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Nutrigenomics and Nutrigenetics - Effect of Dietary Supplementation of *Chromolaena odorata* Leaves Paste on the Intestinal Bacteria of African Mud Catfish (*Clarias gariepinus*) Fingerlings using 16s rRNA Gene Sequencing

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ABSTRACT

Nutrigenomics and nutrigenetics is a new frontier in fish nutrition through dietary supplementation with phytobiotics. A study was conducted to assess the effects of supplementing *Chromolaena odorata* leaves (COL) on the intestinal bacteria of *Clarias gariepinus* fingerlings using 16S rRNA gene sequencing. One hundred and eighty *C. gariepinus* fingerlings (12.37 g, average weight) stocked in 12 net-happas (0.6 m × 1.1 m × 1.2 m) suspended in an earthen pond (30 m × 5 m × 1.2 m) at 15 fish per net-happa under four treatments in three replicates. Fish were fed four iso-nitrogeneous diets containing: Control (0%), COL1 (0.5%), COL2 (1%) and COL3 (1.5%); *ad libitum* for 12 weeks. Molecular identification of culture-dependent bacteria was carried out from the fish fed the COL in the Department of Molecular Biology, Covenant University, Ota, Nigeria. Bacteria counts of intestine were examined in using pour plate method on Nutrient and MacConkey Agar. Bacterial isolates were subjected to DNA extraction, amplification and purified PCR products were subjected to DNA gene sequencing. Bacteria counts significantly differed ($p<0.05$) among the dietary treatments. Total bacteria counts was highest in fish fed the control diet and lowest in fish fed the 1% COL. *Klebsiella pneumoniae* (COL1 and COL2), *Proteus mirabilis* (COL3) and *Shigella flexneri* (control) are the Enterobacteriaceae bacteria found in the fish intestine based on the 16s rRNA gene sequencing. The study recommended the dietary inclusion of *C. odorata* leaves could effectively promote growth of cultured *C. gariepinus* by suppressing the growth of gram negative bacteria.

Keywords: *Chromolaena odorata*, Bacteria, *Clarias gariepinus*, Nutrigenetics, Gene sequencing

Abbreviations: DNA: Deoxyribonucleic Acid; RNA: Ribonucliec Acid; PCR: Polymerase Chain Reaction; BLAST: Basic Local Alignment Search Tool; dNTPs: di Nucleotides Proteins; FASTA: DNA and Protein Sequence Alignment Software Package; MSA: Multiple Sequence Alignment; EMBL: European Molecular Biology Laboratory; EBI: European Bioinformatics Institute; NCBI: National Centre for Biotechnology Information

INTRODUCTION

Plants and weeds have been exploited for their medicinal value throughout the world, Mukherjee and Wahile [1] reported the opinion of World Health Organization, which stated that, '80% of the world's population depend on ancestral medicines for their haleness'. The authors further stated that: for the health care of the remaining 20% population mainly residing in developed countries, therapeutic product of plants and weeds play an important role. Attempting to improve the quality of life, humans have used some of these plants species as sources of food, shelter, clothing, and medicine, cosmetic and for seeking relief from hardship of life. Thus, authors such as Sliva Junior et al. [2] concluded that some of these plants are proven to be medicinal because they contain some active substance that

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bring about remedies to the cure of some peculiar diseases of man. Some communities and ethnic groups in the world made use of knowledge of these medicinal plants as their only therapeutic resource [3].

Furthermore, Odunbaku and Ilusanya [4] reported that searches for substance with antimicrobial activity and healing activities are frequently considered interesting by some researchers since they are frequently used in medicine as remedies for many infectious diseases. It has also been reported by Hostettmann et al. [5] that the African continent has a long history with the use of plants and in some African countries, close to 90% of the population relies on medicinal plants (phytobiotics) as a source of drugs. Demand for phytobiotics, both local and international is continuously growing, as well as the biological improving activities searching for sources of new drugs [6]. Thus, these medicinal plants (phytobiotics) play a promising role in aquaculture by enhancing the resistance of cultured fish against diseases but their biological effects depend on various factors such as time, dosage, method of administration and the physiological conditions of fish.

Chromolaena odorata (L) R.M. King and H. Robinson (plate 2), common name: Siam weed a major perennial shrub which belongs to the family Asteraceae and grows up to 7 m tall [7]. The active compounds present in the leaves of *C. odorata* are in phenolic compound, flavonoid, alkaloid and steroid. Akinmoladun et al. [8] stated that *C. odorata* leaves could be developed as natural antibacterial substance. These compounds have been shown to disturb the function of cytoplasmic membrane and therefore have been described as antibacterial substances [9,10]. Pelczar and Chan [11] reported the activities of a phenolic compound present in *C. odorata* that at low concentration, phenolic compound damage cytoplasm membrane causing the leakage of important metabolite and inactivated bacterial enzymatic system. Also at high concentration, this compound can damage cytoplasm cell membrane and to precipitate the cell protein and the authors further stated that this compound interact with the component of bacterial cell wall which causes higher permeability of the bacterial cell. This compound also diffuses into the cell slowing and even stopping bacterial growth. Moreover, Mori et al. [12] added that some flavonoid substances such as robinetin, myricetin and epigallo catechin interfere with the intercalation bond or hydrogen bond at the nucleic acid assembly preventing the activity of DNA and RNA syntheses. Furthermore, steroid compound have been shown to prevent microbial growth by damaging plasma membrane such that the cell cytoplasm was leaked, thus causing cellular death [13].

Ghormade et al. [14] stated that the emergence of nutrigenomics is to develop foods and feeds that could be linked to genotypes of animals for a better production, productivity and health. Diet on its own or by interaction with other environmental factors could cause epigenetic changes that may turn certain genes on or off [15]. Harland [16] is of opinion that the main role nutrients played in governing the cell content of different proteins has been further investigated and a recognition of their role as regulators of gene transcription, nuclear RNA processing, mRNA stability and mRNA degradation (Ribonucleic Acid) has emerged. Nutrigenomics is a prominent aspect due to its great potentiality for treating chronic disease, to select animals for feed conversion efficiency, production and quality improvement of products [15]. Garg et al. [17] stated that application of genomic principles in a nutrigenomics assist in formulating a specific association between nutrients and genetic factors. Also, nutrigenomics will relate optimal diet to choose from many and different nutritional availability. Nutrigenetics on the other hand, will provide information for identifying the optimal diet for a given subject [18]. In order to define the optimal diets for an individual's; it is important to ascertain the health status at molecular stage of the individual with the consideration of metabolic and epidemiological studies. Nutri correlates diet, health and genomics in term of phenotypic effect also include different -omics such as proteomics and transcriptomics [19,20]. Different molecular and nutritional researches have shown that there are several factors including environmental that are associated with animal health.

To evaluating the interaction between diets and genes, DNA microarray techniques and quantitative real-time Polymerase Chain Reaction (PCR) and DNA sequencing are being employed [21]. These techniques enable researchers to knowing the effects of nutrient which were impossible in the past.

Molecular identification of bacteria culture isolates is a modern technique used to determine the classification and identification of bacteria using its deoxyribonucleic acid (DNA). This is determining through gene sequencing analysis. It's an approach in better understanding and identification of bacteria. Molecular Identification of the work of Ogawa et al. [22] investigated the facultative anaerobes by the 16S rRNA sequence analysis. The basic local alignment search tool, BLAST program was then used to determine homology with other organisms. Twenty-two strains were isolated and identified as *Enterococcus faecalis* or *Enterococcus* sp. from the nutrient agar and GAM agar. Hence, this study entails the assessment of the effect of *C. odorata* leaves on the growth health status of *C. gariepinus* through gene sequencing analysis of the intestinal bacteria of the fish.

MATERIALS AND METHODS

Experimental site

The nutrition aspect of the study was carried out at Aare Amoke Farms, Ifo, Ogun State, Nigeria, which is located

within the Southwestern part of Nigeria at 6°49′ 00°N and 3°12′ 00°E.

Experimental fish

A total of one hundred and eighty African mud catfish (*C. gariepinus*) fingerlings, purchased at a reputable farm was used as the test fish species in the study.

Preparation and processing of *C. odorata* (whole leaves preparation)

Fresh young leaves of *C. odorata* harvested within the premises of the Federal University of Agriculture, Abeokuta, and Ogun State, Nigeria and were authenticated in the Department of Forestry and Wildlife where a voucher specimen was maintained for the plants: *C. odorata* ID No UAHA/08.180002. The leaves were gotten by harvesting the whole plant above a 3 cm stubble height within the mid-vegetative to early flowering stage of development [23]. Thereafter, each leaf was hand-plucked from stems and placed directly into jute-bag. The leaves *C. odorata* were thoroughly rinsed with distilled water to remove dirt and weighed on an electronic scale, cut into smaller pieces with the aid of a knife. The leaves was blended with water which was added at a ratio of 1:1 (1 g of the leaves is equivalent to 1 ml of water.) in a household electric blender (Century, CB-8231-M, China), poured in a glass container and stored in the refrigerator before adding into the basal diets [24].

Experimental system

The experiment was conducted at the Earthen Pond Unit of dimensions 30 m × 5 m × 1.2 m (LBH) of Aare Amoke Farms, Ifo between February 5th-May 20th, 2018. The study was conducted in twelve (12) net-happa of dimensions 0.6 m × 1.1 m × 1.2 m (LBH) which were suspended in the pond supplied with fresh water. One hundred and eighty fingerling were randomly (completely randomized design) stocked to four (4) treatments in the net-happa at a stocking rate fifteen fish per net-happa in three replicates.

Four isonitrogenous diets were formulated at 40% crude protein to containing three varying levels of *C. odorata* leaves-paste and the control **(Table 1)**. The samples of *C. odorata* leaves paste with the three levels (0.5%, 1.0% and 1.5%) were added on top of the basal diets and thoroughly mixed with the use of a mixer.

Table 1. Feed ingredients of the experimental diets (%).

Ingredients	Control	COL1	COL2	COL3
Fishmeal	19.32	19.32	19.32	18.32
Soybean meal	26.32	26.32	26.32	26.32
Groundnut cake	26.32	26.32	26.32	26.32
Yellow Maize	21.04	20.54	20.04	20.54
Vitamin Premix	1.0	1.0	1.0	1.0
Lysine	0.5	0.5	0.5	0.5
Salt	0.5	0.5	0.5	0.5
Vegetable Oil	4.0	4.0	4.0	4.0
Methionine	0.5	0.5	0.5	0.5
DCP	0.5	0.5	0.5	0.5
C. odorata leaves paste (%)	0.0	1.0	2.0	3.0
TOTAL		100	100	100

COL: C. odorata Leaves Paste; DCP: Di Calcium Phosphate

Compounded feeds were pelletized (2 mm) using the pelletizing machine from the feed milling factory, dried to 10% moisture content with the use of a dryer and allowed to cool in an open-air. Feeds were later crushed into smaller particles based on the size of the fingerlings, packed and stored in an opaque nylon bag according to the treatments.

Experimental procedure

The fish was weighed individually at the beginning of the experiment and were acclimated to the experimental system for fourteen days before the commencement of the study and fed two times daily with a commercial diet (40% C.P.).

Fish feeding and monitoring of growth

Before commencing the experiment, fish were starved for 24 h to increase the appetite of the fish and were fed with the

diets at two feeding regimes, in the morning (08:00-09:00) and evening (17:00-18:00), *ad libitum* for 12 weeks. Fish were weighed in each net-happa weekly using a sensitive electronic weighing scale (Mettler Toledo FB602) for the growth of fish and ensuring that the fish are consuming the feeds.

Determination of intestinal bacteria of *C. gariepinus* fingerlings

After the feeding study, fish were selected from each of the treatment supplemented with *C. odorata* and the control for the molecular study. Dissection of the midline in ventral surface of the fish was carried out to remove the gut proximal section. 0.1 g of was sectioned from the fish intestine. This was conducted at the Department of Microbiology, College of Science and Technology, Covenant University, Ota, Ogun state.

The microbial counts and biochemical identification of the bacteria were carried out as follows:

a. 1 g of fish intestine was put into 1 ml of peptone water and incubated for 4 h after which serial dilution was done up to the fifth dilution.

b. 1 ml of each dilution was plated using pour plate method on Nutrient Agar and MacConkey Agar.

c. The plates were incubated for 24-48 h after which the colonies were counted using colony counter (Model KA00-74A, Vision Scientific, Japan). Colony forming unit were calculated for each count. The samples were all done in duplicates.

d. The colonies counted were put on slant for biochemical test. Biochemical test were carried out according to Chessbrough [25] while the identification was done using Bergey's manual of Bacteriology and Manual for the identification of medical Bacteria by Cowan and Steel [26].

Molecular identification of bacteria isolated from the intestine of *C. gariepinus* fingerlings using 16S rRNA gene sequencing

The molecular identification of bacteria isolated from fish intestine using 16s rRNA sequencing were carried out at the Department of Molecular Biology, College of Science and Technology, Covenant University. Five bacteria cultures isolated from the biochemical identification were put forward for the molecular characterization. The stages involved in the molecular characterization of the bacterial isolates are DNA extraction, 16S rRNA Amplification (PCR analysis) and sequencing analysis.

DNA extraction from bacterial cultures isolates

Bacteria possess plasma membrane, a rigid cell wall and an outer membrane. All these mechanical barriers have to be disrupted to release cellular component mainly DNA [27]. Ethylene diaminetetraacetic acid (EDTA) chelates the bivalent ions present in the lipid bilayer weakening the membrane. Sodium Dodecyl Sulphate (SDS) is used to disrupt all membranes followed by the treatment of Chloroform: Isoamylalcohol to denature protein and also to separate SDS and organic phases. Proteinase K is added to denature protein in cell membrane. RNase A is added to avoid RNA contamination. Chilled Isopropanol treatment causes precipitation of DNA from aqueous phase. Precipitation with 70% ethanol eliminates divalent cations. DNA obtained is suspended in TE buffer [28]. DNA from bacterial growth was extracted using the DNeasy Blood and Tissue Kit (QIAGEN; Bechman Instruments Inc., Triton). The following step by step procedures and protocols were observed:

I. 1.5 mL bacteria culture cells was transferred in 2 mL eppendorf tube aseptically and centrifuged for 5 min at 10,000 rpm. Supernatant was drained and the pellet was resuspended in 200 µl PBS (pH 7.2, 50 mM potassium phosphate, 150 mM NaCl).

II. 20 µl proteinase K was added and 200 µl Buffer AL was also added. The tube was mixed thoroughly by vortexing and incubated at 56°C for 10 min.

III. 200 µl ethanol (96-100%) was added to the sample, and mix thoroughly by vortexing again. The resulting mixture was pipetted into the DNeasy Mini spin column placed in a 2 ml collection tube, centrifuged at 8000 rpm for 1 min. The flow-through and collection tube was discarded.

IV. The DNeasy Mini spin column was placed in a new 2 ml collection tube and 500 µl Buffer AW1 was added and centrifuge for 1 min at 8000 rpm. The flow-through and collection tube was discarded.

V. The DNeasy Mini spin column was placed in a new 2 ml collection tube, and 500 µl Buffer AW2 was added and centrifuge for 3 min 14,000 rpm to dry the DNeasy membrane. Flow-through and collection tube was also discarded.

VI. The DNeasy Mini spin column was placed in a clean 1.5 ml micro centrifuge tube and 200 µl Buffer AE was pipetted directly onto the DNeasy membrane, incubated at room temperature for 1 min and then centrifuge for 1 min at 8000 rpm to elute.

VII. Concentration and purity of DNA was measured on Nanophotometer

16S rRNA amplification

One of the most attractive potential uses of 16S rRNA gene sequence informatics is to provide genus and species identification for isolates that do not fit any recognized biochemical profiles, for strains generating only a "low likelihood" or "acceptable" identification [29,30].

Polymerase Chain Reaction (PCR) was performed using the Hi-Media Taq polymerase, Hi-Media 50 mM $MgCl_2$ and Hi-Media 10x buffer and QIAGEN dNTPs (10 mM each). Universal 16S rRNA forward and reverse primer was used. PCR amplifications were performed with an Applied Biosystems Veriti Thermal cycler.

The Master Mixture is presented in **Table 2**. The PCR protocol (conditions which were used for thermal cycling) is shown in **Table 3** [31]. The PCR product is stored at 4°C till infinity.

Table 2. Composition of PCR.

Ingredients	For each reaction
PCR Buffer (10x) $MgCl_2$	2.5 µL
dNTPs (2.5 mM)	2.5 µL
Forward primer (10 pM)	0.5 µL
Reverse primer (10 pM)	0.5 µL
DNA	2.0 µL (50 ng/N)
Taq DNA polymerase (1 U/1 µL)	0.5 µL
H_2O	16.5 µL
Total volume	25.0 µL

Table 3. Thermal cycle conditions.

Stage 1 Initial Denaturation	Stage 2 (30 cycles) Denaturation, Annealing and Elongation	Stage 3 Final Elongation
95°C 5 min	94°C 52°C 72°C 0.30 s 0.30 s 1.25 s	72°C 10 min

Sequencing of partial 16S rRNA gene

Purified PCR products samples were sent to Macrogene Company, Rockville, Maryland, USA for further amplification in one direction with the 16S primers using Big Dye Terminator Ready Reaction Mix (ABI) and Sequencing of amplified product was done with the use of an ABI 3130 Genetic Analyzer version at Macrogene Company.

SEQUENCE ANALYSIS

Partial 16S rRNA gene sequence of studied bacteria was analyzed with nucleotide BLAST search in GenBank from NCBI. Phylogenetic relationship of this species was analyzed with other closely related bacterial species present in GenBank.

Evolutionary relationships of taxa and phylogenetic tree

The sequence generated a table of closely similar organism with test organism. These similar organisms were selected and there sequences were obtained in FASTA format. Once these sequences were collected from BLAST, there sequences were checked for Multiple Sequence Alignment (MSA) using T-Coffee tool from EBI (EMBL). The evolutionary history was inferred using the Neighbor-Joining method [32]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the number of differences method [33] and are in the units of the number of base differences per sequence. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 [34].

DATA ANALYSIS

Analysis of fish growth performance

Fish growth performance was determined illustrated by Agbebi et al. [35] in term of Final Individual Weight, Survival (%), Specific Growth Rate (SGR%/day). The growth parameters were calculated at the end of the experiment is:

$$\text{Percentage weight gain PWG (\%)} = \frac{Final\ mean\ body\ weight}{Initial\ mean\ body\ weight}\ x\ 100$$

$$\text{Specific growth rate, SGR} = \frac{L_nW_2 - L_nW_1}{Time\ (days\ of\ experiment)}\ x\ 100$$

where,

W_1=Initial weight gained

W_2=Final weight gained

L_n=Natural logarithm

$$\text{Survival rate} = \frac{No\ of\ fish\ remaining\ at\ the\ end\ of\ the\ experiment}{No\ of\ fish\ at\ the\ beginning\ of\ the\ experiment}\ x\ 100$$

STATISTICAL ANALYSIS

Primary data obtained from the growth and bacterial load were subjected to one way analysis of variance (ANOVA). Turkey's multiple comparisons test was used for comparison among diets means at a significant level of 0.05. Computations were subjected to GraphPad Prism version 7.04.

RESULTS

Growth performance of fish

The growth performance of *C. gariepinus* fingerlings fed *C. odorata* leaves at three varying levels of dietary supplementation is shown in **Table 4**. The highest final mean weight (1730.46 ± 87.27 g) recorded in fish fed 1.5% *C. odorata* leaves diets was significantly higher ($p<0.05$) than the lowest (711.63 ± 8.78 g) in fish fed the control diet. The weight gain (1583.46 ± 59.33) was highest in fish fed 1.5% *C. odorata* leaves diets and the lowest (526.13 ± 9.26) was observed in fish fed the control diet.

Table 4. Growth performance and nutrient utilization of *Clarias gariepinus* fingerling fed *Chromolaena odorata* leaves (mean ± SEM).

Parameters	Control 0%	COL1 0.5%	COL2 1%	COL3 1.5%
Initial Weight (g)	185.50 ± 0.50^{a}	185.65 ± 0.35^{a}	185.15 ± 0.44^{a}	185.33 ± 0.67^{a}
Final Weight (g)	711.63 ± 8.78^{d}	891.87 ± 5.63^{c}	1419.32 ± 77.71^{b}	1768.61 ± 59.74^{a}
Weight Gain (g)	526.13 ± 9.26^{d}	706.37 ± 5.20^{c}	1233.67 ± 77.62^{b}	1583.46 ± 59.33^{a}
Percentage Weight Gain (%)	283.92 ± 5.99^{d}	380.78 ± 2.00^{c}	664.48 ± 41.45^{b}	855.17 ± 31.25^{a}
Feed Conversion Ratio	1.64 ± 6.35^{a}	1.49 ± 0.01^{b}	1.23 ± 0.09^{c}	1.28 ± 0.02^{b}
Specific Growth Rate (%/day)	2.24 ± 0.02^{c}	2.62 ± 0.01^{b}	3.38 ± 0.09^{a}	3.76 ± 0.06^{a}
Survival Rate (%)	82.22 ± 2.22^{a}	72.22 ± 7.78^{a}	75.56 ± 5.88^{a}	80.00 ± 3.85^{a}

Means along the same row with same letter are not significantly different ($p>0.05$)

Intestinal microflora of the fish

Microbial count of fish: The total bacteria count and fungal counts in the intestine of fish fed *C. odorata* leaves diets and the control is presented in **Table 5**. There was a significant difference ($p<0.05$) in the total bacteria counts among fish *C. odorata* leaves paste and the control having the lowest counts.

Table 5. Microbial load in the intestine of *Clarias gariepinus* fingerlings fed *Chromolaena odorata* leaves.

Treatments	Inclusion Levels	TOTAL Bacteria Counts (CFU/g) × 10^7
Control	0%	2.50 ± 0.30^{a}
COL 1	0.5%	2.30 ± 0.50^{a}
COL 2	1%	0.95 ± 0.56^{b}
COL 3	1.5%	1.00 ± 0.80^{b}

Means along the same column with same letter are not significantly different ($p>0.05$)

Biochemical characterization of the bacteria isolate

Table 6 shows the biochemical test of the bacteria isolated from the intestine of the fish fed *C. odorata* leaves and the control.

Table 6. Biochemical test of the bacteria isolates.

GRM	COA	CAT	CIT	URE	H_2S	MOT	IND	STA	MR	VP	OX	SUSPECTED ORGANISM
+cocci	+	+	-	+	-	-	-	-	+	-	+	*Micrococcus* sp.
-rods	+	+	+	+	-	-	-	-	+	-	-	*Klebsiella* sp.
- rods	+	+	+	+	-	-	-	-	+	-	-	*Shigella* sp.
-rods	+	+	+	+	-	-	-	-	+	-	-	*Shigella* sp.
-rods	+	+	+	+	-	-	-	-	-	-	-	*Klebsiella* sp.

Key: GRM: Gram's Reaction; H_2S: Hydrogen Sulphide; CIT: Citrate Test; MOT: Motility Test; COA: Coagulase Test; IND: INDOLE TEST; CAT: CATALASE TEST; STA: STARCH HYDROLYSIS TEST; URE: Urease Test; MR: Methyl Red Test; VP: Voges Proskaur Test

MOLECULAR CHARACTERIZATION OF BACTERIA CULTURE ISOLATES

Sequencing of bacteria culture isolated from intestine of fish fed the control diet

Partial 16S gene of 536 bp was obtained after sequencing and shown below in FASTA format.

>TGCAAGTCGAACGGTGATCGCGCAGCAGCTTGCT
GCTTCGCTGACGAGTGGCGGACGGGTGAGTAATGT
CTGGGAAACTGCCTGATGGAGGGGGATAACTACTG
GAAACGGTAGCTAACTACCGCATAACAGTCGCAAG
ACCAAAGAGGGGGACCTTCGGGCCTCTCTTGCCAT
CGGATGTGCCCAGATGGGATCTAGCTAGTAGGTGG
GGTAACGGCTCACCTAGGTGCGACGATCCCTAGCT
GGTCTGAGAGGATGACCAGGCCACACTGGAACTGA
GACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGCACAATGGGCGCAAGCCTGATGCAGC
CATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTA
AAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTA
ATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGC
ACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC
GGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGT
AAAGCGCACGGATGGTGA

Sequence analysis

Total 536 bp partial 16S rRNA sequence was retrieved in FASTA format and subjected for BLAST search in GenBank. BLAST result showed that the test organism was similar to *Shigella flexneri* (strain=SIA2) with 97% similarity and E value 0.0.

Figure 1 presented the alignment of BLAST query and the sequences producing significant alignment is shown in **Figure 2**.

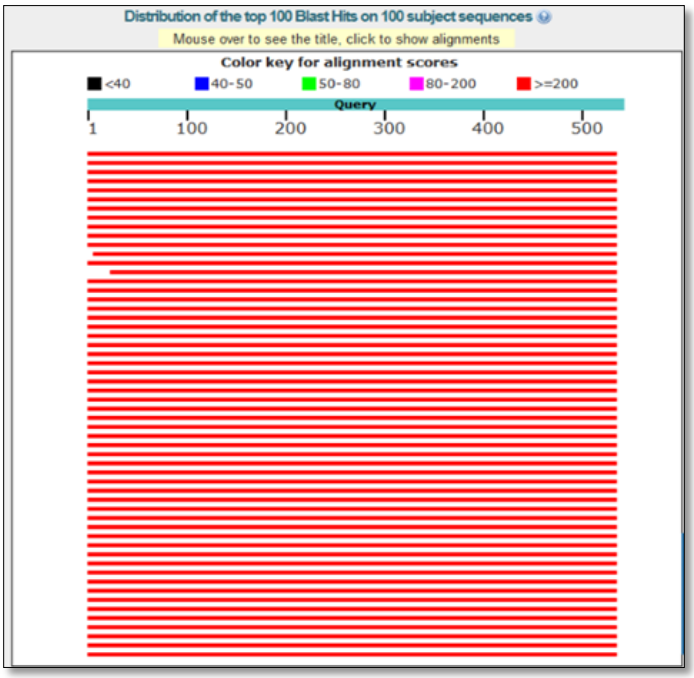

Figure 1. Alignment of BLAST query.

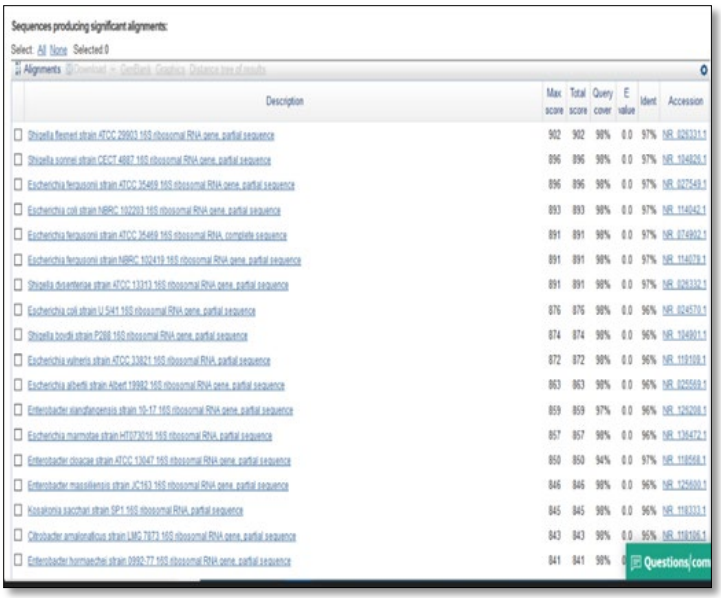

Figure 2. BLAST score, query coverage and E-value for the query sequence.

Evolutionary relationships of taxa

Phylogenetic relationship of test organism was analyzed with other partial 16S rRNA sequence of similar microorganisms. The phylogenetic tree as shown in **Figure 3** has the optimal tree with the sum of branch length=41.81250000 as shown. The analysis involved 11 nucleotide sequences; there were a total of 513 positions in the final dataset.

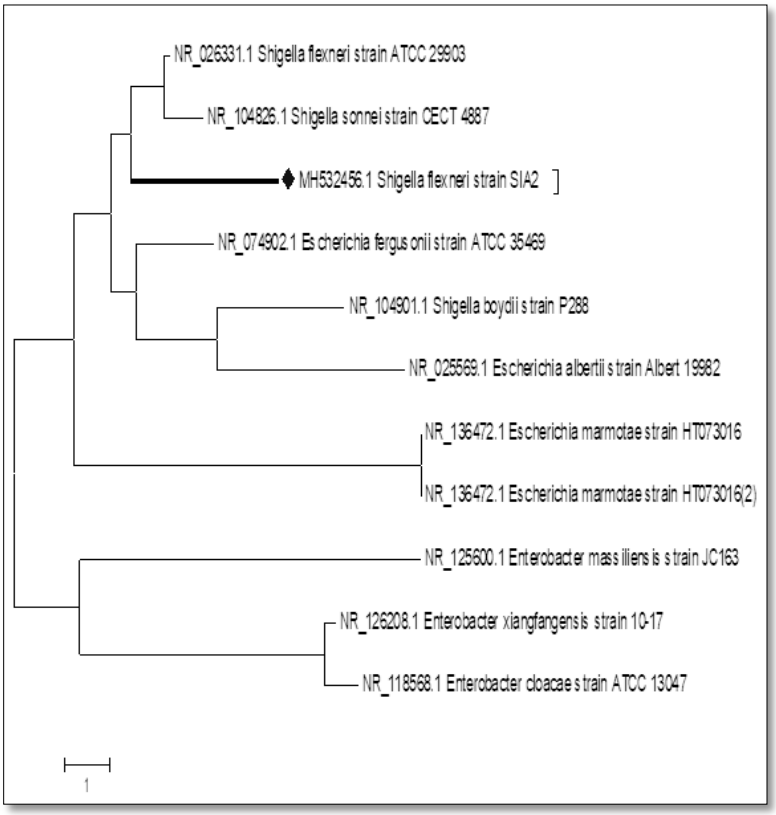

Figure 3. Similarity of the tested organism based on 16S rRNA sequence.

Sequencing of bacteria culture isolated from intestine of fish fed the 0.5% *C. odorata* leaves diet (first replicate)

Partial 16S gene of 517 bp was obtained after sequencing and shown below in FASTA format.

>ACGTCTCGACGTGCAACGCGAAGAACCTTACCTG
GTCTTGACATCCACAGAACTTTCCAGAGATGGATTG
GTGCCTCTCGGGAACTGTGAGACAGGTGCTGCATG
GCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAA
GTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCC
AGCGGTTAGGCCGGGAACTCAAAGGAGACTGCCAG
TGATAAACTGGAGGAAGGTGGGGATGACGTCAAGT
CATCATGGCCCCCTTACGACCAGGGCTACACACGT
GCTACAATGGCATATACAAAGAGAAGCGACCTCGC
GAGAGCAAGCGGACCTCATAGAAGTATGTCGTAGT
CCGGATTGGAGTCTGCAACTCGACTCCATGAAGTC
GGAATCGCTAGTAATCGTAGATCAGAATGCTACGG
TGAATATCGTTCCCGGGCCTTGTACACACCGCCCGT
CACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTA
GCTTAACCTTCGGGAGGGCYTTAGCCT

Sequence analysis

Total 517 bp partial 16S rRNA sequence was retrieved in FASTA format and subjected for BLAST search in GenBank. BLAST result showed that the test organism was similar to *Klebsiella pneumonia* (strain=SIF2) with 99% similarity and E value 0.0.

Figure 4 presented the alignment of BLAST query and the sequences producing significant alignment is shown in **Figure 5**.

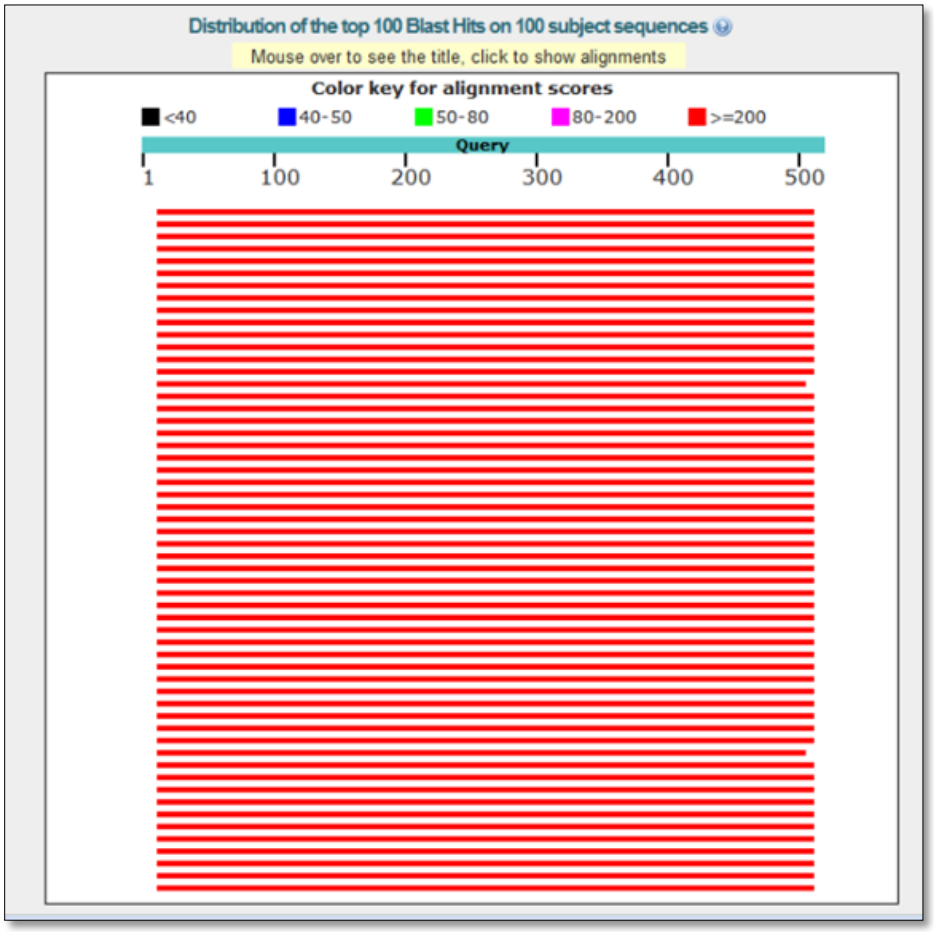

Figure 4: Alignment of BLAST query.

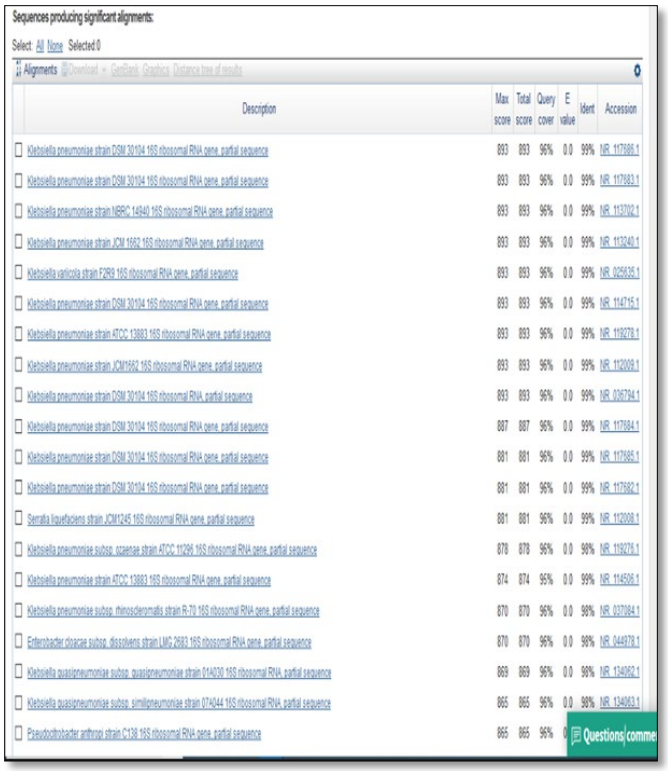

Figure 5. BLAST score, query coverage and E-value for the query sequence.

Evolutionary relationships of taxa

Phylogenetic relationship of test organism was analyzed with other partial 16S rRNA sequence of similar microorganisms. The phylogenetic tree as shown in **Figure 6** has the optimal tree with the sum of branch length=2053.55468750 as shown. The analysis involved 17 nucleotide sequences; there were a total of 468 positions in the final dataset.

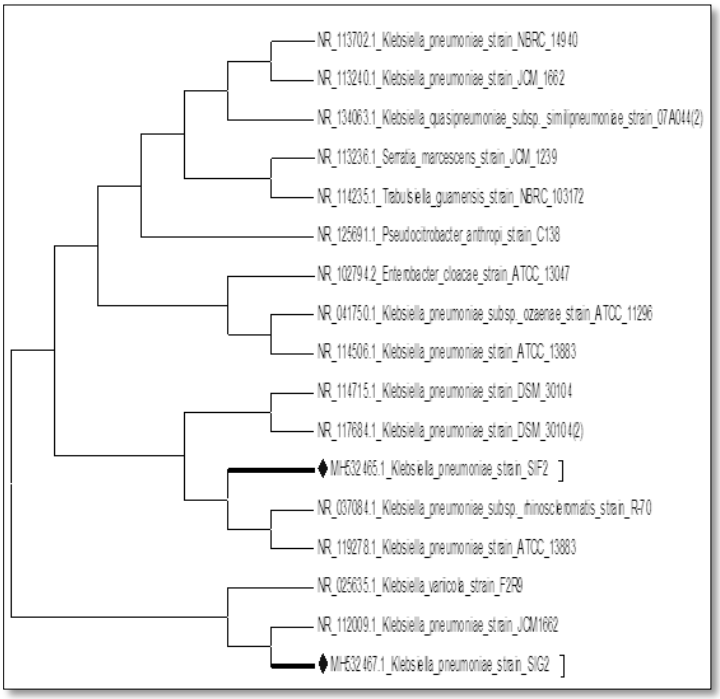

Figure 6. Similarity of the tested organism based on 16S rRNA sequence.

Sequencing of bacteria culture isolated from intestine of fish fed the 0.5% *C. odorata* leaves diet (second replicate)

Partial 16S gene of 401 bp was obtained after sequencing and shown below in FASTA format.

>TCGGGAACTGTAAGACAGGTGCTCCATGGCGGTC
TTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCG
CAACGAGCGCAACCTTTTTCCTTTTTTCCCAGCGGT
TAGGCCGGGAACTCAAAGGAGACTGCCAGTGATAA
ACTGGAGGAAGGTGGGGATGACGTCAAGTCATCAT
GGCCCTTACGACCAGGGCTACACACGTGCTACAAT
GGCAGATACAAAGAGAAGCGACCTCGCGAGAGCA
AGCGGACCTCATAAAGTATGTCGTAGTCCGGATTG
GAGTCTGCAACTCGACTCCATGAAGTCGGAATCGC
TAGTAATCGTAGATCAGAATGCTACGGTGAATACG
TTCCCGGGCCTTGTACACACCGCCCGTCACACCATG
GGAGTGGGTTGCAA

Sequence analysis

Total 401 bp partial 16S rRNA sequence was retrieved in FASTA format and subjected for BLAST search in GenBank. BLAST result showed that the test organism was similar to *Klebsiella pneumoniae* (strain=SIG2) with 98% similarity and E value 0.0.

Figure 7 presented the alignment of BLAST query and the sequences producing significant alignment is shown in **Figure 8**. The phylogenetic tree is shown in **Figure 6**.

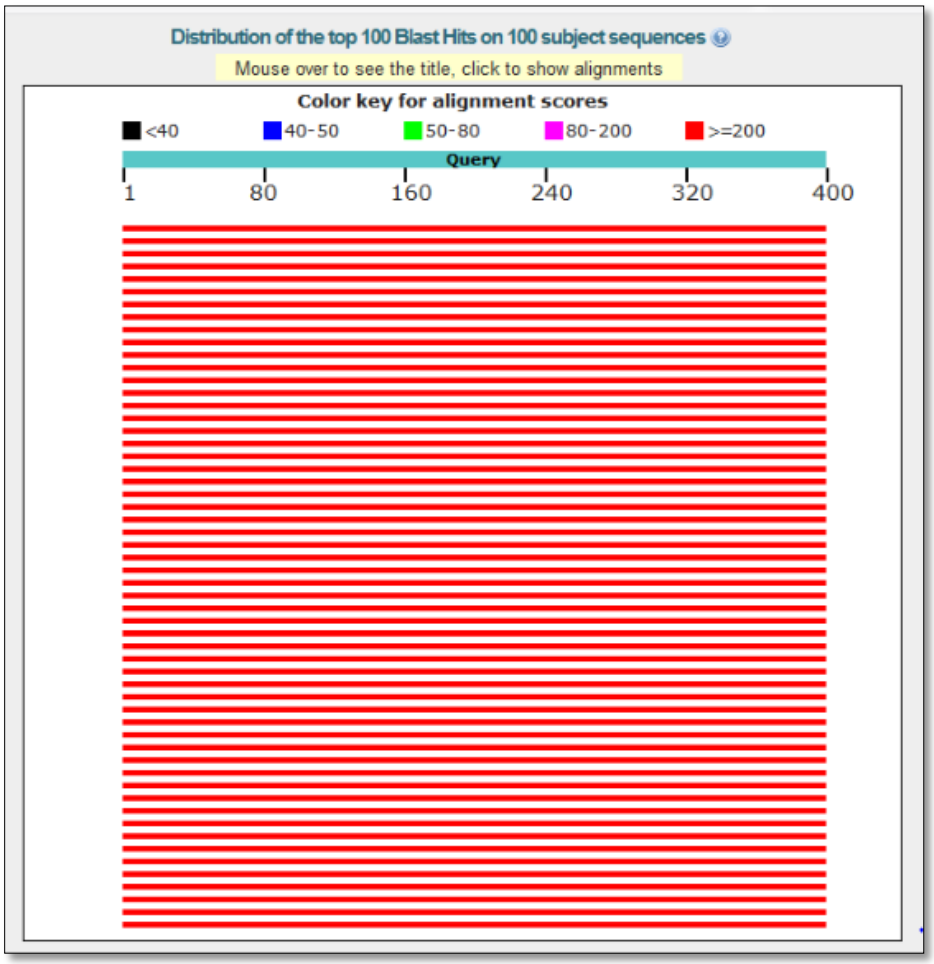

Figure 7. Alignment of BLAST query.

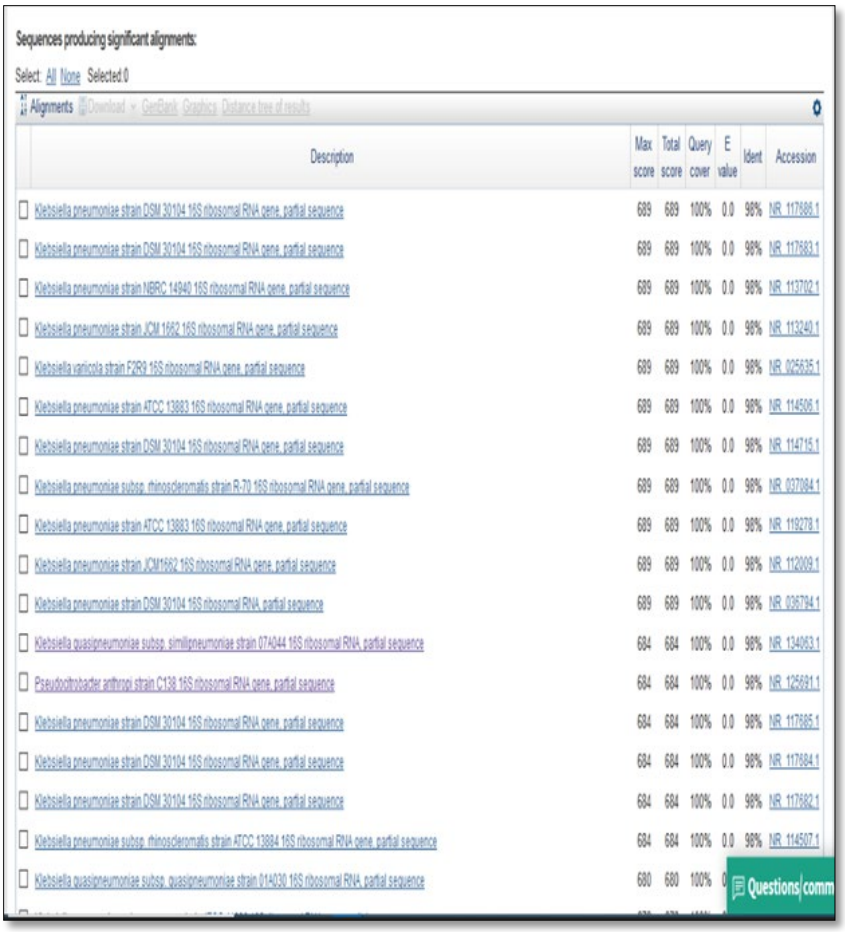

Figure 8. BLAST score, query coverage and E-value for the query sequence.

Sequencing of bacteria culture isolated from intestine of fish fed the 1% *C. odorata* leaves diet

Partial 16S gene of 488 bp was obtained after sequencing and shown below in FASTA format.

AGCGGTAGCACAGAGAGCTTGCTCTCGGGTGACGA
GCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCC
TGATGGAGGGGGATAACTACTGGAAACGGTAGCTA
ATACCGCATAACGTCGCCACAAGACCGCGAAAGTG
GGGGACCTTCGGGCCTCATAGCCATCAGATGTGCC
CAGATGTGGGATTAGCTAGTAGGTGGGGTAACGGC
TCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGG
ATGACCAGCCACACTGGAACTGAGACACGGTCCAG
ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCAC
AATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGT
GTGAAGAAGGCCTTCGGGTTGTGCAAAGCACTTTC
AGACGGGGAGGAAGGCGATAAGGTTAATAACCTCG
TCGATTGACGTTACCCGCAGAAGAAGCACCGGCTA
ACTCCGTGCCAGCAGCCGCTGGTAATACTTGGA

Sequence analysis

Total 488 bp partial 16S rRNA sequence was retrieved in FASTA format and subjected for BLAST search in GenBank. BLAST result showed that the test organism was similar to *Klebsiella pneumoniae* (strain=SIH2) with 97% similarity and E value 0.0.

Figure 9 presented the alignment of BLAST query and the sequences producing significant alignment is shown in **Figure 10**.

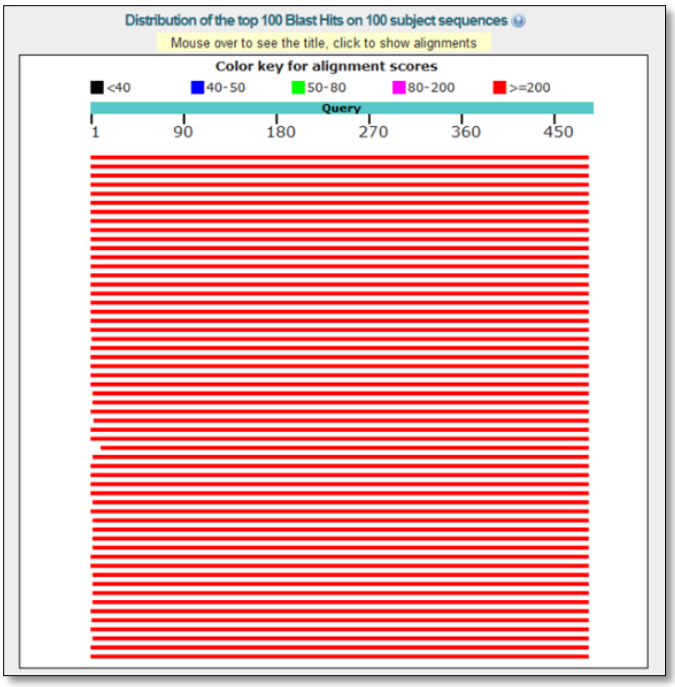

Figure 9. Alignment of BLAST query.

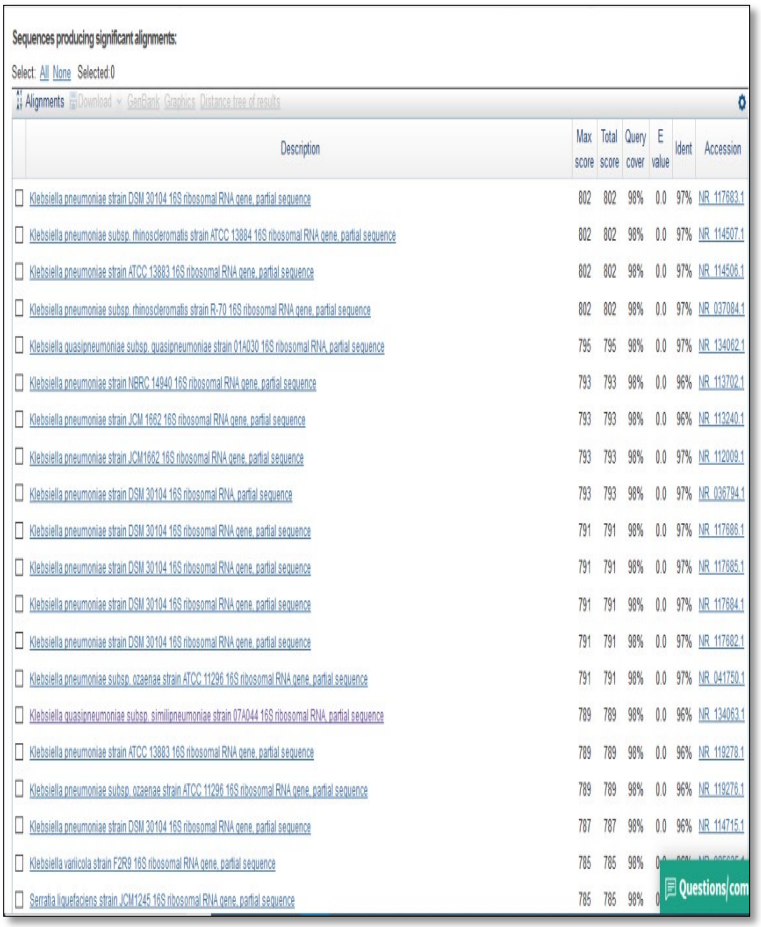

Figure 10. BLAST score, query coverage and E-value for the query sequence.

Evolutionary relationships of taxa

Phylogenetic relationship of test organism was analyzed with other partial 16S rRNA sequence of similar microorganisms. The phylogenetic tree as shown in **Figure 11** has the optimal tree with the sum of branch length=283.94531250 as shown. The analysis involved 20 nucleotide sequences; there were a total of 401 positions in the final dataset.

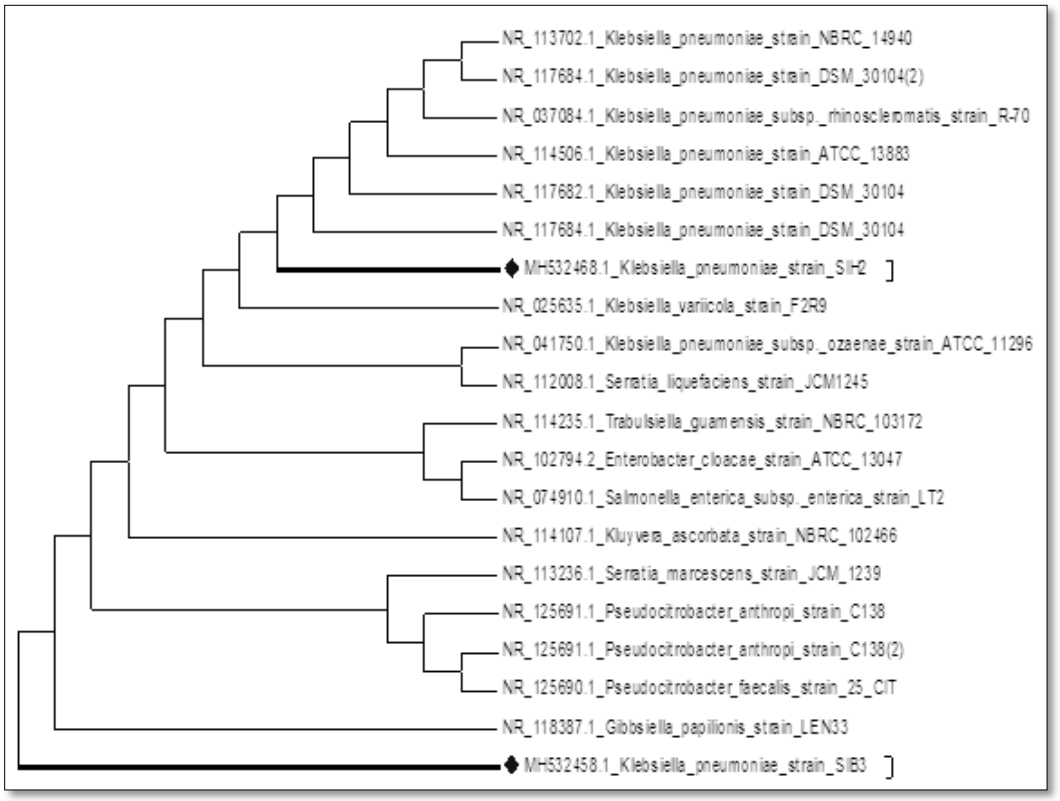

Figure 11. Similarity of the tested organism based on 16S rRNA sequence.

Sequencing of bacteria culture isolated from intestine of fish fed the 1.5% *C. odorata* leaves diet

Partial 16S gene of 579 bp was obtained after sequencing and shown below in FASTA format.

>GAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGAC
GAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTG
CCTGATGGAGGGGGATAACTACTGGAAACGGTAGC
TAATACCGCATAATGTCGCAAGACCAAAGTGGGGG
ACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATG
GGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAG
GCGACGATCCCTAGCTGGTCTGAGAGGATGACCAG
CCACACTGGAACTGAGACACGGTCCAGACTCCTAC
GGGAGGCAGCAGTGGGGAATATTGCACAATGGGCG
CAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAA
GGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGG
AAGGCGTTGAGGTTAATAACCTTGGCGATTGACGT
TACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCA
GCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAAT
CGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTC
TGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCT
GGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTT
GTAGA

Sequence analysis

Total 579 bp partial 16S rRNA sequence was retrieved in FASTA format and subjected for BLAST search in GenBank. BLAST result showed that the test organism was similar to *Klebsiella pneumoniae* (strain=SIA3) with 99% similarity and E value 0.0.

Figure 12 presented the alignment of BLAST query and the sequences producing significant alignment is shown in **Figure 13**.

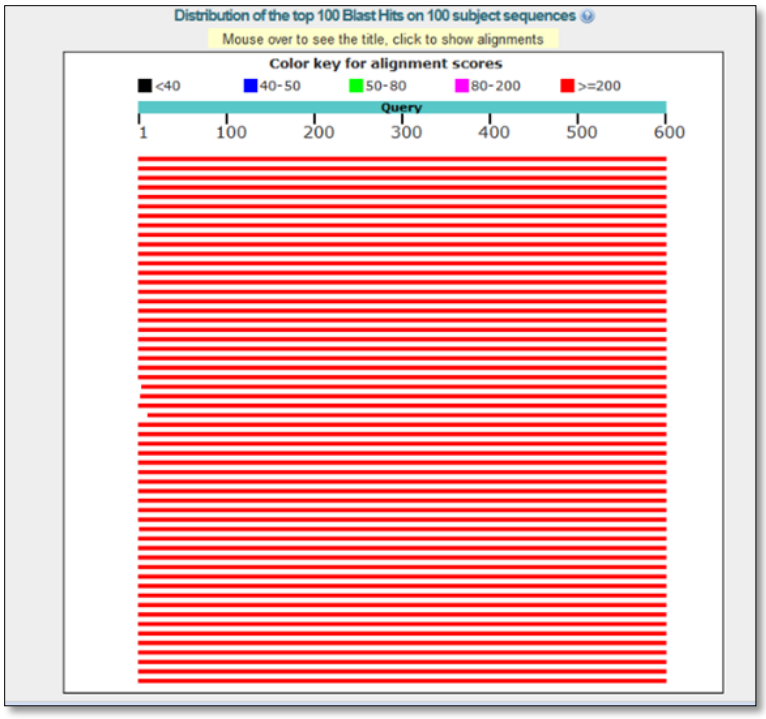

Figure 12. Alignment of BLAST query.

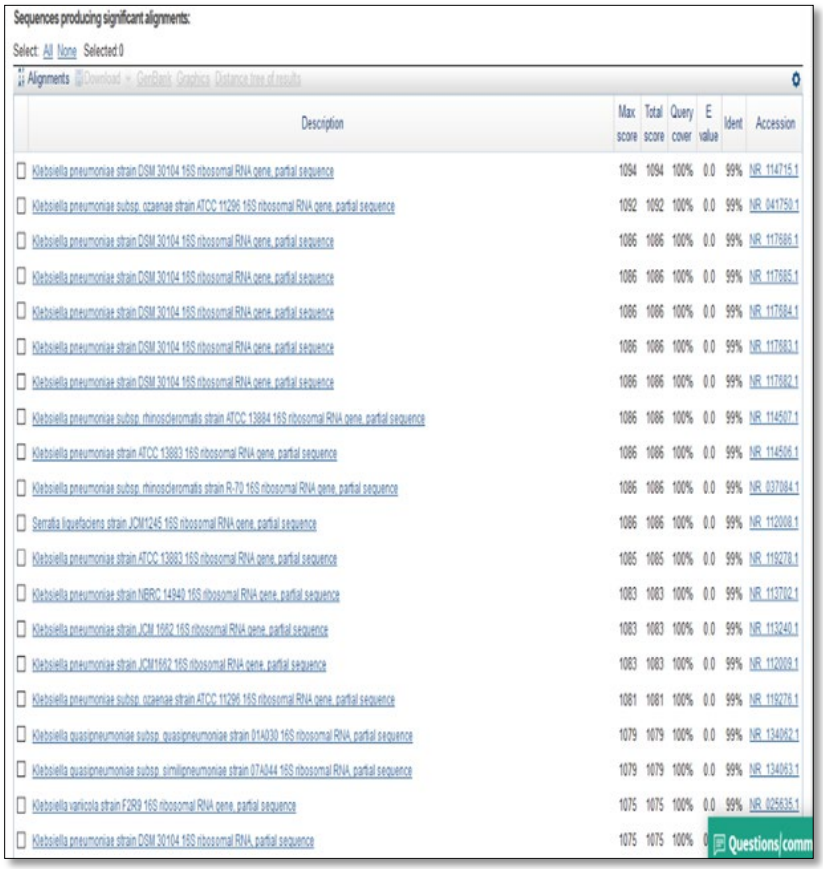

Figure 13. BLAST score, query coverage and E-value for the query sequence.

Evolutionary relationships of taxa

Phylogenetic relationship of test organism was analyzed with other partial 16S rRNA sequence of similar microorganisms. The phylogenetic tree as shown in **Figure 14** has the optimal tree with the sum of branch length=59.31250000 as shown. The analysis involved 19 nucleotide sequences; there were a total of 588 positions in the final dataset.

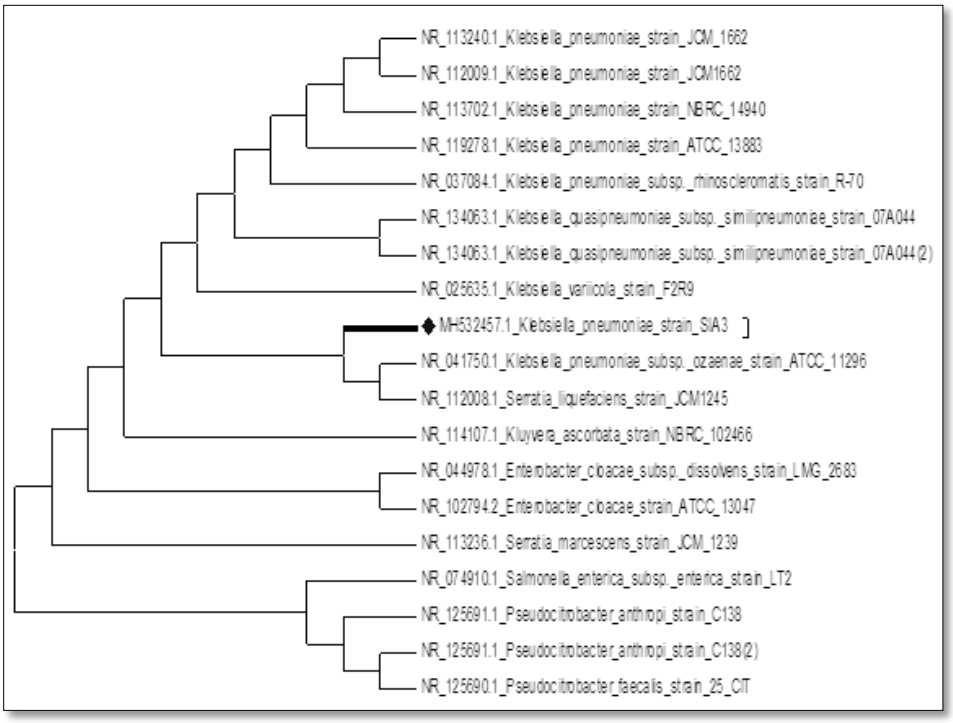

Figure 14. Similarity of the tested organism based on 16S rRNA sequence.

The summary of bacterial species identified is shown in **Table 7**.

Table 7. Bacterial specie identified based on 16S rRNA gene and its acquired GeneBank assession number.

Number	Treatment	Organism	GeneBank Accession Number
SUB4200101	SeqA2 (Control)	*Shigella flexneri*	MH532456
SUB4200101	SeqA3 (COL3)	*Proteus mirabilis*	MH532457
SUB4200101	SeqF2 (COL1)	*Klebsiella pneumoniae*	MH532465
SUB4200101	SeqG2 (COL1(2)	*Klebsiella pneumonia*	MH532467
SUB4200101	SeqH2 (COL2)	*Klebsiella pneumoniae*	MH532468

DISCUSSION

This study has proven that molecular identification is a promising and better approach in identification of microorganism through their DNA. The biochemical identification revealed the presence of Micrococcus sp. in the fish intestine fed control diet while the molecular method revealed *S. flexneri*. Similar variation was observed in fish intestine fed 1% *C. odorata* leaves as biochemical identification revealed *Shigella* sp. while molecular revealed *K. pneumonia*.

Information in this present study has revealed the antibacterial potential of supplementing the leaves of *C. odorata* as it inhibited the growth of some enterobacteriaceae (*K. pneumonia* and *Proteus mirabilis*) found in the intestine of *C. gariepinus*. The report of Vital and Rivera [36] corroborated this present study in that ethanolic extract of *C. odorata* leaves showed antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus* and *S. typhimurium* through inhibiting the growth of these

bacteria when challenged with *C. odorata* leaves extract and the author suggested that the ability of the leaves to inhibit growth of bacteria could be due to presence of flavonoid and tannin found in the phytochemical analysis of the leaves.

The DNA sequencing analysis of the bacterial isolates of fish supplemented with *C. odorata* leaves showed the presence of disease causing enterobacteriaceae but their effects are minimized as indicated in the total bacteria counts of the fish intestine. This could infer that *C. odorata* leaves inhibited the growth of these microorganisms and thus promoting the growth and health of the fish fed the leaves. The highest weight gain, specific growth rate and survival rate and lowest feed conversion ratio were recorded in fish fed the highest inclusion level of *C. odorata* leaves. This indicated that nutrient were better utilized in diet supplemented with *C. odorata* leaves and consequently the dietary inclusion of *C. odorata* leaves inhibited the growth of disease causing pathogen as seen in the gene sequencing analysis. This remarkable achievement is due to the presence of bioactive compounds imbedded in the leaves of *C. odorata*. Several studies have proven that flavonoid, steroid and phenolic compounds are linked to the growth promoting effect and antibacterial potential of the leaves [11-13].

CONCLUSION

This present study has revealed the effect of supplemented diets on the intestinal bacteria of cultured fish based on the results obtained from gene sequence analysis, which is a promising area in fish nutrigenomics and nutrigenetics. *C. odorata* leaves, most especially 1.5% inclusion level have proven to have a significant effect in that it promotes the growth of cultured *C. gariepinus*, reduce the microbial load of the fish and subsequently inhibiting the growth of enterobactriaceae found in the fish intestine through the gene sequencing.

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