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# The Emergence of Tetracycline Resistance in Rumen Bacteria

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**Summary**

**The emergence of antibiotic-resistant bacteria is a major concern for human health because current antibiotics have become ineffective in treating diseases caused by the antibiotic-resistant pathogenic bacteria. The objective of this study was to test the hypothesis that exposing rumen bacteria to tetracycline will gradually lead to the development of tetracycline-resistant bacteria, some of which will become multidrug resistant bacteria. To achieve this objective, rumen fluid containing bacteria were cultured on agar plates containing tetracycline to select for tetracycline-resistant bacteria, which were then cultured on Tryptic Soy Broth (TSB) agar containing chloramphenicol, ampicillin, and kanamycin antibiotics. The results showed that, of the ten tetracycline-resistant bacteria that were previously isolated as resistant to tetracycline at 12 µg/mL, eight isolates were still able to survive in TSB agar supplemented with 25 µg/mL tetracycline. All but one bacterial isolate also grew on TSB agars supplemented with chloramphenicol, ampicillin, and kanamycin. PCR results of 16S rRNA suggest that isolates consisted of the *Sphingobacterium, Stenotrophomonas, and Microbacterium* genuses. These results are significant because they show that some rumen bacteria are capable of developing resistance to tetracycline and may subsequently cause untreatable diseases in humans.**

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## Introduction

The increasing antibiotic resistance of pathogenic bacteria poses a monumental problem for human and animal health. When a resistant bacterium causes a deadly disease in humans or animals, cures will be limited because the original antibiotic treatments no longer work (1). Increasingly, we see bacteria becoming resistant to many antibiotics (2). These bacteria are called "super bugs" or "multidrug resistant bacteria," one of the worst being the methicillin-resistant *Staphylococcus aureus* known by its acronym, MRSA. Feeding antibiotics to animals has been shown to improve animal growth (3). Although the Food and Drug Administration (FDA) has implemented a voluntary approach to stop the use of antibiotics for "production purposes," growth-enhancing antibiotics are still used extensively in animal agriculture (4). This practice results in bacteria in the animal's gastrointestinal tract becoming resistant to the antibiotics in the feed. This can become a significant problem if some of these resistant bacteria are pathogenic to humans and cause infections via contaminated food products derived from the livestock.

Antibiotic resistance in bacteria has been known for many years. The recent emergence of bacteria resistant to widely used antibiotics, such as tetracycline, has raised major concerns for the treatment of life threatening diseases in humans. Tetracycline is an aminoglycoside antibiotic that inhibits translation in the bacterial cell. Plasmids harboring genes encoding resistance against tetracycline enable bacteria to either pump the antibiotic out of the cell or promote ribosomal protection. The introduction of tetracycline can lead to an increase of tetracycline-resistant bacteria via the following mechanisms: 1) the bacteria can modify the antibiotic-binding target site to no longer bind the antibiotic, 2) the bacteria can acquire resistance gene encoding for enzymes that can modify the antibiotic by cleaving off essential parts of its structure, 3) the bacteria can develop an efflux pump system to expel the antibiotics from within the cell (5).

Resistance against antibiotics is a matter of survival for bacteria. When bacteria are exposed to antibiotics, they undergo mutations in their genes, some of which result in one or more of the three molecular mechanisms of resistance to antibiotics. When the same bacterium develops resistance against other antibiotics, the bacterium becomes multidrug resistant. Such bacteria, known as "superbugs," are even more dangerous because infectious diseases caused by these bacteria

may now not be treatable by many antibiotics. If the genes responsible for the multidrug resistance are transferred to other bacteria in their ecosystems, then the superbugs multiply rapidly as more pathogenic bacteria become superbugs.

The rumen microbiota consists of multiple microorganisms including bacteria, fungi, parasites, and protozoa. There are millions of bacteria, both Gram-positive and Gram-negative, in the rumen. These bacteria digest the feed and are sources of proteins and fatty acids, and thus play vital roles in animal development as well as health (6). Exposure to even low levels of antibiotics is known to induce mutations in bacteria that lead to antibiotic resistance. While feeding antibiotics to cattle can help with the growth of certain bacteria that assist in digestion, such practices are also known to cause changes in bacterial DNA that result in resistance to the antibiotics (7).

There is an urgent need to study antibiotic resistance in bacteria. Many lifesaving antibiotics are becoming ineffective for combating pathogenic bacteria because of the emergence of strains resistant to antibiotics (8). The objective of this study was to test the hypothesis that exposing rumen bacteria to tetracycline will gradually lead to the development of tetracycline-resistant bacteria, some of which will become multidrug-resistant bacteria. Experiments were performed to characterize the bacteria in terms of drug resistance, morphology, and genus. The rationale for this study was to generate new knowledge of antibiotic-resistant bacteria and multidrug resistance to advance the fundamental science of antibiotics, bacteria, and gene-environment interactions.

The goal of this study was to test the hypothesis that exposing rumen bacteria to tetracycline will gradually lead to the development of tetracycline-resistant bacteria, some of which will become multidrug resistant bacteria. Our approaches were to expose rumen bacteria first to tetracycline and then to chloramphenicol, ampicillin, and kanamycin followed by Gram Staining and PCR. Our results showed that there were ten bacteria resistant tetracycline at 12 µg/mL of which eight of them were able to grow at 25 µg/mL concentration of tetracycline. While seven of the isolates were Gram-negative, bacteria from only one isolate were Gram-positive. All of the Gram-negative bacteria were able to grow on agar containing chloramphenicol, ampicillin, and kanamycin. The PCR helped us identify the isolates as *Sphingobacterium*, *Stenotrophomonas*, and *Microbacterium* genuses. The findings are significant because they can be used to develop science-based solutions for animal production that affect human health. Such information provides justification for concern and awareness in the use of antibiotics in animal feed.

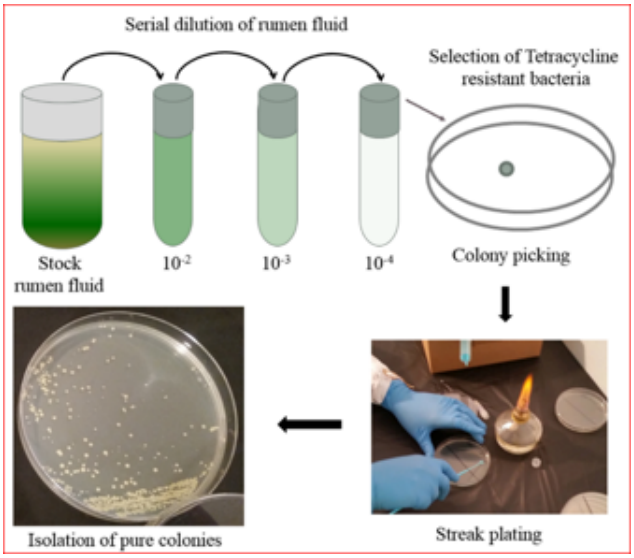


**Figure 1. Isolation of pure bacterial colonies from rumen fluid resistant to tetracycline.**

## Results

### Colony characterization and Gram staining

The bacteria isolated from the rumen were grown for four days with antibiotics, allowing time for them to gain resistance. For the second round of growth on tetracycline plates (25 µg/mL), bacteria were cultured for four days of growth. Following the antibiotic selection, surprisingly there were only eight bacterial colonies resistant to tetracycline. Pure bacterial colonies resistant to tetracycline were isolated by culturing diluted rumen fluid on agar plates containing tetracycline and streak plating (**Figure 1**).

Morphology and cell wall structures are important for characterizing bacteria. There were similarities among the morphologies of the colonies of bacterial isolates. Isolates #1, 2, 3, and 7 formed small gray-white colonies. Isolate #4 displayed large white colonies. Isolates #5, 6, and 8 formed large yellow colonies. Interestingly, all of the bacterial isolates were bacilli. According to the results from Gram staining, bacteria from Isolates #1–7 were Gram-negative and bacteria from Isolate #8 were

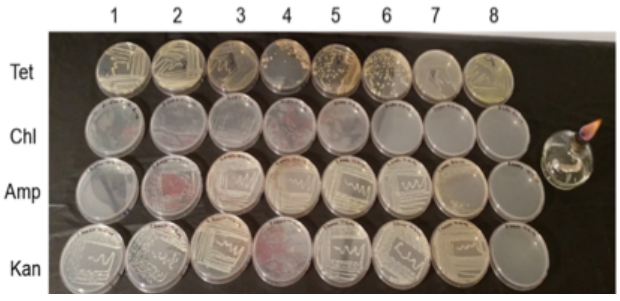


**Figure 2. Multidrug resistance of the bacterial isolates on day 3 of growth.** Lanes 1–8 are bacterial isolates cultured on TSB agar plates containing tetracycline (Tet), chloramphenicol (Chl), ampicillin (Amp), and kanamycin (Kan).

| Bacterial isolates | Gram negative or positive | Shape |
|---|---|---|
| 1 | Negative | Bacillus |
| 2 | Negative | Bacillus |
| 3 | Negative | Bacillus |
| 4 | Negative | Bacillus |
| 5 | Negative | Bacillus |
| 6 | Negative | Bacillus |
| 7 | Negative | Bacillus |
| 8 | Positive | Bacillus |

**Table 1.** Morphological and cell wall characteristics of the bacterial isolates.

Gram-positive (**Table 1**).

### Determining multidrug resistance

To determine if the tetracycline-resistant bacteria were also resistant to other commonly used antibiotics, bacteria from each of the isolates were cultured on Tryptic Soy Broth (TSB) agar supplemented with chloramphenicol (20 µg/mL), ampicillin (100 µg/mL) or kanamycin (100 µg/mL) for four days. Remarkably, all of the bacterial isolates except Isolate #8 grew on TSB agars supplemented with either chloramphenicol, ampicillin, or kanamycin. Isolate #8 grew on tetracycline only (**Figure 2**).

### Identification of tetracycline-resistant bacteria by genotyping

Visualization of the genomic DNA on a 1% agar gel showed that the DNA samples were pure and not degraded (**Figure 3**). The PCR amplifications of the target 16S ribosomal RNA genes were specific and gave a distinct DNA band on the agarose gel (**Figure 4**). The PCR products were sequenced and then, using BLAST, matched to sequences in the NCBI database. Sequencing and bioinformatic analyses of the PCR products of the 16S ribosomal RNA genes showed that the bacterial isolates consisted of *Sphingobacterium* (Isolates #1, 2, 5, and 6), *Stenotrophomonas* (Isolates #3, 4, and 7), and *Microbacterium* (Isolate #8) species

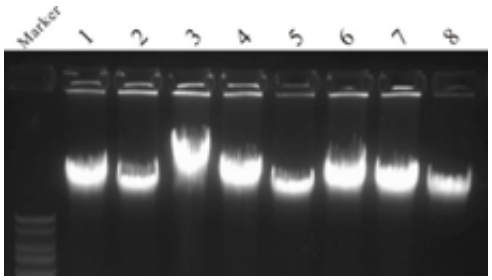


**Figure 3. Genomic DNA of bacterial isolates #1–8 in lanes 1–8.** The Marker lane contains a 1-kb DNA Ladder (Promega Corp. Madison, WI).

| Bacterial isolates | Species |
|---|---|
| 1 | *Sphingobacterium* |
| 2 | *Sphingobacterium* |
| 3 | *Stenotrophomonas* |
| 4 | *Stenotrophomonas* |
| 5 | *Sphingobacterium* |
| 6 | *Sphingobacterium* |
| 7 | *Stenotrophomonas* |
| 8 | *Microbacterium* |

**Table 2.** Identification of bacteria using genetic testing. The bacteria were identified through analysis of 16S rRNA gene sequences using PCR and BLAST approaches.

(**Table 2 and Figure 5**).

## Discussion

Antibiotics are agents that are used for the treatment of bacterial infections and diseases. Most of the widely used antibiotics in modern medicine were discovered decades ago, so bacteria around the world have gradually grown resistant to these precious antibiotics since then. There is a lack of fundamental knowledge about the mechanisms by which antibiotic resistance develops in bacteria and the major negative impacts of resistant bacteria on human health, leading to a dearth of practical solutions derived from original research. The purpose of this study was to test the hypothesis that exposing rumen bacteria to tetracycline will gradually lead to the development of tetracycline-resistant bacteria, some of which will become multidrug-resistant bacteria. The experiments characterized the bacteria in terms of multidrug resistance, morphology, and genus. This was an *in vitro* study simulating the *in vivo* conditions in which cattle are fed antibiotics to promote their growth and development.

In this study, when the rumen bacteria were cultured on agar-containing tetracycline disks, only ten bacterial colonies appeared, and bacteria from two of these colonies did not grow on agar containing higher

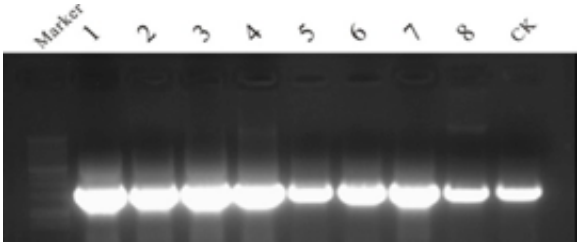


**Figure 4. PCR-amplified 16S rRNA genes of bacterial Isolates #1–8 in lanes 1–8.** *Pseudomonas* fluorescent XW10 was used as positive control, and the corresponding size of 16S rRNA was about 1.5 kb. The 1-kb DNA ladder was used as a marker (Promega, Madison, USA). The "CK" stands for Genomic DNA of *Burkholderia pyrrocinia* strain Lyc2 and was used as a positive control (23).

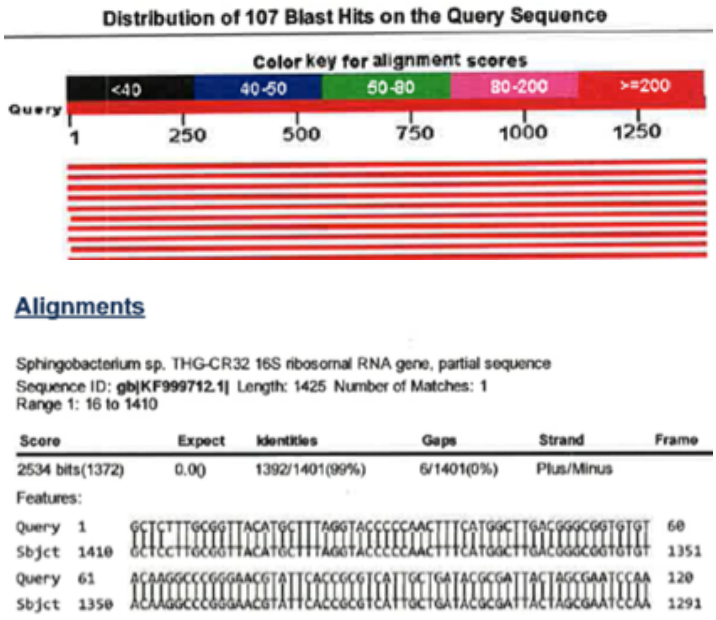


**Figure 5. Representative result of the genetic test.** The bacterial isolates were identified using PCR of 16S rRNA genes, followed by sequencing of the PCR products and bioinformatics database search to determine the matching DNA sequences of known bacteria. The upper panel shows the overall match between the 16S rRNA gene sequences of the bacterial Isolate #1 and the matches in the NCBI's BLAST database. The lower panel shows the base-pair matches between the 16S rRNA gene sequences of the Isolate #1 and the sequences in the database.

The data were generated using the nucleotide comparison tool of the Basic Local Alignment Search Tool (BLAST), which is available at The National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The score is a number that indicates how "well" the sequences correlate with others in the database. "Expect value" is the number of hits one can expect to see according to the size of the sequence. "Identities value" is the number of similar sequences that could be found in the database. "Gaps" refer to the percentage of the sequence that was different from what could be found in the database. "Strand" indicates the direction of the sequence. "Frame" describes which section of the sequence is specifically being observed.

concentrations of tetracycline. This suggests that most of the rumen bacteria are sensitive to tetracycline. Additionally, anaerobic bacteria were not detected, likely because we cultured the bacteria under aerobic conditions. All of the bacterial isolates except Isolate #8 grew on TSB agar plates supplemented with chloramphenicol, ampicillin, or kanamycin. Isolate #8 grew on tetracycline only. These results indicate that all of the bacterial isolates except Isolate #8 had developed multidrug resistance during the experiment, which makes them dangerous for human health because these antibiotics are a commonly prescribed lifeline for many patients. Tetracycline and kanamycin are used to treat pneumonia (9). *Listeria* infections are treated with ampicillin (10). Chloramphenicol is used to control colistin-resistant *Enterobacter cloacae* (11).

The sequence analysis indicated that the isolates consisted of bacteria from the *Sphingobacterium* (#1, 2, 5, and 6), *Stenotrophomonas* (#3, 4, and 7), and *Microbacterium* (#8) genuses. PCR results from the DNA of the 16S ribosomal RNA were clear bands indicating that bacteria from each isolate contained DNA from only one bacterial species (**Figure 3**). Sequence matches between the 16S ribosomal RNA genes and the known sequences in NCBI's BLAST database were 100% similar. These are indicators of pure bacterial isolation and culture. Among the diverse rumen bacteria, many have been shown to be resistant to multiple drugs. Actual resistance or susceptibility to the antibiotics avoparcin, narasin, salinomycin, thiopeptin, tylosin, virginiamycin, and two new ionophore antibiotics has been demonstrated by Nagaraja and Taylor (12).

The findings of our study have significant implications because all of the identified bacteria have pathogenic species. For example, nosocomial outbreaks caused by various *Microbacterium* species have occurred among cancer patients (13). In the same genus, species such as *M. lacticum* can be found in common locations such as home showerheads (14). Any species in the *Microbacterium* genus that is present in livestock can infect humans through contact with an animal's manure or bodily fluid. Previously described as *Flavobacterium* species, some species of the *Sphingobacteria* genus, such as the Gram-negative and bacillus-shaped bacteria, are accountable for infections (15). For example, *S. multivorum* and *S. spiritivorum* are pathogenic to humans, causing peritonitis and rashes, respectively (16). The *Stenotrophomonas* genus includes bacterial species that are present in diverse environments like soil and residential showerheads (14). Resistant to most antibiotics, *S. maltophilia* can contaminate tracheal tubes and urinary catheters, causing nosocomial infections that are very difficult to treat (17-19).

It is possible that the genetic determinants of antibiotic resistance in these cited bacteria can be transferred to other rumen bacteria in the gut, thereby increasing the risks of infection and disease to both animals and humans. Continued applications of antimicrobials to healthy animals initiate dangerous mutations of gut bacteria and pose a major health threat. There seems to be a race between pathogenic bacteria and humans to find ways to "beat" one another. For the bacteria, the race is to develop sufficient mutations quickly enough and before humans can develop another antibiotic to thwart them. As the world human population increases, there is an urgent need to produce corresponding increase of

food animals to feed humans (20).

Multidrug resistance in antibiotic-resistant bacteria is an extremely important problem to address. In this case, we tested the resistance against chloramphenicol, ampicillin, and kanamycin antibiotics, all of which are broad-spectrum antibiotics that are among those most commonly used. In addition, deadly pathogenic bacteria, such as species of *Salmonella,* have been shown to be resistant to tetracycline, chloramphenicol, and ampicillin decades ago by Manten *et al.* (21). Also, bacteria develop resistance against broad range of antibiotics by either acquiring resistance genes to multiple antibiotics or increasing multidrug efflux pumps to pump out diverse antibiotics from the cell (22). While ampicillin is produced by a *Penicillium* fungus species, the others (tetracycline, chloramphenicol, and kanamycin) are produced by species of *Streptomyce*s and target protein synthesis and cell walls in the bacteria, respectively. Thus, the presence of multidrug resistance in the tetracycline-resistant bacteria becomes alarming since some of these bacteria are pathogenic and even a combination of all the tested antibiotics could not cure disease should these bacteria cause infectious disease in humans. It is likely that at least some of these strains are naturally resistant to antibiotics.

The results of this study illuminate the development of bacterial resistance to antibiotics that occurs when rumen bacteria are exposed to the antibiotics so frequently added to animal feed. Considering the fact that there are hundreds of billions of microorganisms, including bacteria, in a cow's rumen fluid (6), the current study has demonstrated that a subset of bacteria are resistant to tetracycline, ampicillin, chloramphenicol, and kanamycin. A more comprehensive study on the effects of any of the other commonly used antibiotics on global microorganisms in the rumen could dramatically extend the knowledge base of this topic.

Our results demonstrate that rumen bacteria do become resistant to tetracycline, a widely used antibiotic for treating diseases in both humans and animals. This new knowledge can be applied to prevent bacteria from becoming resistant to tetracycline. For example, animal producers may have to stop using tetracycline in animal feed. Alternatively, antibiotics fed to animals can be modified such that bacteria will not become resistant. According to a report from the Centers for Disease Control and Prevention (23), approximately two million Americans were hospitalized due to infections caused by bacteria that were resistant to at least one antibiotic. Treatment options for those with heavily resistant bacteria were few, with the result that approximately 23,000 Americans died. Use of antibiotics should be regulated to prevent emergence of even more dangerous bacterial strains like MRSA. Decreased use of antibiotics will, in turn, lower the exposure of the bacteria to these medications, thereby reducing the probability that bacteria will develop "gain of function" or other mutations to become resistant. In conclusion, antibiotics should be used cautiously for therapeutic purposes to protect future animal and human health (24). The results of this study showed that antibiotic exposure of rumen bacteria to tetracycline leads to a population growth of tetracycline-resistant bacteria. Future research directions include ascertaining the diversity of resistance genes and mechanisms among antibiotic-resistant rumen bacteria.

## Materials and Methods

### Isolation of tetracycline-resistant bacteria

Rumen fluid from a fistulated cow was collected, diluted, and plated on Tryptic Soy Broth (TSB) agar containing antibiotic tetracycline disks (12 µg), followed by isolation of ten tetracycline-resistant bacteria in the zone of inhibition on day four. Next, bacteria from the ten colonies were streak-plated on TSB agar supplemented with tetracycline (25 µg/mL). The resistant colonies (8 out of the 10 initial colonies) were picked and streak-plated again to isolate pure bacteria.

### Cryopreservation of bacteria

Well-isolated bacterial colonies for all of the eight isolates were plated on TSB agar supplemented with tetracycline (25 µg/mL). A streak of pure bacteria was resuspended in 1 mL of 15% glycerol in TSB in sterile cryopreservation tubes. The samples were then stored in a freezer at a temperature of -80ºC.

### Gram staining

Day-old pure bacteria cultures were spread on a clean glass slide with two drops of water, and they were fixed using a brief heat exposure. The slides were placed face up on a metal bar and covered with Crystal violet dye, which stains the peptidoglycan layer of the Gram-positive bacteria purple, for 30 seconds. Then, each slide was washed gently with water from a wash bottle. The slides were then covered with Gram's iodine, also known as mordent, which prevents removal of Crystal violet, for 60 seconds. The slides were then held at an angle and rinsed gently with 96% ethanol until all the purple dye ran off. The last dye to be applied was safranin, also known as counterstain, which stains Gram-negative bacteria red or pink, and every slide was covered with the dye for 30 seconds. The slides were then washed with water from a wash bottle and left to air dry. Later they were observed under a microscope, and photographs of each slide under 1000X magnification were taken.

### Multidrug resistance test

Tetracycline (25 µg/mL)-containing TSB agars were

supplemented with chloramphenicol (20 µg/mL), ampicillin (100 µg/mL), and kanamycin (100 µg/mL). The bacteria were then cultured at 37ºC for three days. The bacterial growth on each plate was recorded daily.

### DNA extraction and 16S rRNA gene sequence analyses

Genomic DNA of bacterial Isolates #1 to 8 were prepared with the Wizard® Genomic DNA Purification Kit (Promega Corp., Madison, WI). The nearly full-length 16S rRNA gene was PCR amplified using the universal primers 27F and 1492R, as described previously (25, 26). To assure the quality of PCR amplification, genomic DNA of *Burkholderia pyrrocinia* strain Lyc2 (27) was included as a positive control. The PCR products were cleaned up with Wizard® SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI), as recommended by the manufacturer, and sent to Eurofins MWG Operon (Huntsville, AL) for Sanger sequencing. The SeqMan program in the Lasergene expert sequence analysis software package (DNASTAR) was used to assemble nucleotide sequences. Bioinformatics analyses of the 16S rRNA gene sequence were performed using the Basic Local Alignment Search Tool (BLAST, 28) at the web page (http://www.ncbi.nlm.nih.gov) of the National Center for Biotechnology Information (NCBI). The sequences of 16S RNA genes were matched against sequences in the NCBI database. The matches of sequences that were 100% were considered reliable.

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