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Preliminary Results of Larvicidal Activity and Phylogenic Analysis of Two Strains of Bacteria Isolated in the Demba Tiarki Tara Pond in Mali

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Abstract: ***Faced with the growing resistance of mosquitoes to conventional chemical insecticides, which are often very toxic to humans, it becomes imperative to find alternatives to organic products in order to reduce their nuisance. Actinomycetal bacteria and Bacillus are an opportunity because they have a great capacity for the production of active biological substances. The objective of this study was to isolate and verify the bacteria that produce larvicidal bioactive substances. Two bacteria were isolated from the soil of the DTT pond, tested the culture products on mosquito larvae and analyzed the genetic profile of the 16S rRNA gene. In culture, isolate S226 produced a larvicidal effect on Anopheles gambiae Kisumu of 8% and 14% death in the first hour, 24% and 32% at 48 hours with respective concentrations of 1.5% and 2.5 %. No adult form was visited within 48 hours of culture. As with isolate S296, larvicidal activity was observed at higher concentrations of 44, 46 and 42% for dilutions of 10, 20 and 25%, respectively. Phylogenetic analysis of the 16S rRNA gene from the two bacterial isolates shows that they are apparent to Bacillus and are unknown to the GenBank database. The S226 isolate is 100% similar and 97% identical to Pseudomonas hibiscicola, which of S296 is 100% similar and 95.98% identical to the genus Bacillus amyloliquefaciens. In conclusion, this pond contains microorganisms capable of producing bioactive substances on mosquito larvae. Further studies should be considered in order to better elucidate the active substances.***

Keywords: Bacteria, Phylogeny, Bioactive substances, Larvicidal, *Anopheles gambiae kisumu*

1. Introduction

Insect resistance to chemical insecticides remains a problem in the tropics and subtropics. In the fight against malaria, the control of the vector plays a great importance in the control or even the eradication of the disease. Since 1995, biological control has been introduced in some countries using bacteria such as *Bacillus thuringiensis israelensis* (Bti), [1]. Other bacteria such as the genus Streptomyces [2, 3] and Bacillus have the ability to produce a variety of bioactive substances used as antibiotics, insecticides, antitumors etc. In 2017, Bt strains were isolated and characterized in the Demba Tiarki Tara (DTT) pond, with potential larvicidal power [4]. Thus, strains of bacteria were isolated from the pool of DTT, to test their antimicrobial [5] and larvicidal power. The objective of this study is to identify other bacteria of larvicidal interest that can be used for the control of malaria vectors.

2. Material and Methods

Larvicidal Tests Protocol

The larvicidal tests were carried out on larvae of *Anopheles gambiae kisumu* reared in LaboREM-Biotech according to the WHO protocol [6]. In pots containing 200 ml of water are added 25 immature larvae at stage 3 or 4 of *Anopheles gambiae kisumu*. The water is first left to stand in the open for 10 minutes allowing the chlorine to evaporate. The bacteria were cultured in Bennett liquid medium for 10 days, then the culture is centrifuged to collect the supernatant liquid believed to contain bioactive substances [7]. Different volumes 0.5ml, 1ml, 2ml, 3ml, and 5ml of bacteria culture products are put in these test jars in order to have different concentration series. Each test concentration is duplicated. The larvae were thus observed for 48 hours while noting the dead, the nymphs and the adults at the end of the test. During the first 24 hours no food in the culture medium of the larvae. The averages of the observations were made by concentration, and as a function of time 1 hour, 2 hours, 3 hours, 24 hours and 48 hours. For each test there was a control jar in which the biological test product was not added. Only mortality values between 10 and 90% should be considered important.

Amplification of the 16S rRNA gene

The DNA of the young shoots of bacteria was extracted by the Kit Promega protocol. The gene encoding 16S ribosomal RNA has been used to study genetic diversity [8]. The following primers (primers designed by the firm Eurofins) were used for the amplification of the fragment of the 16S rRNA gene: Gone primer. 5'-AGAGTTTGATCCTGGCTCAG-3 'Return primer. 5'-AAGGAGGTGATCCAGCC-3 'with a TECHNE - PRIME type thermal cycler. The amplification program was as follows: Initial Denaturation 94 ° C in 5mn, 35 cycles of Denaturation 94 ° C in 1mn, Hybridization 55 ° C in 45s, Elongation 72 ° C in 1mn 45s; then a final Elongation 72 ° C

in 10 minutes and at the end the amplifia can be stored at 4 ° C in the thermal cycler. After electrophoresis, the gel is viewed under Ultra-Violet (UV) rays and then photographed with the E-BoX camera and the band size determined with the E-Cap software.

Phylogenic analysis of DNA sequences

The sequences were supplied in the form of an ABI (Applied Biosystems) chromatogram sheet by the Company Inqaba biotec ™ (http://www.inqababiotec.co.za) in South Africa. The results of the sequencing of the 16S DNA fragments were processed by MEGA-X software (Molecular Evolutionary Genetics Analysis), the percentage of GC bonds within the sequences, or Chargaff constant was carried out by the ENDMEMO software (http: //www.endmemo.com/bio/gc.php). Phylogenetic analysis was performed on the NCBI site using Blast (Basic Local Alignment Search Tool) and the dendrogram performed allowed to show the taxonomic position of each isolate in the GenBank database.

3. Results

Larvicidal tests

The larvae of *Anopheles gambiae kisumu* were chosen for two reasons: a) it is the most widespread species in Africa south of the Sahara; b) the high sensitivity of kisumu to insecticides. Bennett's medium was chosen for the test for the production of antibacterial substances because of its high carbon content.

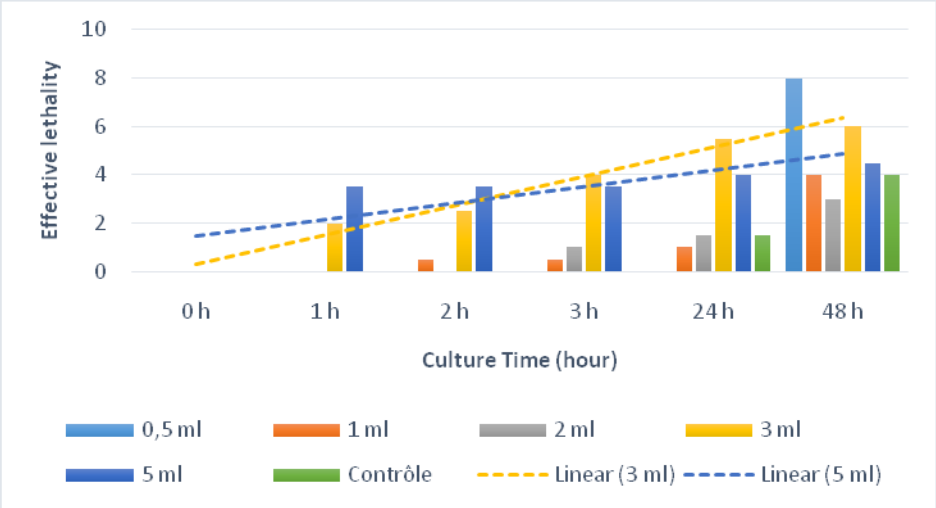

Figure 1: Lethality of *Anopheles gambiae kisumu* larvae in contact with extract of culture of isolate S226 in liquid medium Bennett

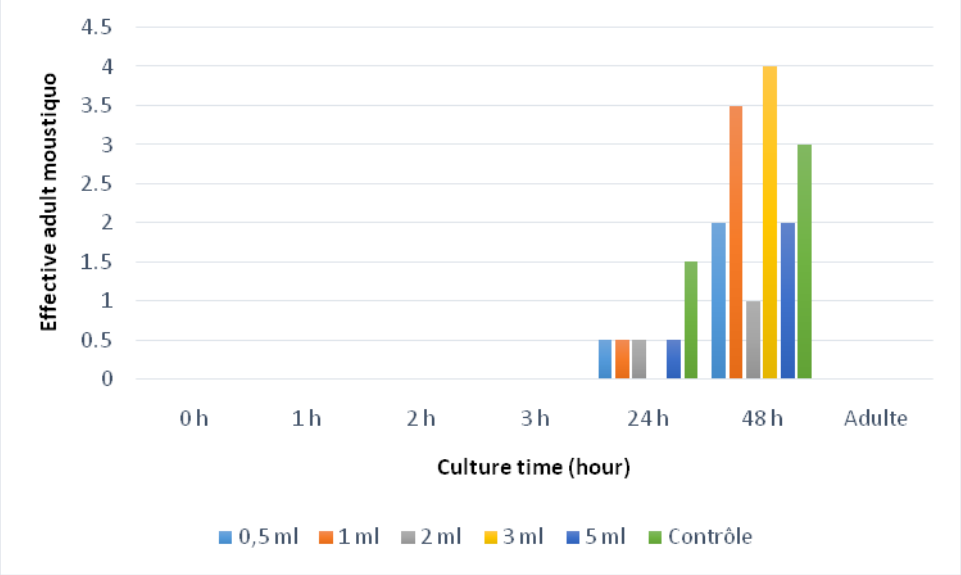

Figure 2: Development of *Anopheles gambiae kisumu* larvae in contact with extract of culture of isolate S226 in Bennett liquid medium

The test with isolate S226 (Figure 1) showed interesting larvicidal properties. From the first hour, larvicidal effects are observed with dilutions of 1.5% and 2.5% (ie 3ml and 5ml in 200 ml of water). This larvicidal effect increased over time up to 48 hours with 32% deaths for the 2.5% dilution and 24% for the 1.5% dilution. Unlike the S296 isolate, that of S226, showed no adult mosquito form at 48 hours and even after (figure 2), but rather nymph forms, the highest rate of which was observed by the 1.5 dilution. % (3ml / 200ml). To this end, the observation was that the lethality

observed in mosquito larvae greatly exceeds the rate recommended by the WHO (10% of 25 larvae or 2.5 larvae) to conclude a larvicidal effect. Despite the melanin-producing capacity as well as the antimicrobial power of isolate S296 [5], its larvicidal effect is more marked than with high concentrations (Figure 3).

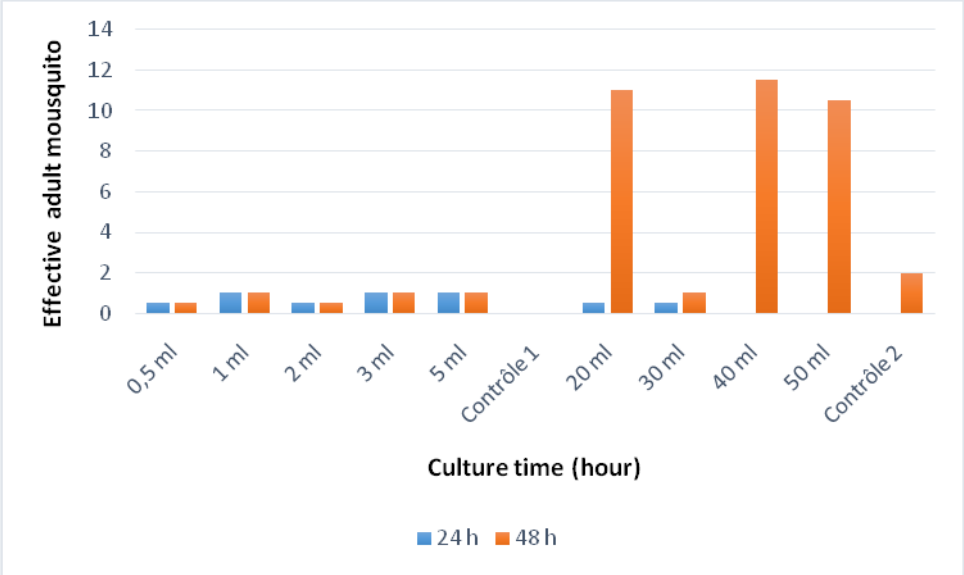

Figure 3: Comparison of the development of *Anopheles gambiae kisumu* larvae in contact with different concentrations of culture extract of isolate S296 in Bennett liquid medium

Molecular analysis

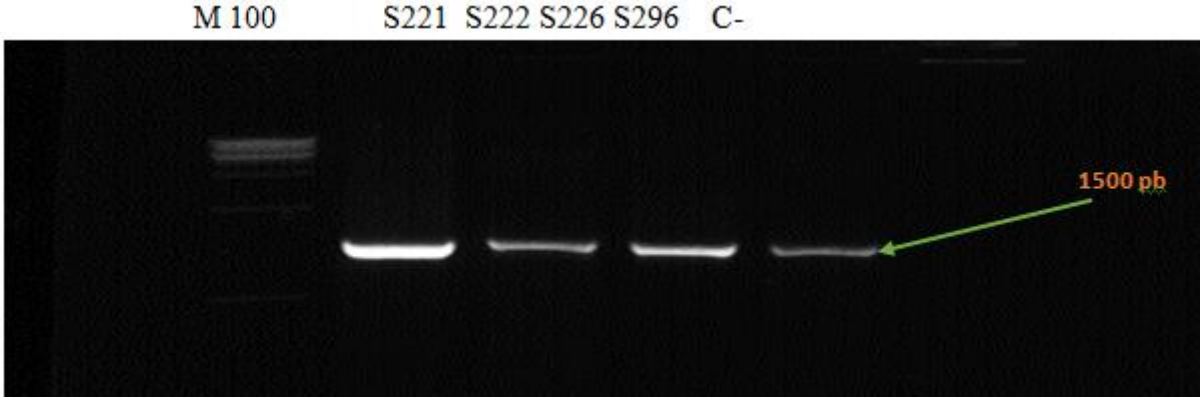

Figure 4: Photo electrophoresis of the amplification of the 16S gene from the selected isolates. The DNAs of the isolates were amplified by a pair of specific primers revealing bands of 1500 base pairs each (Figure 4)

Isolate S226. Format Fasta, length 1061, **Chargaffcoefficient**GC = 53, 53%.

>S226 IBWA-FD1-R F06 18

AGCGCCCTCCCGAAGGTTAAGCTACCTGCTTCTGGT
GCAACAAACTCCCATGGTGTGACGGGCGGTGTGTA
CAAGGCCCGGGAACGTATTCACCGCAGCAATGCTG
ATCTGCGATTACTAGCGATTCCGACTTCATGGAGTC
GAGTTGCAGACTCCAATCCGGACTGAGATAGGGTT
TCTGGGATTGGCTTACCGTCGCCGGCTTGCAGCCCT
CTGTCCCTACCATTGTAGTACGTGTGTAGCCCTGGC
CGTAAGGGCCATGATGACTTGACGTCATCCCCACC
TTCCTCCGGTTTGTCACCGGCGGTCTCCTTAGAGTT
CCCACCATTACGTGCTGGCAACTAAGGACAAGGGT
TGCGCTCGTTGCGGGACTTAACCCAACATCTCACG
ACACGAGCTGACGACAGCCATGCAGCACCTGTGTT
CGAGTTCCCGAAGGCACCAATCCATCTCTGGAAAG
TTCTCGACATGTCAAGGCCAGGTAAGGTTCTTCGCG
TTGCATCGAATTAAACCACATACTCCACCGCTTGTG
CGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGC
GACCGTACTCCCCAGGCGGCGAACTTAACGCGTTA
GCTTCGATACTGCGTGCCAAATTGCACCCAACATCC
AGTTCGCATCGTTTAGGGCGTGGACTACCAGGGTA
TCTAATCCTGTTTGCTCCCCACGCTTTCGTGCCTCA
GTGTCAATGTTGGTCCAGGTAGCTGCCTTCGCCATG
GATGTTCCTCCTGATCTCTACGCATTTCACTGCTAC
ACCAGGAATTCCGCTACCTCTACCACATTTCTAGTC
GCCCAGTATCCACTGCAGTTCCCAGGTTGAGCCCA
GGGCTTTCACAACGGACTTAAACSGACCAYCTACG
CMTCGCTTTACGCCCAGTAATTTCCGAGTAACGCTT
GCACCCTTTCGTATTACCGCGGCTGSYTGGCACGAG
TAARCCGGTGCTTATTCTTTGGGGTACCGTCATCCC
AACCAGGTATTRGCGGCCTGGAARTTCTCTTTCCAA
CAAAGGCCTTAMAACCGGAAGGCYTTTTC

Blast of the 16S DNA sequence of isolate S226, UB20VEF001R, Application: 152169, dated 09/22/2018; is 100% similar with 5 strains including *Pseudomonas hibiscicola* strain ATCC 19867 16S ribosomal RNA (identity score 97%), *Stenotrophomonas maltophilia* strain

IAM 12423 16S ribosomal RNA (Identity score 96.91%), then a score of identity of 96.81 for *Pseudomonas geniculata* strain ATCC 19374 16S ribosomal RNA gene, *Stenotrophomonas maltophilia* strain NBRC 14161 16S ribosomal RNA gene and *Stenotrophomonas pavanii* strain LMG 25348 16S ribosomal RNA gene. The dendrogram shows that the S226 isolate is unknown to the GenBank base (Figure 5).

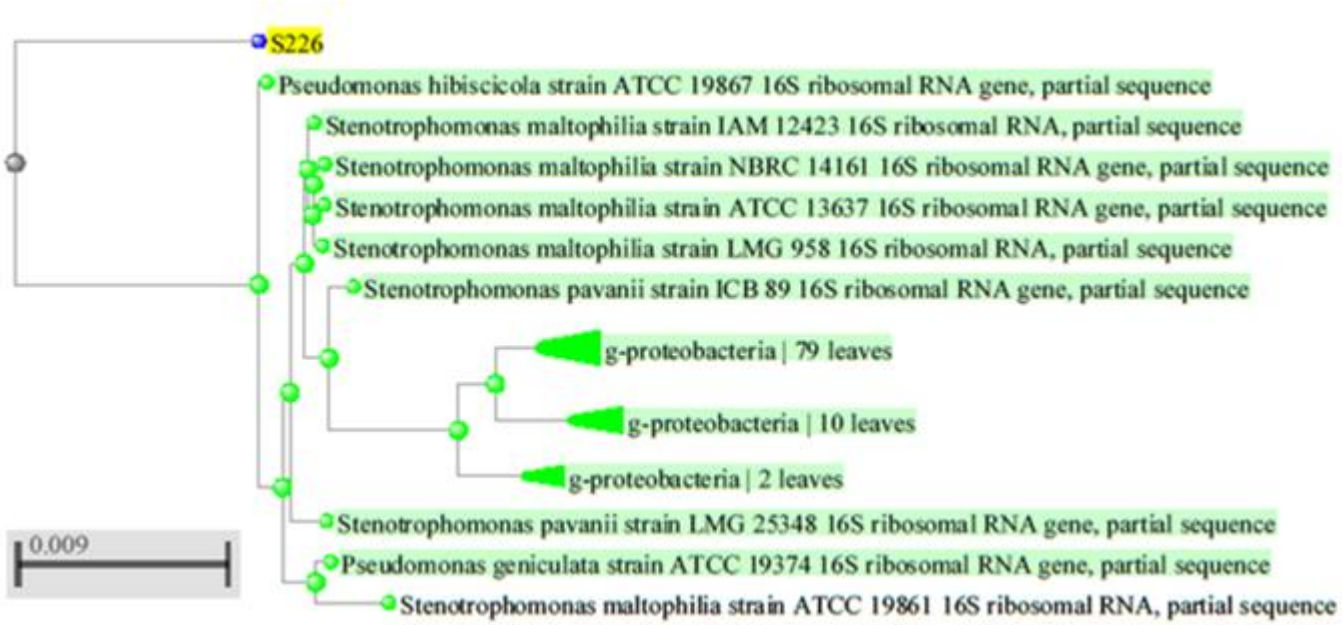

Figure 5: Dendrogram of the DNA sequence of the ribosomal 16S gene of isolate S226 with those found in GenBank, according to the Neighbor-joining method dated 09/22/2018.

The sequence alignment of isolate S296 is that it most closely resembles Bacillus. Among the first 100 Bacillus strains, four have the good score (1766) with 100% similarity and 95.98% identity, of which *Bacillus amyloliquefaciens* strain MPA 1034 is placed in first position then *Bacillus nakamurai* strain NRRL B-41091 16S with a score of 95.89% and *Bacillus vallismortis* strain NRRL B-14890 16S comes in last place with only a similarity of 86%, a score of 1535, but has the highest identity rate 96.10 %. The dendrogram of isolate S296 shows that it is also unknown to the GenBank database (Figure 6).

Isolate S296sequence
Format Fasta, length 1081, Chargaff coefficientGC = 54,11%
>S296 IWA-FD1-R-F11 17
AGGTTCCTTACCGGCTTCGGGTGTTCAATGTCTCGT
GGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACG
TATTCACCGCGGGATGCTGATCCGCGATTACTAGC
GATTCCAGCTTCACGCAGTCGAGTTGCAGACTGSG
ATCCGAACTGAGAACAGATTTGTGGGATTGGCTTA
ACCTCGCGGTCTCGCTGCCCTTTGTTCTGCCCATTG
TAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGA
TGATTTGACGTCATCCCCACCTTCCTCCGGTTTGTC
ACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCT
GGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGA
CTTAACCCAACATCTCACGACACGAGCTGACGACA
ACCATGCACCACCTGTCACTCTGCCCCCGAAGGGG
ACGTCCTATCTCTAGGATTGTCAGAGGATGTMAAG
ACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAAC
CACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATT
CCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCAGG
CGGAGTGCTTAATGCGTTAGCTGCAGCACTAAGGG
GCGGAAACCCCCTAACACTTAGCACTCATCGTTTAC
GGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTC
CCCACGCTTTCGCTCCTCAGCGTCAGTTACAGACCA
GAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCT
CTACGCATTTCACCGCTACACGTGGAATTCCACTCT
CCTCTTCTGGACTCAAGTTCCCAGTTTCCATGACCC
TCCCCGTTGAGCCGGGGGCTTTCACATCAGACTTAA
GAAACCGCCTGCGAGCCCTTTACGCCCATAATTCC
GGACAACGCTTGCCACCTACGTATTACCGCAGCTG
CTGGCACGTAGTTMAGTCGTGGACTWTTCTGGATT
ACGYACYCGTCAAGTGCGGCCTATTGAACGGCACT
TGTTCTTCCCTACGACAGAGCTTTACGATCGAAAAC
GTCATCACCTSCACGCGACGTAGCCTCGTMGACTTT
CGATCCATGCGCAAGA

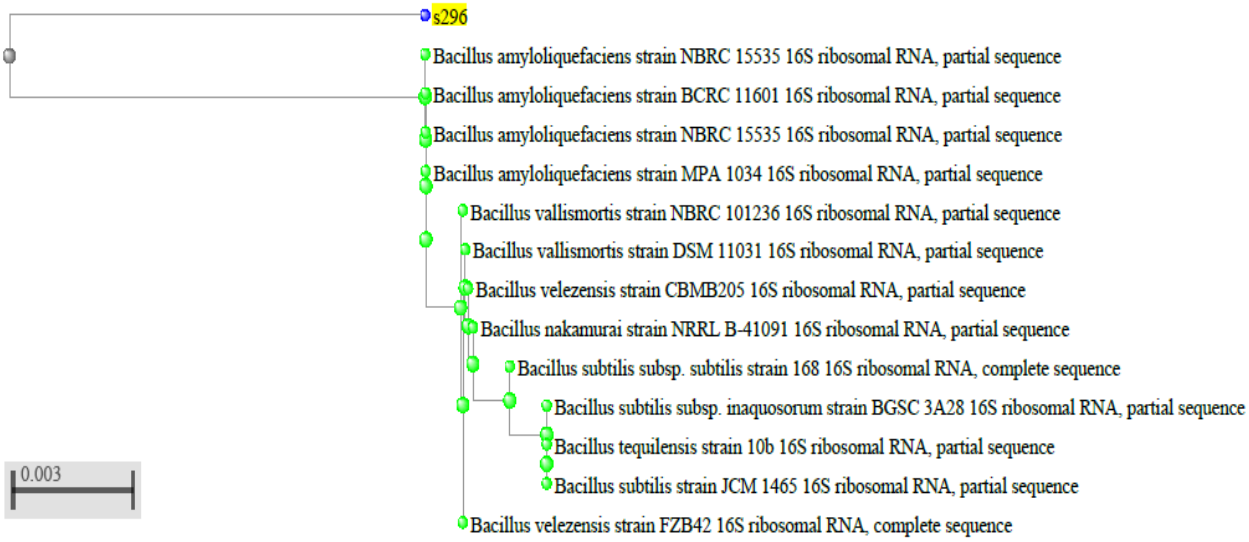

Figure 6: Dendrogram of the 16S ribosomal DNA sequence of isolate S296 with those found in GenBank. (09/22/2018)

4. Discussion

The DTTa pool is an ecosystem favorable to the development of microorganisms. Some of its characteristics such as the pH of the water, the phenomenon of eutrophication and the black color of the soil; are not negligible conditions for the proliferation of actinomycetes. The staining of the soil may also suggest the presence of melanin-producing bacteria, and having a good potential for the production of bioactive substances as announced by certain authors [9, 10]. This melanin pigment most often plays a role in protecting microorganisms, animals and even humans against ultraviolet (UV) rays [11, 12, 13]. The production capacity of bioactive substances remains highly questionable depending on the composition of the culture media [14, 15]. Some authors [15, 16] believe that in addition to carbon sources, pH and incubation temperature have an important influence on the production of secondary metabolites in bacteria. On the other hand, some authors believe that Bennett's medium is proven effective for the production of antibiotics by bacteria [8]. This justifies the choice of this medium in this study, the primary objective of which was to assess the antibiotic production capacity of these bacteria. This study showed antimicrobial activity in 4 isolates, including S296 on *S. aureus* and that of S226 seems to produce a thick and diffusible substance in contact with *S. typhi* and *S. aureus*, [5]. These two isolates have shown promising larvicidal results on mosquito larvae.

In view of these results, morphologically, the isolates appear to be actinomycetes. Studies have shown that actinomycetes have a great potential for producing bio-insecticides [17]

The results of the larvicidal test are promising. Isolate S226 produced a larvicidal effect of 14% (or 3.5 / 25) larval death within the first hour at a concentration of 2.5% (Figure 1). These results were below those observed by Dhansekaran [18], and a study by Prabhakaran [19] showed that the genus *Pseudomonas florescence* has a very important larvicidal activity, almost 100% in less than 24 hours of culture. One of the peculiarities with isolate S226 is the fact that no larvae could develop to reach the adult stage of a flying mosquito. This ability to inhibit larval development by isolate S226 deserves special attention and needs to be further investigated. Isolate S296 is similar to a Bacillus, has a weak larvicidal effect at low concentrations. These results are far less than that of *Bacillus thuringiensis*, which has been shown to have good larvicidal power [20, 21] and remains the benchmark bacterium in biological larvicidal control to this day.

Among the methods for identifying bacteria, the molecular approach appears to be simpler, more convenient, faster and more efficient [22]. This is the basis for the choice of the 16S rRNA gene, which is specific to each species and whose 5 'and 3' ends (first 15 and last 15 bases) are conserved in all bacterial species. Time is most often put forward for the diagnosis or identification of microorganisms, hence the need to invest more in the development of new identification techniques. This is how several rapid diagnostic kits and software such as APIWEBTM, BactecTM, RapidecTM, etc. are on the market, and are most often very oriented and used in the medical field.

The phylogenic comparative analysis of the sequences of the 2 isolates, shows that the S226 isolate has a great similarity (98%) with five bacteria of which the species *Pseudomonas hibiscicola* (gram negative bacteria) has the highest score (1772), and that of S296 is similar to the genus Bacillus, more precisely the gram-positive *Bacillus amyloliquefaciens* with a similarity rate à 95.98%. With this level, it is difficult to say that isolate S296 belongs to the strain *Bacillus amyloliquefaciens*. It is scientifically accepted that any similarity rate of more than 96% to a strain is synonymous with that isolate belonging to that strain. In addition, the dendrogram of the two isolates shows that they are unknown in the Genbank database (Figures 5 and 6)

At the end of this study, two bacteria were isolated from the soil of the DTT pond, having the capacity to produce bioactive substances which exhibited larvicidal properties. These results are of great importance, but remain quite limited, as it would be necessary to pursue additional studies which could be used to develop new biological larvicidal molecules. Isolate S226 could be a potential candidate for the biological control of malaria vectors.

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Conflict of interest
The authors of this article claim that they have no conflict of interest in this work.

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