ACTA MICROBIOLOGICA BULGARICA Volume 40 / 1 (2024)

Comparative Study of the Volatile Organic Compounds Produced by a Mosquito Repellent *Bacillus licheniformis* Strain from Desert Soil Versus a Non-Repellent One

Taha Chouati[1,2], Bahia Rached[1], Serigne Inssa Ngom[4], Aziz Bentis[3], Said Gmouh[3], Christel Béra-Maillet[4], Mohamed Amar[1], Elmostafa El Fahime[1,2,3]*

[1]*Functionnal Genomic Analysis Plateform and CCMM, National Center for Scientific and Technical Research*
[2]*Genopath Research center, ERNN, Faculty of Medicine and Pharmacy, University Mohammed V, Rabat, Morocco*
[3]*Laboratory LIMAT, Faculty of Science Ben M'Sik, Hassan II University, Casablanca, Morocco*
[4]*Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, Jouy-en-Josas, France*

Abstract

Mosquito-borne diseases constitute a major health issue and a high burden for many countries. The biocontrol of mosquitoes is vital for combatting such diseases. Bacteria exhibit huge potential biotechnological uses due to their adaptability and ubiquity. Volatile organic compounds (VOCs) are typically molecules with low molecular weight and high vapor pressure. The volatiles produced by bacteria are complex and can impact insect behavior. This study aimed to compare the volatiles produced by the repellent *Bacillus licheniformis* strain Ba1 with a closely related, yet non-repulsive one (B957). The strains were isolated from extreme environments in Morocco (desert dunes and hot springs) and their resistance to both high temperature and salinity was investigated. After cultivation, the VOCs were extracted and identified by GC-MS. The VOCs detected showed little difference between the two strains, however, their relative abundance varied greatly, suggesting a dose-dependent effect. Ba1 was found to produce both repulsive and attractive compounds as well as molecules with larvicidal effects mainly found in plant extracts. To the best of our knowledge, this is the first study of VOCs emitted by *Bacillus sp.* With a repulsive effect against mosquitoes.

Keywords: *Bacillus licheniformis*; mosquitoes; repellence; VOCs; halotolerance; thermotolerance.

Резюме

Болестите, пренасяни от комари, представляват сериозен здравен проблем и голям проблем за много страни. Биоконтролът на комарите е от съществено значение за борбата с тези болести. Бактериите имат огромен потенциал за биотехнологични приложения поради своята адаптивност и повсеместна употреба. Летливите органични съединения (ЛОС) обикновено са молекули с ниско молекулно тегло и високо парно налягане. Летливите вещества, произвеждани от бактериите, са сложни и могат да повлияят на поведението на насекомите. Целта на това изследване е да се сравнят летливите вещества, произвеждани от репелентния щам *Bacillus licheniformis* Ba1 с близкородствен, но не-отблъскващ щам (B957). Щамовете са изолирани от екстремни среди в Мароко (пустинни дюни и горещи извори) и е изследвана тяхната устойчивост както към висока температура, така и към висока соленост. След култивиране, ЛОС са екстрахирани и идентифицирани чрез GC-MS. Откритите ЛОС показват малка разлика между двата щама, но относителното им количество варира значително, което предполага дозо-зависим ефект. Установено е, че Ba1 произвежда както отблъскващи, така и привличащи съединения, както и молекули с ларвициден ефект, открити главно в растителни екстракти. Доколкото ни е известно, това е първото изследване на ЛОС, отделяни от *Bacillus* sp. с отблъскващ ефект срещу комари.

Corresponding author: melfahime@gmail.com
Acta Microbiol. Bulg. 2024; 40(01). https://doi.org/10.59393/amb24400112

Introduction

Vector-borne diseases constitute an important part of the global burden of infectious diseases. Mosquitoes-borne diseases (MBD) are a great concern because of the wide range of pathogens transmitted ("Vector-borne diseases" n.d.). Particularly, *Anopheles* and *Aedes* genera are responsible for the transmission of malaria, Dengue, Zika Virus, and Chikungunya. These diseases are known for their high morbidity and mortality and can prove to be a significant economic and societal burden on countries, especially developing countries and urban areas (Devine *et al.*, 2019; Daniele *et al.*, 2021). In this context, numerous strategies have been developed to control mosquito populations (Amer and Mehlhorn, 2006; Bhatt *et al.*, 2015). However, population movements and urbanization make these large-scale efforts rather ineffective (Lima-Camara, 2016).

Among synthetic repellents, *N, N*-diethyl-m-toluamide (DEET) has been used since the 1950s for its low cost and great efficacy against mosquitoes and ticks. The repellent effect is obtained when DEET binds to the olfactory receptors of still-in-flight mosquitoes (DeGennaro, 2015). DEET provides an effective and long-lasting repellent activity against both *Culex* and *Aedes* but is slightly less so against *Anopheles*. Despite being considered as the reference repellent, adverse effects from DEET exposure are not unheard of, especially among children *(*Yoon *et al.*, 2015). However, extensive use of insecticide is not without repercussions. Indeed, insecticide resistance is a growing problem that needs to be addressed urgently in order to avoid a resurgence of diseases (Hemingway *et al.*, 2016). Making the insect repellency approach a more interesting preventive measure.

Natural products are favored as a source of repellency due to their low environmental impact compared to synthetic repellents (Sharma *et al.*, 2021). Indeed, essential oils extracted from certain plants can be used for their repellent effect, and a great many have been studied through the years, but their effectiveness is limited by their rapid volatilization, short time of effect, and sometimes their strong odor of the original plant (Benelli *et al.*, 2016).

Whereas plant essential oil use as insect repellent is well documented (Rehman *et al.*, 2014), VOCs produced from other sources are less so. There is however growing evidence of the implication of microorganisms as insect semi-chemicals showing that the microorganism-insect relation is complex and that microbial VOCs can act as both negative and positive cues for a wide variety of insects (Davis *et al.*, 2013). Concerning mosquitoes specifically, there is evidence for attractive effects from different genera (*Bacillus spp.* and *Staphylococcus spp.*) (Takken and Verhulst, 2017) as well as the impact of bacterial abundance rather than microbial diversity (Verhulst *et al.*, 2011). However, to the best of our knowledge, there is little data considering mosquitoes' repellency from microbial VOCs.

Bacillus licheniformis is a Gram-positive, endospore-forming organism with a nearly ubiquitous distribution in the environment (Veith *et al.*, 2004) with the GRAS (Generally Regarded As Safe) status (Schallmey *et al.*, 2004) and a remarkably varied arsenal of enzymes that allows it to thrive under highly diversified conditions (Wiegand *et al.*, 2013). On the industrial level, it is used in an array of processes such as degradation, recycling, or usage as probiotics (Bajaj and Manhas, 2012; Huang *et al.*, 2019; Guo *et al.*, 2020). The extremotolerant *B. licheniformis* strains are of particular interest due to their often-promising properties (Bashir *et al.*, 2018; Ibarra-Villarreal *et al.*, 2021; Thesai *et al.*, 2021). Regarding volatile production, however, *B. licheniformis* is relatively underrepresented, and little documentation exists about its mVOCs or their effects despite its metabolic diversity.

In this study, we focus on two *B. licheniformis* strains (Ba1 and B957) previously isolated in the same study from desert soil on the outskirts of Merzouga, a small Saharan Village in Southeast Morocco (Ba1) and Ain Allah, a hot spring in the same country (Aanniz *et al.*, 2015). Ba1 showed strong repulsive activity against both *Anopheles gambiae* and *Aedes aegypti* whereas B957 did not. Subsequent testing showed that Ba1 had this effect through volatile emissions, allowing for a large area of effect, without a noticeable lethal effect on adult mosquitoes exposed. This repulsion in the case of strain Ba1 has led to a patent deposit (Aanniz *et al.*, 2017). In this study, we used Gas Chromatography with Mass Spectrometry to identify and compare the volatile compounds produced by both strains in order to narrow down the potential repulsive molecules.

Materials and Methods

Strain origins

The studied strains were obtained from the Moroccan Coordinated Collection of Microorganisms (CCMM) at the National Center for Scientific

Research and Technology (Rabat, Morocco). Ba1 was first isolated in 2015 from desert dunes (Merzouga, Morocco) while B957 was collected from the hot spring of Ain Allah. Before this study, both strains were tested for catalase and oxidase activity and repulsive activity against *A. gambiae* and *A. agypti* using a Vertical Landing Bioassay. API gallery 50CH (Biomerieux, USA) was used to identify these strains as *Bacillus sp.* The strains were obtained from CCMM's lyophilized stock, and a fresh culture was prepared before each test in 5 mL TSB and TSA (BioRad, USA).

Thermal and salinity tolerance

Fresh Ba1 and B957 cultures (0.2 mL) were inoculated on 5mL TSB with varying NaCl concentrations (0, 5, 10, 15, and 20%) and homogenized. Then 2 mL of each culture condition were dispatched on 24 wells microplates and incubated at 37°C in constant agitation. Bacterial growth was monitored at 6h intervals through 600 nm OD measurements for 48h. Incubation, agitation, and growth were monitored using an Epoch 2 Microplate Spectrophotometer (Biotek, USA, VE). Multiple plates were used to assess bacterial growth in the different salinity conditions at three temperatures (37, 55, and 60°C). This test was done in triplicate for each condition. Mean absorbance was then calculated and plotted.

DNA Extraction

DNA extraction was performed by combining a mechanical lysis using PRECELLYS (Bertin technologies, France) and a chemical one with a Wizard Genomic DNA Purification Kit (Promega, USA). The obtained DNA was then controlled for quality and quantity using Nanodrop technology (Thermo Scientific, USA).

Identification of strains

Amplification of 16S rRNA gene was done using MyTaq HSDNA Polymerase Kit (Bioline, UK) and the universal primers fd4 (5'-AGAGTTTGATCCTGGCTCAG-3') and rp2 (5'-ACGGCTACCTTGTTACGACTT-3'). Furthermore, Multi-Locus Sequence Typing (MLST) was performed for greater discrimination between same species strains. The sequences generated were controlled by gel electrophoresis (1% agarose) and then purified using an ExoSAP-IT PCR cleanup Kit (Applied Biosystems, USA). The sequence reaction was performed using Big Dye Terminator Cycle Sequencing Kit Version 3.1 (Applied Biosystems, USA). The thermocycler program was as follows: 25 cycles of 96°C for 1 min, 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The products were purified using BigDye Xterminator Purification Kit (Applied Biosystems, USA) and finally analyzed with SeqStudio Genetic Analyzer Capillary Sequencer 3130XL (Applied Biosystems), according to the manufacturer's instructions. The primers suitable for *B. licheniformis*, (Table 1) were obtained from the PubMLST database(Jolley *et al.*, 2018).

Analysis, quality control, and assembly of sequences for all genes were performed using UGENE (V39.2). For phylogenic analysis, complete 16S rRNA sequences were downloaded from NCBI (www.ncbi.nlm.nih.gov), and Maximum Parsimony Tree was generated using MEGA (V11.0.10) with a bootstrap value of 1000.

Headspace GC-MS

Samples were prepared in duplicate in 10 mL vials containing 3 mL of fresh TSB culture incubated for 24h at 37°C. Furthermore, sterile TSB medium was also analyzed for comparative purposes. The analysis was performed using a TriPlus 300 HS-TRACE 1310 gas chromatography equipped with a TSQ 9000 triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc, USA). All samples were analyzed using the Rxi-5ms capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness, RESTEK, USA). Helium gas was used as the carrier gas at a constant flow of 1.0 mL/min. The GC oven programs were held at 50°C for 5 min, and then first increased to 105°C at 5°C/min and

Table 1. List of primer pairs used for MLST and their corresponding Th.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'	T °C
ADK	GGT AAA GGG ACA CAG GCT GA	TCG AGT AAA GGC TGG GTT TG	58
ccpa	TAT GAT GTA GCA CGC GAA GC	TAT CCC CAA GCG CTC TTT TA	58
recF	ACG GTT CTG TTC CCA TTC AG	CAT CAC GGC CAT TGA CAT AG	58
sucC	GGG TCC CGA CGG CCA ACA AA G	GGC CGG TTC CCC TCC GTA GT	58
rpoB	AGG TCA ACT AGT TCA GTA TGG ACG	AAG AAC CGT AAC CGG CAA CTT	58
Spo0A	GAA GTG CTT GGT GTC GCA TA	TGT GTA GCC GAA AAG TGA CG	58

then to 220°C at 10°C/min and held for 5 min, then heated to 300°C with a rate of 20°C min^{-1}. The ion source and transfer line temperatures were maintained at 250°C and 300°C, respectively. The mass range was 35–550 m/z and the scan rate was 0.2 scan per second in full scan mode.

Results

Thermotolerance

Both strains were unable to grow at 60 and 65°C, however, their growth rate varied greatly between 37 and 55°C. Briefly, while both strains grew better at 37°C than at 55°C, OD values measured for Ba1 at any given temperature and time were better than B957. The results are shown in Fig. 1.

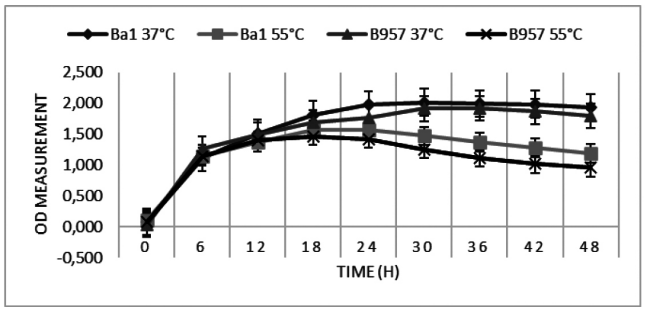

Fig. 1. Growth quantification over time through optic density (OD) measurement of Ba1 and B957 on TSB at 37 and 55°C.

Halotolerance

The growth rate for Ba1 and B957 at different NaCl concentrations followed the same pattern seen for 0% NaCl with the notable exception of the 20% NaCl test, where there was no significant growth registered. Namely, Ba1 and B957 grew better at 37°C than 55°C irrespective of NaCl concentration.

On the other hand, NaCl concentration impacted greatly the growth curve of both strains. As can be seen in Fig. 2 A, B, and C, while Ba1 grew better than B957 at 5% and 15% NaCl, the reverse was true for 10% NaCl. Furthermore, OD measurements for Ba1 varied greatly in function of NaCl concentrations, meanwhile, B957 showed strikingly similar growth between 5 and 10 % NaCl.

Identification of strains

Analysis of the near complete 16S rRNA gene in comparison with other *Bacillus sp.* Type strains revealed that the two studied bacteria were members of *B. licheniformis* (Fig. 3). However, it should be noted that the similarity between *B. licheniformis* and *B. paralicheniformis*, and to a lesser extent, B. *sonorensis* was too high, and thus, 16S rRNA gene sequences alone were considered inconclusive regarding the differentiation of these strains. The sequences were however deposited on

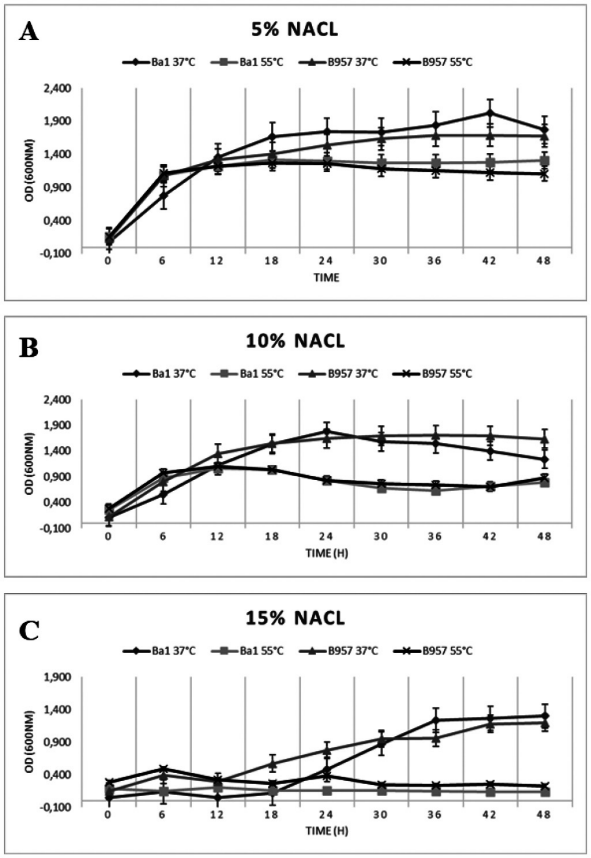

Fig. 2. Growth evolution of Ba1 and B957 600nm Optical Density (OD) measurement for 48h on TSB supplemented with 5 (A), 10 (B), and 15% (C) NaCl at 37 and 55°C

GenBank under the accessions OR195440.1 and OR195519.1 for Ba1 and B957 respectively.

Table 2. Allelic profile of Ba1 and B957 for each MLST scheme

	Ba1		B957	
	Length	Allele	Length	Allele
adk	477	1	475	1
ccpA	596	9	591	**9+**
recF	573	1	586	**1+**
sucC	560	1	575	1
rpoB	535	1	517	1
spo0A	574	1	578	1
ST	41		41*	
Species	*B. lichenifomis*		*B. lichenifomis*	

Each gene is caracterized by its length and allele number. Bold alleles with a plus sign indicate that the allele in question contains additional mutations

To further ascertain the identity of the two studied strains, an MLST scheme was performed, each house-keeping gene was given an allele number according to the PubMLST database, and a final ST was then identified as presented in Table 1. Despite the striking similarity between Ba1 and B957,

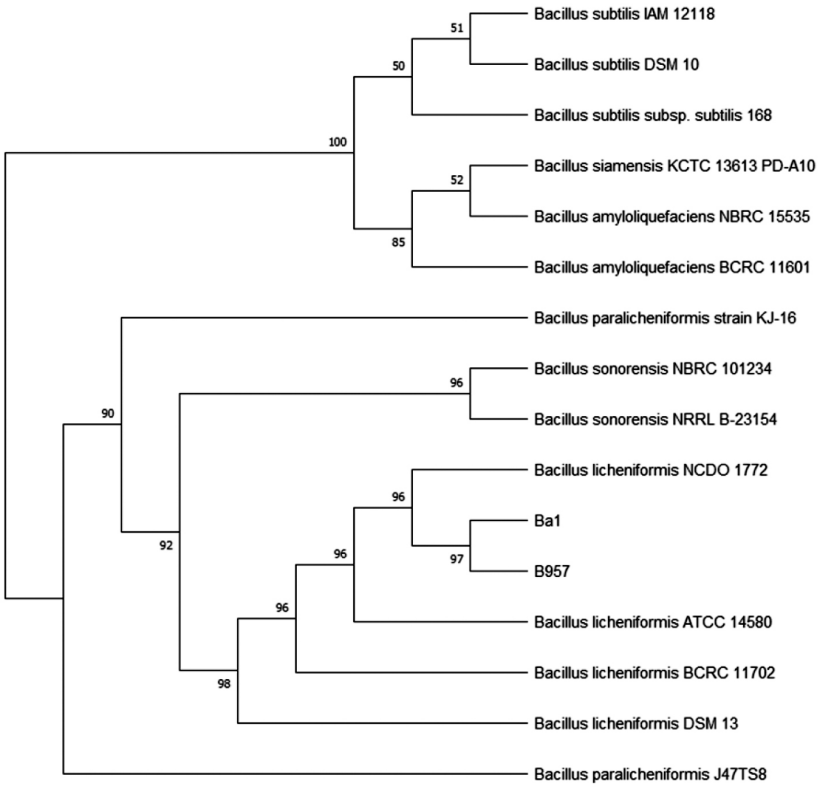

Fig. 3. Maximum Parsimony Tree based on complete 16S rRNA sequences from *B. licheniformis* and its closest relatives showing the relative closeness of Ba1 and B957 as well as their position within the *B. licheniformis* group

ccpa and recF genes in B957 did not conform to alleles 9 and 1 respectively, this difference was however not enough to identify another allele number. thus, we considered that, in the absence of further information, Ba1 and B957 could be considered as part of the same Strain Type (ST 41) albeit with a minimal divergence.

GC-MS analysis

Volatile compounds with an abundance of roughly more than 1% were selected. The comparison with sterile TSB medium showed that only decane and undecane were produced in all samples. Interestingly, the same six compounds (17-Pentatriacontene; 2,6,10-trimethyl-Tetradecane; 1,3-bis(1,1-dimethylethyl)-Benzene; 3-ethyl-5-(2-ethylbutyl)-Octadecane; 2,6,11-trimethyl-Dodecane and 2,6,10,15-tetramethyl-Heptadecane) accounted for around 60% of total emissions by both *Bacillus* strains. The comparison between the abundance of these compounds can be summarized in the figure below, emphasizing the wide variation in the relative abundance of compounds but also the striking similarity in the compounds' nature between the two strains.

With respect to the nature of the analyzed molecules, the majority (13 of 19) were alkanes or alkane derivatives. Furthermore, all alkaloids observed can be considered long-chain compounds (more than 13 carbons) and were not observed in TSB samples. The nature and chemical composition of all the selected compounds is detailed in Table 3 below.

Discussion

The rapid development and ease of bacterial differentiation are important factors in the introduction and study of new species. Indeed, the *B. licheniformis* group has known a number of changes in recent years. Firstly, *Bacillus aerius* was introduced by Shivaji *et al.* (Shivaji *et al.*, 2006), however, later work by Dunlap *et al.* in 2015 led to the disuse of *B. aerius* as a distinct species (Dunlap, 2015) as well as the differentiation between *B. licheniformis* and *B. paralicheniformis* (Dunlap *et al.*, 2015). The work by Dunlap *et al.* was based on genomic comparison and used data from strains considered then as *B. licheniformis* through 16S rRNA gene sequencing. This helps emphasize the ineffectiveness of 16S rRNA gene sequencing as a way to differentiate between *B. licheniformis* and *B. paralicheniformis* in favor of other methods such as MLST or single house-keeping gene sequencing (Jeong *et al.*, 2018). In our study, while the results of 16S rRNA gene sequencing indicated that both strains were *B. licheniformis*, an MLST scheme was considered necessary for clear confirmation of their affiliation.

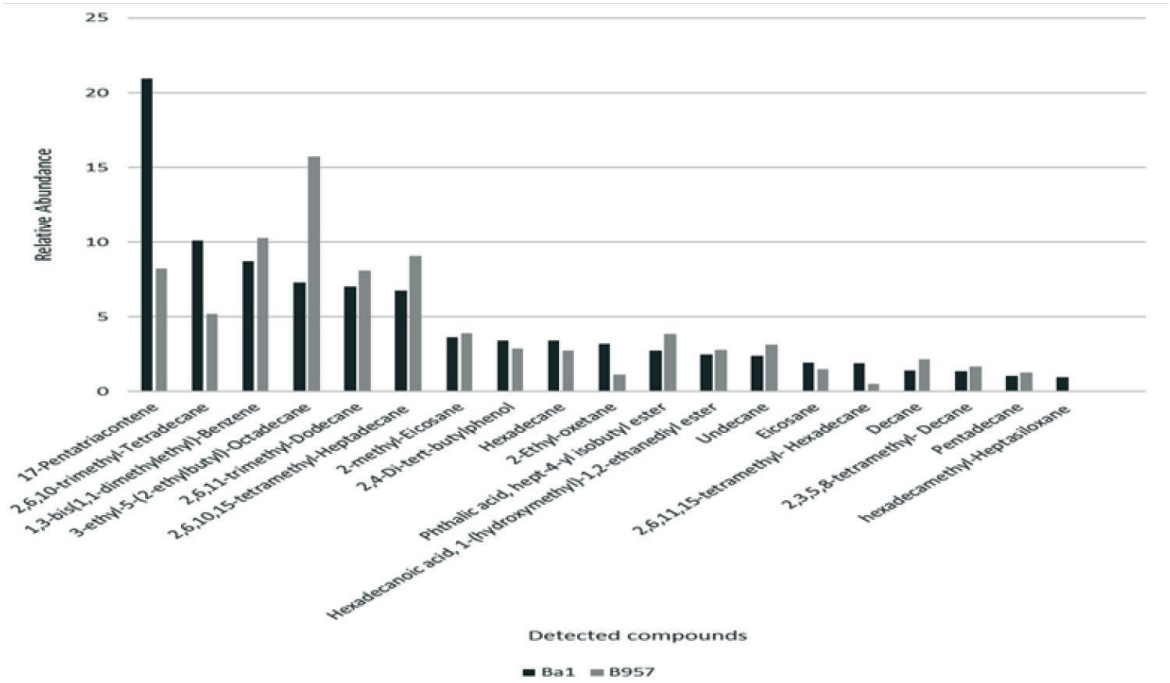

Fig. 4. Identification of detected compounds and their relative abundance in Ba1 as well as B957 using Gas Chromatography coupled to Mass Spectrometry (GS-MS).

Table 3. Name, nature and composition of most important compounds obtained through GC-MS analysis and their relative Abundance in Ba1 and B957

Compound	Nature	Composition	% Ba1	% B957
17-Pentatriacontene	Branched alkene	$C_{35}H_{70}$	20.93	8.22
2,6,10-trimethyl-Tetradecane	Branched alkane	$C_{17}H_{36}$	10.10	5.19
1,3-bis(1,1-dimethylethyl)-Benzene	Alkylbenzene	$C_{14}H_{22}$	8.73	10.28
3-ethyl-5-(2-ethylbutyl)-Octadecane	Branched alkane	$C_{26}H_{54}$	7.27	15.73
2,6,11-trimethyl-Dodecane	Branched alkane	$C_{15}H_{32}$	7.03	8.10
2,6,10,15-tetramethyl-Heptadecane	Branched alkane	$C_{21}H_{44}$	6.75	9.05
2-methyl-Eicosane	Branched alkane	$C_{21}H_{44}$	3.63	3.88
2,4-Di-tert-butylphenol	Phenol	$C_{14}H_{22}O$	3.40	2.85
Hexadecane	Alkane	$C_{16}H_{34}$	3.38	2.72
2-Ethyl-oxetane	Oxetane	$C_6H_{12}O$	3.16	1.14
Phthalic acid, hept-4-yl isobutyl ester	Phtalic Acid Ester	$C_{19}H_{28}O_4$	2.71	3.85
Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	Hexadecanoic acid	$C_{19}H_{38}O_4$	2.47	2.77
Undecane	Alkane	$C_{11}H_{24}$	2.39	3.11
Eicosane	Alkane	$C_{20}H_{42}$	1.94	1.46
2,6,11,15-tetramethyl- Hexadecane	Branched alkane	$C_{20}H_{42}$	1.86	0.51
Decane	Alkane	$C_{10}H_{22}$	1.37	2.14
2,3,5,8-tetramethyl- Decane	Branched alkane	$C_{14}H_{30}$	1.34	1.64
Pentadecane	Alkane	$C_{15}H_{32}$	1.02	1.25
hexadecamethyl-Heptasiloxane	organosiloxane	$C_{16}H_{48}O_6Si_7$	0.94	0

The studied strains showed an ability to resist both 55°C and moderately tolerate salinity (Oren, 2008). This is not surprising considering their origins (Desert soil and hot springs), as well as the previously described *B. licheniformis* strains (Horani and Priest, 1994). This is interesting as naturally thermostable and/or halotolerant enzymes are used in different industrial processes (Saad *et al.*, 2021) ranging from production (Caccamo *et al.*, 2020) to biodegradation (Suganthi *et al.*, 2013). Regarding strain Ba1, while its ability to produce volatiles was not studied in different conditions, its tolerance to

high temperature and salinity is interesting if the production of volatiles is to be adapted in an industrial setting.

Microorganisms are also known to produce complex mixtures of volatiles. Moreover, many volatile "signatures" can be observed and analyzed for different bacteria(Henis *et al.*, 1966). While the 16S rRNA gene sequencing is the easier and more robust method, VOC signatures are able to differentiate between bacterial species (Tracey *et al.*, 1986). VOC signatures have however been regrouped by Lemfack *et al.* (2018) in a single database and showed the remarkable diversity of these volatiles. Moreover, these varied volatiles can act as cues for key insects' behaviors such as aggregation, oviposition, and host location (Davis *et al.*, 2013).

In Table 2, the 7 major compounds (accounting for 64.44% and 60.45% of the total emissions of Ba1 and B957 respectively) were nearly all alkanes (with the only exception being a benzenoid). *Bacillus* strains seem to show a marked production of 4 types of VOCs small volatiles, benzenoids, ketones, and alkanes (Peñuelas *et al.*, 2014).

The nature of volatiles seems to be nearly the same between Ba1 and B957, which could be explained by their phylogenetic closeness (Goelen *et al.*, 2020b). However, their relative abundance in each mix is different. Interestingly, the volatiles produced in higher proportions by Ba1 seem to be part of compositions with effect on mosquitoes.

The most prominent of these is 17-pentatriacontene, which was the most abundant compound produced by Ba1, in contrast to B957. It was identified among 13 compounds from *Rhizophora mucronata's* essential oil. This compound has shown both larvicidal and repellent effects on mosquitoes, specifically *A. stephensi*, *Culex quinquefasciatus,* and *A. aegypti* (Karthi *et al.*, 2020). Specifically, 2,6,11-trimethyl-Dodecane, 2,6,10-trimethyl-Tetradecane, as well as 1,3-bis(1,1-dimethylethyl)-Benzene were among the identified compounds in the patent for mosquitoes' repellency using Ba1 (Aanniz *et al.*, 2017). Interestingly, a study where volatile emissions of chicken (considered as a non-host species for *A. arabiensis*) were analyzed identified Hexadecane among the chicken-specific VOCs with a marked repellent effect against the selfsame mosquitoes' species (Jaleta *et al.*, 2016). Finally, the work of Muttiah *et al.*, where the repellent effect *Jasminum officinale* essential oil was studied, also showed that 2,6,10,15- tetramethyl Heptadecane was part of its composition (Akbar *et al.*, 2022).

Surprisingly, other compounds produced by the two strains are described in the literature as attractive molecules for mosquitoes. Indeed, eicosane and undecane are both reported in the same study concerning the attraction of *A. arabiensis* to sugar-cane pollen (Wondwosen *et al.*, 2018).

Perhaps the most intriguing compounds (if only for their relatively high numbers) are the ones with known larvicidal effects against mosquitoes. 17-Pentatriacontene is again the most prominent compound. Similarly*,* 2,6,10-trimethyl-tetradecane is also identified as one of the main compounds present in the methanol extracts of *Leucas aspera* which were demonstrated in this selfsame study to possess a potent lethal effect on larvae of *A. aegypti* and *A. stephensi* (Elumalai *et al.*, 2017). These two compounds seem to possess both repulsive and larvicidal effects, and also are the most produced by Ba1 as opposed to B957. Additionally, in a study concerning the larvicidal activity of *Magnolia denudata* seed hydrodistillate, 2,4-Di-tert-butylphenol was found to be the most toxic compounds against larvae of the four mosquitoes' species tested (Wang *et al.*, 2016). Garlic essential oils (containing 3-ethyl-5-(2-ethylbutyl)- Octadecane (Hamada *et al.*, 2018) have shown high efficacy against mosquito larvae (Okonkwo and Ohaeri, 2013). Surprisingly, this compound is the most abundantly produced by the non-repulsive strain. Other compounds such as pentadecane (Sutthanont *et al.*, 2010), and 2,6,11,15-tetramethyl-Hexadecane (Ngegba *et al.*, 2022) can also be found in composition with lethal effect on various mosquito larvae species.

Even though the lethal activity of Ba1 and B957 against mosquito larvae was not assessed, the diversity of compounds with a potential larvicidal effect is interesting. Additionally, in Ba1, the two most prominent volatiles are the potentially larvicidal 17-pentatriacontene and 2,6,10-trimethyl-tetradecane. The authors suggest that Ba1 could possess a repellent effect by signaling non-viable, and thus undesirable, oviposition sites for mosquitoes.

As can be seen in Fig. 4, the similarity between Ba1 and B957 VOCs does not extend to their abundance. This might suggest that the repellent effect of Ba1 is dose-dependent. This aligns with previous works who have shown that VOCs impact on insects varies greatly with concentration. As an example, in a study by Goelen *et al.*, changes in compound ratios in otherwise similar VOCs caused a radically different response from aphid parasitoids (Goelen *et al.*, 2020a). Concerning mosquitoes, a basic blend consisting of ammonia, L-lactic acid,

and tetradecanoic acid was previously proven to be attractive to *A. gambiae sensu stricto* (Smallegange *et al.*, 2005). Furthermore, a single compound, even when added at the sub p.p.m level, could reduce the attractiveness of such a blend, as is the case of 2-acetylthiophene,2-nonanone, and 2-phenylethanol (Smallegange *et al.*, 2012).

The challenge of identifying the correct blend of compounds that would have a repulsive effect on mosquitoes is further complicated by the fact that the detection of VOCs by insects is in itself a highly complex and dynamic process (Conchou *et al.*, 2019). For the sake of brevity, we can consider that what causes possible repellence are the compounds and ratios detected by mosquitoes. This means that even trace compounds such as the aforementioned hexadecamethyl-heptasiloxane could play an impactful role provided they are detected by mosquitoes.

The chemical composition of VOCs produced by Ba1 shows a number of interesting compounds that are also present in vegetal essential oils, which could make their production comparatively easier. However, the authors are aware that active formulations of insect repellent need to satisfy a certain number of conditions before being considered efficient (Tavares *et al.*, 2018). While there is no doubt that further studies should explore the viability of the repellence of Ba1, this work is a step in the right direction to broaden the options of prevention of mosquito-borne diseases.

Conclusions

This work shows that repellence against mosquitoes through volatile compounds produced by bacteria is closer to that of vegetal repellence, although it does not necessarily involve specific VOCs. Indeed, there was little difference between compounds produced by the repellent versus the non-repellent bacterial strain, indicating that the repellence against mosquitoes is dose-dependent. Major VOCs produced are well described in the literature but as potential insecticides.

References

Aanniz, T., M. Ouadghiri, M. Melloul, J. Swings, E. Elfahime, J. Ibijbijen, M. Ismaili, M. Amar (2015). Thermophilic bacteria in Moroccan hot springs, salt marshes and desert soils. *Braz. J. Microbiol.* **46**: 443–453. https://doi.org/10.1590/S1517-838246220140219.

Aanniz, T., M. Amar, L. Beladjal, J. Mertens, M. Quadghiri, P. Vandenaele (2017). Insect repellent, WO, WO2016020167A1. Patent, https://patents.google.com/patent/WO2016020167A1/en.

Akbar, R., I. A. Khan, R. A. Alajmi, A. Ali, B. Faheem, A. Usman, A. M. Ahmed, M. El-Shazly, A. Farid, J. P. Giesy, M. A. M. Aboul-Soud (2022). Evaluation of insecticidal potentials of five plant extracts against the stored grain pest, *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Insects* **13**: 1047. https://doi.org/10.3390/insects13111047.

Amer, A., H. Mehlhorn (2006). Persistency of larvicidal effects of plant oil extracts under different storage conditions. *Parasitol. Res.* **99**: 473–477. https://doi.org/10.1007/s00436-006-0183-2.

Bajaj B. K., K. Manhas (2012). Production and characterization of xylanase from *Bacillus licheniformis* P11(C) with potential for fruit juice and bakery industry. *Biocatal. Agric. Biotechnol.* **1**:330–337. https://doi.org/10.1016/j.bcab.2012.07.003.

Bashir, F., M. Asgher, F. Hussain, M. A. Randhawa (2018). Development and characterization of cross-linked enzyme aggregates of thermotolerant alkaline protease from *Bacillus licheniformis*. *Int. J. Biol. Macromol.* **113**: 944–951. https://doi.org/10.1016/j.ijbiomac.2018.03.009.

Benelli, G., C. L. Jeffries, T. Walker (2016). Biological control of mosquito vectors: past, present, and future. *Insects* **7**: E52. https://doi.org/10.3390/insects7040052.

Bhatt, S., D. J. Weiss, E. Cameron, D. Bisanzio, B. Mappin, U. Dalrymple, K. E. Battle, C. L. Moyes, A. Henry, P. A. Eckhoff, E. A. Wenger, O. Briët, M. A. Penny, T. A. Smith, A. Bennett, J. Yukich, T. P. Eisele, J. T. Griffin, C. A. Fergus, M. Lynch, F. Lindgren, J. M. Cohen, C. L. J. Murray, D. L. Smith, S. I. Hay, R. E. Cibulskis, P. W. Gething (2015). The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. *Nature* **526**: 207–211. https://doi.org/10.1038/nature15535.

Caccamo, M. T., C. Gugliandolo, V. Zammuto, S. Magazù (2020). Thermal properties of an exopolysaccharide produced by a marine thermotolerant *Bacillus licheniformis* by ATR-FTIR spectroscopy. *Int. J. Biol. Macromol.* **145**: 77–83. https://doi.org/10.1016/j.ijbiomac.2019.12.163.

Conchou, L., P. Lucas, C. Meslin, M. Proffit, M. Staudt, M. Renou (2019). Insect odorscapes: from plant volatiles to natural olfactory scenes. *Front. Physiol.* **10**: 972. https://doi.org/10.3389/fphys.2019.00972.

Daniele, D. R., D. Montecino-Latorre, S. O. Vanwambeke, M. Marcantonio (2021). Will the yellow fever mosquito colonise Europe? Assessing the re-introduction of *Aedes aegypti* using a process-based population dynamical model. *Ecol. Inform.* **61**: 101180. https://doi.org/10.1016/j.ecoinf.2020.101180.

Davis, T. S., T. L. Crippen, R. W. Hofstetter, J. K. Tomberlin (2013). Microbial volatile emissions as insect semiochemicals. *J. Chem. Ecol.* **39**: 840–859. https://doi.org/10.1007/s10886-013-0306-z.

DeGennaro, M. (2015). The mysterious multi-modal repellency of DEET. *Fly* **9**: 45–51. https://doi.org/10.1080/19336934.2015.1079360.

Devine, G. J., H. J. Overgaard, R. E. Paul (2019). Global vector control guidelines - the need for co-creation. *Trends Parasitol.* **35**: 267–270. https://doi.org/10.1016/j.pt.2018.12.003.

Dunlap, C. A. (2015). The status of the species *Bacillus aerius*. Request for an opinion. *Int. J. Syst. Evol. Microbiol.* **65**: 2341–2341. https://doi.org/10.1099/ijs.0.000271.

Dunlap, C. A., S.-W. Kwon, A. P. Rooney, S.-J. Kim (2015). *Bacillus paralicheniformis* sp. nov., isolated from fermented soybean paste. *Int. J. Syst. Evol. Microbiol.* **65**:

3487–3492. https://doi.org/10.1099/ijsem.0.000441.

Elumalai, D., P. Hemalatha, P. K. Kaleena (2017). Larvicidal activity and GC–MS analysis of *Leucas aspera* against *Aedes aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus*. *J. Saudi Soc. Agric. Sci.* **16**: 306–313. https://doi.org/10.1016/j.jssas.2015.10.003.

Goelen, T., I. S. Sobhy, C. Vanderaa, J. G. de Boer, F. Delvigne, F. Francis, F. Wäckers, H. Rediers, K. J. Verstrepen, T. Wenseleers, H. Jacquemyn, B. Lievens (2020a). Volatiles of bacteria associated with parasitoid habitats elicit distinct olfactory responses in an aphid parasitoid and its hyperparasitoid. *Funct. Ecol.* **34**: 507–520. https://doi.org/10.1111/1365-2435.13503.

Goelen, T., I. S. Sobhy, C. Vanderaa, F. Wäckers, H. Rediers, T. Wenseleers, H. Jacquemyn, B. Lievens (2020b). Bacterial phylogeny predicts volatile organic compound composition and olfactory response of an aphid parasitoid. *Oikos* **129**: 1415–1428. https://doi.org/10.1111/oik.07301.

Guo, Y., X. Qin, Y. Tang, Q. Ma, J. Zhang, L. Zhao (2020). CotA laccase, a novel aflatoxin oxidase from *Bacillus licheniformis*, transforms aflatoxin B1 to aflatoxin Q1 and epi-aflatoxin Q1. *Food Chem.* **325**: 126877. https://doi.org/10.1016/j.foodchem.2020.126877.

Hamada, H. M., M. Awad, M. El-Hefny, M. A. M. Moustafa (2018). Insecticidal activity of garlic (*Allium sativum*) and ginger (*Zingiber officinale*) oils on the cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae). *Afr. Entomol.* **26**: 84–94. https://doi.org/10.4001/003.026.0084.

Hemingway, J., H. Ranson, A. Magill, J. Kolaczinski, C. Fornadel, J. Gimnig, M. Coetzee, F. Simard, D. K. Roch, C. K. Hinzoumbe, J. Pickett, D. Schellenberg, P. Gething, M. Hoppé, N. Hamon (2016). Averting a malaria disaster: will insecticide resistance derail malaria control? *Lancet* **387**: 1785–1788. https://doi.org/10.1016/S0140-6736(15)00417-1.

Henis, Y., J. R. Gould, M. Alexander (1966). Detection and identification of bacteria by gas chromatography. *Appl. Microbiol.* **14**: 513–524. https://doi.org/10.1128/am.14.4.513-524.1966.

Horani, H. K., F. G. Priest (1994). Thermotolerant varieties of *Bacillus licheniformis* isolated from desert environments. *J. Appl. Bacteriol.* **77**: 392–400. https://doi.org/10.1111/j.1365-2672.1994.tb03440.x.

Huang, X., J. Wang, C. Ma, L. Ma, C. Qiao (2019). Diversity analysis of microbial communities and biodegradation performance of two halotolerant and thermotolerant *Bacillus licheniformis* strains in oilfield-produced wastewater. *Int. Biodeterior. Biodegrad.* **137**: 30–41. https://doi.org/10.1016/j.ibiod.2018.11.004.

Ibarra-Villarreal, A. L., A. Gándara-Ledezma, A. D. Godoy-Flores, A. Herrera-Sepúlveda, A. M. Díaz-Rodríguez, F. I. Parra-Cota, S. de los Santos-Villalobos (2021). Salt-tolerant *Bacillus* species as a promising strategy to mitigate the salinity stress in wheat (*Triticum turgidum* subsp. *durum*). *J. Arid Environ.* **186**: 104399. https://doi.org/10.1016/j.jaridenv.2020.104399.

Jaleta, K. T., S. R. Hill, G. Birgersson, H. Tekie, R. Ignell (2016). Chicken volatiles repel host-seeking malaria mosquitoes. *Malar. J.* **15**: 354. https://doi.org/10.1186/s12936-016-1386-3.

Jeong, D. W., B. Lee, H. Lee, K. Jeong, M. Jang, J.-H. Lee (2018). Urease characteristics and phylogenetic status of *Bacillus paralicheniformis*. *J. Microbiol. Biotechnol.* **28**: 1992–1998. https://doi.org/10.4014/jmb.1809.09030.

Jolley, K. A., J. E. Bray, M. C. J. Maiden (2018). Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. *Wellcome Open Res.* **3**: 124. https://doi.org/10.12688/wellcomeopenres.14826.1.

Karthi, S., K. Uthirarajan, V. Manohar, M. Venkatesan, K. Chinnaperumal, P. Vasantha-Srinivasan, P. Krutmuang (2020). Larvicidal enzyme inhibition and repellent activity of red mangrove *Rhizophora mucronata* (Lam.) leaf extracts and their biomolecules against three medically challenging arthropod vectors. *Molecules* **25**:3844. https://doi.org/10.3390/molecules25173844.

Lemfack, M. C., B.-O. Gohlke, S. M. T. Toguem, S. Preissner, B. Piechulla, R. Preissner (2018). mVOC 2.0: a database of microbial volatiles. *Nucleic Acids Res.* **46**: D1261–D1265. https://doi.org/10.1093/nar/gkx1016.

Lima-Camara, T. N (2016). Emerging arboviruses and public health challenges in Brazil. *Rev. Saude Publica* **50**: 36. https://doi.org/10.1590/S1518-8787.2016050006791.

Ngegba, P. M., G. Cui, M. Z. Khalid, G. Zhong (2022). Use of botanical pesticides in agriculture as an alternative to synthetic pesticides. *Agriculture* **12**: 600. https://doi.org/10.3390/agriculture12050600.

Okonkwo, C. O., O. C. Ohaeri (2013). Insecticidal potentials of some selected plants. *J. Chem. Pharm. Res.* **5**: 370-376. https://api.semanticscholar.org/CorpusID:55183931.

Oren, A. (2008). Microbial life at high salt concentrations: phylogenetic and metabolic diversity. *Saline Syst.* **4**: 2. https://doi.org/10.1186/1746-1448-4-2.

Peñuelas, J., D. Asensio, D. Tholl, K. Wenke, M. Rosenkranz, B. Piechulla, J. P. Schnitzler (2014). Biogenic volatile emissions from the soil. *Plant Cell Environ.* **37**: 1866–1891. https://doi.org/10.1111/pce.12340.

Rehman, J. U., A. Ali, I. A. Khan (2014). Plant based products: use and development as repellents against mosquitoes: A review. *Fitoterapia* **95**: 65–74. https://doi.org/10.1016/j.fitote.2014.03.002.

Saad, W. F., A. M. Othman, M. Abdel-Fattah, M. S. Ahmad (2021). Response surface methodology as an approach for optimization of α-amylase production by the new isolated thermotolerant *Bacillus licheniformis* WF67 strain in submerged fermentation. *Biocat. Agric. Biotechnol.* **32**:101944. https://doi.org/10.1016/j.bcab.2021.101944.

Schallmey, M., A. Singh, O. P. Ward (2004). Developments in the use of *Bacillus* species for industrial production. *Can. J. Microbiol.* **50**: 1–17. https://doi.org/10.1139/w03-076.

Sharma, M., A. Alexander, S. Saraf, S. Saraf, U. K. Vishwakarma, K. T. Nakhate, Ajazuddin (2021). Mosquito repellent and larvicidal perspectives of weeds *Lantana camara* L. and *Ocimum gratissimum* L. found in central India. *Biocatal. Agric. Biotechnol.* **34**: 102040. https://doi.org/10.1016/j.bcab.2021.102040.

Shivaji, S., P. Chaturvedi, K. Suresh, G. S. N. Reddy, C. B. S. Dutt, M. Wainwright, J. V. Narlikar, P. M. Bhargava (2006). *Bacillus aerius* sp. nov., *Bacillus aerophilus* sp. nov., *Bacillus stratosphericus* sp. nov. and *Bacillus altitudinis* sp. nov., isolated from cryogenic tubes used for collecting air samples from high altitudes. *Int. J. Syst. Evol. Microbiol.* **56**: 1465–1473. https://doi.org/10.1099/

ijs.0.64029-0.

Smallegange, R. C., G. Bukovinszkiné-Kiss, B. Otieno, P. A. Mbadi, W. Takken, W. R. Mukabana J. J. A. Van Loon (2012). Identification of candidate volatiles that affect the behavioural response of the malaria mosquito *Anopheles gambiae sensu stricto* to an active kairomone blend: laboratory and semi-field assays. *Physiol. Entomol.* **37**: 60-71. https://doi.org/10.1111/j.1365-3032.2011.00827.x

Smallegange, R. C., Y. T. Qiu, J. J. A. van Loon, W. Takken (2005). Synergism between ammonia, lactic acid and carboxylic acids as kairomones in the host-seeking behaviour of the malaria mosquito *Anopheles gambiae sensu stricto* (Diptera: Culicidae). *Chem. Senses* **30**: 145–152. https://doi.org/10.1093/chemse/bji010.

Suganthi, C., A. Mageswari, S. Karthikeyan, M. Anbalagan, A. Sivakumar, K. M. Gothandam (2013). Screening and optimization of protease production from a halotolerant *Bacillus licheniformis* isolated from saltern sediments. *J. Genet. Eng. Biotechnol.* **11**: 47–52. https://doi.org/10.1016/j.jgeb.2013.02.002.

Sutthanont, N., W. Choochote, B. Tuetun, A. Junkum, A. Jitpakdi, U. Chaithong, D. Riyong, B. Pitasawat (2010). Chemical composition and larvicidal activity of edible plant-derived essential oils against the pyrethroid-susceptible and -resistant strains of *Aedes aegypti* (Diptera: Culicidae). *J. Vector Ecol.* **35**: 106–115. https://doi.org/10.1111/j.1948-7134.2010.00036.x.

Takken, W., N. O. Verhulst (2017). Chemical signaling in mosquito–host interactions: the role of human skin microbiota. *Curr. Opin. Insect Sci.* **20**: 68–74. https://doi.org/10.1016/j.cois.2017.03.011.

Tavares, M., M. R. M. da Silva, L. B. de Oliveira de Siqueira, R. A. S. Rodrigues, L. Bodjolle-d'Almeida, E. P. Dos Santos, E. Ricci-Júnior (2018). Trends in insect repellent formulations: A review. *Int. J. Pharm.* **539**: 190–209. https://doi.org/10.1016/j.ijpharm.2018.01.046.

Thesai, A. S., G. Nagarajan, S. Rajakumar, A. Pugazhendhi, P. M. Ayyasamy (2021). Bioaccumulation of fluoride from aqueous system and genotoxicity study on *Allium cepa* using *Bacillus licheniformis*. *J. Hazard. Mater.* **407**: 124367. https://doi.org/10.1016/j.jhazmat.2020.124367.

Tracey, R. P., T. J. Britz, H. J. J. V. Vuuren, P. M. Lategan (1986). Gas chromatographic determination of volatile metabolites formed by *Obesumbacterium proteus* and *hafnia* Species. *J. Inst. Brew.* **92**: 446–451. https://doi.org/10.1002/j.2050-0416.1986.tb04438.x.

WHO. (2023) Fact Sheet: Vector-borne diseases. (accessed 07/06/2023). https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases.

Veith, B., C. Herzberg, S. Steckel, J. Feesche, K. H. Maurer, P. Ehrenreich, S. Bäumer, A. Henne, H. Liesegang, R. Merkl, A. Ehrenreich, G. Gottschalk (2004). The complete genome sequence of *Bacillus licheniformis* DSM13, an organism with great industrial potential. *J. Mol. Microbiol. Biotechnol.* **7**: 204–211. https://doi.org/10.1159/000079829.

Verhulst, N. O., Y. T. Qiu, H. Beijleveld, C. Maliepaard, D. Knights, S. Schulz, D. Berg-Lyons, C. L. Lauber, W. Verduijn, G. W. Haasnoot, R. Mumm, H. J. Bouwmeester, F. H. J. Claas, M. Dicke J. J. A. van Loon, W. Takken, R. Knight, R. C. Smallegange (2011). Composition of human skin microbiota affects attractiveness to malaria mosquitoes. *PLOS ONE* **6**: e28991. https://doi.org/10.1371/journal.pone.0028991.

Wang, Z.-Q., H. Perumalsamy, M. Wang, S. Shu, Y.-J. Ahn (2016). Larvicidal activity of *Magnolia denudata* seed hydrodistillate constituents and related compounds and liquid formulations towards two susceptible and two wild mosquito species. *Pest Manag. Sci.* **72**: 897–906. https://doi.org/10.1002/ps.4064.

Wiegand, S., S. Dietrich, R. Hertel, J. Bongaerts, S. Evers, S. Volland, R. Daniel, H. Liesegang (2013). RNA-Seq of *Bacillus licheniformis*: active regulatory RNA features expressed within a productive fermentation. *BMC Genomics* **14**: 667. https://doi.org/10.1186/1471-2164-14-667.

Wondwosen, B., G. Birgersson, H. Tekie, B. Torto, R. Ignell, S. R. Hill (2018). Sweet attraction: sugarcane pollen-associated volatiles attract gravid *Anopheles arabiensis*. *Malar. J.* **17**: 90. https://doi.org/10.1186/s12936-018-2245-1.

Yoon, J. K., K.-C. Kim, Y. Cho, Y.-D. Gwon, H. S. Cho, Y. Heo, K. Park, Y.-W. Lee, M. Kim, Y.-K. Oh, Y. B. Kim (2015). Comparison of repellency effect of mosquito repellents for DEET, citronella, and fennel oil. *J. Parasitol. Res.* **2015**: 361021. https://doi.org/10.1155/2015/361021.