gltA, ompA and sca4 genes indicated that this new *Rickettsia* sp is closely related to *Rickettsia felis*. No rickettsial DNA was detected from *An. funestus*, *An. arabiensis* or *An. gambiae* molecular form M mosquitoes. Additionally, a BLAST analysis of the gltA sequence from the new *Rickettsia* spp. resulted in a 99.71% sequence similarity to a species previously detected in a blood sample of Senegalese patient with a fever from the Bandafassi village, Kedougou region (Socolovschi *et al*., 2012).

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high abundance in laboratory reared *An. stephensi*, *An. gambiae* and *An. albimanus* mosquitoes (Pumpuni *et al*., 1996). Further, the gut microflora varied depending up on the ecological niche or geographical location of mosquitoes. Straif and his colleagues (1998) identified *Enterobacter agglomerans* and *Escherichercia coli* as the most frequently isolated bacteria, from mid gut of *An. gambiae* and *An. funestus* mosquitoes caught in Kenya and Mali respectively.

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2.4 Mosquito fungal microbiota

Fungi are eukaryotic organisms with distinctive cell wall composed of chitin and they produce asexual non motile spores; conidia in molds and yeasts, such as a medically important genus *Aspergillus* and the commonly used in baker's yeast, *Saccharomyces cerevisiae* (Virginio *et al*., 2014). Although, the term microbiota is primarily used to describe bacteria, fungi are frequently identified and their role in mosquitoes has been a renewed interest of contemporary science (Underhill and Iliev, 2014).

There are fewer studies exploring the mosquito mycobiota on pathogen susceptibility in mosquitoes. Most of the researches focused on interaction of mosquito-fungi and related to the use of pathogenic fungi products to control mosquito populations (Maketon *et al*., 2014). There is evidence to suggested that *Penicillium* molds and other filamentous fungi dominate the mosquito mycobiota without any determinant but other filamentous like *Aspergillus* and *Penicillim* are pathogenic and the generas *Beauveria* and *Metarhizium* can capable of reducing mosquito life span as well as vector competence for pathogens (Graza-Hernandez *et al*., 2013).

In addition to filamentous fungi, many yeasts have been isolated from mosquitoes in both laboratory and field collected mosquitoes (Cappelli *et al*., 2014).

Yeasts are found in many insect species and benefit insects supplying for nutritional dietary deficiency. Yeasts belonging to the genera *Candidia* have been identified in *Aedes* and *Anopheles* mosquitoes in both the laboratory and field collected mosquitoes (Ricci *et al*., 2011). Moreover, *Anopheles* mosquitoes, and it is important against protozoan parasites by releasing a natural anti-plasmodial toxin and used in industrial applications as food and feed bio-preservation agents (Valzano *et al*., 2016).

Yeast generated carbon dioxide from yeast or sugar mixture and its efficacy as attractant in Biogents (BG) traps were evaluated. In this study, the rate of Co_2 production was optimized for different yeast or sugar mixtures which used as bait in BG- sentinel traps and it was compared to octenol baited traps. The results of this study were revealed that, the yeast/sugar (5g: 280g) in 300ml water generated the highest volume of Co_2. Moreover, the carbon dioxide baited traps attracted significantly more mosquitoes than the octenol baited traps (Jerry *et al*., 2017).

2.5 Midgut bacteria and the effect on *Plasmodium* parasites and the mosquito host

There are several somewhat controversial research findings regarding the role of midgut microflora in adult mosquito vector. One study suggests that, some bacteria genus like *Staphylococcus* and *Bacillus* were considered as a symbiont in the midgut of *Anopheles pipiens* and they were important for the normal fecundity of this vector (Fouda *et al.*, 2001). On the other hand, the effect of microflora along with antibiotics were studied for the survival of some mosquito adult vector; *Anopheles aegypti* showed that, *An. aegypti* that fed antibiotics survived a shorter time at low temperature than the vector without antibiotics in food however the mechanism and its action on it was not known (Jadin *et al*., 1966).

Certain species of *Anopheles* are involved in the transmission of various arboviral and filarial diseases to humans and domestic animals and/or are important for their biting in different parts of the world, but the most important disease transmitted by them is malaria. About 70 *Anopheles* species are malaria vectors in which about 40 are important vectors (Service, 1993). Malaria parasite transmission depends on the availability of competent mosquito vectors (Omumbo *et al*., 1998). However, commensal bacteria in the mid gut can reduce the ability of mosquitoes to

transmit disease, either by having direct anti-parasite effects or by stimulating basal immune responses of the insect host. As different bacteria have different effects on parasite development, the composition of bacterial community in the mosquito gut is likely to affect the probability of disease transmission (Osei-Poku *et al*., 2012). Though, these studies have not identified the causal mechanisms through which the presence of bacteria negatively impacts on malaria parasite development (Dong *et al*., 2009).

A comprehensive functional genomic approach to elucidate the molecular interplay between the bacterial co-infection and the development of the human malaria parasite, *Plasmodium falciparum* in its natural vector, *Anopheles gambiae* infection had been analyzed. The result of this study showed that, microbe-free aseptic mosquitoes displayed an increased susceptibility to *Plasmodium* infection while co-feeding mosquitoes with bacteria and *P. falciparum* gametocytes resulted in lower than normal infection levels. Infection analysis also suggested that, the bacteria-mediated anti plasmodium effected was mediated by the mosquitoes' antimicrobial immune response, plausibly through activation of basal immunity (Dong *et al*., 2009). Consequently, the microbiota plays an essential role in modulating the mosquitos' capacity to sustain plasmodium infection.

Mosquito-microbiota interactions have been studied through the maternal transmission route of *Wolbachia* occurs by cytoplasmic incompatibility. Thus, *Wolbachia* has been proposed as a gene drive system for mosquito genetic replacement for the reduction of population size, and for interfering with population age structure to decrease transmission. In particular, this study showed that, the introduction of some strains of *Wolbachia* in *Ae. aegypti* causes a life shortening of mosquito as well as an upregulation of the mosquito immune response that render the mosquito refractory to dengue infection and parasites (Kambris *et al*., 2009).

Besides from *Wolbachia*, the relationship of mosquito with microbiota in their gut mostly studied is related to bacteria. In recent years, different medically important mosquito vectors carry common bacteria of different genera including *Enterobacter, Escherichia, Klebsiella, Serratia, Pseudomonas,* and *Staphylococcus* (Pidiyar *et al*., 2004). These studies described the characterized gut bacteria inhibiting the development of the parasite in the midgut of the mosquitoes, indicating that the mosquito midgut is a site of complex interactions between the mosquito, the malaria parasite and the bacterial microflora in the gut of the mosquito vectors.

Understanding of the molecular mechanisms by which mosquitoes limit the parasite development may lead to new methods for controlling malaria. In this scenario, (Abraham and Jacobs-Lorena, 2004) described that, the mosquito responded to midgut invasion of the malaria parasite and examined the role of mosquito digestive enzymes. They were concluded that, peritrophic matrix and microvillar proteins in the midgut of the mosquito vector were considered as the major barriers to parasite development consequently this lead to the development of novel approach for the malaria control strategy for new of malaria elimination program through molecular approach.

Mosquito-microbiota interactions leads to a complex relationship to innovate new malaria parasite control strategies. Due to the adaptation of different environments by mosquito microbiota, their relationships may have a great impact in better understanding for the development of mosquito-borne disease control strategy. Such relationships exhibited in African and Asian mosquito vectors; the gram negative, α-proteobacteria belonging to the genus *Asaia* was found in high numbers in the midgut, salivary glands and reproductive organs, could be used to express anti-plasmodium molecules and may exert an additional inhibitory effect against pathogens in the salivary glands where the parasites end its life cycle (Favia *et al*., 2007).

Wang *et al*. (2017) explained that, genetically engineered gut bacteria of non pathogenic *Serratia* bacterium strain AS1 isolated from *Anopheles* ovaries that stably colonizes the mosquito midgut, female ovaries, and male accessory glands that secrets anti-plasmodium effector proteins, and the recombinant strains inhibit development of *Plasmodium falciparum* in mosquitoes.

2.6 Bacteria as a source of mosquito semiochemicals

Insects use chemical signals to orient, survive and reproduce in their specific environments. Natural chemicals that transfer information between organisms are generally classified as semiochemicals (Regnier, 1971). These chemicals are called pheromones when utilized for intraspecific communication and allelochemicals when used at interspecific level. It is known that female mosquitoes depend on olfactory cues for sugar feeding, host seeking and oviposition while male mosquitoes mostly respond to odors utilized for sugar feeding (Luntz, 2003).

One possible source of *Anopheles* semiochemicals is the bacteria that exists in their breeding habitats and volatiles from bacteria affects mosquito behavior have been shown in a number of studies. For instance, differences in human skin microflora is believed to be one of the

underlying causes to attract mosquitoes; *Anopheles gambiae*, prefer to bite the foot region of humans and have shown to respond positively to European cheese (Knols and De Jong, 1996).

On the other hand, mosquito vector like *Ae. aegypti* could be attracted by bacterial produced volatiles for oviposition stimulants which is found capric acid made water had shown attractive for a number of ovipositing gravid mosquito. For example, volatiles from *Pseudomonas aeruginosa* and *Bacillus cereus* were the effective in producing volatile attractants for the ovipositing of the *Aedes aegypti* (Poonam *et al*., 2002).

In addition to these; semiochemicals, important cues for selection of the suitable places for female mosquitoes to lay eggs, are key factors for the survival of immature stages of the family Culicidae. Nararro-Silva *et al*. (2009) summarized that, a set of chemicals, visual, olfactory and tactile cues that interact with the female before laying eggs, helping the localization of adequate sites for oviposition. They initiate with the reception of environmental (visual, tactile, and olfactory), which may either attract or repel, limiting the possibilities of finding oviposition sites. The cues include color and optical density, water, texture and moisture, temperature and reflectance of the oviposition substrate.

The role of bacteria on the oviposition and breeding site selection response of *Aedes* mosquitoes were assessed and characterized in India. The results of this study showed that, bacterial cues and water samples which were collected from different natural breeding sites of *Aedes* were the major roles for the vector to response in their breeding habitat and oviposition site selection. Among the seventeen isolates, four isolates of bacteria: *Pseudomonas aeruginosa*, *Acinetobacter calcoaceticus*, *Enterobacter cloacae* and *Acinetobacter anitratus*, were the major ovipositional attractants using bacterial cues. These bacteria were also characterized and processed for ovipositional bioassay and gave a positive result for semiochemicals emitted by these bacteria in selection of breeding and ovipositional sites (Mondal *et al*., 2015).

On the other hand, the bacteria on human body odors play an important role in the host seeking behavior of mosquitoes. In this scenario, Verhulst and his colleagues (2016) observed that, the attractiveness of volatiles from different body parts of humans to malaria mosquito *Anopheles coluzzii*, was assessed. Hence, skin emanations were collected from armpits, hands, and feet; the volatile profile were analyzed and tested for their attractiveness to the malaria mosquito, *An. clouzzii*. The results of the analysis showed that, skin emanations collected from armpits were

less attractive to this vector compared to hands and/or feet. Nevertheless, skin care products, antibacterial soaps and deodorant compound, isopropyl tetradecanoate inhibited mosquito host seeking preference.

The host preference of the anthropophilic mosquito species in the *Anopheles gambiae* complex and *An. arabiensis* responses from skin bacteria were tested. Skin bacteria were collected from human, cow and chicken skin were significantly increased trap catches; and traps containing bacteria collected from human skin showed the highest proportions of *An. gambiae* and *An. arabiensis*. Moreover, traps with bacteria of human origin caught a significantly higher proportion of *An. gambiae* than of *An. arabiensis*, whereas bacterial volatiles from chicken attracted significantly higher numbers of *An. arabiensis* than of *An. gambiae*. Additionally, *An. gambiae* showed specialized response to volatiles from four specific bacteria, whereas *An. arabiensis* respond equally to all species of bacteria tested (Busula *et al*., 2017).

Therefore, in Ethiopia, to our knowledge, no research has been done on the isolation and identification of microflora in the mosquitoes' midgut and salivary gland, though there are some studies that investigated the effect of the midgut microbiota on the development of matured infection in the *Leishmania* species (Bates *et al*., 2015) and a plenty of studies on malaria prevalences and control measures by chemically impregnated bed net usage and destruction of vector breeding sites in the different parts of Ethiopia (Tadesse Dejenie *et al*., 2011; Wakgari Deressa *et al*., 2014).

3. MATERIALS AND METHODS

3.1. Description of the study areas

This study was done in Edo Kontola village Adami Tullu Jiddo Kombolcha district (Figure 1, A) Oromia Regional State. Its altitude between 1500 to 2300mt above sea level and it has an average annual rainfall of 837mm. The main environmental feature of the area is Lake Zeway which covers about 434 km^2 area with average depth of 4 m (Tarekegn Abose *et al*., 1998). The lake supports irrigation farms and fishing, the sole economic activities of the society in this area. The people usually cultivates maize and other cereal crops during rainy season (June to October) and mainly vegetables such as onions, tomatoes, potatoes and green pepper by irrigation during the dry season (November to May) and as well as the rainy season. Many of the inhabitants of the village live in traditional African grass-thatched house locally known as 'mana chita' and some live in houses with corrugated iron roofs. The lake area maintains malaria transmission by creating sustainable potential mosquito breeding sites around the lake shoreline (Oljira Kenea *et al*., 2016). As a result, mosquito abundance increases after the rainy seasons and decreases as the lake volume recedes during the dry months.

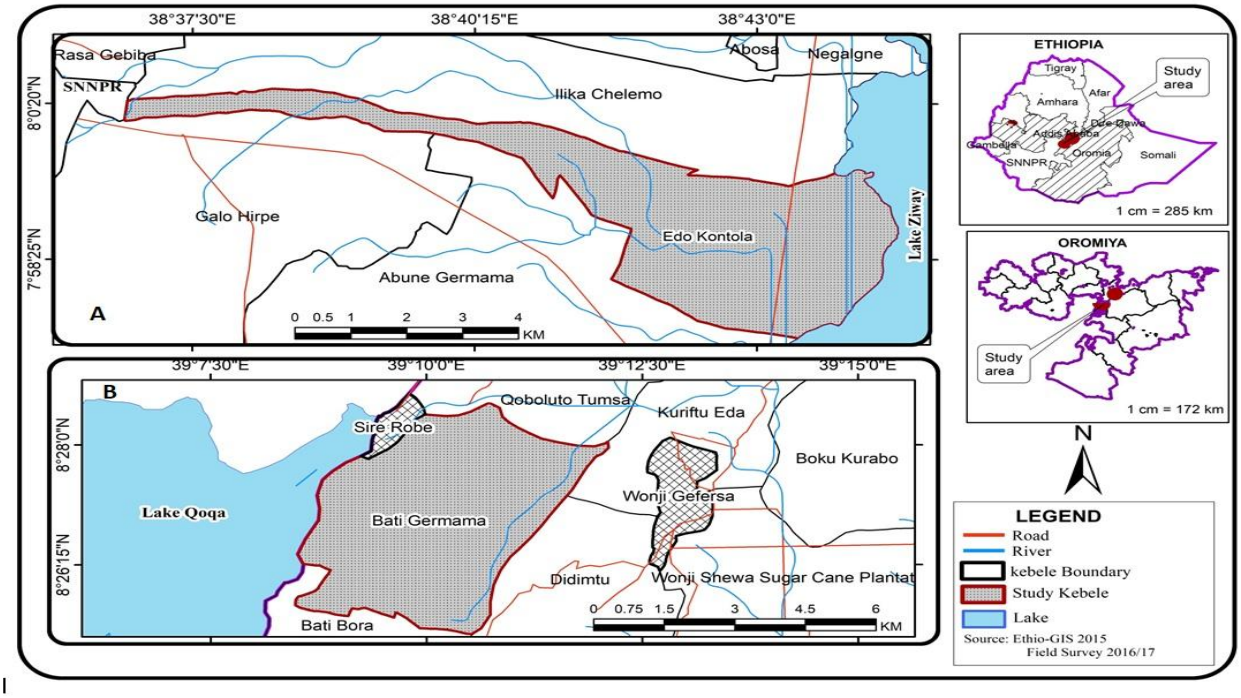

Figure 1: Maps of study areas: (**A**) Edo Kontola; (**B**) Bati Germama

The same study was conducted in Adama Wereda, Bati Germama Kebele (Figure 1, B) Oromia Regional State. Its altitude is 1712mt above sea level and it has an average annual rainfall of 808mm. This kebele is 20 km from the Wereda, situated in the part of East Shewa Zone located in the great rift valley bordered on the south by the Arsi Zone, on the southwest by Koka Reservoir which separates it from Dugda Bora, on the west by Lome, on the north by the Amhara Region, and on the east by Boset. The Awash River, the only important river in this woreda, defines the woreda boundaries on the east and south. The peoples in this area cultivate maize and other cereal crops during rainy seasons. The residents in this area are closer to the mosquito breeding places that are formed by the overflow of Awash river abundant during April to September and short rains in December to January creates standing water, swamp, hoof prints, drains and ponds that considered the village highly malaria endemic from Wereda Kebeles (Firehun Yirefu *et al*., 2007).

3.2 Study periods

This study was conducted from mid of January to June 2017 for laboratory reared *Anopheles arabiensis* and from August to December 2017 for field collected *Anopheles* mosquito species.

3.3 Study design

Ten *Anopheles arabiensis* mosquitoes were dissected as a pool for both midgut and salivary glands for insectary and five fed, five gravid and five unfed mosquitoes for each species were dissected for midgut and salivary glands of field collected of the four *Anopheles* mosquito species. The identification and frequency of microflora isolated pooled midgut and salivary glands in each *Anopheles* species of laboratory reared and field collected were determined and analyzed for comparison.

3.4 Mosquito collection in the field

Anopheles mosquitoes were collected in Edo Kontola village, Adami Tullu Jiddo Kombolcha district, using CDC light traps (indoor) in four houses and Human Landing Catch (HLC) indoor-outdoor respectively. Four houses closed to the lakeshores and irrigation fields were selected for CDC light traps being hanged and mosquitoes were collected within walking distance (≤ 1km) from the lakeshores (Wakgari Deressa *et al*., 2014). For HLC, trained human volunteers catch

mosquitoes which land on their exposed body parts from 19:00 to 06:00 for 50 minute each hour with 10 minute rest for the volunteers.

There were two collection shifts for HLC: one team of collectors worked from 19:00 to 24:00 h followed by the second team from 24:00 to 06:00 h. Every hour, two volunteers rotated between indoor and outdoor positions and carried out the work to reduce position bias. Outdoor collectors were positioned within 10 m from each study house. Each volunteer sat on a chair with the legs exposed from foot to knee and captured mosquitoes as soon as they land on the exposed legs before they commence feeding using a flashlight and mouth aspirator. The principal investigator was coordinated the collection activities and watch volunteers not to fall sleep and bitten by mosquitoes over the study nights (Oljira Kenea *et al*., 2016). The next morning, mosquitoes were transported and identified to species level by morphological characteristics using the standard identification key (Gillies and Coetzee, 1987) at EPHI laboratory.

Anopheles mosquitoes were collected in Adama Wereda, Bati Germama Kebele, using Human Landing Catch (HLC) in two houses closed to the swamp, drains, hoof-prints and small ponds were selected for HLC, human volunteers sat on a chair with legs exposed from foot to knee from 19:00 to 24:00 for 50 minutes each hour with 10 minutes rest for trained human volunteers and captured mosquitoes as soon as they land on before commence feeding using flash light and mouth aspirator. The principal investigator was coordinate collection activities and watch volunteers not to fall sleep and bitten by mosquitoes over the study nights (Oljira Kenea *et al*., 2016). The next morning, mosquitoes were transported and identified to species by morphological characteristics using the standard identification key (Gillies and Coetzee, 1987) at the field laboratory.

3.5 Insectary and Laboratory procedures for *Anopheles arabiensis*

Anopheles mosquitoes were reared and maintained at 28±2^{O}C/Relative Humidity 80% in Aklilu Lemma Institute of Pathobiology (pool 1-9) and Tropical and Infectious Disease Research Center (TIDRC) insectaries in Jimma (pool 10-20) were fitted with a simulated down and dusk machine, essentially required for proper mating and feeding at the insectary. For isolation and identification of microflora from the midgut and salivary glands of laboratory reared *Anopheles* mosquitoes, 200 adult female *Anopheles arabiensis* were transferred and kept in paper cup using

aspirator and fed on a sterile sugar solution (10%) supplied with a sterile cotton swab kept on the cup covered with mesh until they dissected.

3.6 Laboratory processing of adult mosquitoes

3.6.1 Mosquito dissection

Throughout the dissection procedure in the laminar flow; the dissecting stereomicroscope (1X), working area, dissecting needles and forceps were kept sterilized by using 70% ethanol. Prior to the midgut and salivary gland dissection, each pool of *Anopheles arabiensis* from insectary and fed, gravid and unfed mosquitoes from field collected *Anopheles* species were surface sterilized by 70% ethanol followed by washing each pool four times by 1x PBS (Phosphate Buffer Saline) (Gusamo *et al*., 2007).

3.6.1.1 Midgut dissection

A drop of 1x PBS was placed on to a glass slide mounted under the stereomicroscope. Then, a mosquito was transferred on the prepared slide by stabbing the mosquito thorax with a needle tip probe. While holding down the mosquito with the probe, the forceps are being used to grasp the second to the last abdominal segment and pulled gently off the mosquito abdomen in a single motion. Then, abdomen was discarded and forceps were being used to detach the midgut from the thorax. Finally, the midgut was transferred individually to 3ml of Phosphate Buffer Saline, squashed and incubated in water bath (27-31^{o}c) until cultured in enriched Tryptic Soya Broth (Christiansen-Jucht *et al*., 2014). After incubation in water bath, the remaining 2ml of squashed midgut sample was stored in refrigerator at -20^{o}c.

3.6.1.2 Salivary gland dissection

A drop of 1x PBS was placed on a glass slide mounted under a stereomicroscope. Then, a mosquito was picked up by stabling the thorax with a needle-tip probe and mosquito legs were pulled off. Then, it was transferred on to the slide. Then, the head of the mosquito was removed and forceps were being used while the mosquito thorax held down with the probe, another probe is being used to push gently down the thorax. Then, the salivary glands were isolated and severed with needle-tip probe (Coleman *et al*., 2007) and transferred individually to 3ml of Phosphate Buffer Saline, squashed and incubated in water bath (27-31^{o}c) until cultured in enriched Tryptic

Soya Broth (Christiansen-Jucht *et al*., 2014). Finally, after incubation in water bath, the remaining 2ml of salivary gland squashed samples were stored in refrigerator at -20^{o}c.

3.6.2 Culture of midgut and salivary glands, microbiota isolation and identification

One milliliter of each samples of midgut and salivary glands were transferred to 3ml of Tryptic Soya Broth and incubated for 24 hrs. at 35±2 ^{o}c. Then, a loopful of each pool of fed, gravid, unfed (field collected) and midgut and salivary gland (insectaries) mosquitoes' turbid broth were inoculated on Blood, Chocolate, Mac Conkey and Sabouraud Dextrose Agar and incubated in carbon dioxide incubator (Blood and Chocolate media) and aerobic incubator (Mac Conkey and Sabouraud Dextrose media) for 24 hrs. at 35±2 ^{o}c. For isolation of fungi from Sabouraud dextrose media, the culture was incubated for fifteen days as fungi needs more time to grow and isolated based on their colony characteristics. Then, wet film was used to identify yeast and other microbiota of fungi by observing under the microscope.

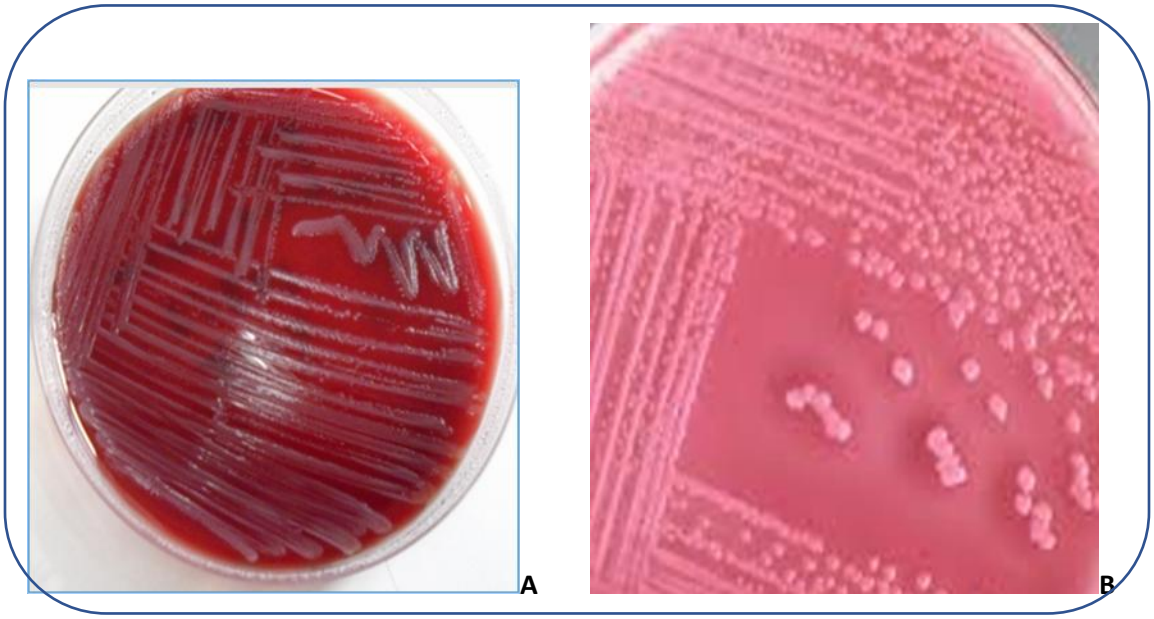

Plate 1: Morphological characteristics of microflora on culture media.

K. pneumonia, *Enterobacter spp*, *Streptococcus* spp. (A)

E. coli, *Ser. marcescens*, *Pseudomonas* spp *Citrobacter* spp. (B)

The sterility of all reagents was checked during the entire procedure. Controls for the efficiency of sterilization were treated like other samples. To isolate single purified colonies of the bacteria, continuous sub-culture of every grown bacterial colony was performed until the pure isolate obtained and microorganisms were first screened based on colony characteristics involving colony size, shape, color, margin, opacity, elevation, and consistency (Plate 1) and the morphology of isolates were studied by gram staining and motility by the hanging drop technique. The identification of microbiotas was performed through conventional biochemical tests such as nitrate reduction, urease activity, citrate utilization, Hydrogen sulfide production, Voges-Proskauer (VP), Methyl Red (MR), ß–galactosidase activity, ornithine and lysine utilization activity and carbon source utilization profile (Table 1) and (Table 2) were also performed according to manufacturer's instruction (Yadav *et al*., 2016).

Table 1. Reaction results of Triple Sugar Iron Agar (TSIA).

No.	Reaction on TSI			Result	Example	
	Butt	Slant	H_2S			
1	Red	Red	Negative	Alk/Alk/- (No action on sugars)	Non fermenter e.g. *Pseudomonas*	
2	Yellow	Red	Negative	A/Alk/- (Glucose fermented without H_2S)	LNF e.g. *Shigella*	
3	Yellow	Red	Positive black in butt	A/Alk/- (Glucose fermented H_2S)	LNF e.g. *Salmonella* *Proteus*	
4	Yellow	Yellow	Negative	A/A/- (three sugars are fermented)	LF e.g. *E. coli* *Klebsiella* *Enterobacter*	

Table 2. Biochemical test results after 24hr. incubation.

***Anopheles* species**	**Biochemical tests**							**Additional**	**Identified bacteria**
	LDC	CIT	TSIA	SIM		UREA	H_2S		
				Motility	Indole				
Anopheles funestus	−	+	−	−	−	−	−	Cat^+, Oxi^+	*Pseudomonase spp.*
Anopheles funestus	−	+	−	−	−	−	−	Cat^+, Oxi^+	*Pseudomonase spp.*
	−	+	+	+	−	+	+	Cat^+, Oxi^-	*Citrobacter spp.*
Anopheles phronesis	−	+	−	−	−	+	−	Cat^+, Oxi^-	*Klebsiella ozenae*
	−	+	−	+	−	+	−	Cat^+, Oxi^-	*Providencia rettegri*
Anopheles phronesis	−	+	−	−	−	−	−	Cat^+, Oxi^+	*Pseudomonase spp.*
Anopheles phronesis	−	+	−	−	−	−	−	Cat^+, Oxi^+	*Pseudomonase spp.*
Anopheles zeimanni	−	+	−	−	−	−	−	Cat^+, Oxi^+	*Pseudomonase spp.*
Anopheles zeimanni	−	+	+	+	−	+	+	Cat^+, Oxi^-	*Citrobacter spp.*
	−	+	−	−	−	−	−	Cat^+, Oxi^+	*Pseudomonase spp.*
Anopheles zeimanni	−	+	+	+	−	+	+	Cat^+, Oxi^-	*Citrobacter spp.*
Anopheles zeimanni	+	+	+	−	−	+	−	Cat^+, Oxi^-	*Klebsiella pneumonia*
Anopheles gambiae s.l	−	+	+	+	−	+	−	Cat^+, Oxi^-	*Entrobacter cloacae*
Anopheles zeimanni	−	+	−	−	−	−	−	Cat^+, Oxi^+	*Pseudomonase spp.*

N.B. (+) is positive; (—) is Negative

Conventionally unidentified microbiotas analyzed and identified in VITEK 2 Compact (Plate 2) (Biomerieux, France) which provides an automatic pipetting and dilution for identification of microbiota from the midgut and salivary gland of *Anopheles* mosquito samples. It has reagent cards with 64 wells for individual biochemical tests and has product type, lot number, expiration date, and a unique identifier that linked with the sample. This machine requires pure colonies to suspend microorganisms in 3ml of sterile aqueous 0.45% saline in 12x75mm clear plastic tubes and turbidity was adjusted accordingly. The tube containing sample suspension was placed in a special rack (Cassette) which accommodate up to 10 tests. All cards were incubated on line at 35±1 ^{o}c. Data were collected at 15 minutes intervals during entire incubation period. In this machine, test reaction results appeared as "+"or "-" and identification levels above 95% was considered as an excellent identification and at least three taxons exhibit the same biopattern or very atypical biopattern or organisms do not correspond any taxon in the data base considered as

unidentified organisms. Finally, all the identified microbiota was stored in 20% tryptic soya broth with glycerol at -80 ^{o}c at Ethiopian Public Health Institute Clinical Bacteriology National Referral Laboratory.

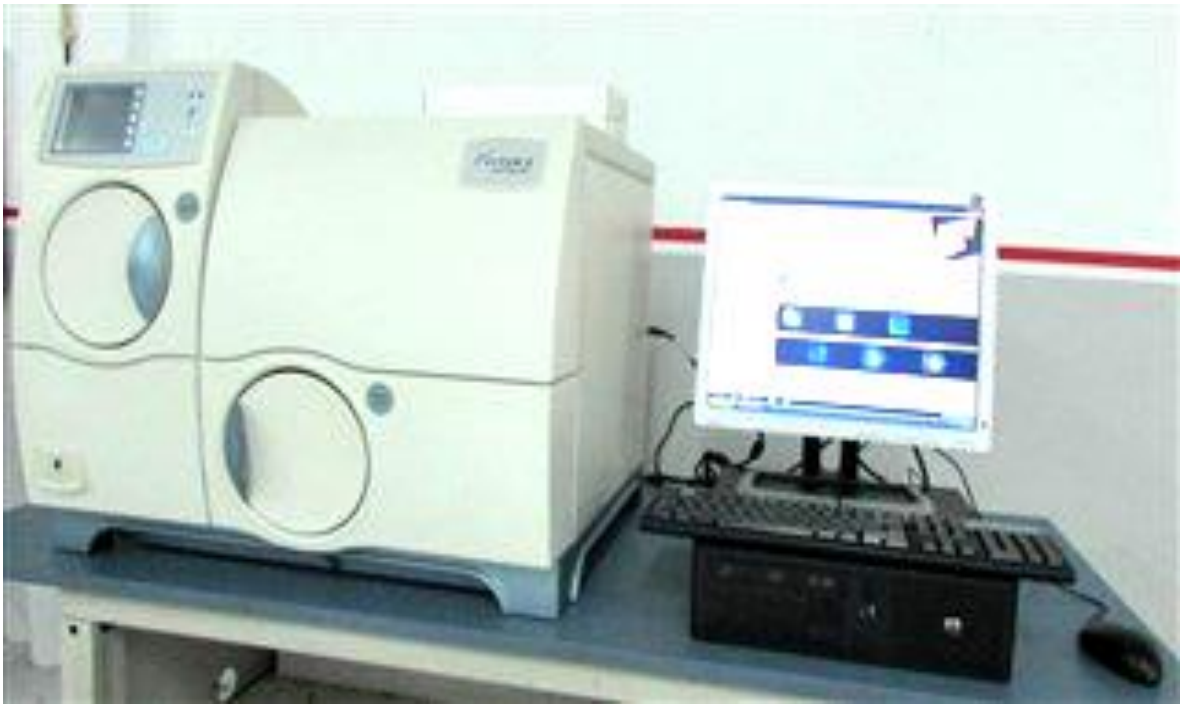

Plate 2: Vitek 2 Compact Automation Machine

3.7 Data Quality Assurance

Identification of *Anopheles* mosquitoes and microflora were carried using the standard Insect Identification keys and verified by repeated crosschecking by experienced medical entomologist and microbiologist respectively. Besides, the identified microflora was compared and checked by Clinical and Laboratory Standard Institute (CLSI) manual. In addition to these, the isolated microflora specimens were preserved for future reference. Moreover, laboratory equipments, reagents and different isolate standards were checked during the entire laboratory work (Appendix 1).

3.8 Ethical clearance

Ethical clearance was obtained from the College of Natural and Computational Science Institutional Review Board (CNS-IRB), Ethiopian Public Health Institute Institutional Review Board (EPHI-IRB) and Adama and Adami Tullu Weredas Health Bureau, in order to get permission for accessing their community and make use of their home for hanging CDC light traps. Moreover, voluntaries were trained on Human Landing Catch collection procedures regarding how to use mechanical aspirators and made aware of the health risks involved during the entire mosquito collection periods, alerted and coordinated every time by the principal investigator.

3.9 Data Analysis

Data from laboratory reared and field collected *Anopheles* mosquitoes were checked for completeness and consistency, and then entered into and analyzed by Statistical Package for Social Sciences (SPSS) version 23 for windows. In the analysis process, descriptive statistic was used to scan the data, assess normality, and identify missing values and outliers. Both descriptive and inferential statistics were integrated in the analysis of study variables. Moreover, it was used to compare the microflora of the laboratory reared and field collected *Anopheles* mosquitoes with their proportion of 5% significance level. Finally, the results were presented using tables, charts and graphs.

4. RESULTS

4.1 Microbiota composition in *Anopheles arabiensis* from insectary

The number of isolates and prevalence of microbiota identified from both midgut and salivary gland of *Anopheles arabiensis* from the two insectaries is presented in Table 1. A total of 200 adult female *Anopheles arabiensis* were dissected, incubated, isolated and screened on four culture media resulting in a total 110 microbiota isolates from both midgut and salivary glands samples. From 97 isolates, 14 microbiota species were identified including: *Klebsiella pneumonia*, *Saccharomyces cerevisiae*, *Serratia marcescens*, *Staphylococcus epidermidis*, *Pseudomonas luteola*, *Pseudomonas aeruginosa*, *Kocuria rhizophila*, *Streptococcus thoraltensis*, *Methylobacterium lacunta*, *Enterococcus casseliflavus*, *Kytococcus sendntarius*, *Lactococcus garvieae*, *Kocuria kristinae* and *Alloiococcus otitis*. The remaining 13 isolates were identified at the genus level including *Pseudomonas* (nine isolates), *Bacillus* (one) and *Acinetobacter* (three). A total of 22 (20%) *Saccharomyces cerevisiae* (nine species) and *Pseudomonas* species (9 genera) were identified from insectaries. *Serratia marcescens* was the second abundant microbiota comprises 13% of the total species which were isolated from 14 isolates. *Acinetobacter* and *Streptococcus thoraltensis* were comprised 6% of the total identified bacteria. Moreover, *Klebsiella pneumonia*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Bacillus* species were the least identified microbiota.

Table 3. The total number of isolates and microbiota identified from midgut and salivary gland of laboratory reared *Anopheles arabiensis.*

Species identified	organ of microbiota identified		Total # of isolates	Percent
	midgut	salivary gland		
Klebsiella pneumonia	---	1	2	2%
Saccharomyces cerevisiae	3	6	22	20%
Serratia marcescens	3	3	14	13%
Staphylococcus epidermidis	---	1	2	2%
Pseudomonas luteola	1	---	2	2%
Pseudomonas aeruginosa	1	---	2	2%
Kocuria rhizophila	---	1	2	2%
Streptococcus thoraltensis	2	1	8	6%
Methylobacterium lucunta	---	1	2	2%
Enterococcus casseliflavus	2	---	5	5%
Kytococcus sendntarius	1	---	2	2%
Lactococcus garvieae	3	2	12	10%
Kocuria kristinae	1	---	2	2%
Alloiococcus otitis	1	---	2	2%
Pseudomonas spp.	4	5	22	20%
Bacillus spp.	---	1	2	2%
Acinetobacter spp.	---	3	7	6%

Moreover, *Lactococcus* and *Enterococcus* were gram positive cocci that are catalase negative, usually facultative, anaerobic bacteria that grow in 6.5% NaCl, and 40% bile salts at pH 9.6. These microorganisms were identified both in midgut and salivary gland of *Anopheles arabiensis* comprises 10% and 5% respectively. All the above microbiota identified from insectary were screened by Vitek 2 Compact automation analyzer (Plate 2).

4.2 Microbiota composition in field collected *Anopheles* mosquitoes

During a night collection of CDC light trap and Human Landing Catch (HLC) (Edo Kontola) and two nights collection of HLC (Bati Germama kebele), a total of 520 female anopheline mosquitoes were captured. *Anopheles gambiae s.l* (260) was the dominant species followed by *An. zeimanni (*140), *An. phareonsis* (85) and *An. funestus* (35). Overall, 320 mosquitoes were captured outdoors and 200 were indoors. For this microflora study from the midgut and salivary glands of *Anopheles* mosquitoes, a total of ten pools of mosquitoes, thirty mosquitoes per pool (five fed, five gravid and five unfed) for each midgut and salivary glands relation to each species. Moreover, four species of mosquitoes; gravid and unfed (*An. gambiae s.l*), gravid and unfed (*An. phareonsis*), fed, gravid and unfed (*An. zeimanni*) and unfed (*An. funestus*) were left from the total dissected mosquitoes.

Each pools and left mosquitoes were dissected, incubated, isolated and screened on four culture media resulting in a total of 128 bacterial isolates. Sixty-five isolates were members of four genera and 7 species of gram negative bacteria and the remaining 63 isolates comprises 5 species belonged to gram positive bacteria: *Staphylococcus epidermidis*, *Streptococcus thoraltensis*, *Streptococcus mitis/oralis*, *Staphylococcus aureus* and *Enterococcus feacium* (Table 4) and (Table 5). Both gram negative and gram positive bacteria were isolated from midgut and salivary glands of fed, gravid and unfed mosquitoes. Table 5 shows the feeding stage, sources of samples and *Anopheles* mosquitos' species with their conventional biochemical test results along with their identified bacteria species.

Table 4. The summarized result of microbiota identified by Vitek 2 Compact from Adami Tullu, Jido Kombolcha District and Adama Wereda, Bati Germama Village/Kebele.

***Anopheles* species**	**Abdominal conditions**	**Identified microbiota**	
		Midgut	**Salivary glands**
An. funestus	Fed	------	*Unidentified*
	Gravid	*Ser. marcescens*	-------
	Unfed	------	*Str. thoraltensis*
An. pharoensis	Fed	*Unidentified*	-------
	Unfed	*Staph. epidermidis*	
	Unfed	------	*Serratia fonticola*
An. zeimanni	Fed	*Ser. marcescens*	-------
	Gravid	*Str. thoraltensis*	-------
	Gravid	*Str. thoraltensis*	-------
	Fed	-------	*Str. thoraltensis*
	Unfed	-------	*Str. thoraltensis*
	Unfed	*Ser. fonticola*	-------
An. gambiae s.l	Fed	*Str. thoraltensis*	*Str. thoraltensis*
	Gravid	*Str. mitis/oralis*	-------
	Gravid	-------	*Staph. aureus*
	Unfed	*Ser. fonticola*	-------
	Unfed	-------	*Str. thoraltensis*
	Fed	*Enterococcus feacium*	-------

Table 5. Biochemical test results of field collected *Anopheles* mosquito after 24hr incubation.

Anopheles species	**Abdominal conditions**	**Sources of samples**	**Identified bacteria spp.**	**# of identified bacteria**	**% of identified bacteria**
An. funestus	Fed	Midgut	*Pseudomonas spp.* (1)	4	5%
	Gravid	Salivary gland	*Citrobacter spp.* (1) *Pseudomonas spp.* (1)		
	Unfed	Salivary gland	*Pseudomonas spp.* (1)		
An. pharoensis	Fed	Salivary gland	*Klebsiella ozenae (1)* *Providencia rettegri (1)*	9	10%
	Gravid	Midgut	*Pseudomonas spp.* (2)		
		Salivary gland	*Pseudomonas spp.* (1)		
	Unfed	Midgut	*Pseudomonas spp.* (1) *Acinetobacter spp.* (1)		
		Salivary gland	*Pseudomonas spp.* (1) *Acinetobacter spp.* (1)		
An. gambiae s.l	Fed	Salivary gland	*Pseudomonas spp.* (5) *Enterobacter cloacae* (1) *Acinetobacter spp.* (1)	59	69%
		Midgut	*Pseudomonas spp.* (3) *Klebsiella pneumonia* (1) *E. coli* (1) *Enterococcus feacium* (1)		
	Unfed	Salivary gland	*Klebsiella pneumonia* (5) *Citrobacter spp.* (1) *E. coli* (5) *Acinetobacter spp.* (5) *Pseudomonas spp.* (4)		
		Midgut	*Pseudomonas spp.* (5) *Bacillus spp.* (1) *E. coli* (3) *Acinetobacter spp.* (3) *Klebsiella pneumonia* (3)		
	Gravid	Salivary gland			
		Midgut	*Klebsiella pneumonia* (2) *Pseudomonas spp.* (3) *E. coli* (1) *Pseudomonas spp.* (3) *Bacillus spp.* (1) *E. coli* (1)		

An. zeimanni	Fed	Midgut	*Acinetobacter spp.* (1) *Pseudomonas spp.* (1)		
		Salivary gland	*Pseudomonas spp.* (1)	14	16%
	Unfed	Midgut	*Pseudomonas spp.* (1) *Klebsiella pneumonia* (1) *Citrobacter spp.* (1)		
		Salivary gland	*Pseudomonas spp.* (1) *Klebsiella pneumonia* (1)		
	Gravid	Midgut	*Pseudomonas spp.* (1) *Klebsiella pneumonia* (1)		
		Salivary gland	*Pseudomonas spp.* (1) *Klebsiella pneumonia* (1) *Citrobacter spp.* (1) *Acinetobacter spp.* (1)		

A total of 12 bacterial species were isolated and identified from the four collected *Anopheles* species (Table 4) and (Table 5). According to the number of isolated and identified species, it was shown that, *Anopheles gambiae s.l* had a higher diversity of bacteria with 9 species isolated from fed midgut and salivary gland, unfed midgut and salivary gland, and gravid midgut and salivary gland; divided in 11 genera of bacteria: *Pseudomonas*, *Enterobacter*, *Streptococcus*, *Staphylococcus*, *Serratia*, *Enterococcus*, *Acinetobacter*, *Klebsiella*, *Escherichia*, *Citrobacter* and *Bacillus*. In contrast, *Anopheles funestus* had only two species from four genera were identified: *Citrobacter*, *Pseudomonas*, *Serratia* and *Streptococcus*.

Surprisingly, *Serratia fonticola* was identified from unfed *Anopheles pharoensis*, *An. zeimanni* and *An. gambiae s.l* (Table 4). The genus *Pseudomonas* 59/86 (69%) was the most frequently isolated bacterial genus in this field study (Table 5). In wet film microscopic examination, there was no any fungal or yeast isolates were identified from Sabouraud Dextrose agar media. Moreover, conventionally unidentified microorganisms were identified by VITEK 2 Compact (Biomerieux, France) automation machine (Table 4).

Additionally, the overall microbiota analysis was done and each microflora count was assessed (Figure 2). *Pseudomonas* species were the dominant identified bacteria from midgut and salivary gland of laboratory reared and field collected *Anopheles* mosquitoes. *Acinetobacter* species were the next dominant bacteria identified followed by *Saccharomyces cerevisiae* and *Kelbsiella pneumonia*. In contrast, the genus *Enterobacter*, *Providentia*, *Staphylococcus* and species such

as; *Enterobacter cloacae*, *Klebsiella ozenae*, *Kocuria rhizophila* and *Enterococcus feacium* were the least identified bacteria.

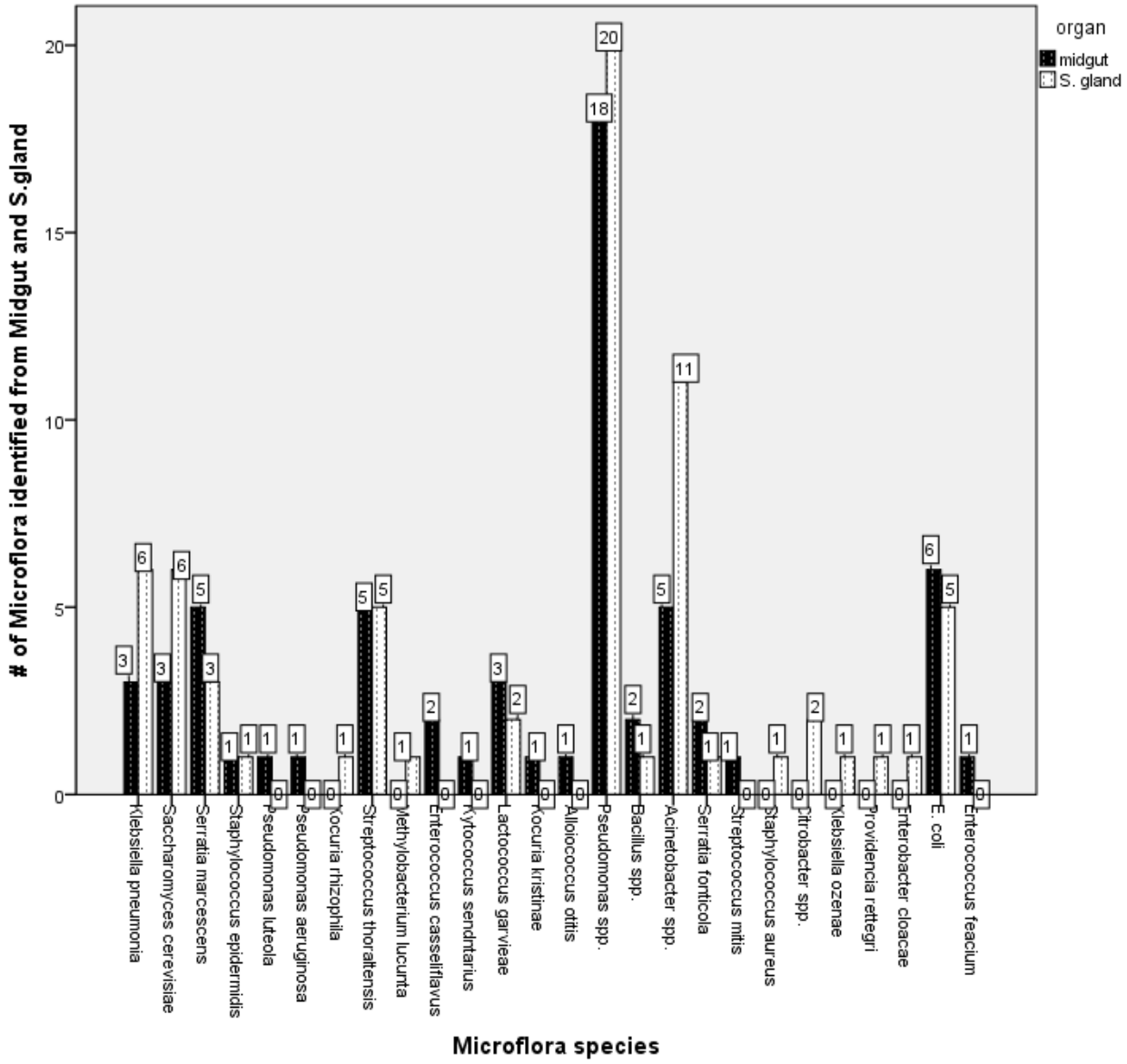

Figure 2: The total number of microflora species identified from the midgut and salivary glands of *Anopheles* mosquito species in Oromia region of Ethiopia.

Moreover, both laboratory reared and field collected *Anopheles* mosquitoes were assessed and their microflora proportion were analyzed. The major malaria vector, *Anopheles gambiae s.l* was found to be the highest number of microflora isolated from its midgut and salivary gland among field collected *Anopheles* species (figure 3).

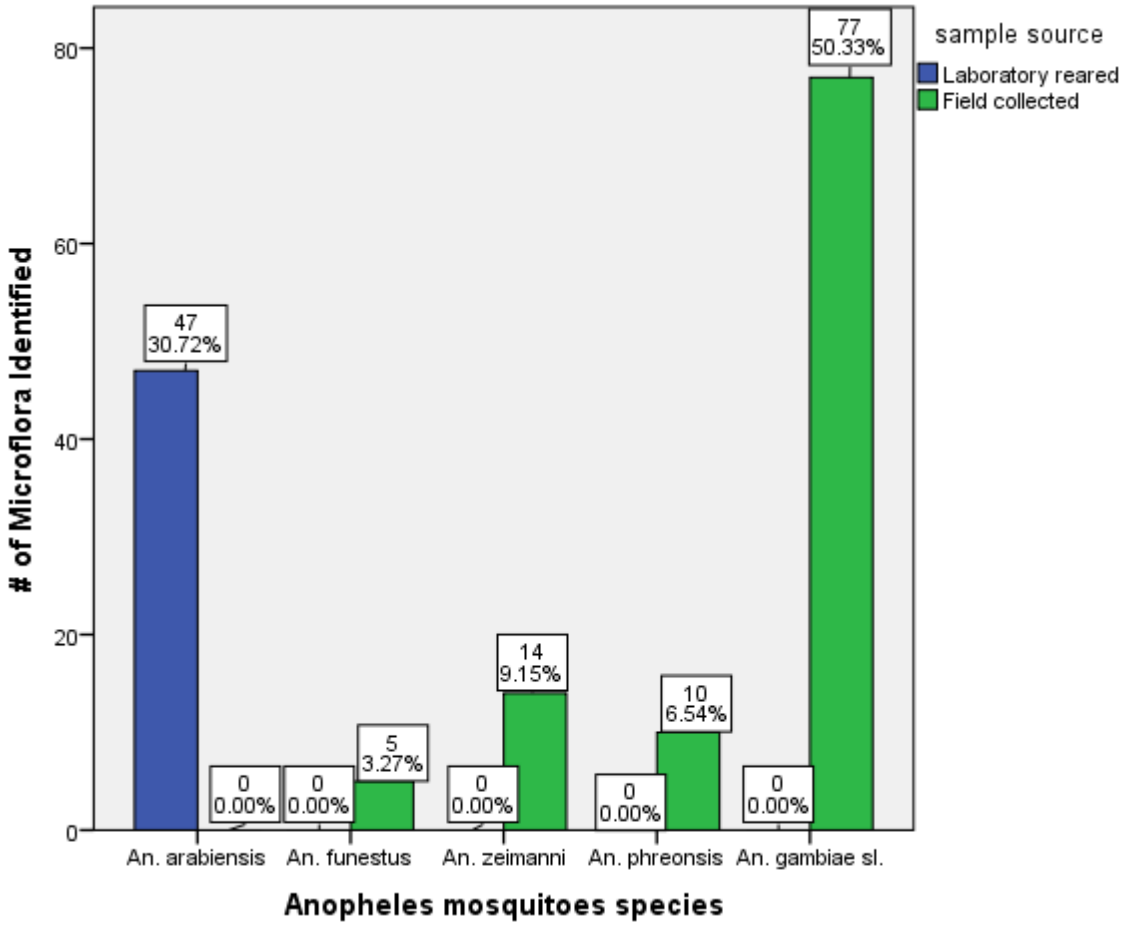

Figure 3: Isolation and identification of microflora from midgut and salivary gland of laboratory reared and field collected *Anopheles* species from insectary and field collected from Edo Kontola village and Batu Germama Kebele, Oromia Regional State, Ethiopia were assessed. From field collected, *Anopheles gambiae s.l* was the largest number of bacteria isolated 77(50.33%) followed by *Anopheles arabiensis* 47 (30.72%) from the insectary. But, *Anopheles funestus* 5 (3.27%) was the least microflora count among all *Anopheles* species from field collected *Anopheles* mosquitoes.

4.3 Comparison of microflora between laboratory reared and field collected *Anopheles* mosquitoes

A total of 26 microflora were identified from laboratory reared and field collected *Anopheles* species. The number of microflora from midgut and salivary gland were counted and the total number of each sources were counted (Table 6).

Table 6. The number of all microflora types identified from laboratory reared and field collected *Anopheles* species.

Microflora types	**Sample source**						**Total**
	Laboratory reared			*Field collected*			
	Midgut	S. gland	total	Midgut	S. gland	total	
Klebsiella pneumonia	0	1	1	6	9	15	16
Saccharomyces cerevisiae	3	6	9	0	0	0	9
Serratia marcescens	3	3	6	2	0	2	8
Staphylococcus epidermidis	0	1	1	1	0	1	2
Pseudomonas luteola	1	0	1	0	0	0	1
Pseudomonas aeruginosa	1	0	1	0	0	0	1
Kocuria rhizophila	0	1	1	0	0	0	1
Streptococcus thoraltensis	2	1	3	3	5	8	11
Methylobacterium lucunta	0	1	1	0	0	0	1
Enterococcus casseliflavus	2	0	2	0	0	0	2
Kytococcus sendntarius	1	0	1	0	0	0	1
Lactococcus garvieae	3	2	5	0	0	0	5
Kocuria kristinae	1	0	1	0	0	0	1
Alloiococcus otitis	1	0	1	0	0	0	1
Pseudomonas species	4	5	9	18	16	29	38

Bacillus species	0	1	1	2	0	2	3
Acinetobacter species	0	3	3	4	8	12	15
Serratia fonticola	0	0	0	2	1	3	3
Streptococcus mitis	0	0	0	1	0	1	1
Staphylococcus aureus	0	0	0	0	1	1	1
Citrobacter species	0	0	0	1	3	4	4
Klebsiella ozenae	0	0	0	0	1	1	1
Providencia rettegri	0	0	0	0	1	1	1
Enterobacter cloacae	0	0	0	0	1	1	1
E. coli	0	0	0	5	6	11	11
Enterococcus feacium	0	0	0	1	0	1	1
Total			47			90	137

At 5% level of significance, only *Serratia marcescens* was statistically significant difference of proportions between laboratory reared and field collected *Anopheles* species. On the other hand, all others bacterial species have no statistically significant difference of proportions between laboratory reared and field collected *Anopheles* species (Table 7).

Thus, the P-value compared with the level of significance (5%) and summarized. (Table 7 and Appendix 2).

Table 7. The result of two sample independent proportions test.

Bacterial species	**Null Hypothesis**	**Alternative Hypothesis**	**P-Value**	**Decision**
Klebsiella pneumonia	$H_o: P_L = P_F$	Ha: $P_L \neq P_F$	0.1085	No significant difference
Serratia marcescens	$H_o: P_L = P_F$	Ha: $P_L \neq P_F$	0.0173*	significant difference
Staphylococcus epidermidis	$H_o: P_L = P_F$	Ha: $P_L \neq P_F$	0.6748	No significant difference
Streptococcus thoraltensis	$H_o: P_L = P_F$	Ha: $P_L \neq P_F$	0.6868	No significant difference
Pseudomonas species	$H_o: P_L = P_F$	Ha: $P_L \neq P_F$	0.0629	No significant difference
Bacillus species	$H_o: P_L = P_F$	Ha: $P_L \neq P_F$	0.9259	No significant difference
Acinetobacter species	$H_o: P_L = P_F$	Ha: $P_L \neq P_F$	0.1274	No significant difference

Where P_L is proportion of Laboratory reared and P_F is proportion of field collected *Anopheles* species.

Proportion is significant if $P < 5\%$

5. DISCUSSION

Identification of midgut and salivary gland microbiota in vector mosquitoes has received notable attention since recent studies suggest that the composition microflora of the vector midgut and salivary gland affect the outcome of the mosquito infection with the *Plasmodium* parasites (Gonzalez-Ceron *et al*., 2003). Although a number of studies have been carried out to identify the bacterial microbiota of *Anopheles* mosquitoes, several important vectors remain unstudied in Ethiopia.

Among those were *An. arabiensis*, *An. zeimanni*, *An. gambiae s.l*, *An. pharoensis* and *An. funestus* which have been investigated in the current study, and from which aerobic midgut and salivary gland bacteria have been isolated and identified. Strikingly, as seen lack of differences between the identified bacteria despite coming from different species and feeding stages. This suggests that the bacteria acquired by *Anopheles* mosquitoes is not random selection, but instead points to be possible co-adaptation between feeding stages and bacteria which interact in mosquitos' midgut and salivary gland (Gonzalez-Ceron *et al*., 2003).

The number of isolates in this study still showed that, gram negative bacteria dominate the flora of *Anopheles* mosquito species (Table 3,4 & 5). This agrees with earlier results from culture based studies on *An. stephensi* and *An. maculipennis* in Iran (Djadid *et al*., 2011) and *An. gambiae s.l* and *An. funestus* in Kenya and Mali (Straif *et al*., 1998), and also data from sequence based studies on *An. darlingi* in Brazil (Terenius *et al*., 2008) and *An. gambiae* in Kenya (Gillies and Coetzee, 1987) all showing predominance for gram negative bacteria.

Some bacteria are common among several important vectors. For instance, the genus *Serratia*, which was isolated from *An. stephensi* (Rani *et al.,* 2009) , *An. culicifacies* (Chavshin *et al*., 2012) and *An. gambiae* (Cirimotich *et al.,* 2011) have now been identified from laboratory reared *An. arabiensis* in midgut and salivary gland as well as all field collected *Anopheles* mosquitoes' species in this study due to its closest in the midgut and salivary gland microenvironment modulate the life cycle of the vectors and sporogonic development of the malaria parasite (Cirimotich *et al*., 2011; Tchioffo *et al*., 2016).

Another common bacterial genus is *Pseudomonas,* which has been found among several mosquito vectors in Asia and the Americas (Terenius *et al*., 2008; Chavshin *et al.,* 2012).

However, a recent study found that *Pseudomonas* only at a low level in Kenyan mosquitoes (Osei-Poku *et al.*, 2012). In our study, we identified *Pseudomonas* as the most frequently isolated bacteria from laboratories reared *Anopheles arabiensis* in midgut and salivary glands; from field collected fed midgut, gravid and unfed salivary gland of *An. funestus*; gravid midgut and salivary glands, unfed midgut and salivary glands of *An. pharoensis*; fed midgut and salivary glands, unfed midgut and salivary glands and gravid midgut and salivary glands from *An. zeimanni*; and fed midgut and salivary glands, gravid midgut and salivary glands, unfed midgut and salivary glands of *An. gambiae s.l.*

The same finding was made previously from malaria mosquito's epithelia especially the genera of *Pseudomonas*, *Serratia* and *Acinetobacter* in the midgut while *Pseudomonas* and *Acinetobacter* from salivary glands of *Anopheles* mosquito species (Tchioffo *et al.*, 2016) and this suggests that some bacterial taxa were more associated with one or another tissue at a particular time point of physiology and breeding site. A note of caution is that among the more than 100 *Pseudomonas* species some are pathogenic; for example, *Pseudomonas aeruginosa* is an opportunistic human pathogen in severely immunocompromised patients (Gellatly and Hancock, 2013). Therefore, care must be taken so that for a paratransgenesis approach only *Pseudomonas* species that are nonpathogenic should be selected.

Although the term microbiota is primarily used to describe bacteria, fungi are frequently identified from the larvae in water, and as adults either by ingesting fungi in a sugar meal or by external physical contact with fungal spore (Lynch*et al*., 2012). As a result, the mosquito midgut and salivary glands have a common fungus include molds and yeast (Virginio *et al.*, 2014). In this study, a total of nine pools of mosquito samples, three from midgut and six from salivary gland were isolated from laboratory reared *Anopheles arabiensis* and this result is in line with the finding that yeasts have been isolated from both laboratory reared and field collected *Anopheles* mosquitoes (Cappelli *et al*., 2014).

Several previously studies have already analyzed mosquito microbiota and field of their respective breeding sites using molecular approach, mainly based on analyzing sequences of the 16 ribosomal RNA gene and cultures of fed and gravid *An. gambiae* microbiota indicated that, *Serratia marcescens* and *Streptococcus mitis* were the dominant isolates from *An. gambiae* (Tandina *et al*., 2016). However, in our study *Serratia marcescens* and *Streptococcus mitis* were identified from gravid *An. funestus* and midgut of *An. gambiae sl* respectively. Moreover, the

midgut of fed *An. gambiae* showed that, *Staphylococcus epidermidis* and *Enterobacter cloacae* were the dominant isolated bacteria. But in our study, these isolates were identified from the midgut of unfed *An. pharoensis* and similar with fed salivary gland of *An. gambiae s.l* respectively this is due to the different breeding places that affects their adaption of different microbiota in their midgut as well as salivary gland of *An. gambiae* in southern France.

Anophelines harbor a diverse microbiota which affects the insect physiology, metabolism and immune processes. A recent study showed that, current metagenomic tools combined with conventional culture-dependent techniques provided an insight in the elucidation of the role of the *Anopheles*-associated microbiota and this meta-analysis reported 105 genera of bacteria. Among these, *Lactococcus garvieae*, *Kocuria kristinae*, *Enterococcus casselifavus* and *Methylobacterium lacunta* were identified from different organs of *Anopheles* mosquitoes from different geographical locations (Villegas and Pimenta, 2014). This result is correlated with our study, theses microbiota was identified from both midgut and salivary gland (*Lactococcus garvieae*), midgut (*Kocuria kristinae*), midgut (*Enterococcus casseliflavus*) and salivary gland (*Methylobacterium lacunta*) from laboratory reared *Anopheles arabiensis* respectively this suggests that the food source and the environment from which larva transported to the laboratory to be reared in insectaries.

Moreover, the number of bacterial genera were reported per human malaria vectors. A current count on bacterial genera reported for a multiple anopheline species and a total of 109 unique genera were identified amongst 218 published reports using inclusion criteria (Villegas, 2014). In this study, *Anopheles gambiae* (71 genera) was the most reported associated bacterial genera followed by the Asian human malaria vector *Anopheles stephensi* (46 genera); *Anopheles arabiensis* (23 genera) and *Anopheles funestus* (16 genera). However, in the case of our study, *Anopheles arabiensis* (14 genera) was most bacteria genera associated with; *Anopheles gambiae s.l* (9 genera) and *Anopheles zeimanni* and *Anopheles phreonsis* 7 and 6 genera respectively.

6. CONCLUSION

This study describes isolation and identification of microbiota in the midgut and salivary glands of *Anopheles arabiensis* from insectary and fed, gravid and unfed mosquitoes of four species from the field collected *Anopheles* species. This study provided an in depth description of the microbiota diversity in midgut and salivary glands of *Anopheles* mosquitoes in some malaria endemic areas of Ethiopia.

From this report, forty genera were identified and can be a milestone for studying relationship between microbiota and mosquitoes and for the development of a new malaria control strategy. Our findings indicated that, *Pseudomonas* species had the dominant microbiota identified from all species of field collected mosquitoes and laboratory reared *Anopheles* mosquitoes and *An. arabiensis* had got a diversified microbiota from the rest of species. *An. gambiae s.l* had got the largest microbiota identified from all species of *Anopheles* mosquitoes.

Microbiota which was dominantly isolated and identified from the midgut and salivary glands of laboratory reared and field collected *Anopheles* mosquitoes have been the new option for mosquito vector management in endemic areas of Ethiopia. Therefore, this report is important for researchers to develop a new options of malaria vector control strategy.

7. RECOMMENDATIONS

- *Pseudomonas* was the dominant isolated and identified bacteria and its mechanisms, positive or negative contributions on the development of *Plasmodium* parasites should be studied further in Ethiopian context.
- The mechanism how gram negative bacteria secrets anti-plasmodium substance, midgut enzymes and peritrophic proteins should be investigated in order to introduce and successfully maintain these microfloras in the field collected *Anopheles* mosquito species. This may be more optimal for paratransgenic application in implementation of vector control strategies in endemic areas of Ethiopia.
- Unidentified microbiotas should examine more through the use of culture independent sequencing of bacterial 16S rRNA for identifying substantial diversity of microbiota among the mosquito species in malaria endemic areas of Ethiopia.
- This result may provide to implement integrated vector management for researchers in malaria elimination program and must be studied more on the contribution of parasite-vector control options in malaria endemic areas of Ethiopia.

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9. APPENDICES

Appendix 1: Quality control for equipments and chemical reagents done in Ethiopian Public Health Institute (EPHI)

Equipments/ chemical reagents	**Vitek 2 Compact**	Incubator (5% CO_2)	Aerobic incubator	Biochemical test reagents	**Culture media**	**P^H of DH_2o**
Quality control tests	**G-ve *E. coli*; G+ve *S. aureus*; *P. aeruginosa* (NLF) (ATCC: 25922); (ATCC: 25923); (ATTC: 27853)**	**Fixed T^o: 35±2^0C ; Co2 reading, RH (80%) via back of Incubator (Tap water) External Thermometer- compared with the fixed T^o**	**Testing its temperature by using thermometer (External thermometer by lab personnel)**	Using ATCC for each **LDC**: Test for *E. coli* Test for *Proteus* **Citrate**: Test for *E. coli* Test for *Kleb/Prot* **Motility**: Test for *E. coli* Test for *Kleb/Shi* **KIA**: Ac/Ac *E. coli/Kleb* Ac/Alk *Shig/Proteus* Alk/Alk *Pseud/Acine* **TSIA: SIM: H_2S *Salmo/Proteus* Motility: indole *E. coli* *K. pneumonia***	**(Blood, Mac, SDA, CHO) *Sterility*: incubate without any micro organism *Growth support:*** Hemolysis; Blood **Beta Alpha Gamma**	**Measurement of P^H**
Results of quality control tests	***E. coli* 98%; *S. aureus* 99%; *P. aeruginosa* 98%**	***St. pneumonia* *St. pyrogen* *H. influenza* *Neisseria* spp. *Gonorrhea* *meningitis* if all are grown**	***Pseudomonas spp.* *Acinetobacter spp.* *Enterobacteriaceae* If all are grown**	**LDC:** Positive for *E. coli* Negative for Proteus **Citrate:** Negative for *E. coli* Positive for *Kleb. /Pro.* **Motility**: Positive for *E. coli* Negative for *Kleb. /Shig.* **KIA**: Positive for *E. coli/Klebsiella* Positive for *Shigella/Proteus* Positive for *Pseudomonas/Acinit* **SIM**: Positive for *Salmonella/Proteus* **Indole**: Positive for *E. coli* Negative for *K. pneumo.*	**No growth** **Complete**: *Staph* **Partiall**y: *St. pneum* **No hemolysis**: *E. faecalis*	**7.0 – 7.4**
Remarks	**The machine is working properly or it identified the bacterial isolates correctly.**	**The 5% incubator is working properly**	**The aerobic incubator working properly**	**If all these tests were confirmed accordingly, they are ready to use for each biochemical test.**	**Pure Properly working of growth supporting media**	**Used for prepare media**

Appendix 2. Statistical Analysis Result

The result of two sample independent proportion test

Number of bacteria from laboratory reared = **47**

Number of bacteria from field collected = **90**

***Anopheles* species**	**# of bacteria in laboratory reared**	**# of bacteria in field collected**	**Proportion in laboratory reared**	**Proportion in field collected**
Klebsiella pneumonia	1	8	0.021277	0.088889
Serratia marcescens	6	2	0.12766	0.02222
Staphylococcus epidermidis	1	1	0.021277	0.011111
Streptococcus thoraltensis	3	7	0.06383	0.07778
Pseudomonas species	9	29	0.191489	0.322222
Bacillus species	1	2	0.021277	0.022222
Acinetobacter species	3	13	0.06383	0.14444

Proportion of ***Klebsiella pneumonia*** for laboratory reared **is 0.0212765957**

Proportion of ***Klebsiella pneumonia*** for field collected **is 0.0952380952**

Two sample test proportion

Variable	Proportion	Stand. Error	Z	P>|z|	[95% Conf. interval]
Lab-reared	0.021277	.021049			-.0199787 .0625319
Field collected	0.088889	.0320282			.0324639 .1580123
Diff.	-0.067612 Under Ho:	.0383258 .0460762	-1.61	0.108	-.1490787 .0011557

Ho: Proportion of lab-reared – Proportion of field collected

Ho= difference; **Ho= PL=PF=0 Vs Ha: PL≠PF**

P value = **0.1085**>**α 5%** We **do not reject Ho**, i.e. **no difference**

Proportion of ***Serratia marcescens*** for laboratory reared **is 0.12766**

Proportion of ***Serratia marcescens*** for field collected **is 0.02381**

Two sample test proportion

Variable	Proportion	Stand. Error	Z	P>|z|	[95% Conf. interval]
Lab-reared	.1276596	.0486767			.0322551 .2230641
Field collected	0.02222	.0166342			-.008793 .056412
Diff.	.1054374 Under Ho:	.0514404 .0436188	2.38	0.017	.0030287 .2046714

Ho: Proportion of lab-reared – Proportion of field collected

Ho= difference; **Ho= PL=PF=0 Vs Ha: PL≠PF**

P value = **0.017 < α 5% We reject the Ho, i.e. significance difference**

Proportion of ***Staphylococcus epidermidis*** for laboratory reared **is 0.021277**

Proportion of ***Staphylococcus epidermidis*** for field collected **is 0.011905**

Two sample test proportion

Variable	Proportion	Stand. Error	Z	P>|z|	[95% Conf. interval]
Lab-reared	0.021277	.021049			-.0199787 .0625319
Field collected	0.011111	.0118337			-.0112888 .0350984
Diff.	.010166 Under Ho:	.0241474 .022335	0.42	0.675	-.0379562 .0566999

Ho: Proportion of lab-reared – Proportion of field collected

Ho= difference; **Ho= PL=PF=0 Vs Ha: PL≠PF**

P value = **0.675 > α 5% We do not reject the Ho, i.e. no difference**

Proportion of *Streptococcus thoraltensis* for laboratory reared **is 0.06383**

Proportion of *Streptococcus thoraltensis* for field collected **is 0.083333**

Two sample test proportion

Variable	Proportion	Stand. Error	Z	P>|z|	[95% Conf. interval]
Lab-reared	0.06383	.0356566			-.0060559 .1337155
Field collected	0.07778	.0301561			.0242284 .1424382
Diff.	-.01395 Under Ho:	.0466989.0483692	-0.40	0.687	-.1110317 .0720246

Ho: Proportion of lab-reared – Proportion of field collected

Ho= difference; **Ho= PL=PF=0 Vs Ha: PL≠PF**

P value **= 0.687 > α 5% We do not reject the Ho, i.e. no difference**

Proportion of *Pseudomonas species* for laboratory reared **is 0.191489**

Proportion of *Pseudomonas species* for field collected **is 0.345238**

Two sample test proportion

Variable	Proportion	Stand. Error	Z	P>|z|	[95% Conf. interval]
Lab-reared	0.191489	.057394			.0789992 .3039795
Field collected	0.322222	.0518754			.2435642 .446912
Diff.	-.130733 Under Ho:	.0773636 .0826626	-1.86	0.063	-.3053786 -.0021189

Ho: Proportion of lab-reared – Proportion of field collected

Ho= difference; **Ho= PL=PF=0 Vs Ha: PL≠PF**

P value **= 0.063> α 5% We do not reject the Ho, i.e. no difference**

Proportion of *Bacillus species* for laboratory reared **is 0.021277**

Proportion of *Bacillus species* for field collected **is 0.02381**

Two sample test proportion

Variable	Proportion	Stand. Error	Z	P>|z|	[95% Conf. interval]
Lab-reared	0.021277	.021049			-.0199787 .0625319
Field collected	0.022222	.0166342			-.008793 .056412
Diff.	-.000945 Under Ho:	.0268283.0272484	-0.09	0.926	-.0551155 .0500496

Ho: Proportion of lab-reared – Proportion of field collected

Ho= difference; **Ho= PL=PF=0 Vs Ha: PL≠PF**

P value **= 0.926 > α 5% We do not reject the Ho, i.e. no difference**

Proportion of *Acinetobacter species* for laboratory reared **is 0.06383**

Proportion of *Acinetobacter species* for field collected **is 0.154762**

Two sample test proportion

Variable	Proportion	Stand. Error	Z	P>|z|	[95% Conf. interval]
Lab-reared	0.06383	.0356566			-.0060559 .1337155
Field collected	0.14444	.0394623			.0774173 .2321065
Diff.	-.08061 Under Ho:	.0531852 .0596465	-1.52	0.127	-.1951732 .0133089

Ho: Proportion of lab-reared – Proportion of field collected

Ho= difference; **Ho= PL=PF=0 Vs Ha: PL≠PF**

P value **= 0.127> α 5% We do not reject the Ho, i.e. no difference**