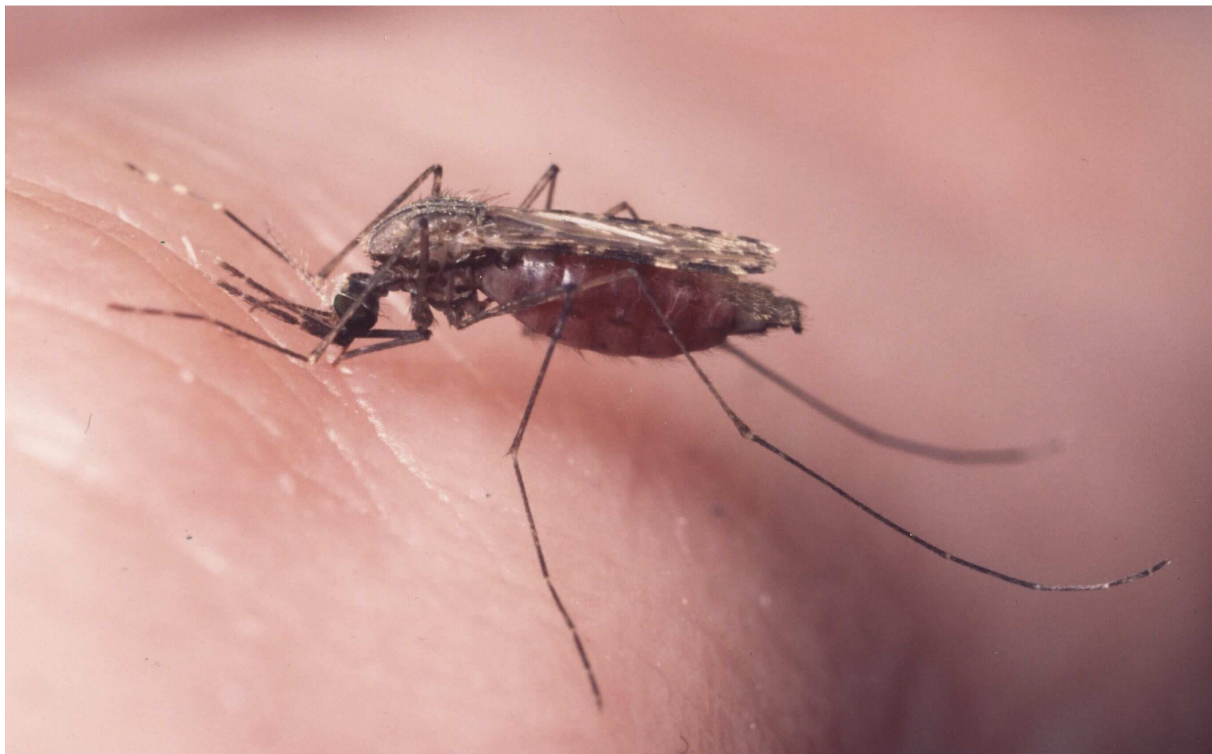
WAGENINGEN UNIVERSITY
LABORATORY OF ENTOMOLOGY

The attractiveness of different species of skin microorganisms to the malaria vector *Anopheles gambiae s.s.*



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Abstract

The malaria vector *Anopheles gambiae s.s.* is guided by human odors when searching for its host. Research showed that incubated sweat is more attractive than fresh sweat and that this difference is caused by the volatiles produced by bacteria. A recent study showed that a mix of microorganisms from the human skin grown on agar were attractive to *A. gambiae*. Many different types of microorganisms can be found on the human skin. While much is still unknown, a number of them have been studied in detail, especially those related to infections and odor production. In this study it was determined which bacterial species common on human skin affect the behavior of *A. gambiae.* Several skin microorganism species were cultured on a standard liquid medium. The effects of five successfully cultivated bacteria species on the behavior of *A. gambiae* were studied in a dual-port olfactometer experiment. Logistic growth phase and stationary phase concentrations of the bacteria were used while using medium as a negative control and a mix of bacteria from a human foot as a positive control. This resulted in the mix from a human foot and the stationary phase concentrations of *Staphylococcus epidermidis*, *Bacillus subtilis*, *Brevibacterium epidermidis* and *Corynebacterium minutissimum* being significantly more attractive than the medium while the logistic growth phase concentrations of all the bacteria species, the stationary phase concentration of *Pseudomonas aeruginosa* and the medium were not. In a subsequent olfactometer experiment the stationary phase concentrations of the four attractive bacteria species plus the mix from a human foot were tested against ammonia. This resulted in a ranking showing the relative attractiveness compared to ammonia. *Corynebacterium minutissimum* and the mix from a human foot were significantly more attractive than *Staphylococcus epidermidis* and *Bacillus subtilis*. The stationary phase concentrations of *Staphylococcus epidermidis*, *Bacillus subtilis*, *Brevibacterium epidermidis* and *Corynebacterium minutissimum* were furthermore tested in an MM-X experiment with the medium as a control. In this experiment none of the bacteria species was significantly more attractive than the control. This study provides interesting insights in the attractiveness of skin bacteria to *A. gambiae*. Further studies, especially analyses of the odors secreted by the bacteria, need to be carried out, aiming at the development of new mosquito trapping and control systems.

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Introduction

Malaria is one of the deadliest diseases in the world and continues to infect approximately 500 million people each year, especially in tropical Africa. About one million of them die (Greenwood et al., 2008). While the actual cause of the disease is the *Plasmodium* parasite, the mosquitoes that carry this parasite are responsible for transferring it. The most important malaria vector is *Anopheles gambiae* Giles *sensu stricto* (henceforth referred to as *A. gambiae*). This species is highly antropophilic, i.e. it feeds almost entirely on humans. While also other mosquito species can transmit the disease, they usually feed on other host species also and are thus less instrumental in spreading malaria among humans. Malaria was eliminated in the United States in the middle of the twentieth century and has since long been banished from Europe. The Global Malaria Eradication Program, founded by the World Health Organization in 1955 failed however to eradicate malaria from the third world due to the development of resistance against pesticides in both the *Plasmodium* parasite and the mosquitoes (Greenwood et al., 2008). Recently, more studies have been conducted on mosquito host-seeking behavior. New keys in the fight against malaria may be found in this field. When it is known by which mechanisms mosquitoes choose and locate their hosts, this knowledge can be used to our advantage by developing repellents and traps to catch the mosquitoes, for example in association with newly developed baits.

Olfaction in mosquitoes

Mosquitoes locate their hosts, as well as their mating partners, mainly by olfactory cues. They have receptors for semiochemicals on their antennae and palps (Bowen, 1995;Lu et al., 2007). Studies using tests in which the only information from hosts was provided by odors showed that odors secreted by host species attract mosquitoes (Costantini et al., 1993). Many mosquitoes are attracted by carbon dioxide (CO_2) (Costantini et al., 1996), presumably because of the presence of CO_2 in the breath of hosts. *A. gambiae* however is highly anthropophilic and therefore uses other cues than carbon dioxide alone to locate its host. Carbon dioxide is especially attractive to antropophilic mosquitoes in combination with other compounds like L-lactic acid and ammonia, which are known to be present in the human odour blend. Ammonia in itself has shown to be attractive to *A. gambiae* however too high concentrations are repulsive (Dekker et al., 2002;Smallegange et al., 2005). L-lactic acid is a component of human sweat. This is another indication that sweat plays a role in mosquito host-seeking behavior. A study by Knols and De Jong (1996) showed that Limburger cheese, chosen because its smell resembles the smell of human feet, attracts female *A. gambiae*. An explanation for the similar smell of Limburger cheese and human feet, and the attractiveness of this cheese for *A. gambiae*, may lay in the fact that related bacteria can be found in both the cheese and on human feet (*Brevibacterium* sp) (Knols and De Jong, 1996). These bacteria emit fatty acids causing the distinguished sweat smell. This may indicate that mosquitoes are, at least partly, attracted by the odors produced by bacteria. Research showed that incubated sweat is more attractive than fresh sweat and that this difference is caused by the volatiles produced by bacteria (Braks et al., 2000;Meijerink et al., 2000). It could explain why certain people are more attractive to mosquitoes then others since there are large differences in microorganism composition between individuals, caused by differences in the physical characteristics of the skin, pH, the availability of nutrients and other factors (Braks et al., 1999;Noble, 2004).

A recent study showed some interesting results on the attractiveness of skin bacteria to malaria mosquito *A. gambiae* (personal communication N. Verhulst). In this study microorganism samples were collected from a human foot and the attractiveness of these

samples was tested in a dual-port olfactometer with a sample on blood agar in one port and sterile blood agar in the other. Agar plates with microorganisms proved to be significantly more attractive than control agar plates. Odors were collected from agar plates containing the microorganisms and chemically analyzed to detect compounds emitted by the microorganisms. This resulted in fourteen compounds which were significantly more present in the air collected from above the agar plates with skin microorganisms compared to control samples of air. These compounds were then presented to *A. gambiae* in a synthetic mixture and proved to be attractive for the mosquitoes. A chemical analysis was also performed for only *Staphylococcus epidermidis.* This resulted in five compounds which were significantly more present than in the control. These five components were also present in the analysis of the foot microorganisms. *S. epidermidis* was however less attractive than the foot samples, indicating that there are more attractive compounds in the foot samples, possibly secreted by other bacteria species.

Sweat glands of the human skin

Several types of glands can be found on the human skin. These glands excrete compounds with different functions, like pheromones, sweat and fat. Fat is secreted by sebaceous glands. They can be found everywhere on the body except for the hand palms and the soles of the feet. The main purpose of fat is to keep the skin waterproof. Sweat glands can be divided in two subgroups: apocrine and eccrine sweat glands. Eccrine sweat glands can be found all over the body but especially on feet soles and serve mainly to control body temperature. Apocrine sweat glands can be found mainly in the axilla and in the genital area. Apocrine glands open in a hair follicle. They secrete lipids and pheromones and are supposed to be the main contributors to sweat odour (Zeng et al., 1992). Knowledge of these glands is important since they may have influence on the volatiles produced by skin microorganisms.

Human skin microorganisms

Many different types of microorganisms can be found on the human skin. While much is still unknown, a number of them have been studied in detail, especially those related to infections and odor production. An important group of bacteria on the human skin comprises the *Staphylococcus* genus, which are aerobic, Gram-positive bacteria. *S. epidermidis* is the most abundant on the skin except for the arms and legs where *S. hominis* is predominating. Several other species can be found on more isolated areas, like *S. aureus* which can be found mainly in the nostrils (Noble, 2004).

Another group commonly found on human skin consists of the coryneform bacteria, most notably the *Brevibacterium, Propionibacterium* and *Corynebacterium* genera (Braks et al., 1999;Noble, 2004). This is a heterogeneous group consisting of Gram-positive bacteria. The group can roughly be divided in two subgroups: coryneforms whose growth depends on lipids and coryneforms whose growth does not depend on lipids. The lipid-dependent coryneforms are the most abundant on human skin (Noble, 2004). The lipid-dependent aerobic species are found mainly in de wetter areas of the skin, like the space between the toes, nostrils and axilla. Though these areas do not contain many sebaceous glands, they seem to provide enough lipids to maintain the bacteria (McGinley et al., 1985). Knols et al. (1997) suggested that bacteria in Limburger Cheese are responsible for the production of the 'human-specific' odors, involved in host seeking of *A. gambiae*. *Brevibacterium linens* is involved in the ripening of Limburger cheese and closely related to *Brevibacterium epidermidis*, which is a resident of the microflora on human feet (Anthony et al., 1992;Noble, 2004). It is suggested that dairy *Brevibacterium,* like *B. linens,* may have originated from either humane or bovine skin (Jackman, 1982). *B. epidermidis* could be one of the bacteria on human skin involved in the production of odors attractive to malaria mosquitoes (Knols and De Jong, 1996). Research

showed that coryneforms, especially brevibacteria and micrococci, in the armpits are responsible for the production of volatile fatty acids (VFAs) which cause the axillary malodour (James et al., 2004a). The most important anaerobic lipophilic coryneform is *Proponibacterium acnes* (McGinley et al., 1978;Noble, 2004). It can be found in areas with many sebaceous glands, like the face. Another microorganism which can be found near high densities of sebaceous glands is the lipophilic fungus *Malassezia furfur* (Wilde and Stewart, 1968).

Bacteria of the *Bacillus* genus are common on human feet and have been detected more frequently on people with strong foot odour (Ara et al., 2006). *Bacillus* species have been detected in relatively high numbers on children and during months with relatively high temperatures (Kloos and Musselwhite, 1975).

Recently, a molecular study analyzing the diversity of human skin microorganisms has shown that bacteria from the Gram-negative, aerobic genus *Pseudomonas* are by far the most abundant species on human arms. Grice et al. (2008) examined the arms of human subjects and found that bacteria of this genus were flourishing on the more moist parts of the arm, like the antecubital fossa. However these bacteria are more known as invasive species of wounds and not as permanent skin residents, a single *Pseudomonas* species was responsible for 59 % of the sequences while only less than 5 % of the microorganisms consisted of *S. epidermidis* and *Pr. acnes* (Grice et al., 2008). (Gao et al., 2007) also examined human arms and did not find such a distinguished high level of *Pseudomonas*, possibly because they only examined the volar forearm.

Microorganism growth has two important stages: a stage in which logistic growth can be seen as the microorganisms multiply exponentially in an abundance of resources, and a stage in which the maximal density has been reached (Figure 1)(Zwietering et al., 1990). A spectrophotometer can be used to determine the stage of a certain microorganism culture by measuring optical densities for different incubation times.

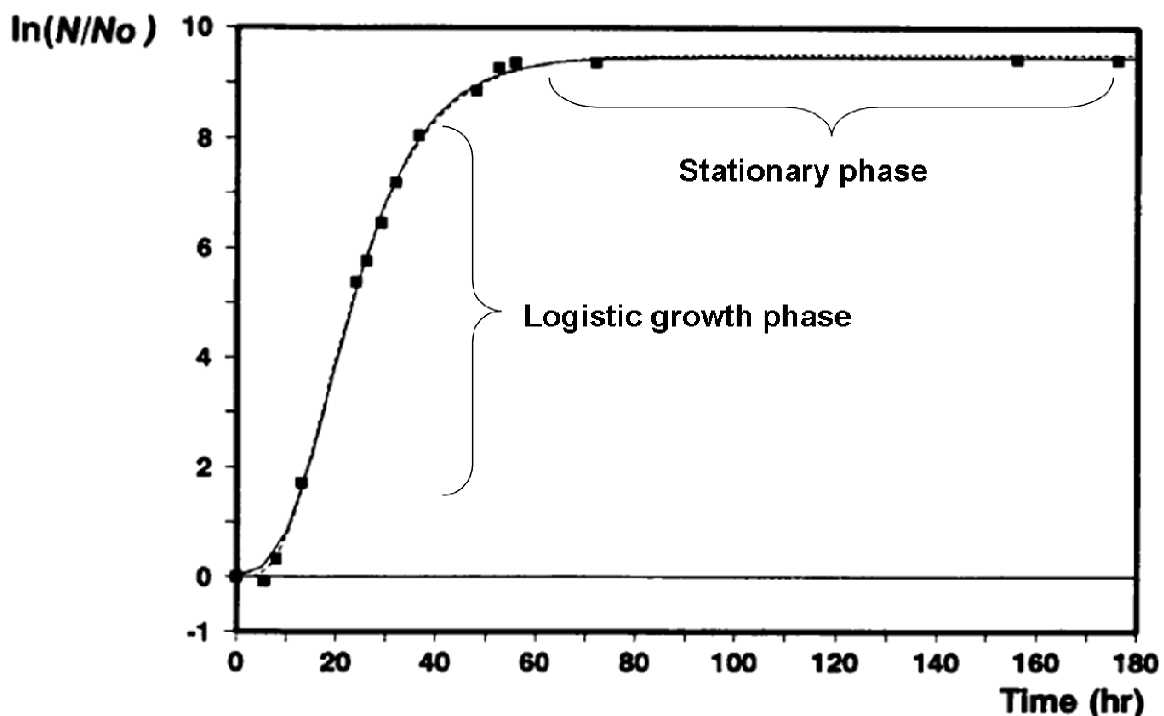

Figure 1: example of a growth curve showing the logistic growth phase and the stationary phase for *Lactobacillus plantarum* (modified after Zwietering et al., 1990).

Volatile fatty acids

The compounds secreted by the sweat glands are in itself not very odourous. Studies have shown that the bacteria at the glands consume the compounds and the excreted volatile fatty acids (VFAs) are responsible for the sweat smell. Examples of these fatty acids are acetic acid, propionic acid, isovaleric acid and isobutyric acid (Ara et al., 2006). While the vast majority of the metabolized fatty acids consists of acetic acid, isovaleric acid and isobutyric acid also contribute greatly to foot odour because of their sensory detection thresholds being much lower than that of acetic acid. Bacteria of the *Bacillus* genus produce isovaleric acid and are thought to be great contributors to foot odour (Ara et al., 2006). Different *Propionibacterium* and *Staphylococcus* species of axillary bacteria have been tested for their ability to produce C_2-C_3 VFAs, acetic and propionic acid under anaerobic conditions (James et al., 2004b). Results show that most of the bacterial strains produce these VFAs as products of the fermentation of glycerol and lactic acid. In another experiment several axillary bacteria species, including *Staphylococcus*, *Brevibacterium* and *Propionibacterium* species, were tested for their ability to produce VFAs from valine, leucine and isoleucine. Results show that only the *Staphylococcus* species can use these amino acids to produce the highly odourous methyl-branched C_4-C_5 VFAs. No direct link however was found between the numbers of *Staphylococcus* bacteria and the malodour intensity in the axilla, possibly because of the variability in VFA generation for different *Staphylococcus* strains (James et al., 2004b). It has however been suggested that lipid dependent corynebacteria are the major contributors to axillary malodour because they are unable to fully degrade isostearic and isopalmic acids, leaving high levels of VFAs as a result while *Micrococcus* and *Brevibacterium* completely degrade the substrate, leaving smaller amounts of VFAs (James et al., 2004a). An association between the number of corynebacteria and the intensity of axillary malodour has been found (Taylor et al., 2003). VFAs are also interesting because they have already shown to be attractive to *A. gambiae* (Knols et al., 1997). Smallegange et al. (2005) indicates that *A. gambiae* relies on the combination of CO_2, lactic acid and VFAs to find its human host, however the VFAs alone, carboxylic acids in this case, were repellent to the mosquito.

Scientific relevance

Research considering the attractiveness of skin microorganisms to mosquitoes is still in its infancy. It is only in the last decade that the link between mosquitoes and skin bacteria has been clearly laid. Unraveling these mechanisms provides many challenges. When we want to identify human odors that play a role in mosquito behavior, knowledge about the skin microorganisms and the volatiles they emit is essential. The link between microorganisms and mosquito behavior furthermore provides an interesting combination of different disciplines, linking microbiology with behavior experiments.

Social relevance

Since mosquitoes infected with the *Plasmodium* parasite continue to infect and kill millions of people, research which leads to new ways of controlling malaria has a very high social relevance, especially if this can lead to practical control mechanisms which can be used on a large scale. The increasing resistance of mosquitoes for insecticides increases the urgency of developing alternative control mechanisms. Odors attracting mosquitoes may be used in traps, providing a relatively simple method to reduce the impacts of malaria. Before odors produced by skin bacteria can be used in practical control mechanisms or monitoring tools it first has to become clear which bacteria emit attractive compounds and which compounds this are. Then the suitable microorganisms can be selected and cultured for use in traps, or the compounds produced by the microorganisms can be analyzed and synthetically produced.

Objectives

Determine which bacterial species common on human skin affect the behavior of *A. gambiae s.s.*

- *Is it possible to cultivate seven skin microflora species on liquid medium?*

- *What is the growth rate of the microorganisms in liquid medium?*

- *What is the effect of volatiles emitted by the microorganism species on* A. gambiae *in a dual port olfactometer?*

- *What is the effect of volatiles emitted by the microorganism species on* A. gambiae *in MM-X traps?*

Methods

In this study behavioral experiments have been performed in which female *A. gambiae* were released in a dual port olfactometer in order to study the effect of different skin microorganism species on *A. gambiae* behavior. These species were *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Corynebacterium minutissimum*, *Brevibacterium epidermidis, Micrococcus luteus*, *Malassezia furfur* and *Bacillus subtilis* (Table 1). These species were chosen because they represent a wide range of different microorganism species which are all found regularly on human skin. Anaerobic species like *Propionibacterium acnes* were not included because it would not be possible to test them in an anaerobic environment in the olfactometer. The microorganism species came from DSMZ (Germany) or, in case of *P. aeruginosa* and *B. subtilis*, from the laboratory of Microbiology at Wageningen University and Research centre.

Table 1: The microorganism species. NCTC and ATCC numbers are international standard reference numbers.

Species	NCTC Number	ATCC Number	DSMZ Number
Staphylococcus epidermidis	11047	14990	20044
Corynebacterium minutissimum	10288	23348	20651
Micrococcus luteus	2665	4698	20030
Brevibacterium epidermidis	11083	35514	20660
Malassezia furfur		12078	6170
Pseudomonas aeruginosa			
Bacillus subtilis			

Cultivation of the microorganism species

All microorganism species were cultivated on Petri-disks containing species-specific agar media to enable optimal growth (see appendix 1). They were kept at 34° C, the average temperature of the human skin. Single colonies of successfully cultivated species were later again incubated in tubes containing 5 ml standard liquid medium (Table 2) to prevent the different media from having influence on the outcome of the experiments. Liquid medium was chosen to be able to determine microorganism concentrations. The tubes containing the medium and the microorganisms were placed in an incubator (Innova^{tm}4000) at 34° C shaking at 225 rpm. After incubation, for each successfully cultivated species a glycerol stock was made containing 300 µl glycerol and 700 µl medium. These glycerol stocks were then placed in a freezer at -80° C.

Table 2: ingredients of the liquid medium

ingredient	***Per liter medium***
Infusion from heart muscle	2.0 g
Pancreatic digest of casein	13.0 g
Yeast extract	5.0 g
Sodium chloride	5.0 g
Destilled water	1000 ml

Microorganism growth curves

To determine growth rates of the microorganisms, bacterial densities were measured at different incubation times using a spectrophotometer (BioRad SmartSpec^{tm} 3000) at 620 nm, with the medium as a blanc. The spectrophotometer gives the extinction coefficient, representing the fraction of light lost to scattering and absorption while going through a cuvet filled with medium. The higher this value, the more turbid the medium is. Therefore this value is a measure for the bacterial biomass in the medium. Bacteria concentrations giving a spectrophotometer value above 1 had to be diluted 10 times because the spectrophotometer is not accurate at optical densities above 1. For these diluted concentrations, again optical density was measured and the value was multiplied by 10. Furthermore, for each species a high dilution was made and this dilution was plated out on an agar plate while the optical density of the original concentration was measured. After incubation, colonies were counted. Since optical density values for these concentrations were known, reference curves showing the number of bacteria for a given spectrophotometer value could now be produced.

Next, the stationary phase and the logistic growth phase were determined for each species. Two graphs were made: one showing incubation time on the X-axis and the extinction value on the Y-axis and one showing incubation time on the X-axis and bacteria numbers on the Y-axis. For the latter the equations from the optical density reference curves were used.

Mosquitoes

The *Anopheles gambiae* Giles *sensu stricto* colony at Wageningen University, The Netherlands, originated from Suakoko, Liberia. The mosquitoes have been cultured in the laboratory since 1988 with blood meals from a human arm twice a week. The adult mosquitoes were maintained in 30 · 30 · 30 cm gauze cages at 27 ± 1° C, 80 ± 5% relative humidity, and a photo-scotophase of 12:12 light:dark. They had access to a 6% glucose solution on filter paper. The larvae were reared in tap water in plastic trays and fed daily with Tetramin® baby fish food. Pupae were collected daily and placed in adult cages for emergence (Smallegange et al., 2005).

Olfactometer experiments: attractiveness of the bacteria species

A dual port olfactometer, consisting of a Perspex flight chamber of 1.48 · 0.50 · 0.49 m, was used to study the behavioral responses of female *A. gambiae* to the odors emitted by the bacteria species that were cultured successfully. Pressurized air was charcoal filtered, humidified and led through two Perspex mosquito trapping devices, which are linked to two ports (diameter 5 cm, 25 cm apart) into the flight chamber with a speed of 0.21 ± 0.02 m/s. Dim light was provided in this room. The experimental room was maintained at a temperature of 28 ± 2 °C and a relative humidity of 55 ± 10%. The temperature inside the flight chamber was equal to that of the room and the relative humidity was maintained at 60 ± 10%. The relative humidity of the air flowing out of the ports was maintained above 80% and the temperature is kept at 28 ± 1.5 °C. (Smallegange et al., 2005).

No sugar water but only water was provided to the mosquitoes 18 hours before the experiments. Female mosquitoes were 6-8 days old. Before releasing the mosquitoes temperature and relative humidity were measured in the olfactometer: at one of the ports and in the middle of the flight chamber. Temperature and relative humidity in the room were also measured. Air flow was measured occasionally to affirm a constant air flow.

Two bacteria species were tested in one series of experiments. For each species, two concentrations were used: a concentration at which the species shows logistic growth and a concentration at which the species has reached its stationary phase. Bacteria from the glycerol stocks were placed in 5 ml standard medium and then incubated until they had reached either their previously determined logistic growth optimum or their stationary phase. Then the

optical density of the concentrations was measured. Afterwards the media with the bacteria were pipetted in 1.5 ml Eppendorf tubes. Those tubes were placed in the fridge (4° C) until they were used in the olfactometer experiments. Also samples from a human foot were taken by using a sampling ring and washing buffer (see appendix 2) and incubated on the standard medium at 34° C for 30 hours. This mix of foot bacteria was used as a positive control. As a negative control medium without bacteria was used.

Every experimental day started with a dummy test in which no odors were used; only clean moist air was released into the flight chamber. In the other experiments glass blasted sand slides containing 100 µl of the medium with the bacteria, and 100 µl medium as a control, were placed in the ports (Table 3). In each experiment, 30 female *A. gambiae* were released. The experiments lasted for 15 minutes. During the experiments only dim light was allowed in the room. After each experiment, remaining mosquitoes and the trapping devices were removed from the olfactometer and the numbers of mosquitoes remaining in the trapping devices and the release cage were counted. Then new trapping devices were placed and a new experiment could start.

Table 3: olfactometer experiments scheme.

Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**
Log 1 L	Stat 1 R	Log 2 R	Stat 2 L	Mix L	Medium L
Stat 1 R	Stat 2 R	Medium L	Mix R	Log 1 R	Log 2 R
Log 2 L	Mix L	Stat 1 L	Log 1 L	Medium R	Stat 2 L
Stat 2 R	Medium R	Mix R	Log 2 R	Stat 1 L	Log 1 L
Mix L	Log 1 L	Stat 2 L	Medium L	Log 2 L	Stat 1 R
Medium R	Log 2 L	Log 2 R	Stat 1 R	Stat 2 R	Mix R

Log = logistic growth phase, Stat = stationary phase, 1 = first bacteria species, 2 = second bacteria species, L = left side of the olfactometer, R = right side of the olfactometer. In the other port the control was placed. This scheme was executed three times to test all the bacteria species.

Each day from 9 am until about 12 am, seven experiments were performed including the dummy test at the start with only clean moist air. During this time the mosquitoes, which are kept under an alternative day-night rhythm, are most active. After the experiments mosquitoes in the trapping devices were counted and the devices were cleaned in the dishwasher. The sand blasted glass slides were cleaned with soap and ethanol every day. In the last series only one bacteria species was tested; a worn sock was tested as a positive control because there was now space in the scheme to include an extra treatment.

During three days a series of control experiments was performed to examine the effects of the medium on mosquito behavior.

Olfactometer experiments: relative attractiveness of the bacteria species compared to ammonia

The bacteria species that were significantly attractive to *A. gambiae* were further studied in a series of experiments in which they were tested against medium plus a solution of 2.5 % ammonia in water. Ammonia is known to be attractive (Smallegange et al., 2005). The response of *A. gambiae* on a bacteria species compared to the response on ammonia served as a measure for the attractiveness of that bacteria species. All the attractive bacteria species were tested in a single series of experiments, so each bacteria species was tested under equal environmental circumstances. With these results a ranking of the attractiveness of the species compared to ammonia could be made.

Ammonia was tested in 0.1 mm low-density polyethylene (LDPE) sachets to ensure a constant distribution of ammonia volatiles. 100 µl of the ammonia solution was pipetted into these sachets. The sachets were placed on a wire structure so that they could hang in the

middle of the trapping devices. The ammonia sachets were placed in a trapping device together with a sand blasted glass slide with the medium. Sachets filled with 100 µl water were placed in the trapping device together with a sand blasted glass slide with the medium with bacteria to act as a control for the ammonia sachets.

The following treatments were tested: stationary phase concentrations of the four attractive bacteria species, the mix from a human foot and a worn sock. The sequence of the treatments was randomized on the same day and between days. After the experiments, the mosquitoes in the trapping devices were counted and the LDPE sachets were weighted using a balance (Mettler AC 100) to determine the level of evaporation of the ammonia and water.

The MM-X experiment

Stationary phase concentrations of the attractive bacteria species were tested in an experiment using two Mosquito Magnet X (MM-X) traps (Kline, 1999) in a large cage (233x250x330 cm) inside a climate-controlled room to examine the attractiveness of the bacteria species in a different setting and on a larger scale. In the first two series a filter paper was placed in a small bottle filled with 5 ml medium with bacteria while a bottle containing only the medium with a filter paper was used as a control. These bottles were then attached in the black air outlet of the MM-X trap with tape. For the last two series another setup was used: a filter paper dipped in medium with the bacteria was rolled up and placed on a wire hook which was placed in an MM-X trap. A filter paper dipped in only medium was used as a control in the other trap. For four days, every day 75 female *A. gambiae* were released in the cage at 9 am. Trapped mosquitoes were counted after four hours (personal communication N. Verhulst). The treatments were randomized to prevent positional effects of the cage.

Statistical analysis

Log growth curves for each bacteria species were made by fitting the observed datapoints in Genstat (release 11.1.0.1789), with a logistic S-shaped curve. From this curve, the logistic growth optimum could be calculated.

For each two-choice test in the olfactometer and MM-X setup a χ^2-test was used to analyze whether the total (i.e. sum of all replicates) number of mosquitoes that was trapped in the treatment trapping device and the total number that was trapped in the control trapping device differed from a 1:1 distribution. A Generalized Linear Model with binomial function (GLM Genstat) was used to investigate the effect of treatments on the total response, which is defined as the number of female mosquitoes caught in both trapping devices as the percentage of mosquitoes that flew out of the release cage (personal communication N. Verhulst). Furthermore in this analysis the effects of other factors, for example the time of the day on which the experiment was executed and the temperature, were determined.

For the olfactometer experiments with ammonia, the differences between the number of mosquitoes trapped in the trapping device containing the medium with bacteria was delivered and the number trapped in the trapping device from which ammonia-containing air was delivered were analysed with a generalized linear model (GLM; Binomial, linked in logit; the dispersion was estimated to account for heterogeneity, Genstat, Release 11.1.0.1789)(Qiu et al., 2006).

Results

Cultivation of the microorganism species
Of the seven microorganism species, six were cultivated successfully on their specific agar medium. *Malassezia furfur* did not grow on the medium; agar plates containing *Ma. furfur* showed no growth, or infection with an unidentified bacteria. Also on specific liquid medium *Ma. furfur* did not grow. Therefore it was decided to exclude *Ma. furfur* from the rest of the experiments. *Micrococcus luteus* seems to have grown at first but cultivation later proved to be problematic: the tubes that should contain *Mi. luteus* were infected with other bacteria or there was no bacterial growth at all. Also on the specific liquid medium it failed to grow successfully. After several attempts to grow *Mi. luteus* it was decided to exclude the species from the rest of the experiments.

Microorganism growth curves
For each successfully cultivated bacteria species an optical density reference curve was made. These graphs show the number of bacteria for a certain spectrophotometer value (Figure 2 and Figure 3). See appendix 3 for the curves of *Bacillus subtilis*, *Brevibacterium epidermidis* and *Staphylococcus epidermidis.*

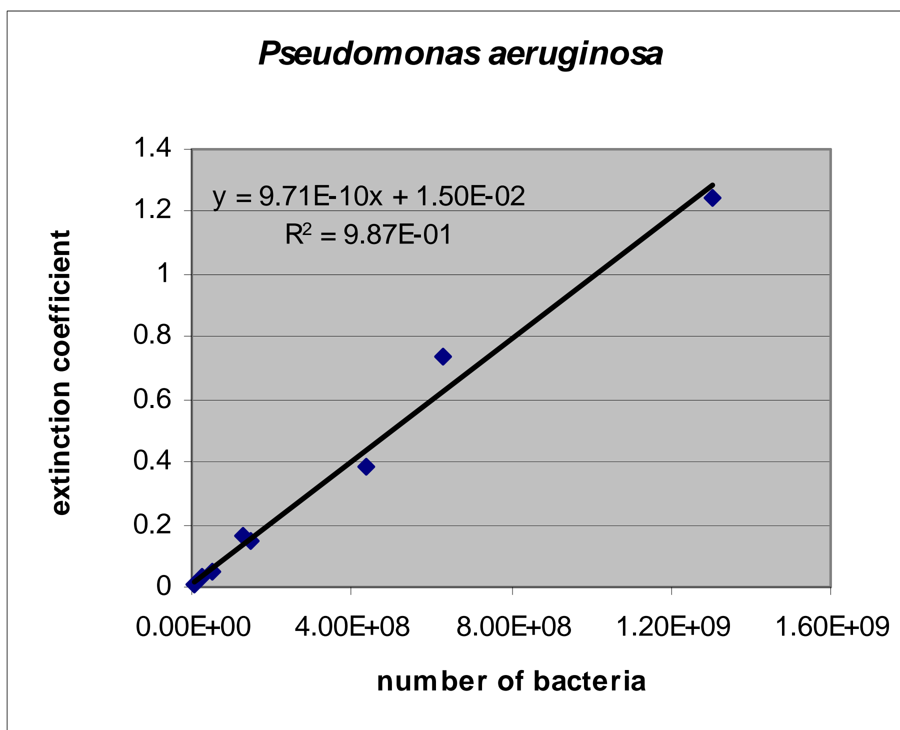

Figure 2: optical density reference curve for *Pseudomonas aeruginosa*

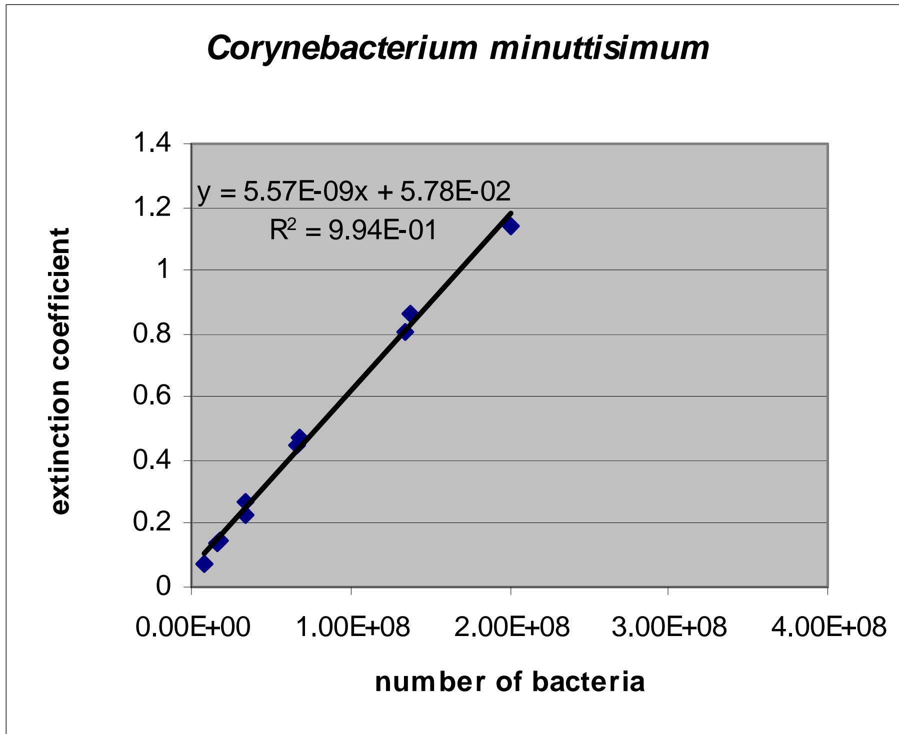

Figure 3: optical density reference curve for *Corynebacterium minuttisimum*

For all five cultured bacteria species growth curves were made. *B. epidermidis* showed the fastest growth; *B. subtilis* the slowest. *C. minutissimum* showed the highest extinction values at the stationary phase while *P. aeruginosa* and *B. subtilis* show the lowest optical density (Figure 4). Bacteria numbers were highest for *P. aeruginosa* and lowest for *C. minutissimum* (Figure 5). The growth curves with the bacteria numbers were made using the equations from the optical density reference curves. With the growth curves the optimal logistic growth optima were determined (Table 4) (Appendix 4).

Table 4: logistic growth optima

Bacteria species	**Logistic growth optimum (hours)**	**Equation**
Bacillus subtilis	12.9	=-0.0132 + 0.8035/(1 + EXP(-0.426*(Time – 12.839)))
Brevibacterium epidermidis	5.8	=-0.0477 + 1.0318/(1 + EXP(-0.5767*(Time – 5.69)))
Corynebacterium minutissimum	11.2	=-0.1063 + 1.1281/(1 + EXP(-0.2361*(Time – 11.138)))
Pseudomonas aeruginosa	9.9	=-0.0386 + 0.8071/(1 + EXP(-0.3334*(Time - 9.892)))
Staphylococcus epidermidis	9.3	=0.0028 + 0.9393/(1 + EXP(-0.6232*(Time - 9.2141)))

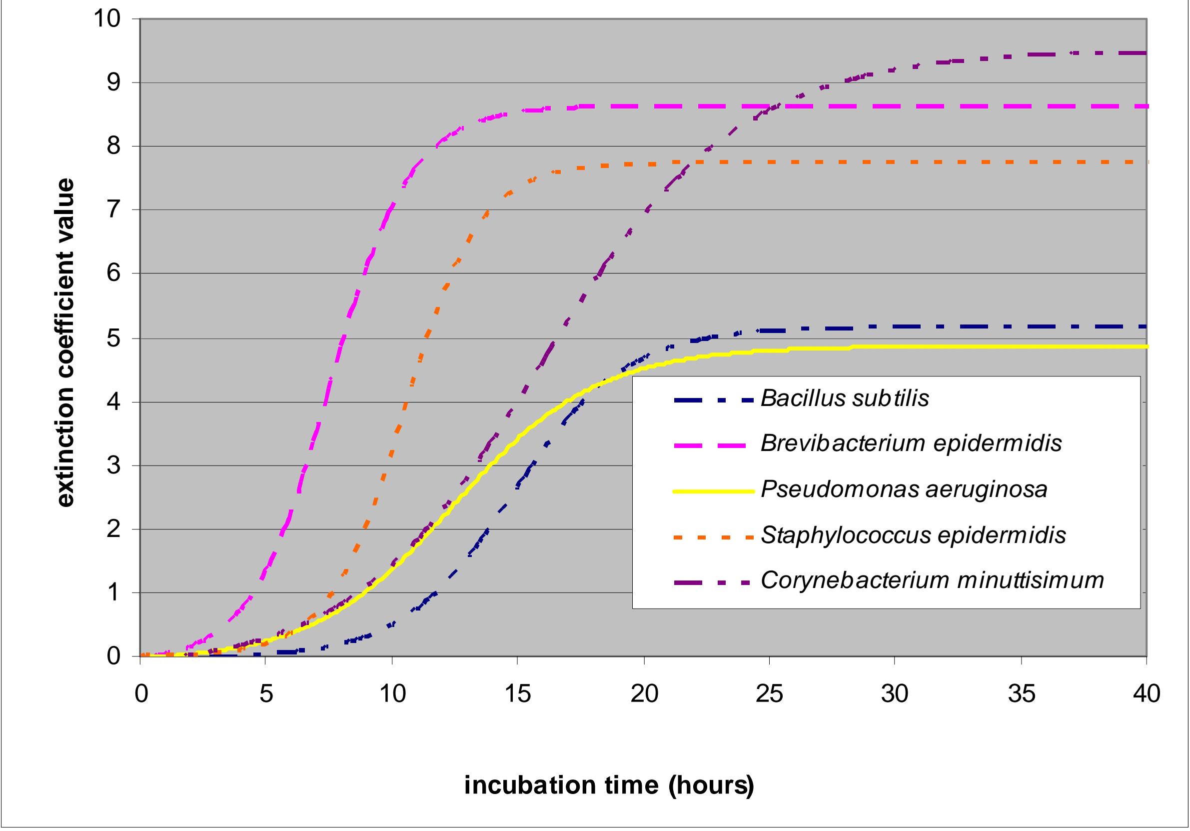

Figure 4: growth curves of the bacteria species showing the incubation time and the extinction coefficient value.

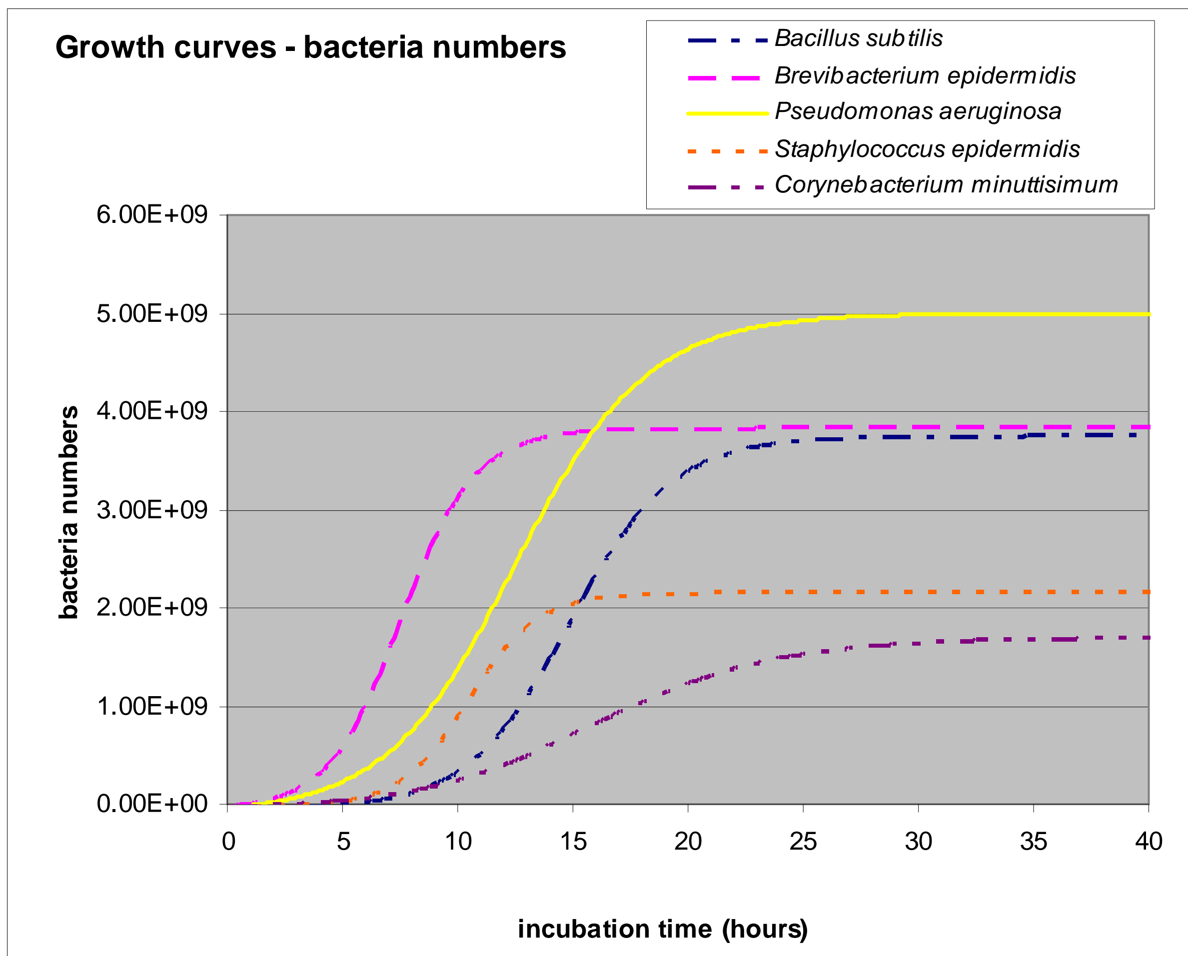

Figure 5: growth curves showing the incubation time and the numbers of bacteria.

Olfactometer experiments: the attractiveness of the bacteria species

The concentration representing the stationary phase of *Bacillus subtilis* was significantly more attractive to *A. gambiae* than the control (P<0.001) with a total response of around 20% (Figure 6). The logistic growth phase of *B. subtilis* was not attractive. Both *Pseudomonas aeruginosa* concentrations were not attractive. The mix from a human foot was significantly more attractive than the control (P<0.001), showing a total response of 25-30%. The total response on the control was in this series of experiments low, with a maximum total response of around 5%. Total response on the control was lower than the total response of two of the treatments and there was no side effect. The GLM analysis showed that the total response was significantly higher on the mix than the total responses on *P. aeruginosa*, the control and the logistic phase of *B. subtilis*. The total response on *B. subtilis* in the stationary phase was also significantly higher than the responses on *P. aeruginosa* and the control (Figure 6; Appendix 5). In this series of experiments, the time at which a treatment was tested had a significant effect on the results (Appendix 5).

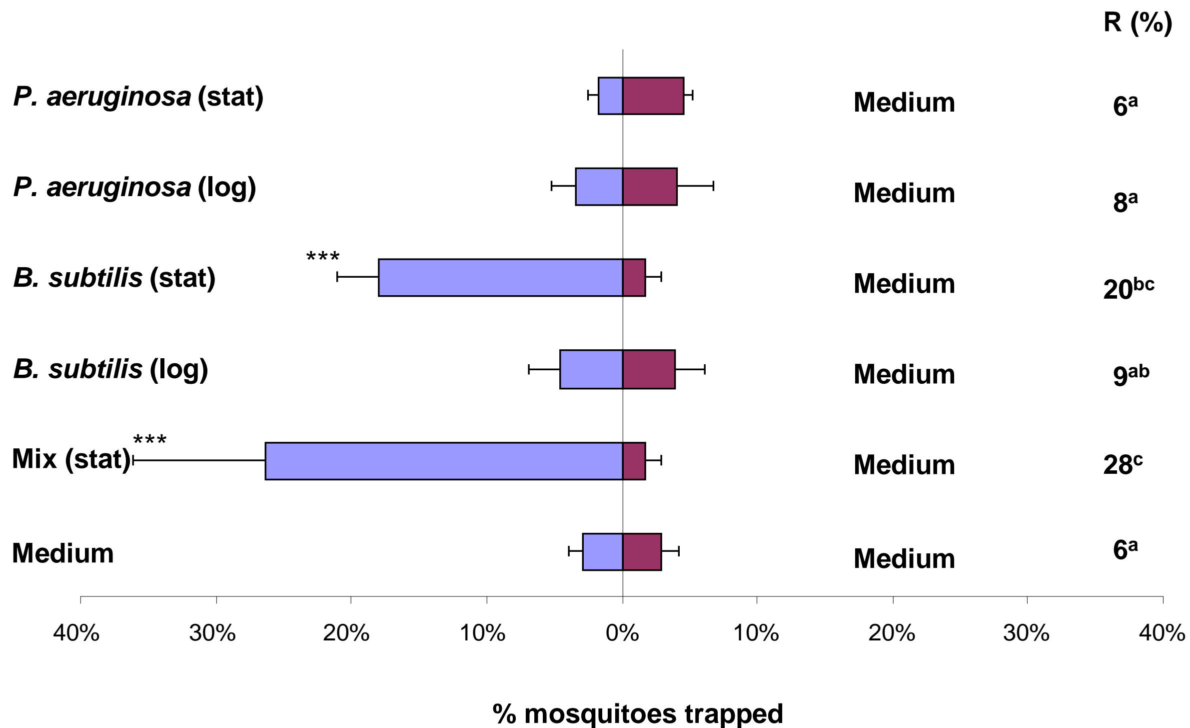

Figure 6: response of *A. gambiae* to *Pseudomonas aeruginosa*, *Bacillus subtilis* and a mix from a human foot. Log = logistic growth phase, stat = stationary phase. The x-axis shows the percentage response for each treatment versus the response on the control. ***: χ^2-test P<0.001. R=Total response to both trapping devices. Data not sharing the same superscript letter differ significantly at P<0.05 (GLM).

For both *Brevibacterium epidermidis* and *Staphylococcus epidermidis* the logistic growth phase was not attractive. For both species the stationary phase was significantly more attractive than the control (P<0.001 for *B. epidermidis*, P<0.01 for *S. epidermidis*)(Figure 7). The mix from a human foot was again significantly more attractive than the control (P<0.001). With the logistic phase of *S. epidermidis*, the control was significantly more attractive than the treatment (P<0.05). The GLM analysis showed that the total responses on the mix and on the stationary phase of *B. epidermidis* were significantly higher than the total response on the logistic phase of *B. epidermidis* (Figure 7; Appendix 5).

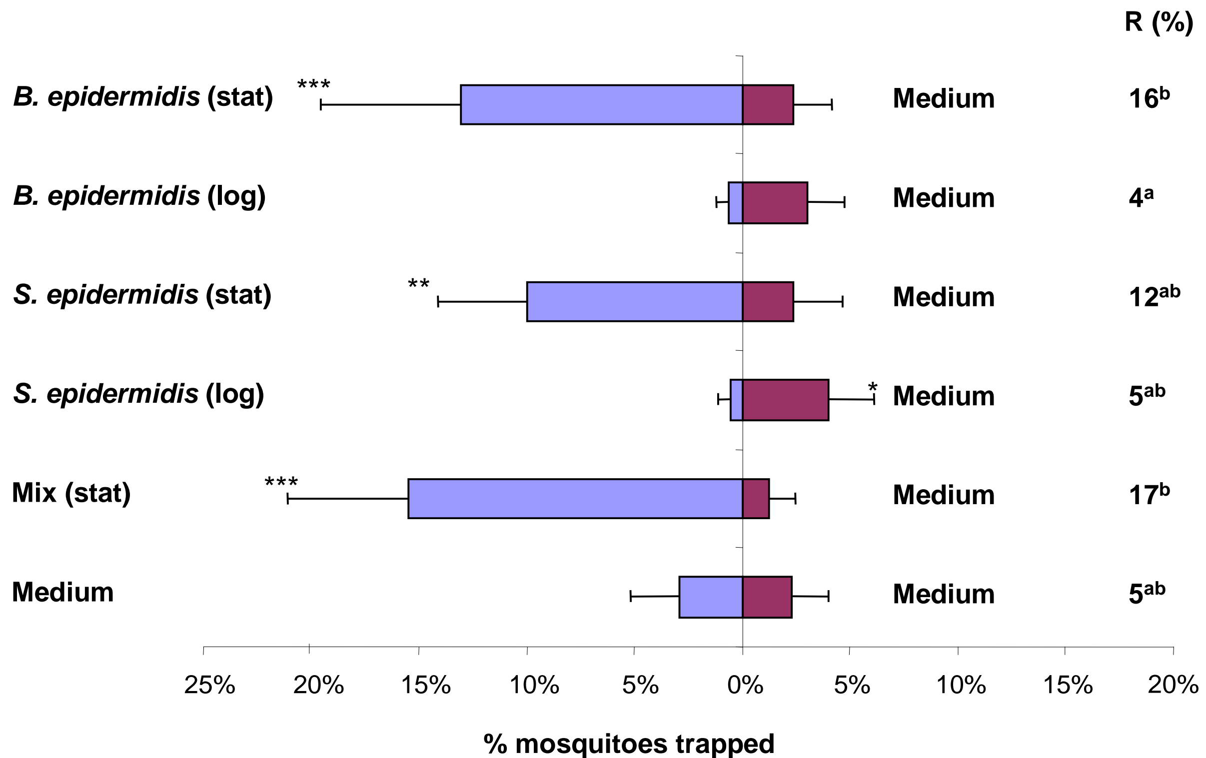

Figure 7: response of *A. gambiae* to *Brevibacterium epidermidis, Staphylococcus epidermidis* and a mix from a human foot. Log = logistic growth phase, stat = stationary phase. The x-axis shows the percentage response for each treatment versus the response on the control. ***: χ^2-test P<0.001; **: χ^2-test P<0.01; *: χ^2-test P<0.05. R=Total response to both trapping devices. Data not sharing the same superscript letter differ significantly at P<0.05 (GLM).

The stationary phase of *C. minutissimum* was significantly more attractive than the control (P<0.001)(Figure 8). The logistic phase was not attractive. Again the mix was significantly more attractive than the control (P<0.001). The worn sock was much more attractive than the control (P<0.001), showing a total response of almost 80%. Not a single mosquito was found in the trapping device containing the control when the sock was tested. GLM analysis showed that the total response on the sock was significantly more attractive than the total responses on the other treatments. Furthermore, the total responses on the mix and the stationary phase of *C. minutissimum* were significantly more attractive than the total responses on the logistic growth phase of *C. minutissimum* and the control (Figure 8; Appendix 5).

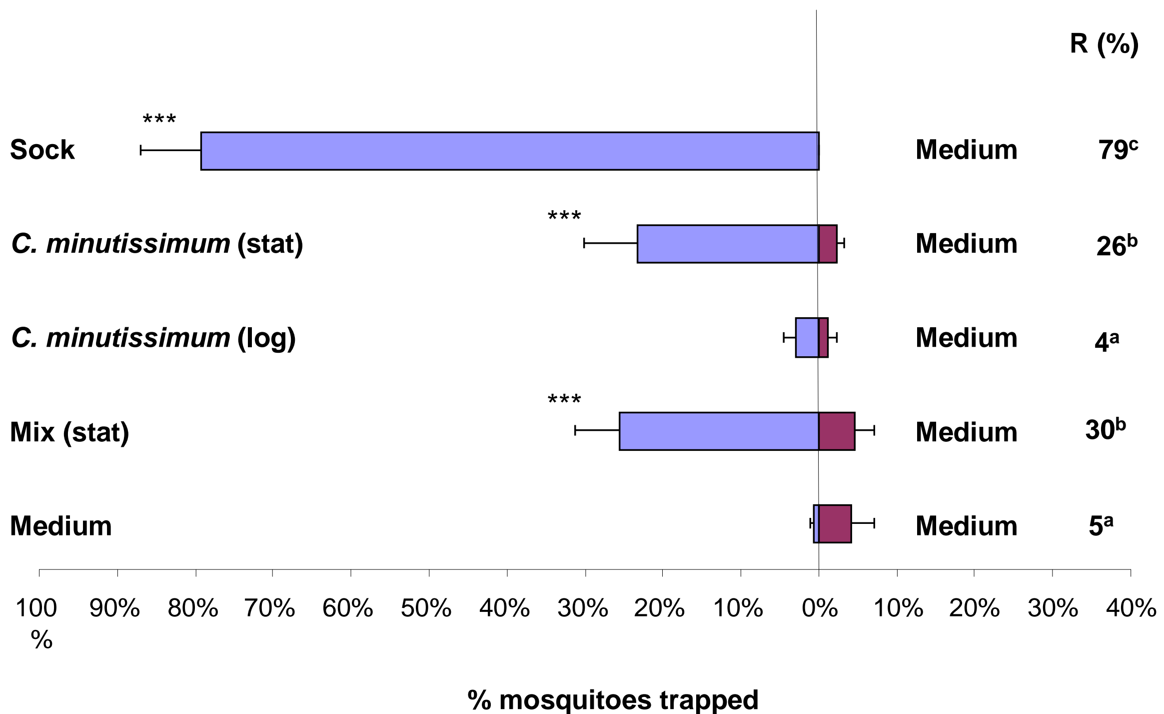

Figure 8: response of *A. gambiae* to *Corynebacterium minutissimum*, a mix from a human foot and a worn sock. Log = logistic growth phase, stat = stationary phase. The x-axis shows the percentage response for each treatment versus the response on the control. ***: χ^2-test P<0.001. R=Total response to both trapping devices. Data not sharing the same superscript letter differ significantly at P<0.05 (GLM).

In the last series of experiments, in which the effects of the medium itself were tested, no treatment was significantly attractive to *A. gambiae*. In all cases response was very low. GLM analysis showed no significant differences in response between the treatments (Figure 9; Appendix 5).

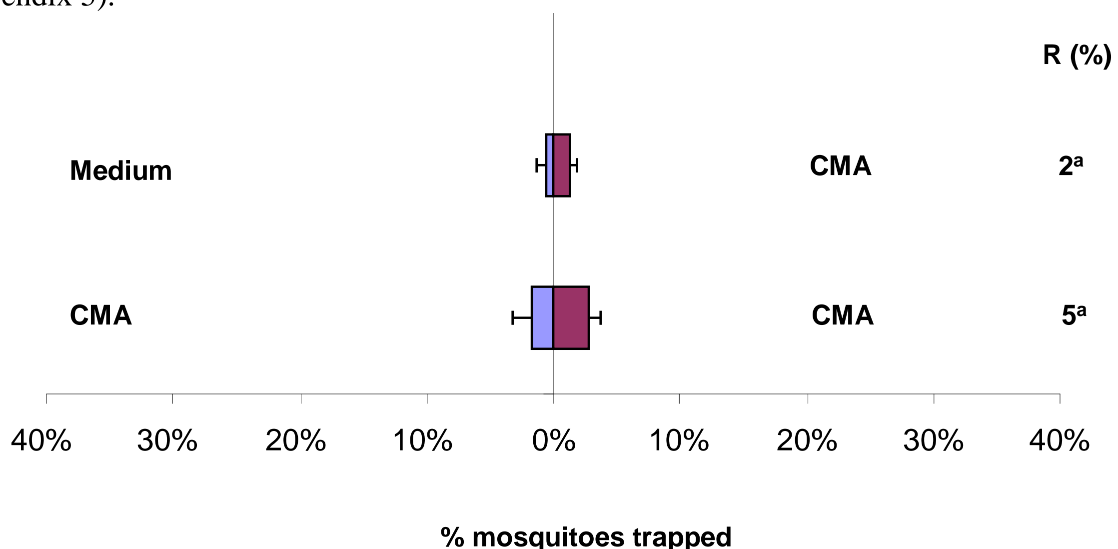

Figure 9: response of *A. gambiae* to the medium and clean moist air (CMA). The x-axis shows the percentage response for each treatment versus the response on the control. R=Total response to both trapping devices. Data not sharing the same superscript letter differ significantly at P<0.05 (GLM).

Olfactometer experiments: relative attractiveness of the bacteria species compared to ammonia

In the experiments in which ammonia was used as a control, the response of *A. gambiae* on *Corynebacterium minutissimum*, the mix and the worn sock was higher than on the control (Figure 10). Responses on *Brevibacterium epidermidis*, *Bacillus subtilis* and *Staphylococcus epidermidis* were lower than on the control. A ratio of 60%, as seen for *C. minutissimum*, means 60% of the total response was on the treatment while 40% was on the control. For the other three bacteria species the average ratio was not higher than 30%. The mix shows an average ratio of about 80%. In the case of the worn sock the ratio was 100%, i.e. not a single mosquito was found on the control (Figure 10). GLM analysis showed that the ratio when *C. minutissimum* was tested against ammonia was significantly higher than the ratio of *B. subtilis* and *S. epidermidis.* The ratio of the mix was significantly higher than the ratio of *B. epidermidis*, *B. subtilis* and *S. epidermidis*. The ratio of the worn sock was significantly higher than the ratios of all the other treatments (Figure 10; Appendix 6).

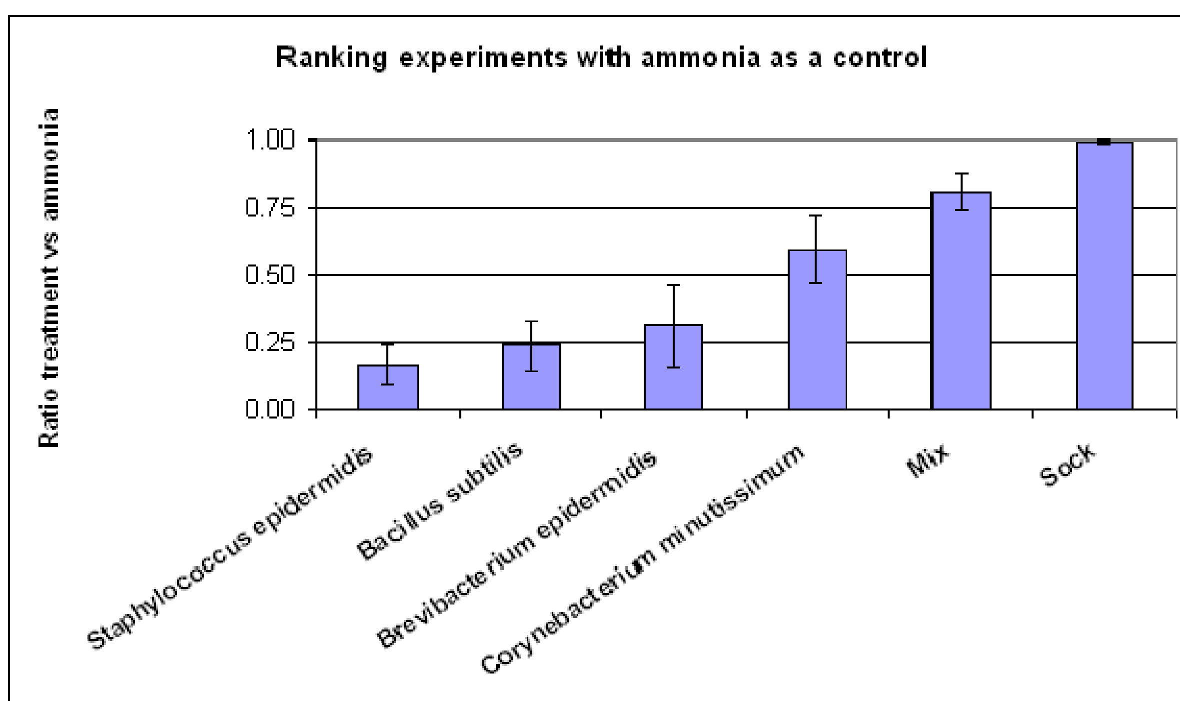

Figure 10: response of *An. gambiae* to four species of bacteria, a mix of bacteria an a worn sock compared to ammonia. The value 1 indicates that all mosquitoes were caught on the treatment and none on the ammonia. Data not sharing the same superscript letter differ significantly at $P<0.05$.

The LDPE sachets with the ammonia and water were weighted to measure the evaporation. A very small amount of weight loss was observed (Table 5).

Table 5: ammonia and water evaporation in the LDPE sachets

	Weight after first day (g)	Weight after last day (g)
Water sachet 1	0.2974	0.2955
Water sachet 2	0.3010	0.2995
Ammonia sachet 1	0.2730	0.2709
Ammonia sachet 2	0.2566	0.2549

The MM-X experiment

None of the four bacteria species found to be attractive in the olfactometer experiments, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Brevibacterium epidermidis* and *Corynebacterium minutissimum*, was significantly more attractive than the control to *A. gambiae* in the MM-X experiment. This was the case for the method with the filter paper in the glass bottle in the MM-X trap(Figure 11A) as well as for the method with the filter paper placed on a hook in the trap (Figure 11B). Average response was around 12-22 % for the treatments and around 15-25 % for the control (Figure 11A+B).

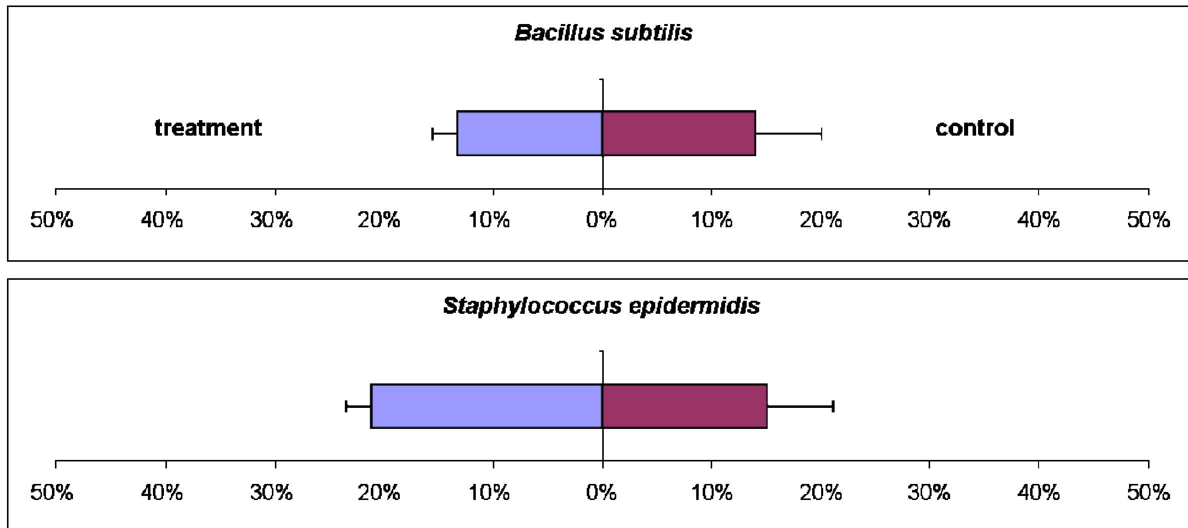

Figure 11A: response of *A. gambiae* to *Bacillus subtilis* and *Staphylococcus epidermidis*. The left side of the bars shows the response on the bacteria, the right side shows the response on the control.

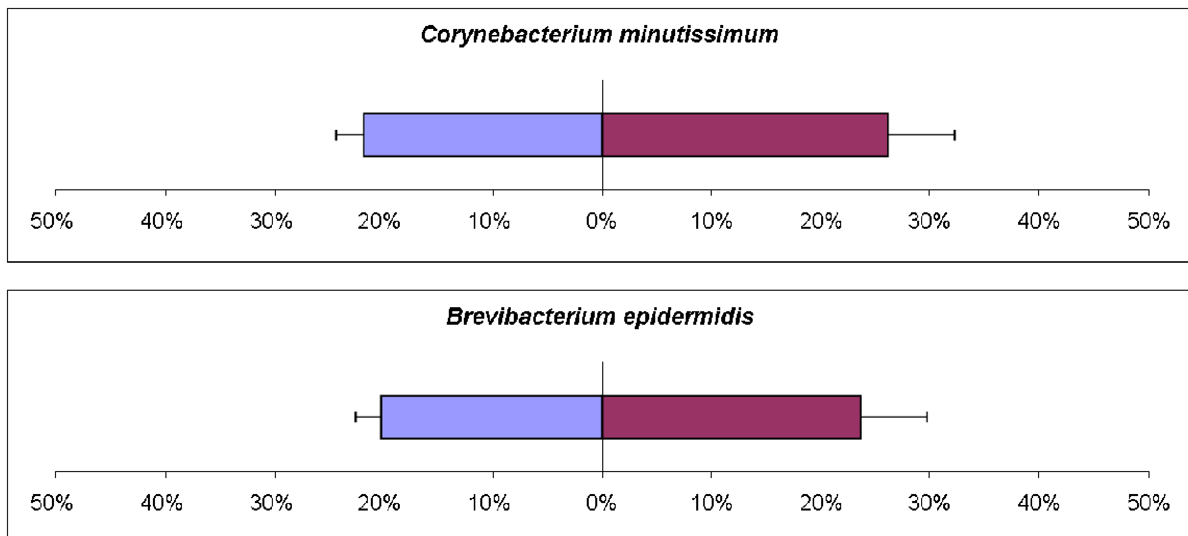

Figure 11B: response of *A. gambiae* to *Corynebacterium minutissimum* and *Brevibacterium epidermidis*. The left side of the bars shows the response on the bacteria, the right side shows the response on the control.

Discussion

Cultivation of the microorganism species
Five of the seven microorganism species were cultivated successfully. These were *Pseudomonas aeruginosa, Staphylococcus epidermidis*, *Bacillus subtilis*, *Brevibacterium epidermidis* and *Corynebacterium minutissimum.* These species were first successfully cultured on species-specific agar plates and then successfully cultured in the standard liquid medium. The fungus *Malassezia furfur* failed to grow on both the specific agar plates and the specific liquid medium. An explanation for this might be that the agar plates got infected with a bacteria that prevented the growth of the fungus, or that the growth conditions or the medium were still not optimal for this fungus.

Micrococcus luteus was at first successfully cultivated on both the specific agar and the standard medium, though it grew slowly. In a later stage however, growth of *Mi. luteus* almost stopped. *Mi. luteus* is known to be a difficult species to cultivate (personal communication P. Verbaarschot). A possible explanation for this might be that the bacteria in the glycerol stock were in bad condition because of repeated freezing and defrosting. Maybe *Mi. luteus* is more vulnerable to this than the other species. Since *Mi. luteus* is well known as a skin bacteria, the attractiveness of this species is still worthwhile to study so in further studies this species should not be overlooked.

The growth curves show that the highest optical density at the stationary phase was measured with *Corynebacterium minutissimum* (Figure 4). Since the bacteria numbers for these species were lower than for the other species (Figure 5), *C. minutissimum* might be relatively large so that few bacteria still result in a high optical density. Another possibility is that these bacteria are less transparent than the other species, leading to less light being let through. The opposite may be true for *Pseudomonas aeruginosa*: while it has the highest number of bacteria per ml at the stationary phase, the optical density for this species is the lowest. Therefore *P. aeruginosa* may be relatively small or relatively transparent, although no information on this could be found in literature.

Attractiveness of the bacteria species
Two main conclusions can be drawn from the first series of olfactometer experiments:

First, four of the five species were significantly attractive to *A. gambiae*. *Staphylococcus epidermidis*, *Bacillus subtilis*, *Brevibacterium epidermidis* and *Corynebacterium minutissimum* all attracted significantly higher numbers of mosquitoes than the control in their stationary phase (Figure 6; Figure 7; Figure 8). This implies that all of these species secrete compounds attractive to *A. gambiae* when cultured on the liquid medium. These species are all well-established as skin bacteria and are known to produce volatile fatty acids, often using sweat as a substrate (James et al., 2004b). Especially interesting is that the bacteria do not need human skin to produce attractive volatiles; when cultivated on the medium they also attract mosquitoes.

To determine which compounds secreted by these bacteria are actually attractive to the mosquitoes, it would be very interesting to perform odour analyses of samples taken from these bacteria. When the volatiles secreted by the bacteria are known, these compounds can be tested individually in an olfactometer.

The only species that was not attractive was *Pseudomonas aeruginosa*. This species is not typically known as a skin bacteria but was found on human arms in large numbers (Grice et al., 2008). *Pseudomonas* species are known to be very versatile and can live in a wide variety of habitats. But since *P. aeruginosa* is not specialized to life on human skin, it may be possible that compounds produced by *P. aeruginosa* are different from the compounds

attracting *A. gambiae*. From an evolutionary point of view: it would not be useful for *A. gambiae* to develop a preference for compounds secreted by *P. aeruginosa* since these compounds would not necessarily lead directly to human hosts. Compounds produced by *Pseudomonas* sp. have shown antibacterial activity, making this species interesting for odour analysis (Padilla et al., 2006). Maybe these compounds are involved in the unattractiveness of *P. aeruginosa* to *A. gambiae*.

Since the bacteria densities used in the olfactometer experiments were the highest for *P. aeruginosa* (Figure 5), the unattractiveness of this species is unlikely to be the result of simply a low number of bacteria at the stationary phase. However, since *P. aeruginosa* shows the lowest optical density of all the examined bacteria species it is a possibility that the bacteria are too small to secrete sufficient amounts of volatile fatty acids to be attractive to the mosquitoes. It must be stated here however that *B. subtilis* shows a comparable optical density and lower bacteria numbers and still the species was significantly attractive.

The second interesting result of the attractiveness olfactometer experiments is that, for the four attractive bacteria species, only the concentration representing the stationary phase was attractive while the concentration representing the logistic growth phase was not. One possible explanation for this might be that bacteria in their logistic growth phase produce different volatiles than bacteria in their stationary phase. A more straightforward explanation however is that the bacteria numbers at their growth optimum are still much lower than at the stationary growth phase and therefore produce lower amounts of volatiles.

To examine this, the stationary phase concentrations can be diluted until they have reached the optical density level of the logistic growth phase. It then can be assumed that the stationary phase concentrations consist of the same amount of bacteria as the logistic growth phase concentrations. When there are still differences between the attractiveness of these concentrations, it might be worthy to study the differences in volatiles secreted by those concentrations.

In one case, the medium seemed to be significantly attractive compared to *S. epidermidis* in its logistic phase ($P<0.05$). In theory this could mean that *S. epidermidis* in its logistic phase is slightly repellent, but seen the results of the control experiments and the other experiments with this species, this result should be regarded as an artifact.

Mix from a human foot

The mix from a human foot, used in all experiments as a positive control, was always significantly more attractive than the control. This mix has been incubated for 30 hours and is therefore considered to represent the stationary growth phase, though no growth curve could be made for the mix because the mix consists of different bacteria species which all have different logistic growth optima. Because of the mix' attractiveness, it could be expected that the mix consists of at least one but probably several attractive microorganism species. It would be interesting to investigate the microorganism species composition of this mix. This was not done in this study because it is an intensive process for which specialized techniques are required. Furthermore, also from the mix an odour analysis can be executed to compare the odors secreted by the bacteria from the mix with the odors secreted by the five examined species individually.

A drawback of the use of the mix is its variability: the ratios of the microorganism species present in the mix, or even the number of species, may fluctuate depending on coincidental densities of bacteria at a certain time on the foot. Furthermore foot samples from different people may vary greatly in microorganism composition. Therefore it is difficult to compare the attractiveness of the mix, as a control, to the attractiveness of the bacteria species.

Worn sock
The sock was more attractive than all other treatments, even when worn for only two hours (Figure 8). Possibly the volatiles arising from these socks are more attractive than the individual bacteria species. These could be secreted by microorganisms not examined in this study. Therefore it would be valuable to determine which microorganism species can be found on those socks, however this would be a rather difficult and intensive process. Again, odour analysis would be a possible method to get more information about the volatiles released from these socks. It would be especially interesting to compare odour profiles of worn socks with those of the mix from samples of the same persons to determine possible differences between those two.

There might be an obvious reason why the sock is so much more attractive than the other treatments: the total surface of the sock, as placed in the trapping device of the olfactometer, is much bigger than the surface of the glass slide containing the other treatments. Therefore it is possible that the amount of volatiles released to the air flow in the olfactometer is simply much larger compared to the other treatments and hence attracts more mosquitoes. This can be examined by using other methods to present the medium with the bacteria to the mosquitoes, for example by dipping a filter paper in the medium and placing that filter paper in the trapping device. In this way the surface on which the medium is exposed will be increased.

Control experiments
The medium in itself was not attractive (Figure 9). This indicates that *A. gambiae* is attracted purely by compounds produced by the bacteria. In a study by N. Verhulst, agar medium proved to be attractive to *A. gambiae* (personal communication N. Verhulst). Since the liquid medium was not attractive, the use of liquid medium in future olfactometer studies would be recommended. The fact that the liquid medium was not attractive advocates The fact that the mosquitoes were not attracted by the clean moist air indicates that the air is not polluted with mosquito-attracting compounds and therefore the olfactometer is a useful way to study olfactory behavior in mosquitoes.

The relative attractiveness compared to ammonia
The reasoning behind using the ammonia as a positive control and not for example the mix from a human foot was that ammonia, placed in an LDPE sachet at a known concentration, would provide a constant level of attractiveness to which the attractiveness of the bacteria could be compared. Though the number of mosquitoes attracted by the ammonia showed large variation during the experiments, the ranking provided valuable results (Figure 10). *Corynebacterium minutissimum* was significantly more attractive than *Staphylococcus epidermidis* and *Bacillus subtilis*. *C. minutissimum* was the only bacteria species that attracted more mosquitoes than the ammonia control and was comparably attractive as the mix. Apparently *C. minutissimum* emits the most attractive volatiles, or the highest amount of attractive volatiles. This leads to *C. minutissimum* being the most interesting candidate for further studies in search of attractants to *A. gambiae*.

The MM-X experiment
None of the bacteria species were attractive in the MM-X experiment (Figure 11). Both used methods gave comparable results. The second method was inclined because the idea was that this would lead to higher evaporation and therefore more volatiles released in the air. However also this method failed in attracting mosquitoes. Why the results obtained in the olfactometer are not continued in the MM-X traps is not clear. When a worn sock is used in the MM-X trap, response is usually high (personal communication N. Verhulst) so the setup

was expected to work. Furthermore bacteria cultured on agar medium have shown to be attractive to *A. gambiae* in the MM-X setup (personal communication N. Verhulst). The bacteria were incubated for the desired period and optical densities were comparable to those for the stationary phase in the olfactometer experiments. A possible explanation might be that the volatiles produced by the bacteria are distributed too fast into a relatively large volume, much larger than the volume of the olfactometer, and that therefore the level of attractive volatiles was too low to attract the mosquitoes.

Conclusion and recommendations for further studies

Now that it is known that *Staphylococcus epidermidis*, *Bacillus subtilis*, *Brevibacterium epidermidis* and *Corynebacterium minutissimum* are individually attractive to *A. gambiae* and that *C. minutissimum* seems to be the most attractive of these species, the question is what the next step in finding attractants should be. Since *C. minutissimum* is the most promising species, performing an odour analysis for this species is highly recommendable. If this analysis leads to the discovery of specific volatiles which are responsible for the attractiveness of *C. minutissimum*, it may provide scientists working on malaria control with useful tools in mosquito traps. Studying the odour profiles of other *Corynebacterium* species might be worth the effort because of the variability in volatile fatty acid secretion that has been found in different *Staphylococcus* strains (James et al., 2004b). This indicates that different strains of the same genus can produce different compounds. Odour analyses for the other attractive bacteria species might also provide useful information., maybe leading to the discovery of other attractive compounds. Furthermore odour analysis from *Pseudomonas aeruginosa* samples might give insight in the unattractiveness of this species, possibly showing differences in volatile composition between this species and the attractive species. Other skin microorganism species, for example *Micrococcus luteus* and the fungus *Malassezia furfur*, may be of interest since nothing is known yet of their attractiveness to *A. gambiae*.

In this study the attractiveness of combinations of bacteria species has not been examined. Combinations of several attractive bacteria species may lead to enhanced attractiveness compared to the separate species and therefore are worth testing in olfactometer experiments.

A major concern in this study is the use of the medium to cultivate the bacteria. The level to which the medium can be compared with the human skin is questionable. The volatiles produced by bacteria cultured on this medium might be different from the ones produced by the same bacteria on the human skin. Therefore olfactometer experiments testing skin bacteria cultured on several types of medium and subsequent odour analyses may give insight in volatile production in relation to the substrate.

Overall, interesting results have been achieved in this study showing that several skin bacteria species, but not all, attract *A. gambiae* while being cultured on a liquid medium. However, further research is needed to determine attractive compounds secreted by these bacteria in order to develop mosquito attractants usable in monitoring tools or control mechanisms.

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Appendix

Appendix 1: species-specific media (according to N. Verhulst, personal communication and the DSMZ website)

Staphylococcus epidermidis

Casein peptone, tryptic digest	10.0	g
Yeast extract	5.0	g
Glucose	5.0	g
NaCl	5.0	g
Agar	15.0	g
Distilled water	1000.0	ml

Corynebacterium minutissimum

Brain heart infusion	18.5	g
Glucose	5.0	g
Agar	12.0	g
Distilled water	1000.0	ml

Micrococcus luteus

Peptone	5.0	g
Meat extract	3.0	g
Agar	15.0	g
Distilled water	1000.0	ml

Brevibacterium epidermidis

Casein peptone, tryptic digest	10.0	g
Yeast extract	5.0	g
Glucose	5.0	g
NaCl	5.0	g
Agar	15.0	g
Distilled water	1000.0	ml

Malassezia furfur

Malt extract	40.0	g
Ox-bile (desiccated)	20.0	g
Tween 40	10.0	g
Glycerol mono-oleate (techn.)	2.5	g
Agar	15.0	g
Distilled water	1000.0	ml

Pseudomonas aeruginosa

Peptone	5.0	g
Meat extract	3.0	g
Agar	15.0	g
Distilled water	1000.0	ml

Bacillus subtilis

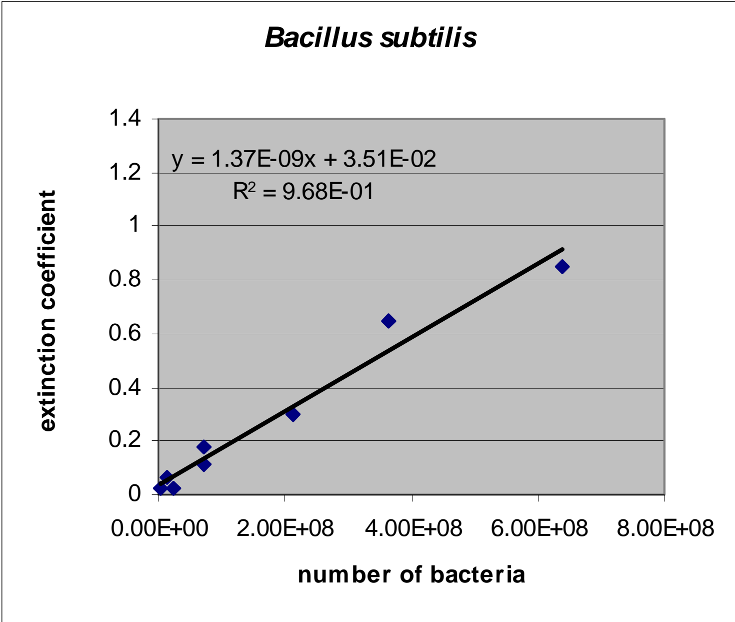
Peptone	5.0	g
Meat extract	3.0	g
Agar	15.0	g
Distilled water	1000.0	ml

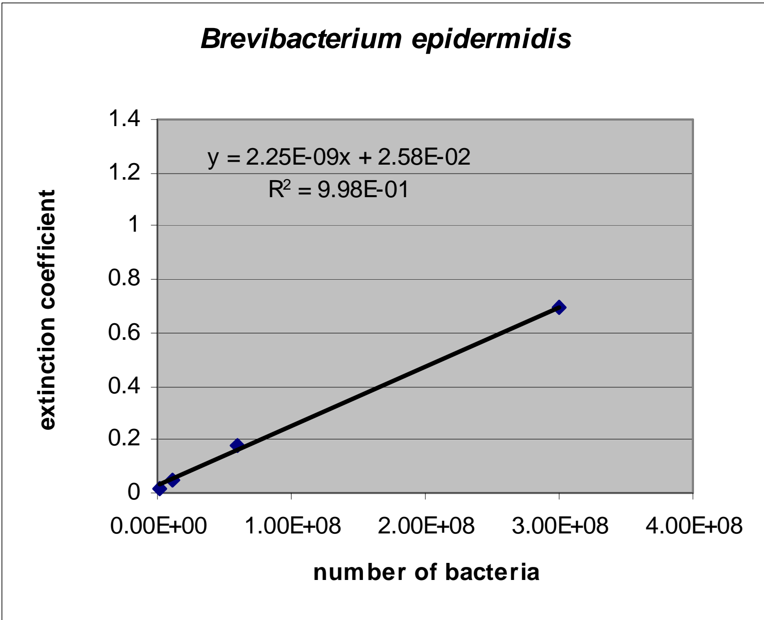
Appendix 2: protocol for the collection of skin samples (according to (Taylor et al., 2003))

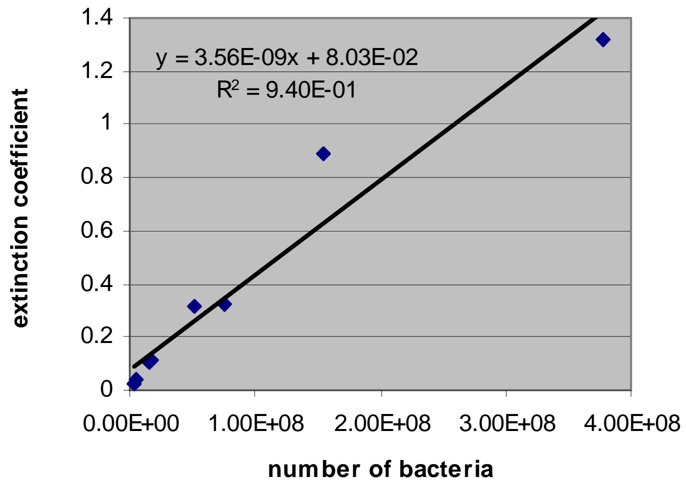
- A sterile Teflon scrub cup (internal diameter 1.9 cm) is placed on the sole of the foot.
- 0.75 mL of full-strength wash fluid is added.

  Wash fluid: 75 mM sodium phosphate (Na_2-NO_3) buffer (pH 7.9), 0.1% (v/v) Triton X-100 (autoclaved)
- The surface of the skin, within the cup, is gently scrubbed with a glass stick for 1 min.
- The fluid is aspirated to an Eppendorf tube.
- The process is repeated with another 0.75 mL of sampling fluid at the same site, and the two samples are pooled.
- 100 µL aliquots are made and the samples are diluted 5 times in sterile half-strength wash fluid

Appendix 3: optical density reference curves for *Bacillus subtilis, Brevibacterium epidermidis* and *Staphylococcus epidermidis*







Staphylococcus epidermidis
y = 3.56E-09x + 8.03E-02
R2 = 9.40E-01
extinction coefficient
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0.00E+00
1.00E+08
2.00E+08
3.00E+08
4.00E+08
number of bacteria

Appendix 4: Genstat logistic growth curves results

Bacillus subtilis

Nonlinear regression analysis

Response variate: C4
Explanatory: C3
Fitted Curve: A + C/(1 + EXP(-B*(X - M)))

Summary of analysis

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	3	2.38956	0.796522	189.65	<.001
Residual	22	0.09240	0.004200		
Total	25	2.48196	0.099279		

Percentage variance accounted for 95.8
Standard error of observations is estimated to be 0.0648.

Message: the following units have large standardized residuals.

Unit	Response	Residual
4	0.9138	2.55
22	0.3916	2.66

Estimates of parameters

Parameter	estimate	s.e.
B	0.426	0.130
M	12.839	0.475
C	0.8035	0.0412
A	-0.0132	0.0247

Brevibacterium epidermidis

Nonlinear regression analysis

Response variate: C4
Explanatory: C3
Fitted Curve: A + C/(1 + EXP(-B*(X - M)))

Summary of analysis

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	3	2.25209	0.750698	345.99	<.001
Residual	12	0.02604	0.002170		
Total	15	2.27813	0.151875		

Percentage variance accounted for 98.6
Standard error of observations is estimated to be 0.0466.

Message: the following units have large standardized residuals.

Unit	Response	Residual
6	0.8756	-2.07
10	1.0682	2.49

Estimates of parameters

Parameter	estimate	s.e.
B	0.5725	0.0775
M	5.677	0.262
C	1.0364	0.0579
A	-0.0517	0.0391

Corynebacterium minutissimum

Nonlinear regression analysis

Response variate: C4
Explanatory: C3
Fitted Curve: A + C/(1 + EXP(-B*(X - M)))

Summary of analysis

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	3	3.33982	1.113275	926.79	<.001
Residual	18	0.02162	0.001201		
Total	21	3.36145	0.160069		

Percentage variance accounted for 99.2
Standard error of observations is estimated to be 0.0347.

Message: the following units have large standardized residuals.

Unit	Response	Residual
1	0.0228	2.24

Message: the following units have high leverage.

Unit	Response	Leverage
1	0.0228	0.53

Estimates of parameters

Parameter	estimate	s.e.
B	0.2361	0.0232
M	11.138	0.485
C	1.1281	0.0601
A	-0.1063	0.0495

Pseudomonas aeruginosa

Nonlinear regression analysis

Response variate: C4
Explanatory: C3
Fitted Curve: A + C/(1 + EXP(-B*(X - M)))

Summary of analysis

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	3	1.99085	0.663616	132.48	<.001
Residual	14	0.07013	0.005009		
Total	17	2.06098	0.121234		

Percentage variance accounted for 95.9
Standard error of observations is estimated to be 0.0708.

Message: the following units have large standardized residuals.

Unit	Response	Residual
18	0.6274	-2.23

Estimates of parameters

Parameter	estimate	s.e.
B	0.3334	0.0892
M	9.892	0.776
C	0.8071	0.0661
A	-0.0386	0.0507

Staphylococcus epidermidis

Nonlinear regression analysis

Response variate: C2
Explanatory: C1
Fitted Curve: A + C/(1 + EXP(-B*(X - M)))

Summary of analysis

Source	d.f.	s.s.	m.s.	v.r.	F pr.
Regression	3	2.38853	0.796176	435.48	<.001
Residual	10	0.01828	0.001828		
Total	13	2.40681	0.185139		

Percentage variance accounted for 99.0
Standard error of observations is estimated to be 0.0428.

Message: the following units have large standardized residuals.

Unit	Response	Residual
14	1.0386	2.64

Message: the following units have high leverage.

Unit	Response	Leverage
12	0.3105	0.60

Estimates of parameters

Parameter	estimate	s.e.
B	0.6232	0.0794
M	9.214	0.296
C	0.9393	0.0341
A	0.0028	0.0230

Appendix 5: Genstat GLM results: attractiveness experiments

Bacillus subtilis and *Pseudomonas aeruginosa*

Regression analysis

Response variate:	%_respons
Binomial totals:	1['rsave'][2][2]
Distribution:	Binomial
Link function:	Logit
Fitted terms:	Constant + treatment + time_nr

Summary of analysis

Source	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
Regression	10	3.796	0.37962	4.68	<.001
Residual	25	2.028	0.08113		
Total	35	5.824	0.16641		
Change	-5	-1.725	0.34490	4.25	0.006

Dispersion parameter is estimated to be 0.0811 from the residual deviance.

Message: the following units have large standardized residuals.

Unit	Response	Residual
2	0.286	2.33
19	0.793	2.59
23	0.107	-2.30

Estimates of parameters

Parameter	estimate	s.e.	t(25)	t pr.	antilog of estimate
Constant	-3.576	0.698	-5.12	<.001	0.02799
treatment Bacillus 30	1.001	0.521	1.92	0.066	2.720
treatment Medium	-0.453	0.659	-0.69	0.498	0.6354
treatment Mix Rob	1.515	0.504	3.01	0.006	4.548
treatment Pseudomonas 30	-0.357	0.644	-0.55	0.584	0.7000
treatment Pseudomonas 8	-0.143	0.614	-0.23	0.818	0.8672
time_nr 3	1.203	0.672	1.79	0.086	3.330
time_nr 4	0.228	0.767	0.30	0.769	1.256
time_nr 5	1.216	0.672	1.81	0.082	3.373
time_nr 6	2.167	0.636	3.41	0.002	8.733
time_nr 7	1.262	0.669	1.89	0.071	3.532

Message: s.e.s are based on the residual deviance.

Parameters for factors are differences compared with the reference level:

Factor	Reference level
treatment	Bacillus 12
time_nr	2

Accumulated analysis of deviance

mean deviance approx

Change	d.f.	deviance	deviance	ratio	F pr.
+ treatment	5	2.07170	0.41434	5.11	0.002
+ time_nr	5	1.72452	0.34490	4.25	0.006
Residual	25	2.02818	0.08113		
Total	35	5.82441	0.16641		

```
 167  RPAIR[PRINT=tprobabilities] !p(treatment)
```

Pairwise differences

Regression analysis

Response variate: %_respons
Binomial totals: 1['rsave'][2][2]
Distribution: Binomial
Link function: Logit
Fitted terms: Constant + treatment + time_nr

t probabilities of pairwise differences

Bacillus 12	*				
Bacillus 30	0.066	*			
Medium	0.498	0.021	*		
Mix Rob	0.006	0.220	0.002	*	
Pseudomonas 30	0.584	0.026	0.891	0.003	*
Pseudomonas 8	0.818	0.045	0.649	0.004	0.748
	Bacillus 12	Bacillus 30	Medium	Mix Rob	Pseudomonas 30

Pseudomonas 8	*
	Pseudomonas 8

Brevibacterium epidermidis and *Staphylococcus epidermidis*

Regression analysis

Response variate: %_respons
Binomial totals: 1
Distribution: Binomial
Link function: Logit
Fitted terms: Constant, treatment

Summary of analysis

Source	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
Regression	5	1.268	0.25368	2.54	0.050
Residual	30	2.999	0.09996		
Total	35	4.267	0.12192		

Dispersion parameter is estimated to be 0.1000 from the residual deviance.

Message: the following units have large standardized residuals.

Unit	Response	Residual
6	0.414	2.16

Estimates of parameters

Parameter	estimate	s.e.	t(30)	t pr.	antilog of estimate
Constant	-1.707	0.358	-4.77	<.001	0.1814
treatment Brevibacterium 6	-1.661	0.803	-2.07	0.047	0.1900
treatment Medium	-1.209	0.686	-1.76	0.088	0.2983
treatment Mix Rob	0.109	0.497	0.22	0.829	1.115
treatment Staphylococcus 30	-0.266	0.533	-0.50	0.621	0.7662
treatment Staphylococcus 9	-1.321	0.712	-1.86	0.073	0.2668

Message: s.e.s are based on the residual deviance.

Parameters for factors are differences compared with the reference level:

Factor	Reference level
treatment	Brevibacterium 30

Accumulated analysis of deviance

Change	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
+ treatment	5	1.26840	0.25368	2.54	0.050
Residual	30	2.99889	0.09996		
Total	35	4.26729	0.12192		

```
 154  RPAIR[PRINT=tprobabilities] !p(factor)
```

Fault 2, code VA 11, statement 19 in procedure RPAIR

Command: & NlevFact[1...#NTreatFc] = NLEV(TREATFACTORS[])
Invalid or incompatible type(s).
Structure factor is not of the required type.

```
 155  RPAIR[PRINT=tprobabilities] !p(treatment)
```

Pairwise differences

Regression analysis

Response variate: %_respons
Binomial totals: 1['rsave'][2][2]
Distribution: Binomial
Link function: Logit
Fitted terms: Constant, treatment

t probabilities of pairwise differences

Brevibacterium 30	*		
Brevibacterium 6	0.047	*	
Medium	0.088	0.630	*

Mix Rob	0.829	0.034	0.062	*	
Staphylococcus 30	0.621	0.099	0.191	0.480	*
Staphylococcus 9	0.073	0.722	0.896	0.052	0.159
	Brevibacterium 30	Brevibacterium 6	Medium	Mix Rob	Staphylococcus 30

Staphylococcus 9	*
	Staphylococcus 9

Corynebacterium minutissimum and the worn sock

Regression analysis

Response variate: %_respons
Binomial totals: 1
Distribution: Binomial
Link function: Logit
Fitted terms: Constant, treatment

Summary of analysis

Source	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
Regression	4	11.090	2.7726	22.10	<.001
Residual	25	3.136	0.1254		
Total	29	14.226	0.4906		

Dispersion parameter is estimated to be 0.125 from the residual deviance.

Estimates of parameters

Parameter	estimate	s.e.	t(25)	t pr.	antilog of estimate
Constant	-3.094	0.702	-4.41	<.001	0.04533
treatment Corynebacterium 30	2.052	0.776	2.65	0.014	7.780
treatment Medium	0.080	0.977	0.08	0.935	1.084
treatment Mix Rob	2.249	0.770	2.92	0.007	9.478
treatment Sock Rob	4.386	0.785	5.58	<.001	80.29

Message: s.e.s are based on the residual deviance.

Parameters for factors are differences compared with the reference level:

Factor	Reference level
treatment	Corynebacterium 11

Accumulated analysis of deviance

Change	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
+ treatment	4	11.0903	2.7726	22.10	<.001
Residual	25	3.1359	0.1254		
Total	29	14.2262	0.4906		

```
138  RPAIR[PRINT=tprobabilities] !p(treatment)
```

Pairwise differences

Regression analysis

Response variate:	%_respons
Binomial totals:	special['rsave'][2][2]
Distribution:	Binomial
Link function:	Logit
Fitted terms:	Constant, treatment

t probabilities of pairwise differences

Corynebacterium 11	*				
Corynebacterium 30	0.014	*			
Medium	0.935	0.015	*		
Mix Rob	0.007	0.668	0.008	*	
Sock Rob	0.000	0.000	0.000	0.000	*
	Corynebacterium 11	Corynebacterium 30	Medium	Mix Rob	Sock Rob

Control experiments

Regression analysis

Response variate:	%_respons
Binomial totals:	1
Distribution:	Binomial
Link function:	Logit
Fitted terms:	Constant, treatment

Summary of analysis

Source	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
Regression	2	0.0801	0.04006	0.87	0.440
Residual	15	0.6928	0.04619		
Total	17	0.7729	0.04547		

Dispersion parameter is estimated to be 0.0462 from the residual deviance.

Estimates of parameters

Parameter	estimate	s.e.	t(15)	t pr.	antilog of estimate
Constant	-3.027	0.418	-7.24	<.001	0.04845
treatment Medium	-0.977	0.776	-1.26	0.227	0.3764
treatment Micrococcus Medium	-0.367	0.648	-0.57	0.579	0.6925

Message: s.e.s are based on the residual deviance.

Parameters for factors are differences compared with the reference level:

Factor	Reference level
treatment	CMA

Accumulated analysis of deviance

Change	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
+ treatment	2	0.08013	0.04006	0.87	0.440
Residual	15	0.69279	0.04619		
Total	17	0.77292	0.04547		

```
198  RPAIR[PRINT=tprobabilities] !p(treatment)
```

Pairwise differences

Regression analysis

Response variate: %_respons
Binomial totals: special['rsave'][2][2]
Distribution: Binomial
Link function: Logit
Fitted terms: Constant, treatment

t probabilities of pairwise differences

CMA	*		
Medium	0.227	*	
Micrococcus Medium	0.579	0.469	*
	CMA	Medium	Micrococcus Medium

Appendix 6: Genstat GLM results: ammonia experiments

Bacillus subtilis, Brevibacterium epidermidis, Corynebacterium minutissimum, Staphylococcus epiderimidis and the worn sock versus ammonia

Regression analysis

Response variate:	nr_treatment
Binomial totals:	nr_total
Distribution:	Binomial
Link function:	Logit
Fitted terms:	Constant, treatment

Summary of analysis

Source	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
Regression	5	181.11	36.222	21.56	<.001
Residual	30	50.41	1.680		
Total	35	231.52	6.615		

Dispersion parameter is estimated to be 1.68 from the residual deviance.

Message: the following units have high leverage.

Unit	Response	Leverage
13	5.00	0.37

Estimates of parameters

Parameter	estimate	s.e.	t(30)	t pr.	antilog of estimate
Constant	-1.170	0.495	-2.37	0.025	0.3103
treatment Brevibacterium+H20	0.382	0.856	0.45	0.659	1.465
treatment Corynebacterium+H20	1.545	0.709	2.18	0.037	4.687
treatment Mix Rob+H20	2.601	0.659	3.95	<.001	13.47
treatment Sock Rob	6.01	1.39	4.33	<.001	406.0
treatment Staphylococcus+H20	-0.439	0.730	-0.60	0.552	0.6444

Message: s.e.s are based on the residual deviance.

Parameters for factors are differences compared with the reference level:

Factor	Reference level
treatment	Bacillus+H20

Fitted values and residuals

Unit	Binomial total	Response	Fitted value	Standardized residual	Leverage
1	12	1	2.84	-1.30	0.32

2	3	1	0.71	0.30	0.08
3	4	0	0.95	-1.20	0.11
4	3	1	0.71	0.30	0.08
5	4	1	0.95	0.05	0.11
6	12	5	2.84	1.28	0.32
7	2	0	0.62	-1.01	0.13
8	3	1	0.94	0.07	0.19
9	4	0	1.25	-1.54	0.25
10	3	2	0.94	1.08	0.19
11	3	2	0.94	1.08	0.19
12	1	0	0.31	-0.69	0.06
13	10	5	5.93	-0.57	0.37
14	1	1	0.59	0.80	0.04
15	3	3	1.78	1.45	0.11
16	5	4	2.96	0.85	0.19
17	2	0	1.19	-1.52	0.07
18	6	3	3.56	-0.40	0.22
19	7	7	5.65	1.43	0.12
20	8	6	6.46	-0.33	0.14
21	9	4	7.26	-2.02	0.16
22	11	10	8.88	0.80	0.19
23	7	7	5.65	1.43	0.12
24	15	12	12.11	-0.06	0.26
25	25	25	24.80	0.54	0.20
26	20	19	19.84	-1.20	0.16
27	19	19	18.85	0.46	0.15
28	25	25	24.80	0.54	0.20
29	19	19	18.85	0.46	0.15
30	19	19	18.85	0.46	0.15
31	6	0	1.00	-1.23	0.14
32	5	1	0.83	0.16	0.12
33	8	1	1.33	-0.28	0.19
34	10	0	1.67	-1.69	0.24
35	4	2	0.67	1.24	0.10
36	9	3	1.50	1.06	0.21
Mean				0.02	0.17

Accumulated analysis of deviance

Change	d.f.	deviance	mean deviance	deviance ratio	approx F pr.
+ treatment	5	181.112	36.222	21.56	<.001
Residual	30	50.408	1.680		
Total	35	231.520	6.615		

```
 226  RPAIR[PRINT=tprobabilities] !p(treatment)
```

Pairwise differences

Regression analysis

Response variate: nr_treatment
Binomial totals: nr_total
Distribution: Binomial
Link function: Logit
Fitted terms: Constant, treatment

t probabilities of pairwise differences

	Bacillus+H20	Brevibacterium+H20	Corynebacterium+H20	Mix Rob+H20	Sock Rob
Bacillus+H20	*				
Brevibacterium+H20	0.659	*			
Corynebacterium+H20	0.037	0.188	*		
Mix Rob+H20	0.000	0.011	0.125	*	
Sock Rob	0.000	0.001	0.003	0.018	*
Staphylococcus+H20	0.552	0.359	0.012	0.000	0.000

	Staphylococcus+H20
Staphylococcus+H20	*