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Bacterial infections in the early phase of Nile tilapia production in Zambia

Bakterielle infeksjoner i tidlig fase av Nil tilapia produksjon i Zambia

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Foreword

The world population is growing, leading to an increasing demand for food. Globally, Nile tilapia (*Oreochromis niloticus*) is the third most farmed fish species, behind silver carp and grass carp (2017) (El-Sayed, 2019). This makes tilapia a very important food source in many parts of the world and contributes to food security in many developing countries.
Comparing Atlantic salmon (*Salmo salar*) production in Norway, with the production of Nile tilapia, is both exciting for us and useful for discovering and understanding some of the challenges the Nile tilapia producers are facing. One of the main reasons for us choosing this study is that we want to contribute to improvement of the production of this important source of protein and micronutrients (Nölle et al., 2020). Our goal is to evaluate factors and difficulties the farmers of Nile tilapia are facing, while focusing on the early production phase. We are also eager to see which bacteria occurs in the samples collected from Nile tilapia in Zambia.

Summary

Title: Bacterial infections in the early phase of Nile tilapia production in Zambia

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Globally tilapia is the third most farmed group of fish, and it is an important food source in many parts of the world. Tilapia is a fast-growing freshwater fish, which tolerates more than many other fish species in terms of environmental variabilities like changes in temperature and salinity, as well as low dissolved oxygen levels in the water.

The main object of this thesis was to assess the production principles and environments in tilapia production in Zambia, including characterization of potential pathogenic bacteria in fingerlings and fry in the early stages of production.

22 bacterial isolates previously collected in Zambia, were characterized by Gram-staining and biochemical characterization with API 20 E and API 20 NE, and further analyzed using molecular methods including PCR, followed by sequencing, and combined with Maldi-Tof analyses. During our field study in Zambia, we used a structured questionnaire to collect information about production principles and routines from tilapia managers and farmers. Some potentially pathogenic and non-pathogenic bacteria were found during our laboratory work, suggesting the fish lives in suboptimal environment, and are exposed to stressors making them less resilient to disease. Increased mortality in the early phase of production may cause massive economic consequences because the entire production chain is affected. The tilapia farms we visited during our field study is still under development with little resources and economic input, producing cheap fish to the local market.

Definitions and abbreviations

NMBU	Norwegian University of Life Sciences
UNZA	University of Zambia
Nile tilapia	*Oreochromis niloticus*
Catfish	*Clarias gariepinus*

Lake Kariba	One of the largest man-made reservoirs and lakes in the world. It lies along the border between Zambia and Zimbabwe.
Broodstock	The male and female fishes used for reproduction.
Fry	The first stage in the life of a Nile tilapia, hatched from eggs.
Hapas	Small square shaped cages for keeping tilapia together, used in ponds.
Fingerlings	Larger than fry, big enough for transfer to grow-out ponds or cages.
XX females	Normal female Nile tilapia.
YY males	Male Nile tilapia with two Y chromosomes
TSA	Tryptic Soy Agar, a non-selective media with enough nutrients allowing growth of a wide selection of microorganisms.
TSB	Tryptic Soy broth, the broth counterpart of TSA.
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
Polyculture	Polyculture is the production of multiple fish species within a particular aquaculture environment.

Methyl-testosterone	Methyl-testosterone is a synthetic male hormone which closely mimics the naturally produced hormone testosterone. Commonly used for sex-reversal of tilapia fry, often given orally mixed into the feed.
Gram negative	Gram-negative bacteria lose the crystal violet stain (and take the color of the red counterstain) in Gram's method of staining. This is characteristic of bacteria that have a cell wall composed of a thin layer of a particular substance (called peptidoglycan).
Gram positive	Gram-positive bacteria are bacteria classified by the color they turn into by staining with crystal violet dye. This is characteristic of bacteria that have a cell wall composed of a thick layer of a particular substance (called peptidoglycan).
Saprolegnia	Saprolegnia is a genus of water molds that typically feeds on waste from fish or other dead cells, they will also take advantage of creatures that have been injured.
Methemoglobinemia	Methemoglobin is a form of hemoglobin that cannot carry oxygen. In methemoglobinemia, tissues cannot get enough oxygen.
Virulence	Virulence is defined as the degree of pathogenicity of a pathogen (bacteria, fungi, or viruses).
Seed production	Production of fertilized fish eggs.

Introduction

Tilapia

Tilapia is the general name given to all fish in the cichlid group consisting of three genera: *Oreochromis*, *Sarotherodon* and *Tilapia* (Trewavas, 1983). The first scientific record of tilapia culture is from Kenya in 1924, and later it spread throughout Africa and in the early 1940s towards Far East and to America (Gupta & Acosta, 2004). The genus *Oreochromis* consists of more than 31 species with 3 cultured aquaculture species: Nile tilapia (*O. niloticus*), Mozambique tilapia (*O. mossambicus*) and blue tilapia (*O. aureus*). These species have been introduced to more than 100 countries worldwide (He et al., 2011; Romana-Eguia et al., 2004). Tilapiine fishes are freshwater fish, which are resilient and tolerates more than many other fish species in terms of environmental conditions such as changes in temperature and salinity, as well as low dissolved oxygen levels in the water. They tolerate stress well and appear quite resistant to infection diseases. The generation intervals are short, which means that they can reproduce fast and reach slaughter weight within months. This in combination with Tilapiine fishes being hardy, make these fish relatively easy to produce under various conditions. Tilapiines accepts artificial feeds immediately after absorption of the yolk-sac, which makes it easy to feed (El-Sayed, 2019).

Environmental requirements

Wild tilapia species are for the main part found in freshwater. They inhabit shallow streams, ponds, rivers, lakes, and brackish water, and as mention above they seems to tolerate a wide range of environmental conditions (DAF, 2020). However, even though they tolerate changes in environmental factors, suboptimal conditions may affect their physiology, growth, reproduction, metabolism, and resistance/susceptibility to infections (El-Sayed, 2019).

Tilapiines can grow, reproduce, and develop normally in water temperatures between 20-35 °C but outside this range, they show poorer growth and utilization of feed. Mortality occurs when temperatures are under 11-12 °C and over 42°C (El-Sayed, 2019).

Despite tilapias being a freshwater fish, they tolerate a wide range of water salinity, and optimum salinity is between 5-10 ‰ but they can grow and survive in a wider range depending on acclimation (El-Sayed, 2019).

Dissolved oxygen affects fish feeding, growth and metabolism. Tilapias tolerate levels as low as 0,1-0,5 mg/L over shorter periods, and if allowed to reach surface air they can survive at 0 mg/L. However, low levels of dissolved oxygen in the water limits respiration and metabolic activities. At normal oxygen levels, between 6,0-6,5 mg/L, growth, feed utilization and immunity are improved. Smaller fish tolerate low dissolved oxygen levels less than larger fish (El-Sayed, 2019).

The toxicity of ammonia increases with decreasing levels of dissolved oxygen and decreases with increasing CO_2. Ammonia toxicity also depends on the pH level in the water, size of the fish, acclimation time and culture system. Levels between 0,07 and 0,14 mg/L are toxic and affect the growth negatively. Nitrification is a process where bacteria use inorganic nitrogen compounds as an energy source, by oxidizing ammonia into nitrite (NO_2) and further into nitrate (NO_3). Nitrate is not lethal to tilapia but may decrease immune functions and nitrite is highly toxic because it disturbs multiple physiological functions (methemoglobinemia) (El-Sayed, 2019).

Tilapia farming

The tilapia culture has been expanding rapidly, especially the last two decades. In 1970 only 12 countries practiced tilapia culture, while in 2017 the number of countries was 125 (El-Sayed, 2019). In 2018 Asia accounted for 68,8 % of the world's tilapia production (FAO, 2020). Despite tilapias being native to Africa, tilapia culture in Africa is relatively new, as

mentioned and Africa contribute to a relatively low percentage of the world's overall production (21 % in 2017). Farmed tilapia in Africa, however, have had a rapid increase in recent years and today Nile tilapia is by far the most cultured fish species in Africa (El-Sayed, 2019). See table 1 for an overview of the increase in tilapia production in Africa since 1950.

Table 1. Tilapia production in Africa in tons in different years (El-Sayed, 2019).

Year	**Tilapia production in tons in Africa**
1950	980 t
1999	119,299 t
2010	644,403 t
2017	1,220,320 t*

*The main producer in Africa is Egypt.

The republic of Zambia, which is the country our samples are collected from, is a landlocked country on the Central African plateau. There are three seasons, the rainy season from November to April (27-38 °C), the cool dry season from May/June to August (14-21 °C) and the hot dry season from September to November. Zambia has an average elevation of 1200 meters above sea level, modifying the tropical climate during the cool, dry season. (Wikipedia, s.a.-a; Wikipedia, s.a.-c)

Despite being landlocked water resources are plenty, mainly contributed by the Zambezi and Congo River basins, for agriculture, drinking water, energy production, industry and fish farming (Nkuwa et al., 2013).

It is estimated that fish represents more than 20 percent of the animal protein intake in Zambia (Musumali et al., 2009). For many years capture fisheries met the demand for fish, but a decrease in supply created an opportunity for an increase in fish farming. Today Zambia is the 4th largest producer of farmed tilapia in Africa (numbers from 2017), a total production of

30,100 tons in 2017 (El-Sayed, 2019). There are small-scale farms, mainly producing for the local markets, and large-scale farms supplying urban markets.

Figure 1. Nile tilapia ready to be sold at the market. (Private photo)

Farming systems

According to El-Sayed (2019), tilapia farming can be divided into intensive and semi-intensive productions. These are definitions that reoccur in the literature, without any clear definition of the difference between the two production types. It is reasonable to believe that a semi-intensive system typically takes place in ponds of different sizes with low density of fish, while intensive farming is often based on cages and tanks where fish is stocked in much higher density, and where processed commercial feed is supplied. Gupta and Acosta (2004) divided the production into socio-economic groups, where resources invested in the operation

separate between groups. Josupeit, H. (2005) divides the aquaculture production into three methods: Local Pond culture, small-scale commercial systems, and industrial aquaculture systems. The small-scale commercial systems are often semi-intensive, and the broodstock may not be of the best quality. Fish produced in these systems often target the local market, but some also include export markets.

In the "Review of feeds and fertilizers for sustainable aquaculture development in sub-Saharan Africa" (Hecht, 2007) production systems are divided into extensive, semi-intensive and intensive. Extensive production being polyculture in earthen ponds, the work mainly being done within the family with low investment and low production costs. Intensive production is described as monoculture, with management and employment opportunities. Semi-intensive is described as something in between the two production forms. See table 2 for a full overview of characteristics.

Table 2. Characteristics of different production systems.

CATEGORIES and CHARACTERISTICS	**Extensive**	**Semi-intensive**	**Intensive**
Culture systems	Earthen ponds	Earthen ponds and cages	Cages, raceways, tanks and earthen ponds
Species	Polyculture (various tilapiine species, catfish and carp)	Mainly polyculture (Nile tilapia and African catfish), some monoculture (tilapia)	Mainly monoculture
Management input	Low to medium	Medium to high	High
Labour needs	Family labour to low requirement for external labour	Medium	Low to high (More capital intensive)
Capital costs	Low to medium	Medium	Medium to high
Operational costs	Zero to low	Medium	High
Business orientation	Low to medium	Medium to high	High
Integration with other farm activities	Medium to high	Low to high	Low
Feeding	Zero to supplementary	Scheduled to unscheduled using mainly farm-made feeds	Scheduled intensive feeding using pellets or farm-made feeds

Fertilisation	Zero to medium level	Medium to high level	Zero to high level

Source: Hecht, T. (2007). Review of feeds and fertilizers for sustainable aquaculture development in sub-Saharan Africa.

Without going further into detail about what separates the small-scale commercial system from the two others, the point is to show that there is no accepted consensus as to how to divide the different production systems.

A multitude of approaches are employed for tilapia farming. Keeping male and female tilapia in the same pond is possible, but the harvest must be done before the fish reaches reproductive age to avoid breeding in the ponds, which results in too high density and huge variability in size at slaughter. Harvesting fish before they reach the reproductive age gives the fish less time to grow, and results in fish being harvested at a smaller size than most farmers want, and the market prefers. The way most farmers avoid these challenges is to stock only male fish in the same pond, also called “all-male production” or sex-reversion (Rakocy et al., 2009). The male tilapia grows faster than the females, and using this approach make the production as effective as possible (The Fish Site, 2005). All-males can be produced by feeding the fry methyl-testosterone in the feed for 14-28 days (El-Sayed, 2019).

Hatching time of fertilized tilapia eggs ranges from <3 days to >6 days. Several factors affect the hatching time including water temperature, salinity, pH, water flow and broodstock nutrition. Temperatures from 25 to 32 °C are considered optimal for best hatching and survival rates. Temperatures under 22 and over 35 °C have various negative effects on seed production and egg quality. The goal is therefore to keep the water temperature between 25 to 32 °C in the hatchery (El-Sayed, 2019). Water flow is also affecting spawning efficiency and larval growth of tilapia. Higher water flow makes the yolk absorption time shorter. A flow rate of approximately 8 L min^{-1} in a 20-liter hatching unit has been considered optimal for yolk sac absorption in Nile tilapia eggs (El-Sayed et al., 2005).

Salinity is also a factor that affects hatching success. One study (Watanabe et al., 1985) showed that 5‰ salinity did not affect hatching of Nile tilapia and gives the same hatching percentage as in freshwater. Salinity over 10‰ gave a lower hatching success, while seawater prevented hatching from occurring.

Broodstock nutrition also affects spawning efficiency, egg hatchability and larval growth. Higher dietary levels of protein have been proven to reduce hatching time of *O. niloticus* eggs (El-Sayed, 2019).

Many factors affect the growth of Nile tilapia fry. The most important ones are stocking density, food quality and feeding regime, photoperiods, water flow and water replacement. Larval stages of Nile tilapia are more sensitive to the length of the photoperiod than the older developing stages. Long photoperiods (24 and 18h) made the fry perform better than fry exposed to shorter photoperiods (12 and 6h). The endogenous rhythm of the fry must synchronize with the external environment, and in shorter photoperiods this requires more energy for a shorter time where light is present. This may be one explanation of why shorter photoperiods lead to reduction of somatic fish growth (El-Sayed, 2019).

Bacterial infections in freshwater fish

There are several species of bacteria in aquatic ecosystems, some of them are symbiotic bacteria positively influencing host organisms (Grossart et al., 2013) plus pathogenic and opportunistic disease-causing bacteria. The development of bacterial diseases in fish depends on the interaction between the bacteria, the host, and the environment. A change in the environment could cause an invasion of an otherwise harmless bacteria and development of a bacterial infection (Raman et al., 2013). Bacteria earlier known to be non-pathogenic have been isolated from diseased fish. For example, change in the environment or in the presence of stress, like increased temperatures or transport may cause disease from bacteria which are harmless under optimal environmental conditions.

Aeromonas spp.

The most common bacteria in freshwater fish are motile aeromonads and can be found worldwide, in freshwater and marine ecosystems. They can cause diseases in humans, frogs, pigs, cattle, birds, and marine animals (Jorgensen & Pfaller, 2015). *Aeromonas* spp. have been acknowledged as a human pathogen since the late 1960s. This bacterial genus is known for causing gastroenteritis, and the most common source is seafood and drinking water (FHI, 2019). A significant number of virulent aeromonads have been taxonomically identified in freshwater fish, but proving their pathogenicity is not always easy (Dong et al., 2017).

Aeromonas jandaei and Aeromonas veronii

Aeromonas jandaei and *Aeromonas veronii* are facultative anaerobic, motile, gram-negative rod bacteria with oxidase-positive, catalase-negative and glucose-fermenting properties. They are resistant to the vibriostatic agent O/129 (Carnahan et al., 1991). Disease in freshwater fish caused by *Aeromonas* spp. have for a long time believed to be caused by *A. hydrophila*, a species well known to be pathogenic for fish. Nonetheless, *Aeromonas jandaei* and *Aeromonas veronii* have been isolated from diseased Nile tilapia (Dong et al., 2017). The clinical findings included dark bodies, abnormal swimming, and loss of appetite, which also can be seen in infections with *A. hydrophila*. The virulence of *A. jandaei* and *A. veronii* has been confirmed by experimental infections with different dosages.

Disease outbreaks are associated with stress-related conditions such as transportation, handling, changes in the environment and co- infections. However, a study (Dong et al., 2017) described disease associated with both *A. jandaei* and *A. veronii* without any stressor present. In the same study it was seen that fish, which survived the lowest dose of infection were able to resist a second infection. This may suggest that there is a possibility for the development of a vaccine in the future.

Other aeromonads that have been linked to causing disease in tilapia include *Aeromonas sobria* and *Aeromonas dhakensis* (Dong et al., 2017).

***Staphylococcus* spp.**

Staphylococcus is a genus with at least 40 species (Wikipedia, s.a.-b). A fair amount of these are commensal bacteria that colonize the skin and mucous membranes of most mammals, particularly those living in close contact with humans. *S. epidermis* is frequently isolated from human epithelia and known for being a contaminant at laboratories (Ghayoor et al., 2015).

Stapylococcus epidermidis

Staphylococcus epidermidis has been described as a fish pathogen in farmed, freshwater fish. It is a facultative anaerobe, gram-positive, non-motile, non-spore-forming cocci (De Vos et al., 2009). The bacteria tolerate harsh conditions, like high salt concentrations (Otto, 2009). Under the microscope it occurs in irregular grape-like clusters. When applied to agar, it grows in grayish white, smooth and raised colonies that may be mucoid or slimy (De Vos et al., 2009).

The pathological changes described in freshwater fish with *S. epidermidis* infection includes hemorrhages on pelvic fins, petechial hemorrhages internally and ascites (Austin & Austin, 2016). A study done on naturally and experimentally infected Nile tilapia described macroscopical white nodules and microscopical granulomatous formations in various internal organs (Huang et al., 1999).

Formation of and adhesion to existing biofilm, when polysaccharide intracellular adhesin (PIA) is expressed by the bacteria, is one of the most important virulence factors (Rohde et al., 2010). This may indicate that *S. epidermidis'* biofilm can be a long-term reservoir in fish farms and be a potential source of infection.

***Streptococcus* spp.**

Streptococcus spp. has been described to be the cause of substantial morbidity and mortality in fish. They can infect a wide range of hosts and are important pathogens in human and veterinary medicine. Streptococci are Gram-positive, non-motile, non-spore-forming, catalase-negative cocci (Amal & Zamri-Saad, 2011).

S. agalactiae and *S. iniae* are emerging fish pathogens both in freshwater and saltwater. Dead or infected fish or feces from infected fish is believed to be the sources of infection. Fish farmed in high densities or fish with abrasions or wounds are more susceptible. Also, environmental factors as too high or too low water temperatures, decreased dissolved oxygen concentration and high nitrite or ammonia concentrations are contributing factors. The clinical signs of a *Streptococcus* spp. infection include loss of appetite, abnormal swimming, hemorrhages at the base of the fins and in the eyes, distended abdomen, opaque cornea, and exophthalmia. Pathological findings include ascites, enlarged liver and spleen and inflammation in heart and kidney (Amal & Zamri-Saad, 2011).

Generating knowledge as regards presence of disease-causing bacteria in the early stages of tilapia production is both important and necessary, especially when it comes to understanding possible causes of increased mortality in the early production phase. Understanding which bacteria that play a role as potential pathogenic agents in tilapia production, makes it easier to understand how these affect fish and which diseases they lead to. Screening for presence of bacteria present at the different sites, makes it easier to evaluate which biosecurity measurements the farmers should include in their production strategy, and thereby be in position to prevent occurrence of bacterial infections in the future.

One study focusing on mortality in early stages of tilapia production in the Siavonga area in Zambia was performed by veterinary students at NMBU a few years back, where they found

indications of potential pathogenic bacteria in samples from fingerlings (Donbæk et al., 2019). The samples collected in their study were prepared for long time storage and kept at -80 °C. In this study we have used these samples for further analysis, including methods of biochemical characterization and a more thorough characterization using molecular methods, PCR followed by sequencing combined with Maldi-Tof analysis.

Objective

The main objective in this study was to obtain a more detailed characterization of the production principles and environments in tilapia production in Zambia including characterization of bacteria detected as potential disease-causing in fingerlings and fry in the early stages of production. We had three sub-aims:

1. Characterize the bacterial isolates in samples collected from hatcheries, and in samples taken after transfer to hapas.
2. Field study with main focus on explaining the effects of increased mortality in the early production phase, before and after transferring the fry into hapas.
3. Collect information about production principles and routines through a questionnaire of tilapia managers and farmers in Zambia

Material and methods

Collection of sample material

Because of the COVID-19 pandemic and restricted time available, we were unable to collect new samples from Zambia for characterization. Therefore, the samples used in this study were previously collected by Christine Donbæk, Anniken Mork and Ingelin Ravlo during their visit to Zambia in 2019, and stored at -80 ºC at NMBU, Faculty of Veterinary Medicine.

The samples were collected from the fingerlings by swabbing the inside of the abdominal cavity with sterile swabs. The water-samples were collected by submerging a 250 mL container at approximately 0,4 meters depth before being opened and closed when the container was completely filled (Donbæk et al., 2019).

We allowed the samples to keep their tags (number and a letter) to make it easier to keep track of the samples. The samples were also given a number from 1-22.

Table 3 shows the tagging of the samples (Donbæk et al., 2019), what farm they were from and when they were taken, and what number we gave them. The farms the samples are collected from may not be the same farms we visited. The farms from which the samples were collected from, were given a number from the roman numeral system (*e.g.*, "farm I", "farm II" etc.) to avoid misunderstanding.

Table 3. Explanation of when and where the samples were collected (Donbæk et al., 2019) and what number we gave them in this present study.

Number	Sample	When the sample was taken (Farm number)
1	3A	Sample taken from the pond at the time of collection of the fingerlings (Farm I)
2	30B	Fish samples were taken from the transportation box at the end of transportation (Farm II)
3	34A	Samples were taken day 4 and 5 after transportation (Farm IV[1,2]).

4	24A	Fish samples were taken from the transportation box at the end of transportation (Farm II)
5	18A	Day 1 after transportation the fish samples were taken before the arrival of the fingerlings (Farm IV[1,3])
6	3C	Sample taken from the pond at the time of collection of the fingerlings (Farm I)
7	W2-A	Water sample (Farm I)
8	25B	Fish samples were taken from the transportation box at the end of transportation (Farm II)
9	9B	Sample taken from the pond at the time of collection of the fingerlings (Farm I)
10	38A	Samples were taken day 4 and 5 after transportation (Farm IV[1,2]).
11	21A	Fish samples were taken from the transportation box at the end of transportation (Farm II)
12	30D	Fish samples were taken from the transportation box at the end of transportation (Farm II)
13	27A	Fish samples were taken from the transportation box at the end of transportation (Farm II)
14	25D	Fish samples were taken from the transportation box at the end of transportation (Farm II)
15	35C	Samples were taken day 4 and 5 after transportation (Farm IV[1,2]).
16	2A	Sample taken from the pond at the time of collection of the fingerlings (Farm I).
17	28C	Fish samples were taken from the transportation box at the end of transportation (Farm II)
18	23A	Fish samples were taken from the transportation box at the end of transportation (Farm II)
19	4A	Sample taken from the pond at the time of collection of the fingerlings (Farm I)
20	14B-2	Day 1 after transportation the fish samples were taken before the arrival of the fingerlings (Farm IV[1,3])
21	1C	Sample taken from the pond at the time of collection of the fingerlings (Farm I)
22	40A	Samples were taken day 4 and 5 after transportation (Farm IV[1,2]).

1) Farm IV gets fingerlings from both farm I and farm II.

2) Fingerlings from both farm I and farm II are mixed in the same cage.

3) Fingerlings from farm I.

Laboratory work

Creating our own bank of bacterial isolates

Frozen samples stored at -80 ºC had been collected earlier (Donbæk et al., 2019). These samples were transferred into a bucket with ice to avoid temperature fluctuation during initial preparation of the samples, and the ice buckets were then placed in a hood, previously disinfected with 70 % alcohol. A sterile single-use inoculating loop was used to streak the stored bacteria (in liquid) from the sample tube onto the surface of a TSA agar plates. Each sample was taken out of the ice bucket, streaked onto the surface of one agar plate and the tubes were then transferred back to ice. The agar plates were incubated at 22 ºC and 28 ºC (in air) for 24 hours until bacterial colonies had formed on the surface of the agar. The bacteria incubated at 28 ºC grew faster than the bacteria incubated at 22 ºC. We then collected bacterial colonies with a sterile single-use loop and put them carefully into a 1,5 mL tube with 1,0 mL TSB-medium. The tubes were marked, then thoroughly mixed with a vortex mixer, and stored at -80 ºC.

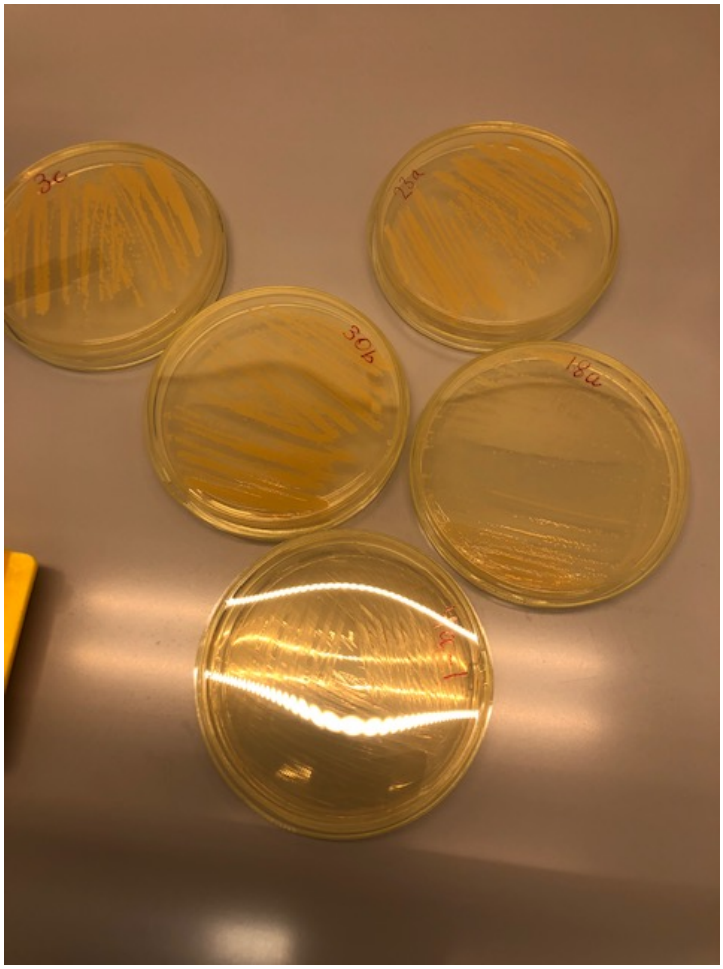

Figure 2. TSA plates with bacterial growth. (Private photo)

Gram-staining

Purified bacteria grown in broth at 37 °C were used for gram staining. A drop of broth with bacteria was placed on a glass slide using a 30-µL pipette and smeared out using a sterile single-use inoculating loop, followed by air-drying. The slides were put in crystal violet solution for 60 seconds and then gently rinsed with water. After rinsing the slides were put into an iodine solution for 60 seconds and gently rinsed with water again. Next, the slides were rinsed with a decolorization solution (Grams Decolorization Solution) until all the excessive crystal violet solution was washed off from the slides. Lastly the slides were put in a Safranin solution for 60 seconds, gently rinsed with water and air-dried.

A selected number of samples was stained and examined by microscopy, motivated by the fact that all samples had been stained in a previous study (Donbæk et al., 2019). Gram staining was included to get an understanding of how the method is performed and how results are read, since this is a simple method (level 1) often used in diagnostic labs.

Biochemical characterization

For biochemical characterization we used API 20E and API 20NE, two bacterial identification systems that combine biochemical tests for gram-negative Enterobacteriaceae or non-Enterobacteriaceae, respectively. The test systems contain 20 tubes with different substrates, each giving negative or positive results dependent on the bacteria characteristics. The 20 reactions are represented in Tables 4, 5, 6, and 7.

API 20E were used to determine if the bacteria were enteric gram-negative rods, and if an API 20NE were necessary to do further biochemical characterization.

API 20E

First the incubation box was prepared, marked, and filled with 5 mL of distilled water into the bottom of the tray to avoid dehydration of tests during incubation. Then the test strip was placed into the tray.

10 mL PBS was filled in a small single-use tube. 3-4 colonies from bacterial isolates grown on TSA-agar at 28 ºC for 24 hours were scooped up with a sterile single-use inoculating loop and put into the 10 mL tube with PBS. The bacteria-PBS-suspension was mixed into a homogenous mix with a turbidity equivalent to 1,0 McFarland. 3 bacterial isolates were analyzed by API 20 E (6= 3C, 18= 23a, 2= 30b).

The strip was inoculated by filling of both the tube and cupule of the tests [CIT], [VP] and [GEL] with the bacterial suspension. On the other tests only the tubes were filled, not the cupules. To create an anaerobic environment in the tests ADH, LDC, ODC, H2S and URE the cupules were filled with mineral oil. Then the incubation box was closed and incubated at 37 °C for 20 hours.

Table 4. Overview of the enzymatic tests in the API 20 E, and what they are detecting.

Test	**Detection of**
OPNG	β -galactosidase
ADH	Arginine dihydrolase
LDC	Lysine decarboxylase
ODC	Ornithine decarboxylase
CIT	Citrate utilization
H_2S	Hydrogen-sulphide production
URE	Urease
TDA	Tryptophane deaminase

IND	Indole production (from tryptophan)
VP	Acetoin production (Voges Proskauer)
GEL	Gelatinase

Table 5. Overview of fermentation tests in the API 20 E, and what sugars are tested.

Fermentation test*:* to determine if the bacteria can ferment the sugar or not. If sugar is fermented the pH changes and the color will change from blue to yellow.	
Test	**Sugar**
GLU	D-glucose
MAN	D-mannitol
INO	Inositol
SOR	D-sorbitol
RHA	L-rhamnose
SAC	D-sucrose
MEL	D-melibiose
AMY	Amygdalin
ARA	L-arabinose

The results of the API 20 E are found after the incubation period is complete. The API 20 E manual (BioMérieux, 2002) comes with a Reading Table (see appendix II) that is used for reading and understanding the results. The Reading Table contains the expected colors for negative and positive reactions for all the spontaneous reactions. The spontaneous reactions are ONPG, ADH, LDC, ODC, CIT, H2S, URE, GEL, GLU, MAN, INO, SOR, RHA, SAC, MEL, AMY and ARA. If three or more of the spontaneous reactions are positive, the results are to be noted on the result sheet. After all the spontaneous reactions have been read and written down, it is time to reveal the test which requires the addition of reagents.

- For reading the TDA Test one must add 1 drop of TDA reagent into the tube. Reddish brown color indicates a positive reaction, while any other color meant the reaction was negative.
- To read the VP Test one drop of VP 1 is added, followed by one drop of VP 2 reagent. It is important to wait for at least 10 minutes, to allow the reaction to happen before reading the results. A strong pink or red color indicates a positive reaction, while a slightly pink color after 10 minutes means that the reaction was negative.
- For reading the IND Test one drop of JAMES reagent is added to the tube. Pink color development indicates that the reaction is positive, while other colors mean the reaction was negative. It is important to perform the indole production test at last (IND) and to avoid closing the lid after adding JAMES reagent, because the reaction releases gaseous products that may interfere with interpretation of other tests on the strip.

If there are less than 3 positive spontaneous reactions, re-incubation of the strip for 24 +/- 2 hours is necessary. After the extra incubation time the tests can be read, followed by adding the reagents to the remaining tests.

The numeric profile is used for identification of the bacteria in the sample. The tests are separated into groups of 3 on the result sheet, and a value of 1, 2 or 4 is indicated for each test. By adding these values of the positive tests together, a 7-digit profile number is obtained for the total 20 tests of the API 20 E strip. The oxidase reaction counts as test 21 and has a value of 4 if positive.

A database (V4.0) with the Analytical Profile Index is used to look up the numerical profile in the list of profiles. If using the identification software, it is possible to type in the 7-digit code manually and search for it in the database. In some cases, doing this is sufficient to determine what type of bacteria the sample contains. In other cases, the code will not be sufficient, and

further tests must be carried out before any conclusions can be drawn. The results of the follow up tests will give you a 9-digit code, to be entered into the identification software.

API 20NE

API 20 NE were used to determine if the bacteria were non-fastidious, non-enteric, Gram-negative rods. This was used on the samples that did not show 4 positive results on the API 20 E. Three isolated bacteria were analyzed by API 20 NE. (11= 21A, 15= 35C, 16= 2A). These were inoculated in TSB and incubated at 37 °C for 20 hours. The incubation method was the same as for the API 20 E test. 1 mL of the inoculated broth was transferred to 1,5 mL microcentrifuge tubes and then centrifuged at 60 rpm for 1 minute at 4 °C. This separated the bacteria from the broth. Then the supernatant was removed, and 100 µL API NaCl 0.85% Medium was added and mixed thoroughly by using a sterile, single-use pipette. 60 µL were then extracted from the saline bacteria suspension and added into a new 2 mL API NaCl 0.85% Medium ampoule and mixed thoroughly to make a suspension with a turbidity equivalent to 2,0 McFarland.

The strip was inoculated by first filling the tests from NO_3 to PNPG by distributing the saline suspension into the tubes, and not the cupules, with a pipette. Then the ampoule of API AUX Medium was opened and 200 µL of the remaining saline suspension was added to the ampoule. This was mixed thoroughly with a pipette and formation of bubbles were avoided. Next the tubes and cupules of tests [GLU] to [PAC] were filled with the suspension until a flat meniscus was formed. Mineral oil was added to tests GLU, ADH and URE until a convex meniscus was formed. Then the incubation box was closed and incubated at 29 °C for 24 hours.

Table 6. Overview of the enzymatic tests in the API 20 NE, and what they are detecting.

Test	**Detection of**
NO_2	Reduction of nitrates to nitrites
N_2	Reduction of nitrates to nitrogen
TRP	Indole production (from tryptophane)
GLU	Fermentation of glucose
ADH	Arginine dihydrolase
URE	Urease
ESC	Beta-glucosidase
GEL	Gelatinase
PNPG	β -galactosidase

Table 7. Overview of the assimilation tests in the API 20 E, and what substrates are tested.

Assimilation tests: to determine if the bacteria can assimilate the substrate. The ability to assimilate the substrate means the bacteria can grow, and that is represented by an opaque color.	
GLU	D-glucose
ARA	L-arabinose
MNE	D-mannose
MAN	D-mannitol
NAG	N-acetyl-glucosamine
MAL	D-maltose
GNT	Potassium gluconate
CAP	Capric acid
ADI	Adipic acid
MLT	Malic acid

CIT	Trisodium citrate
PAC	Phenylacetic acid

As the API 20 E, the results of the API 20 NE are found after the incubation period is completed. Also, the API 20 NE manual (BioMérieux, 2003) comes with a reading table (see appendix III) that is used for reading and understanding the results. The reading table contains the expected colors for negative and positive reactions for all the spontaneous reactions, which are GLU, ADH, URE, ESC, GEL and PNPG. All the reactions are recorded and written down on the result sheet.

The NO_3 test requires one drop of NIT 1 and one drop of NIT 2 reagents to change color. After 5 minutes the content of the NO_3 -tube will either turn pink-red, which indicates a positive reaction, or have a negative reaction indicated by no change in color. The pink-red color confirms that the nitrates in the tube has been reduced to nitrites by the bacterium. The negative reaction may be due to nitrogen production, and 2-3 mg of Zn reagent are added to confirm if nitrogen have been produced or not. After 5 minutes the content of the NO_3 -tube will either turn pink, indicating a negative result, as the tube contains nitrates that the zinc reduced to nitrite. Or the content of the tube will remain colorless indicating a positive result.

The TRP test is performed by adding 1 drop of JAMES reagent. The reaction is immediate if a pink color appears in the tube the reaction is positive. If the content of the tube turns colorless, pale green or yellow the result is negative.

Both the NO_3 and TRP tests should be done protecting the assimilation tests from contamination from the air. We covered the tube with the box lid while adding additional reagents and reading to prevent that.

The assimilation tests were read by observing if the tube had turned opaque or not. An opaque tube indicated a positive reaction, the bacteria had grown. If the tube showed a weak result, it was recorded as +/-.

The tests are separated into 7 groups on the result sheet, each tube in the group having a value of 1, 2 or 4. Oxidase test is performed independently from the test strip and has a value of 4. If positive the value is added to the total value of the group. The total value of each of the seven groups makes a 7-digit code, a numeric profile used for identification of the bacteria in the sample. See figure 3 for an example where the 7-digit-code is 2154575, highlighted in grey.

Test	NO_3	TRP	GLU	ADH	URE	ESC	GEL	PNPG	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC	Ox
Result	-/-	+	-	+	-	-	+	-	+	-	-	+	+	-	+	+	+	+	+	-	+
	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4	1	2	4
	2			1			5			4			5			7			5		

Figure 3. An example of how one will add up the values from the results on the test strip.

Identification is performed using a database (V6.0). Either looking up the numerical profile in the list of profiles using the Analytical Profile Index or typing in the 7-digit profile in the identification software.

The API 20 NE test is only intended for non-fastidious, non-enteric, Gram-negative rods included in the database. In some cases, further and different tests must be done before concluding what bacteria the sample contains.

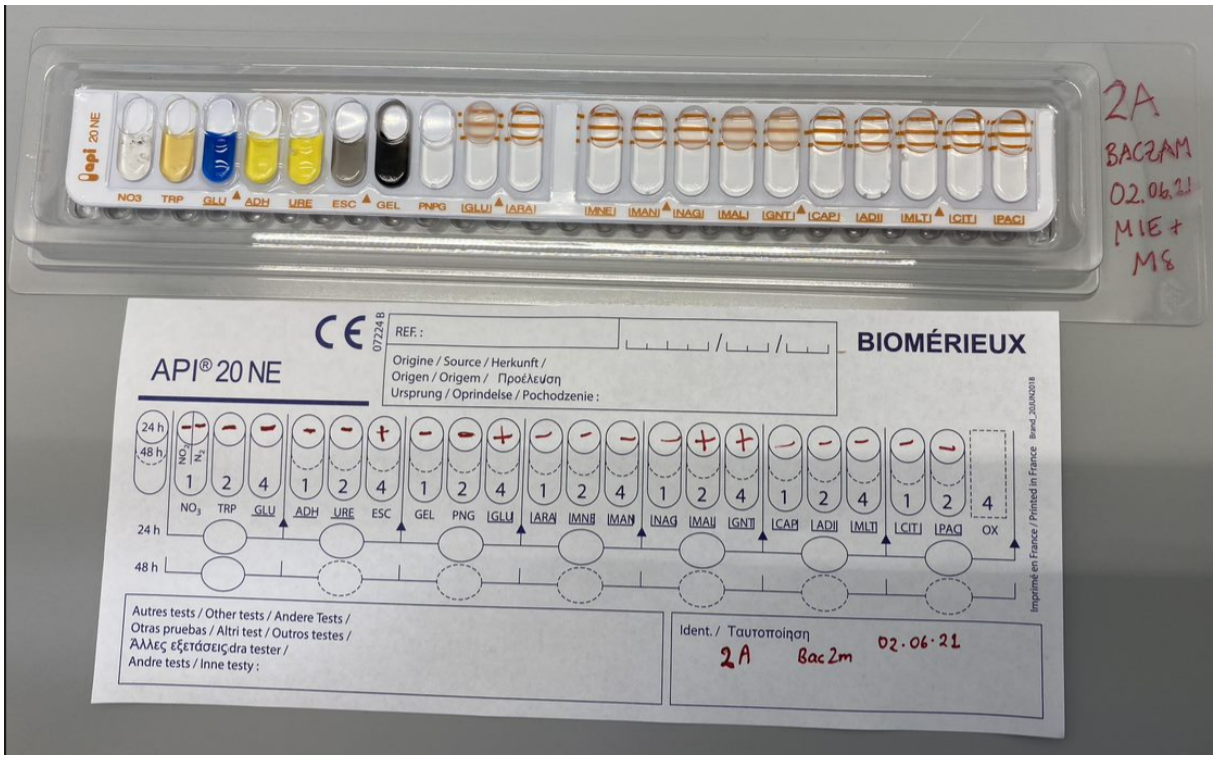

Figure 4. An incubation box with a test strip after incubation. Underneath, a result sheet. (Private photo)

Maldi-Tof

Maldi-Tof is an acronym for Matrix-Assisted Laser Desorption Ionization, the ionization technique and Time-Of -Flight, a type of mass spectrometer. In bacteriology Maldi-Tof is used to detect and characterize organic molecules such as bacteria. A laser is used to strike a matrix of small organic molecules to transfer the bacterial biomolecules into gaseous ions without fragmenting them. The Tof (Time-of-Flight) then separates the ions by measuring the time it takes for the ions to reach a detector. The identification of the different bacterial analytes is then done by comparing their Maldi-Tof-mass-spectrum with a database containing different known mass-spectra of bacteria. This is also the limiting factor of the Maldi-Tof because only bacteria with known mass-spectra are identified (Singhal et al., 2015).

The bacteria isolates are placed on a slide with 48 small wells, called a Maldi-slid. An *E. coli* colony was put in the middle as a reference colony. The Maldi-Tof uses the *E. coli* to

calibrate and compare the known against the bacterial analytes. Lastly, a matrix (VITEK MS-CHCA matrix) was put on top of the wells filled with bacteria. The matrix is a small organic molecule which facilitates the ionization process by absorption of UV-light (ARCC-Chem, 2016). The slide was air-dried, and then put into the Maldi-Tof.

When the Maldi-Tof has created a vacuum inside, a laser is fired at the wells. The laser triggers an ablation and desorption of the sample and matrix. The bacterial molecules are ionized and accelerated into a time-of-flight mass spectrometer. The Tof detector then records the time it takes for the ions to travel a certain distance. The velocity of the ions depends on the mass-to-charge-ratio. In a vacuum to ions of the same mass will move in the same path at the same velocity, and a heavier ion will move at a lower speed. The mass-spectrometer is then able to calculate the mass of the ions it detects and compares the results with a database with help of different algorithms (Singhal et al., 2015).

Genomic DNA isolation (extraction)

To extract the DNA the DNeasy Blood & Tissue Kit Quick- Start Protocol (Qiagen, 2016) (see appendix IV) was used, with slight modifications. The bacterial isolates were incubated on TSA overnight at 27 ºC. We transferred enough colonies from each isolate to an Eppendorf-tube filled with 200 µL of PBS and made sure the bacterial concentration in the tube was at an OD of 1 (Optical density). The tubes were marked 1- 22. 20 µL of protein kinase and 200 µL of AL buffer were added to each tube. Then the tubes were mixed by vortexing and incubated at 56 Cº for 10 minutes. This was followed by adding 200 µL of ethanol (96-100%), before mixing thoroughly by vortexing again. Centrifugation at 8000 rpm for 8 seconds was done to make non-lysed bacteria fall to the bottom of the tube. The supernatant was gently transferred to sterilized DNeasy Mini spin columns placed in 2 mL collection tubes and centrifuged at 8000 rpm for 1 minute at 4 °C. Tubes with unfiltered DNA-samples were gently mixed again using a pipette, and centrifuged for 8800 rpm, 4

minutes at 3 °C. The flow-through and collection tubes were discarded. The spin columns were placed in new 2 mL collection tubes and 500 µL Buffer AW 1 was added to each tube before centrifugation at 8000 rpm for 1 minute at 3 °C. Tubes with unfiltered DNA-samples were gently mixed again using a pipette, and centrifuged for 8800 rpm, 4 minutes at 3 °C. The flow- through and collection tubes were discarded. The spin columns were placed in new 2 mL collection tubes and 500 µL Buffer AW 2 was added to each tube before centrifugation at 14 000 rpm for 3 minutes at 4 °C. The flow-through and collection tubes were discarded before the spin columns were transferred to new 2 mL collection tubes, then centrifuged at 8000 rpm for 1 minute at 4 °C. The spin columns were transferred to new 2 mL microcentrifuge tubes. Then the DNA was eluted by adding 40 µL nuclease free water to the center of each spin column membrane, incubated for 1 minute at room temperature (15-25 °C) and centrifuged for 1 minute at 8000 rpm at 4 °C and stored at -20 °C. We added 40 µL instead of 200 µL as it says in the protocol, because we did not want the DNA sample to become too diluted.

Spectrophotometry

The DNA concentration and purity were determined using a NanoDrop spectrophotometer. The samples were scanned in the spectral range from 230- 320 nm wavelength, and the NanoDrop then measured the amount of light that each sample absorbed. The NanoDrop spectrophotometer was first calibrated with 2 µL of the nuclease free water used when DNA was extracted, then a 2 µL sample was analyzed, and the result was recorded. Each sample was analyzed, and the NanoDrop was cleaned with a tissue after each analysis.
The NanoDrop spectrophotometer was used to determine if the DNA samples were sufficiently pure for further analysis and amplification by PCR. This was done by measuring the 260/280 and 260/230 nm ratios. Nucleic acids have an absorbance maximum at 260 nm and a steep drop in absorbance at 280 nm. A pure DNA sample is indicated by an absorbance

ratio between 1,8-2,0. If there is contamination this ratio will be below 1,8. The 260/280 is an absorbance ratio that is a good indicator of protein contamination or contamination by reagents used in the extraction protocol. The 260/230 absorbance ratio is an indicator of contamination, most likely by organic compounds, if below 1,8 (Asami, 2015; Thermo Scientific, 2012).

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a laboratory technique used to increase the number of copies of a segment of DNA or other genetic material. The number of copies increases exponentially, as each DNA-strand is separated (by heat, denatured) and synthesized into two new, identical strands (Britannica, 2021) (NCBI, s.a.). In this study PCR were used to amplify the bacterial DNA for sequencing.

In the present study the 16S rRNA gene was targeted to be amplified, using 27F and 1492R primers, because the 16S rRNA gene contains hypervariable regions that depicts a specific sequence distinct to a bacterial species (Britannica, 2020).

<u>Components of PCR:</u>

- DNA template, the purified DNA from our bacterial isolates. The DNA contains the target sequence, the sequence of interest.
- DNA polymerase, an enzyme that synthesizes the new strands of DNA
- Forward and reverse primer, short sequence of nucleotides complementing the 3'-end of the two strands from the target sequence. The primers provide an initiation site where the DNA polymerase can bind.
- Nucleotides (dNTPs), the "building blocks" used to make new copies of DNA. A mixture of the bases adenine (A), guanine (G), cytocine (C) and thymine (T).
- Buffers, making an ideal chemical environment for DNA-polymerase activity.

100 ng of each DNA-sample were diluted in sterile water to a total volume of 10 µL. The amount of sterile water and DNA were calculated in this way:

100/ (x ng/µL DNA) = µL of DNA sample

10 µL - x µL of DNA sample = µL of sterile water

See appendix V for results of calculations. If the amount of DNA sample exceeded 10 µL, only 10 µL of the sample and no sterile water were put into the tube. Eight by eight tubes were filled to avoid evaporation. Then the tubes were spun for approximately 5 seconds and put into a bucket of ice also to avoid evaporation.

The rest of the PCR-components were mixed. 0,5 µL of 27F primer (F: 5'-AGA GTT TGA TCA TGG CTC A-3)', 0,5 µL of 1492R primer (5'- TAC GGT TAC CTT GTT ACG ACT T-3') and 10 µL of mastermix. The mastermix used contained $MgCl_2$-buffer, Taq DNA polymerase and nucleotides. The mixture was then diluted with 11,5 µL of sterile water to make the mixture the total volume of 22,5 µL. To make enough for all the samples, we made 30 times the amount.

22,5 µL of the mixture and 2,5 µL of the diluted DNA is put into a tube, and carefully mixed using a pipette. The samples were placed in a PCR machine, and the DNA was amplified using the protocol shown in table 8.

Table 8. The thermal cycling protocol used for DNA-amplification with PCR.

Cycle step	Time (min:sec)	Temperature (C°)		Number of cycles
1	0:10	98	Initial denaturation	1
2	0:30	98	Denaturation	34
3	0:30	55	Annealing	
4	2:00	72	Extension	
5	5:00	72	Final extension	1

Gel electrophoresis

1 gram agarose ultrapure was added to 100 mL of 1xTAE-buffer and heated in a microwave at 720 watts for about 2 minutes until boiling, clear mixture, making 1 % agarose gel. When the gel reached 65 °C, 10 µL of a fluorescent DNA gel stain were added to the gel and gently mixed. The gel mixture was then poured into a gel tray with 2 x 14 lanes and left for 40 minutes.

Each DNA sample was mixed with 5 µL of DNA Gel Loading Dye (6X) from Thermo Scientific using a pipette. 230 µL of each sample were put into each well, starting from well number two. 50 µL of 0,1 µg/µL GeneRuler 100 bp Plus DNA Ladder from Thermo Scientific was loaded into the first well and used as both a positive control and reference as it consists of DNA fragments of known lengths. An electric current with the electric potential of 90 V was put on the gel for 40 minutes, making an electric field where the negatively charged DNA will move from the negatively charged end towards the positively charged end.

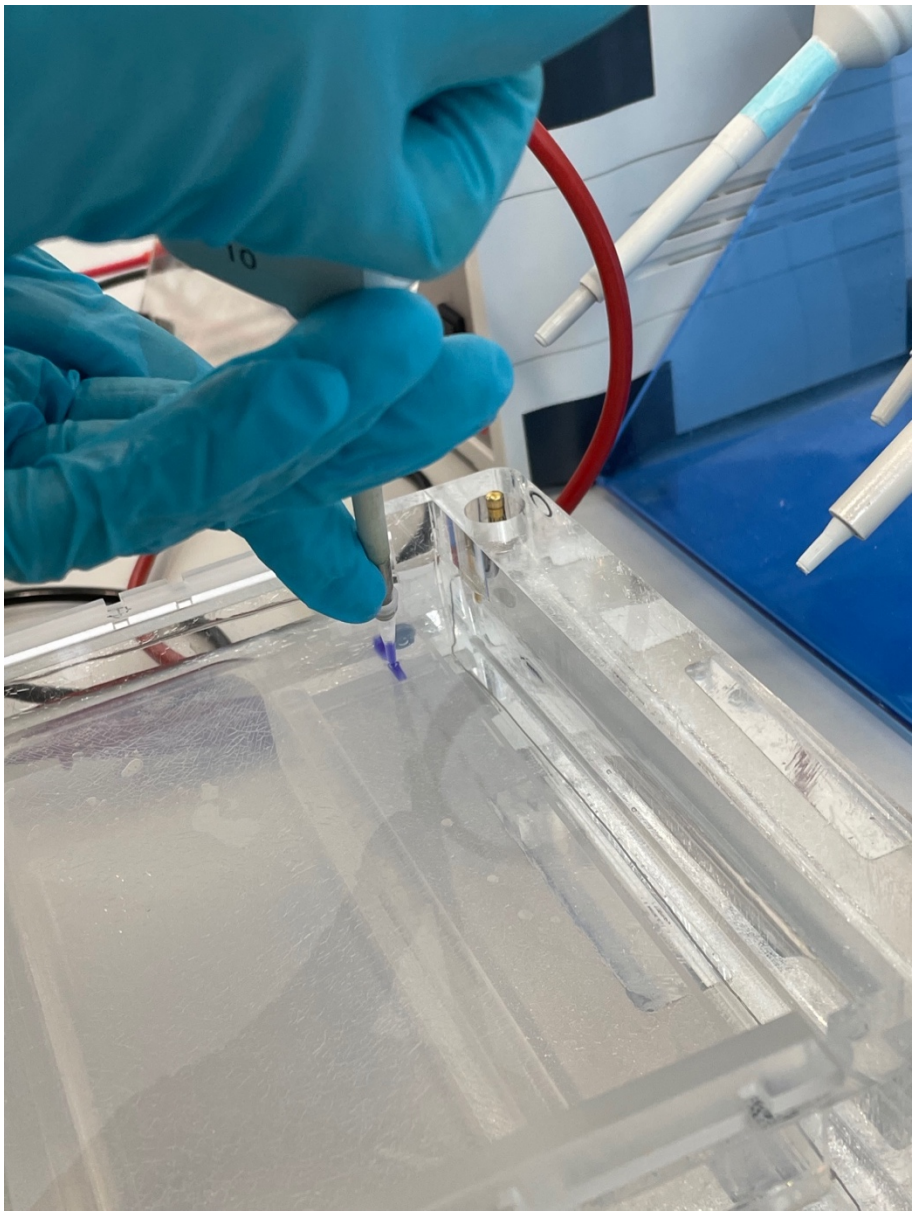

Figure 5. Filling the wells with DNA-sample using a sterile, single-use pipette. (Private photo)

DNA is negatively charged because the backbone of the DNA, made of deoxyribose-sugar and phosphate groups, is negatively charged. As the agarose gel is porous the DNA fragments will move through, and the bigger fragments (more base pairs) will move slower, and the shorter fragments (fewer base pairs) move faster. Therefore, the smallest fragments will be placed closer to the positive end and the larger fragments will be closer to the negative end after incubation (Rogers, 2017).

The bonds were visible under UV-light as the DNA was stained with fluorescent stain. The

gel was only exposed to UV-light for å short amount of time for us to see if the gel electrophoresis was successful, considering that UV-light alters the DNA-structure.

Each gel slice, containing DNA, was cut out with a sterile scalpel, and put into a tube. The gel slices weighing approximately 100 µg per piece, 300 µL of Buffer QE were added to each tube making a 3:1 buffer-gel-ratio. The tubes were then incubated at 50 °C for 10 minutes in a shaking incubator, until the gel slices were completely dissolved. Then 100 µL of isopropanol was added to each tube (1:1 isopropanol-gel-ratio). The dissolved gel was placed into a spin column in a collection tube and centrifuged at 10 000 RPM for 1 minute. The content of the collection tube was discarded, and the collection tube reused. 500 µL of Buffer QG was added to each spin column in collection tubes, and then the tubes were centrifuged at 10 000 RPM for 1 minute. To wash the samples, 750 µL of Buffer PE was added to each tube which were centrifuged at 10 00 RPM for 1 minute. The flow-through was discarded, and the tubes centrifuged at 10 000 RPM for 1 minute again. The spin columns were placed into new microcentrifuge tubes (1,5 mL). To elute the DNA 20 µL of nuclease free water (Ambion) were pipetted onto the center of the membrane in the spin column, and the tubes were centrifuged at 10 000 RPM for 1 minute.

The DNA-concentration in the tubes were then measured in a spectrophotometer, the value had to be between 10-50 ng/µL. Diluted primers (9:1 water-primer-ratio) and our DNA-samples were sent to EUROFINS in Germany for sequencing.

Interviews and observations (Questionnaire)

To gain a better understanding of tilapia farming in Zambia, we visited a total of five tilapia farms, one farm per day, and interviewed 1-2 workers at each production site. The farms we visited were handpicked by Prof. Bernard Hang'ombe, University of Zambia. Prof. Stephen Mutoloki and Prof. Øystein Evensen from NMBU joined us for all visits. The farms included one hatchery and four commercial farms. Among the commercial farms, one bought fingerlings, while the remaining three had their own hatchery or their own way of collecting fry. To protect the privacy of the fish farms, we have replaced the farm's actual names with numbers from 1- 5.

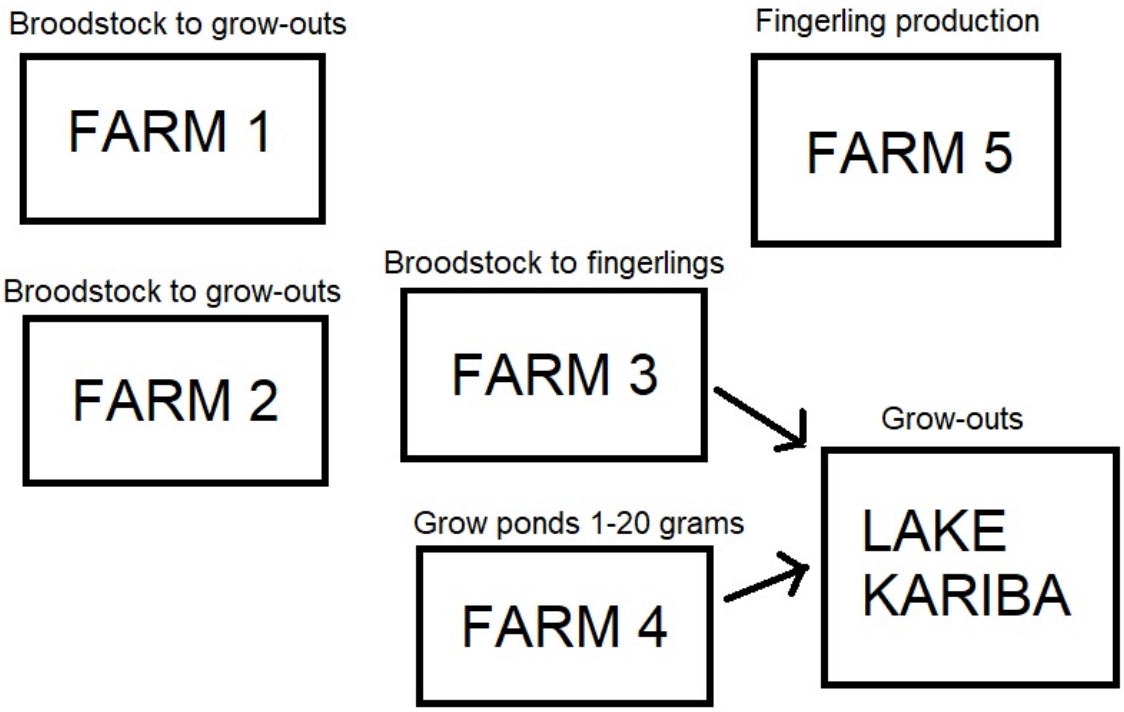

Figure 6. The relationship between the five different farms visited for our study.

We had a standardized list of questions (prepared prior to the visits) that we went through at each production site. Before going through the questionnaire with the managers, we toured the farm guided by one of the farm workers/managers. During the presentation of the farm, the information provided covered the questions prepared. We also experienced that we had to ask the same question more than once, since the respondent did not provide sufficient details or too short answers were given. We experienced that repeating the question, changed their responses, probably because they had more time to think through what was actually asked or that they better understood the question when it was repeated. We asked open questions to the extent possible, to avoid putting words in their mouths, with an aim to provide explanations rather than yes/no responses. The questions covered the entire production chain, so we had to drop some questions not relevant for the different farms. The questionnaire is attached as Appendix I.

Statistical analysis

An interrater agreement test was used to compare outcome of Maldi-Tof and 16S sequencing using Stata17.

Results

Laboratory work

Gram staining

Only 13 slides were stained, and only 5 of them were examined under a microscope. See table 9 for results.

Table 9. The results of the gram staining.

Sample number	Sample name	Gram positive or negative	Shape
1	3A	Gram negative*	Short rods (coccobacillus)
3	34A	Gram positive	Cocci
7	W2-A	Unreadable*	Short rods (coccobacillus)
11	21A	Gram positive	Short rods (coccobacillus)
14	25D	Gram negative*	Rods

*weak staining

Biochemical characterization

The results from API 20 E and API 20 NE were non-conclusive and did not provide any conclusive results as to what genus or species for the different bacterial isolates. We did not find any known genus or species of bacteria fitting into the results. The results from API 20 E, and API 20 NE are shown in the tables 10 and 11 below.

Table 10. Results from API 20 E. pos = positive reaction, neg = negative reaction

Sample number		2	6	18
Sample name		30B	3C	23A
Reactions	ONPG	pos	pos	pos*
	ADH	neg	neg	neg
	LDC	neg	neg	neg
	ODC	neg	neg	neg
	CIT	neg	neg	neg
	H_2S	neg	neg	neg
	URE	neg	neg	neg
	TDA	pos*	pos*	neg
	IND	neg	neg	neg
	VP	pos	pos	pos
	GEL	pos	pos	pos
	GLU	neg	pos	neg
	MAN	neg	neg	neg
	INO	neg	neg	neg
	SOR	neg	neg	neg
	RHA	neg	neg	neg

	SAC	pos*	neg	neg
	MEL	pos	neg	neg
	AMY	pos*	neg	neg
	ARA	neg	neg	neg

*Slightly positive

Table 11. Results from API 20 NE. neg= negative reaction, pos= positive reaction

Sample number		1	8	9	11	14	15	16	20
Sample name		3A	25B	9B	21A	25D	35C	2A	14B-2
Reactions	NO_2	neg	neg	neg	neg	neg	neg	neg	neg
	N_2	neg	neg	neg	neg	neg	neg	neg	neg
	TRP	neg	neg	neg	neg	neg	neg	neg	neg
	GLU	neg	neg	neg	neg	neg	neg	neg	neg
	ADH	neg	neg	neg	neg	neg	neg	neg	neg
	URE	neg	neg	neg	neg	neg	neg	neg	neg
	ESC	pos	pos	pos	pos	pos	pos	pos	pos**
	GEL	pos	pos	pos	pos	pos	neg	neg	pos
	PNPG	pos	pos	pos	pos	pos	neg	neg	neg
	GLU	neg	neg	neg	*	neg	neg	pos	neg
	ARA	***	***	***	*	***	neg	neg	***
	MNE	***	***	***	neg	***	neg	neg	***
	MAN	***	***	***	neg	***	neg	neg	***
	NAG	***	***	***	neg	***	neg	neg	***
	MAL	***	***	***	pos**	***	pos	pos	***
	GNT	***	***	***	pos**	***	neg	pos	***

	CAP	***	***	***	neg	***	neg	neg	***
	ADI	***	***	***	neg	***	neg	neg	***
	MLT	***	***	***	*	***	neg	neg	***
	CIT	***	***	***	*	***	neg	neg	***
	PAC	neg	neg	neg	*	neg	*	neg	neg

* Dried out after 48 hours
**Slightly positive
*** Misunderstanding, did not test

Maldi-Tof

Out of 22 samples analyzed by Maldi-Tof, 9 samples came back with inconclusive results. Eight samples came back as *Exiguobacterium acetylicum* and one as *Exiguobacterium aurantiacum*. Two samples were from the *Aeromonas*-family, but genus could not be determined. One sample was a species from *Brevibacillus*-family, and one sample was *Bacillus idriensis*. See Table 12 for complete results.

Table 12. Results from Maldi-Tof

Number	Well number	Sample name	Result	Confidence level
1	E1	3A	*Exiguobacterium acetylicum*	99,9 %
2	E2	30B	*Exiguobacterium acetylicum*	99,9 %
3	E3	34A	*Brevibacillus spp.*	98,9 %
4	E4	24A	*Exiguobacterium acetylicum*	99,9 %
5	F1	18A	*Exiguobacterium acetylicum*	99,9 %
6	F2	3C	*Exiguobacterium acetylicum*	99,9 %
7	F3	W2-A	*Exiguobacterium aurantiacum*	99,9 %
8	F4	25B	*Exiguobacterium acetylicum*	99,9 %
9	G1	9B	None	
10	G2	38A	*Bacillus idriensis*	99,9 %
11	G3	21A	None	
12	G4	30D	*Exiguobacterium acetylicum*	99,9 %
13	H1	27A	*Aeromonas veronii/Aeromonas sobria*	50 %
14	H2	25D	None	
15	H3	35C	None	
16	H4	2A	None	
17	I1	28C	*Aeromonas sobria/Aeromonas veronii/Aeromonas jandaei*	33,3 %
18	I2	23A	*Exiguobacterium acetylicum*	99,9 %
19	I3	4A	None	
20	I4	14B-2	None	
21	J1	1C	None	
22	J2	40A	None	

Genomic DNA isolation and spectrophotometry

After DNA isolation, the spectrophotometer showed that 9 out of 22 samples had a 260/280 ratio over 1,8. The 260/280 is an absorbance ratio used for determining how pure the sample is, and degree of protein contamination. For DNA a ratio of at least 1,8 is generally accepted as a pure sample, as mentioned in material and methods.

For PCR we needed a total concentration of 10 ng/µL. Most of the samples had a concentration over 10 ng/µL and could be diluted. Four of 22 samples had a concentration under 10 ng/µL, meaning the total concentration of the sample was acceptable for our use but

not ideal. The low 260/280 ratio of the nine samples was acceptable because they were of such high concentration. See table 13 for full results.

Table 13. Showing results from the spectrophotometry.

Number	**Sample**	**ng/µl**	**A260**	**A280**	**260/280**	**260/230**
1	3A	10,86	0,217	0,104	2,09	0,70
2	30B	63,73	1,275	0,950	1,34	0,68
3	34A	12,69	0,254	0,139	1,83	0,67
4	24A	40,44	0,809	0,620	1,30	0,69
5	18A	87,03	1,741	1,349	1,29	0,72
6	3C	42,79	0,859	0,616	1,39	0,71
7	W2-A	216,66	4,333	2,262	1,92	1,96
8	25B	167,05	3,341	2,566	1,30	0,75
9	9B	117,63	2,353	1,870	1,26	0,74
10	38A	10,42	0,208	0,117	1,79	0,58
11	21A	6,84	0,137	0,055	2,49	0,47
12	30D	51,39	1,028	0,783	1,31	0,68
13	27A	152,58	3,052	1,571	1,94	1,88
14	25D	39,18	0,784	0,600	1,31	0,67
15	35C	38,40	0,768	0,497	1,54	0,69
16	2A	37,35	0,747	0,454	1,64	0,64
17	28C	186,48	3,730	1,928	1,93	1,84
18	23A	32,33	0,647	0,455	1,42	0,65
19	4A	5,47	0,109	0,045	2,43	0,65
20	14B-2	3,99	0,080	0,033	2,42	0,48
21	1C	5,17	0,103	0,041	2,54	0,57
22	40A	57,77	1,155	0,795	1,45	0,68

Polymerase chain reaction (PCR) and gel electrophoresis

After PCR and gel electrophoresis with 27F primer and 1492R primer, every sample except number 7 (sample W2-A) showed clear DNA bands in the agarose gel. The remaining bands is roughly 1000 base pairs in size. See table 14 and figure 7.

Table 14. Showing the placement of the samples in the different wells in the agarose gel during gel electrophoresis. The one highlighted in grey is sample number 7 (sample W2-A) in well 8 on the upper row, the one sample that did not show a band after gel electrophoresis.

Upper row															
Well number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Sample number	Ladder	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sample name		3A	30B	34A	24A	18A	3C	W2-A	25B	9B	38A	21A	30D	27A	25D
Lower row															
Well number	1	2	3	4	5	6	7	8	9						
Sample number	Ladder	15	16	17	18	19	20	21	22						
Sample name		35C	2A	28C	23A	4A	14B-2	1C	40A						

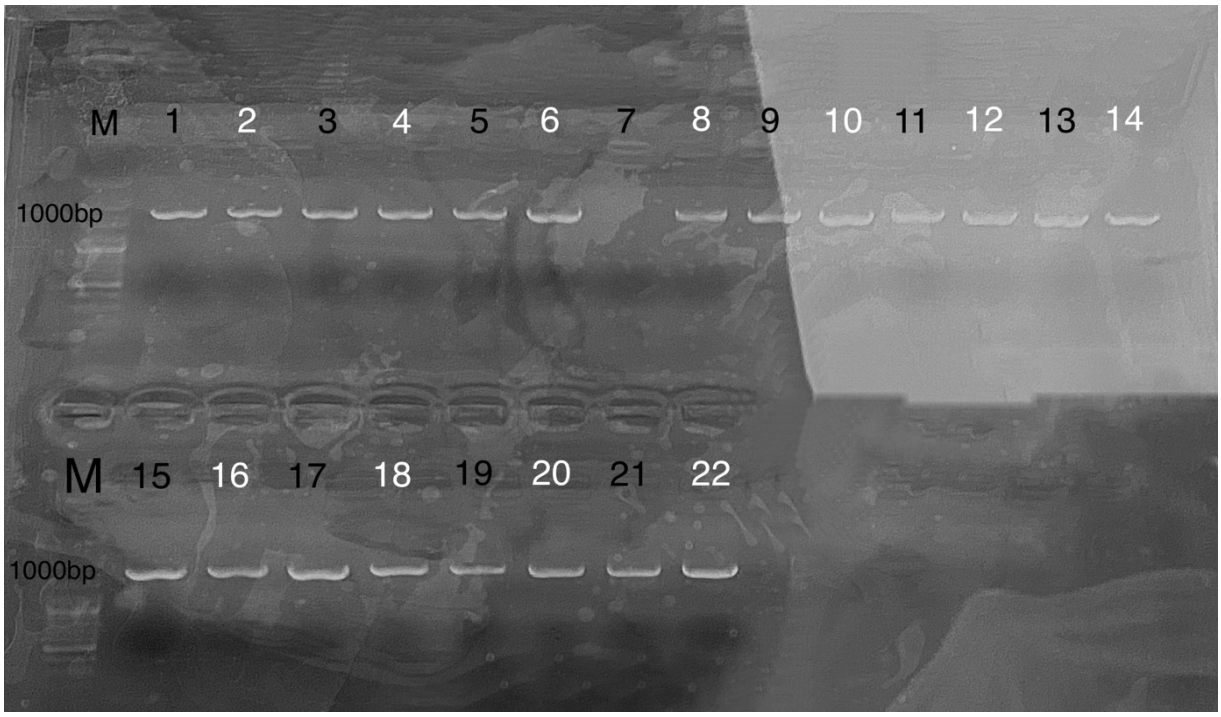

Figure 7. Results after PCR and gel electrophoresis. Every well except number 8 on the upper row has visible bands. Well number 8 is sample number 7 (sample name W2-A).

Sequencing results

All the samples were sent for DNA sequencing (at Eurofins), and results were obtained for all samples except sample number 7 (sample name W2-A). The results from the gene sequencing at Eurofins are slightly different from the results from the analysis with Maldi-Tof. See table 15 for comparison of the results and appendix IX for full results from the DNA sequencing.

Table 15. Comparison of the results from Maldi-Tof analysis and gene sequencing at Eurofins. The samples that had different results from the two methods are highlighted in grey, except the samples where one method gave inconclusive results.

Sample number	**Sample name**	**Results from Maldi-Tof**	**Results from gene sequencing at Eurofins**
1	3A	*Exiguobacterium acetylicum*	*Exiguobacterium acetylicum*
2	30B	*Exiguobacterium acetylicum*	*Exoguobacterium indicum*
3	34A	*Brevibacillus spp.*	*Macrococcus equipercicus*
4	24A	*Exiguobacterium acetylicum*	*Exiguobacterium acetylicum*
5	18A	*Exiguobacterium acetylicum*	*Exiguobacterium acetylicum*
6	3C	*Exiguobacterium acetylicum*	*Exiguobacterium acetylicum*
7	W2-A	*Exiguobacterium aurantiacum*	None
8	25B	*Exiguobacterium acetylicum*	*Exiguobacterium acetylicum*
9	9B	None	*Exiguobacterium acetylicum*
10	38A	*Bacillus idriensis*	*Bacillus indicus*
11	21A	None	*Exiguobacterium acetylicum*
12	30D	*Exiguobacterium acetylicum*	*Exiguobacterium acetylicum*
13	27A	*Aeromonas veronii/Aeromonas sobria*	*Aeromonas veronii*
14	25D	None	*Exiguobacterium indicum*
15	35C	None	*Fictibacillus nanhaiensis*
16	2A	None	*Fictibacillus* sp.
17	28C	*Aeromonas sobria/Aeromonas veronii/Aeromonas jandaei*	*Aeromonas jandaei*
18	23A	*Exiguobacterium acetylicum*	*Exiguobacterium indicum*
19	4A	None	*Fictibacillus* sp.
20	14B-2	None	*Bacilllus indicus*
21	1C	None	*Staphylococcus epidermis*
22	40A	None	*Planomicrobium* sp.

A simple interrater agreement test was carried out and the results are shown in table 16. As can be seen, the agreement between the two methods with regard to species identification is fair to moderate, percent agreement being 40.9% with a kappa value of 0.28 (Cohen's kappa), p=0.016 indicating that the methods differ significantly.

Table 16. Results from interrater test, comparing agreement of the two methods regarding species identification.

```
. kappaetc toff pcrr

Interrater agreement                            Number of subjects =      22
                                                  Ratings per subject =       2
                                       Number of rating categories =      14
```

	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
Percent Agreement	0.4091	0.1073	3.81	0.001	0.1860	0.6322
Brennan and Prediger	0.3636	0.1155	3.15	0.005	0.1234	0.6039
Cohen/Conger's Kappa	0.2814	0.1078	2.61	0.016	0.0572	0.5056
Scott/Fleiss' Pi	0.2533	0.1200	2.11	0.047	0.0037	0.5028
Gwet's AC	0.3708	0.1156	3.21	0.004	0.1304	0.6112
Krippendorff's Alpha	0.2702	0.1200	2.25	0.035	0.0207	0.5198

```
     Benchmark scale

           <0.0000      Poor
     0.0000-0.2000      Slight
     0.2000-0.4000      Fair
     0.4000-0.6000      Moderate
     0.6000-0.8000      Substantial
     0.8000-1.0000      Almost Perfect
```

When compared at bacterial genus level, the agreement between the tests increased, with percent agreement of 50%, table 17, *i.e.,* moderate agreement but with relatively low kappa values.

Table 17. Results from interrater test, comparing agreement of the two methods regarding genus identification.

```
. kappaetc tt pp

Interrater agreement                            Number of subjects =      22
                                                  Ratings per subject =       2
                                       Number of rating categories =      14
```

	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
Percent Agreement	0.5000	0.1091	4.58	0.000	0.2731	0.7269
Brennan and Prediger	0.4615	0.1175	3.93	0.001	0.2172	0.7059
Cohen/Conger's Kappa	0.4039	0.0915	4.41	0.000	0.2136	0.5943
Scott/Fleiss' Pi	0.3665	0.1121	3.27	0.004	0.1334	0.5996
Gwet's AC	0.4677	0.1179	3.97	0.001	0.2224	0.7130
Krippendorff's Alpha	0.3809	0.1121	3.40	0.003	0.1478	0.6140

The samples tested originated from different farms (farm 1, 2 and 4), and from water (ponds or boxes used for transport of fish) or fish. As seen from Table 18 below, *Exiguobacterium* spp. were identified water (from ponds) and fish. There are no obvious differences between different farms. *Aeromonas* spp. originate from fish and are not found in water.

Table 18. Bacterial species detected from different sites/part of the transport, environments and in water or fish based on PCR results.

Bacteria	Site	Enviro~t	Farm
Exiguobacterium acetylicum	Pond	Water	Farm 1
Fictibacillus sp.	Transport start	Water	Farm 1
Staphylococcus epidermis	Pond	Water	Farm 1
Exiguobacterium acetylicum	Pond	Water	Farm 1
Fictibacillus sp.	Pond	Water	Farm 1
Exiguobacterium acetylicum	Pond	Water	Farm 1
None	Water	Water	Farm 1

Bacteria	Site	Enviro~t	Farm
Exiguobacterium indicum	Transport end	Fish	Farm 2
Aeromonas jandaei	Transport end	Fish	Farm 2
Exoguobacterium indicum	Transport end	Fish	Farm 2
Aeromonas veronii	Transport end	Fish	Farm 2
Exiguobacterium acetylicum	Transport box	Fish	Farm 2
Exiguobacterium acetylicum	Transport end	Fish	Farm 2
Exiguobacterium indicum	Transport end	Fish	Farm 2
Exiguobacterium acetylicum	Transport end	Fish	Farm 2
Exiguobacterium acetylicum	Transport end	Fish	Farm 2

Bacteria	Site	Enviro~t	Farm
Planomicrobium sp.	Post transport	Water	Farm 4
Fictibacillus nanhaiensis	Post transport	Water	Farm 4
Exiguobacterium acetylicum	Before arrival	Fish	Farm 4
Bacillus indicus	Post transport	Water	Farm 4
Bacilllus indicus	Before arrival	Fish	Farm 4
Macrococcus equipercicus	Post transfer	Water	Farm 4

Interviews and observations from farms visited

By our definition, the farms we visited in Zambia are intensive small to large commercial farms, but questions can be raised as whether they are to be defined as intensive or semi-intensive. All farms have monoculture tilapia, which according to Hecht (2007) is one of the main criteria that defines intensive tilapia farming. All visited farms fed commercial feeds (except one farm making their own pellets), and the fish was fed with the same feeding regime every day.

See table 19 for summary of results from interviews and observations done at the different farms.

Table 19. Summary of results from interviews and observations done at the different farms.

Farm	**Production**	**Number of employees**	**Species kept at the farm and sold**	**Production system**	**Feed used**
Farm 1	Small-scale, commercial farm	35	Nile tilapia and Catfish	Ponds	Commercial feed – Aller Aqua Feed
Farm 2	Medium-scale, commercial farm	242	Nile tilapia, pigs and cattle	Ponds	Make their own feed
Farm 3	Large-scale, commercial farm	90	Nile tilapia	Cages	Commercial feed (not specified)
Farm 4	Medium-scale, commercial farm	44	Nile tilapia	Cages	Commercial feed – Skretting
Farm 5	Medium-scale hatchery	28	Nile tilapia, goats and sheep	Cages	Commercial feed – Aller Aqua Feed.

Farm 1

Farm 1 is a small-scale commercial farm located near Kafue town, close to Kafue River. It was established over 20 years ago and has been producing Nile tilapia *(Oreochromis niloticus)* since the start. There are 15 permanent workers at the farm, and 20 seasonal workers, a total of 35 employees. In 2019 they had a total production of 17 tons of tilapia.

Figure 8. Hapas in pond at farm 1. (Private photo)

They had some problems in 2020 with environmental contamination of the ponds, likely due to heavy rain. This resulted in 2020 being a bad production year for Farm 1. Last year they introduced catfish into their production, and now they are breeding, producing, and selling both tilapia and catfish at the site. They started putting catfish into the grow-out ponds as they had problems with tilapia breeding in those ponds, causing a population of different sized tilapia in the same pond. Different sized fish in the same population often results in the fish growing less than it would if all the fish were the same size. By introducing catfish to the ponds, they found an easy and effective way of getting rid of fry that hatched in the ponds (catfish will predate on tilapia fry).

The interview was done with two workers. One of them had worked there for 2 years, and the other one for 3 years, both daily. Farm 1 has their own tilapia and catfish broodstock, and their own on-site hatchery. The farm gets its water from a dug out “arm” of Kafue River, and

the water is not treated before pumped into the ponds. Before the water enters the hatchery, it is cleaned by a simple gravel filter. Oxygen is also added to the hatchery water. Further, they add salt to the water (3-4 ppm) that is used to clean the eggs. After hatching and when the fry has absorbed the yolk sac, the fry is transferred into small hapas in small ponds. Farm 1 admitted using oral antibiotics (Oxytetracycline) prophylactic to prevent bacterial infections the first 21 days after transferring to hapas. The fry is also sex reversed using methyltestosterone over a period of 21 days, and during this period, antibiotic treatment is also used. The fry stays in the small start-feeding hapas until they reach a size around 1-2 grams. Then they are transferred into large ponds (150 x 50 meter). The fish stay in these ponds for around 6 months until they are ready to be harvested. The tilapia at Farm 1 is harvested and sold at around 780 grams.

Farm 1 had a challenge with some of the fry getting sick in the small hapas. In 2020 they had a disease outbreak where they observed fry with abnormal circular swimming patterns and dark pigmentation. They didn't treat the sick fry in any way, they just removed all the fry, emptied the pond, applied lime and let it stay dry for a year before fertilizing and introducing new fish to the pond. One year later, during our field study, the new fry began to show the same clinical signs as the year before.

Farm 2

Farm 2 is a medium-scale commercial farm located right next to Kafue River, about one and a half hours drive from Lusaka. The farm was first established in the 1970s and has a yearly production of 1200 tons of Nile tilapia. There are around 242 workers on site. The owner of the farm was the one showing us around and he was the person we interviewed. The owner likes to say that they do "plankton farming", because one of the main food sources of tilapia is zooplankton. To increase the amount of zooplankton in the water, it is necessary to fertilize the ponds. Most farmers use different kinds of bought fertilizer, but this farmer has found his

own way of fertilizing his ponds. He has placed a pig farm with 25 000 pigs next to the lake, with large pipes bringing the pig feces from the barn into the ponds, allowing easy fertilization of the water in the ponds. He has placed some single pig bins around the intake water as well, and in this way, he is able to fertilize some of the water before it has even entered the production site.

Farm 2 uses water from the Kafue River that is cleaned through a natural type of biofilter system. The way this works is by natural filtration of river water thanks to a man-made sandbar next to the river's flood plain. This sandbar works as a large-scale gravel filter, that cleans the water passing from Kafue River and the flood plain, through the sandbar and into a large wetland area. The wetland area is filled with different types of weed and plants that also play a role in cleaning the water. From this wetland area water flows in rivers into the farmland area and is in this way distributed to the entire farm. Water is then pumped from these small rivers into the ponds when needed.

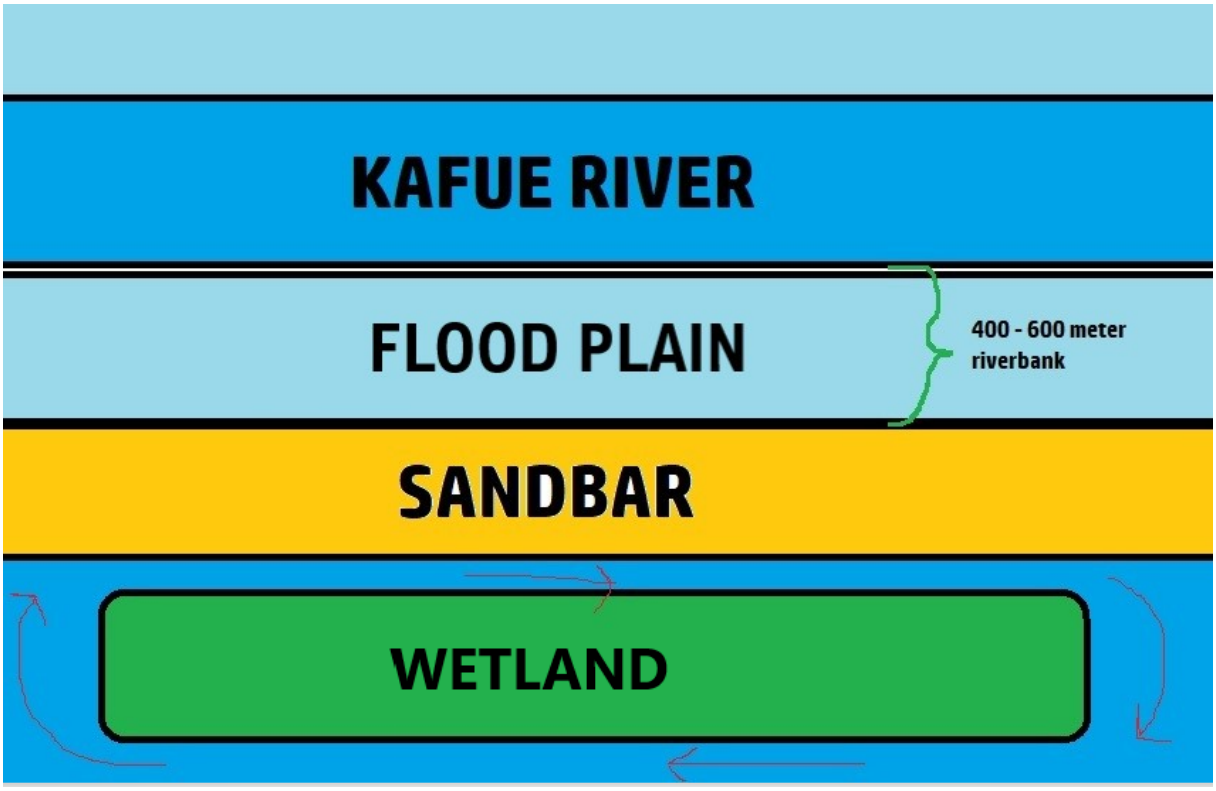

Figure 9. Showing the natural biofilter that has been built right next to the Kafue River.

Farm 2 has its own broodstock consisting of normal XX females and imported YY males imported from Stirling University, UK, resulting in the offspring being all males. This means they do not use hormones for sexual reversal of the fry. They let the broodstock reproduce naturally in the ponds and collect eggs from the females mouths and transfer the fertilized eggs into the hatchery. Extra sperm is added for possibly increasing the fertilization percentage. After the eggs have hatched and the fry have absorbed the yolk sac, they transfer the fry out into large plastic tanks (d = 2 meters, h = 1 meter, cylinders) for start feeding. They stay there until ready to be transferred into large ponds (details of weeks/months not given). All the fish in the same pond are harvested and sold on day 270. At this time the fish variates in size, but all the fish is sold at the local marked anyway.

Figure 10. Plastic tanks where the fry stays after absorbing the yolk sac. (Private photo)

Farm 3

Farm 3 is a large-scale commercial farm with 90 employees. The farm is located a 40 km drive away from Siavonga and has been producing Nile tilapia for the last decade. They have a monthly production of 12 million fingerlings, and harvest 40 tons of full-grown tilapia daily 6 days per week (12480 tons per year). Farm 3 gets their water from Lake Kariba and the water is not treated prior to pumping it into the ponds. The interview was done with one of the fish health managers as well as the safety manager on site. Farm 3 have their own broodstock, which they in the cold season mix in the ratio 3:1 females to males in a breeding hapas. In the warm season they use a 2:1 ratio of females to males. They use natural breeding and collect fry directly from the pond using a small-masked net that they drag around the sides of the ponds. Using this method, they can collect around 700 000 fry every single day. The fry is sorted by size, and the largest ones are put down, because they are past the point where sex reversal is possible. The fry with similar size is put into the same tank (1 x 1 x 1 meter), and feed is introduced. The average fry size at this step is around 0,02 grams. After staying in these start feeding tanks for a while, the largest fry swims to the surface, and these are the female fry. The female fry is collected, and half of them are put into a pond for sex reversal, while the other half is put into a different pond as part of the farm's breeding program. When they consider the fry to be big enough, they transfer them from the tank into an open pond. When the fingerlings in the ponds reach 4 grams, they are collected

Figure 11. Sorting of fry by size. (Private photo)

by using a fishing net, and are then transferred by car down to Lake Kariba where they are put into cages (10 x 10 meters), and harvested when they reach slaughter size, around 400 - 600 grams.

Farm 4

Farm 4 is a medium-scale commercial farm located around one hour drive from Siavonga, not so far from where Farm 3 is located. The farm produces Nile tilapia and has 44 employees. They harvest around 8 tons of tilapia daily, five times a week (2080 tons per year). We interviewed one of the female workers that had been working there for six months. The farm was built in the 1980s and has been producing fish ever since. There is no broodstock or hatchery on site, the farm buys 1-gram fingerlings from a sister company located 80 minutes away by boat (on the Zimbabwean side of Lake Kariba). When the fingerlings arrive at the farm they are put into juvenile cages (400 000 fish/cage), where they stay until they reach a weight of around 20 grams. How long it takes for the fingerlings to reach 20 grams depends on management, feeding and temperature. In the summer it takes around 20 weeks for them to reach this weight, while it can take up to 25 weeks in the winter. When the fish reach 20 grams, they are transferred into cages in Lake Kariba. The fish are harvested at around 450 grams, since the best marked price is achieved at this size.

Figure 12. Cages on Lake Kariba. (Private photo)

Farm 5

Farm 5 is a medium-size hatchery, and the only hatchery we visited in Zambia. Farm 5 produces and sells fingerlings and has 28 workers in total. They first started their production in 2017 and produce and sell 2 million Nile tilapia fingerlings at 1-2 grams each month. We interviewed two workers, who had worked at the farm from the start. The hatchery and farm in general get their water from the Kafue River. The water is not treated before being pumped into the ponds, but they change the pond water every two weeks. The water is cleaned prior to being pumped into the hatchery. They have an outdoor biofilter consisting of a gravel filter made of small rocks, sand, and charcoal, as well as a part that cleans the water thanks to UV-rays from the sun. They monitor some of the water parameters, like the oxygen level, both inside the hatchery and in the ponds.

Figure 13. View over farm 5. (Private photo)

Farm 5 produces its own broodstock, which they allow to breed naturally in the ponds in a ratio of 3:1 females to males. They collect eggs manually by catching the females and holding them upside down. A finger is used to open the female's mouth, and gently shake the eggs out of the mouth and into a bucket. They collect an average of 400-500 eggs per female broodstock. The harvested eggs are taken into the hatchery where they are cleaned with formalin (1 mL/L water), followed by water with salt (5 ppt). After being cleaned, the eggs are weighed and sorted after what stage they are in. Eggs in stage 1 and 2 are yellow-orange, while eggs in stage 3 are orange. Well-developed eggs and fry with some yolk sac remaining, count as stage 4. The staging is done quickly and manually, and the eggs in the same stage are put into the same batch and tank. When the eggs have hatched, they put the entire batch into a box that can hold up to 10 000 fry and wait for them to use up their yolk sac. After the yolk sac has been absorbed, the fry is moved out into an open pond, where they stay for 2 weeks until they reach the size of 1-2 grams, at which size they are ready to be sold. Farm 5 talked about Saprolegnia outbreaks they sometimes face in the fingerling ponds, often seen after handling the fish. They don’t treat the affected fish; they just move the healthy-looking fishes to a new pond and get rid of the sick ones before draining, applying lime, and drying the old pond.

Discussion

Bacterial infections, along with viral, parasitic and fungal infections, play an important role when it comes to infectious diseases and disease outbreaks in tilapia farming (Abdel-Latif et al., 2020). It is important for the farmers to know about presence of different disease-causing pathogens. This may be helpful for preventing disease as well as foresee the consequences of a potential disease outbreak. Another advantage of farmers being familiar with different bacteria and diseases is that they can take measures to make the impacts of an outbreak as small as possible.

Laboratory findings

Through this study we were able to obtain a more detailed characterization of the bacteria detected in the samples. We kept the focus on describing the potential disease-causing bacteria in fingerlings and fry in the early stage of the production. The bacteria we found in our samples that could be pathogenic to tilapia are *A. veronii*, *A. jandaei* and *Staphylococcus epidermidis* (Dong et al., 2017; Huang et al., 1999; Raj et al., 2019). We also found several other bacteria like *Exiguobacterium acetylicum*, *Exiguobacterium indicum*, *Fictibacillus nanahaiensis*, *Fictibacillus sp*., *Bacillus indicus*, *Macrococcus equipercicus* and *Planomicrobium sp.* We believe these are non-pathogenic to tilapia as we could not find any sources describing them as disease-causing bacteria. Most of our samples contained *Exiguobacterium* which is strange considering that members of this genus have been isolated from remarkably diverse habitats from glacial ice to thermal springs (Miller & Whyte, 2011). *Exiguobacterium acetylicum* may anyway have a probiotic and bioremediation application in aquaculture (de Mello Júnior et al., 2021).

Our findings cannot be directly linked to the farms we visited, as we don't know from which farms the samples were collected and they were also collected two years back under a production setting that likely differed from what is the practice today.

We cannot draw any firm conclusions as regards the pathogenic potential of the bacteria we detected and if this can be linked to increased mortality in the farms. However, the abdominal cavity is usually sterile, and the anticipation is that bacteria should not be present. It is interesting that the same bacterial species were found in the environment as in fish samples. Potentially pathogenic bacteria may cause disease if the host is more stressed/susceptible than normal (Dong et al., 2017). A stressor in the environment might be a logical explanation for this.

As mentioned, we did detect potentially pathogenic or disease-causing bacteria in some of the samples, *Staphylococcus epidermidis* in pond water and two *Aeromonas* spp. in fish samples. This may explain the increased mortality in the early production phase, seen on the farms where the samples were collected from.

Sources of error

Among the biochemical characteristics and molecular methods, we did notice some differences. The API systems are designed for bacteria grown at 37 °C and thus deviations may occur when lower incubation temperatures are used or when testing bacteria not having optimal growth at 37 °C.

Gram staining was hard to interpret and trust as we did not have the opportunity to heat-fixate our samples before staining them. We chose not to Gram-stain all the samples because of time restrictions and because we were going to characterize them in more detail later. The results from the Gram staining showed that the shape of the bacteria was correct. All the bacteria should be presented as Gram-stain-positive, however sample number 1 and 14 had Gram-negative staining.

We chose to do API 20 E and API 20 NE on some of the samples to gain more laboratory experience. Sample number 2, 6 and 18 were selected for API 20 E, and sample number 1, 8, 9, 11, 14, 15, 16, 20 were selected for API 20 NE biochemical characterization. However, the test results that should be identical, came back as different. Even though bacteria number 2 and 18 contained the same bacteria (*Exiguobacterium indicum*), the API 20 E showed a different TDA result between the two samples. Bacteria number 2 showed a positive TDA result, while bacteria number 18 showed a negative TDA result although both samples contained *Exiguobacterium indicum*.

Sample number 1, 8, 9 and 11 were all *Exiguobacterium acetylicum* and should be identical. However, the results we obtained differed, probably because of our misinterpretation of the test manual, which made it impossible for us to compare the results between sample 1, 8, 9 and 11 on these tests. In addition, we also got no results in GLU and PAC for sample 11, because these tubes dried out after 48 hours.

We could also see a differences in the results from the Maldi-Tof compared to the DNA sequencing. Sample 2, 3, 10 and 18 showed different results between the Maldi-Tof and the sequencing. For samples 9, 11, 14, 15, 16, 19, 20, 21 and 22 the Maldi-Tof results were not conclusive. The reason for this could be that the database, which the Maldi-Tof compared the bacterial analyses to, did not contain the mass-spectra of the bacteria in our samples. The Maldi-Tof is a sensitive analytical method that requires good technique and experience when preparing a Maldi-Tof slide. It is also important to avoid contamination it because contaminants may interfere with the Maldi-Tof process and complicate the mass-spectrum (bioMérieux-University, 2021)

This was our first attempt using the Maldi-Tof, and it is possible that we contaminated the samples unintentionally. However, still, the Maldi-Tof did analyze sample number 1, 4, 5, 6, 8, 9, 12, 13 and 17 correctly within hours showing that the Maldi-Tof has a great potential to

correctly characterize bacterial samples if the database is detailed enough. Running the tests more than once makes the result even more reliable.

During the preparations for the DNA sequencing, we experienced that some of the bacteria had properties that made them viscous during centrifugation. This led to clogging of the tubes, which caused problems with extracting the DNA but repeating this part of the laboratory work allowed us to get good results.

The results from the spectrophotometer showed us that there were good quantity and concentration of DNA in all the samples. In the gel electrophoresis we noticed that sample number 7 did not run through the gel as expected, even though the concentration was high. Therefore, we decided to cut out a piece of sample 7 at the 1000bp-band in the gel and sent it for sequencing, but it came back with no result.

Effects of increased mortality in the early production phase

Consequence of an increase in mortality in the hatchery

Eggs are vulnerable to Saprolegnia infection (Ali et al., 2019). All the hatcheries used salt in the water to prevent Saprolegnia and bacterial infections and none of the farms we visited had experienced any problems with mortality in the hatchery. If there was to be an increase in mortality in the hatcheries the consequences would be huge, because this is the first step of the production, and an increase in mortality here would affect the entire production chain. Mortality in the hatchery would lead to less fry surviving, which again results in lower numbers of fingerlings and less fish in the grow-outs. Lower number of fish at the production site would result in big economical losses, considering that there would not be any fish to feed, and the production volume would be suboptimal. For the farms collecting fry to use as broodstock, an increase in fry mortality will also result in less broodstock.

Consequence of an increase in mortality after the fry is transferred into hapas

After the fry is transferred to hapas for start feeding, a lot of time and work has already been invested during this early production phase. The hatchery in farm 5 had continuous supervision and farm 1 had one employee devoted to the hatchery during working hours. This means that the resources used in the hatchery will be lost if the fry dies after transport from the hatchery to hapas. This may be an argument for saying that mortality of the fry (after transport to the hapas) would have even bigger economic consequences. Time and resources have already been invested, without getting anything back in an event resulting in increased mortality. All the farms we visited had a vision of not using any kind of treatments or medications in the event of a disease outbreak. Farm 1 admitted using antibiotics, Oxytetracycline, prophylactic. This was to prevent bacterial infections from establishing after transferring the fry to small hapas. None of the farms admitted using antibiotics or other medications as a therapeutical substance. In case of an increase in mortality, all the farms would avoid treating the fish. This means that the only option would be moving the clinically healthy-looking fish to a new pond or stamping out the entire fish population in the sick pond. Both resulting in big economical losses. The impacts of less fish produced on the farm would also impact the society in general. Less food produced means less food to the local markets, less money traded and less workplaces to the Zambians.

Information about production principles and routines

From the interview conducted, we conclude that the farms in general demonstrated good management practices. They knew their production well, the production was well planned, and problem solving was part of their daily management practices. Importantly, most of the tilapia farmers we interviewed use simple approaches and not the fanciest technology on the market. A lot of work is manual, and many of the workers do not have a formal education

within the field. A key point here is the production is simple and still under development. Farmers learn from experience, new knowledge is gained which results in improved methods, and new technology come into use continuously. Tilapia production is a low-cost industry, and the product is sold at relatively low price in local markets. Thus, we are still at a phase where they produce as many tons of fish as possible with as little resources and economic input as possible.

Tilapia farmers produce fish according to market demand, and they adjust their production based on the customers' requests and wishes. The market wants fish with red gills (a sign of freshness), without medicine or treatment, and at the cheapest price. This means the fish is not bled out during slaughter, to prevent the gills of turning pale. The fish is therefore also not treated for any infections and farmed in the cheapest possible to keep the price down. The tilapia production has not developed to the stage where animal welfare is the highest priority because the market is not prioritizing animal welfare. The main focus is food security, and a cheap and fresh product. Avoiding stressing the fish is part of good animal welfare. The workers on the farms tried to avoid stressing the fish, but as we observed this on the farms, most of the time this had low priority and management practices were not set up to take this into account. Stressing the fish in different settings during the production phase may cause the fish to become more susceptible to disease without the workers knowing or considering this. Our interpretation of the farmers responses is that the workers cared about the fish being healthy and with as low losses as possible, but that is not the same as saying that animal welfare is the farmer's number one priority. As mentioned above, all the farmers claimed that they did not treat sick fish, because the consumers did not want to buy fish that had been treated. We cannot ascertain whether this claim is fact-based or not, but an impression was given that the first choice was not antibiotic treatment when mortality increased.

Conclusion

We did detect potentially pathogenic bacteria and bacteria believed to be non-pathogenic in water samples from hatcheries and from fingerlings raised in the same hatcheries, which represent a risk of infection disease outbreaks. An interesting finding is that the same bacterial species, considered as non-pathogenic, was found in samples both from water and from the abdominal cavity of the fish.

Increased mortality due to infection diseases in the early production phase represent a significant risk of economic losses because increased mortality in the hatchery will affect the entire production chain. If the increase in mortality happens after transfer to hapas, time and resources invested in the hatchery would be lost, as well as affecting the rest of the production chain.

The tilapia production is mainly influenced by the demands and requests from the market, making the production low-cost to produce cheap fish. We visited farms demonstrating good management practices with little resources and economic input, in a still developing industry.

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Sammendrag

Tittel: Bakterielle infeksjoner i tidlig fase av tilapia produksjon i Zambia

Forfattere: Hege Holgersen, Mie Sofie Holen Munkelien and Maria Breivik Skarshaug

Veileder: Øystein Evensen, Institutt for parakliniske fag

Globalt er tilapia den tredje største oppdrettsfisken, og en viktig matkilde i mange deler av verden. Tilapia er en hurtigvoksende ferskvannsfisk som tåler mer enn mange andre fiskearter når det gjelder miljøvariasjoner.
Hovedformålet med denne oppgaven var beskrive produksjonsprinsippene i tilapiaproduksjonen i Zambia, samt å utføre en detaljert karakterisering av bakterier tidligere mistenkt som sykdomsfremkallende hos yngel i tidlige stadier av produksjonen.
22 bakterieisolater, tidligere innsamlet i Zambia, ble karakterisert ved Gram-farging og kjemisk testing med API 20 E og API 20 NE. Videre ble prøvene analysert ved bruk av molekylære metoder, PCR, etterfulgt av sekvensering kombinert med Maldi-Tof. Under

feltstudien vår i Zambia samlet vi inn informasjon om produksjonsprinsipper og rutiner fra ansatte ved fem tilapia-farmer ved hjelp av et spørreskjema.

Noen potensielt patogene og ikke-patogene bakterier ble funnet under vårt laboratoriearbeid. Dette kan tyde på at fisken lever i et suboptimalt miljø med rikelig med bakterier til stede, og det kan også tyde på at fisken kan ha blitt utsatt for stress som har gjort den mer mottagelig for bakterieinfeksjoner. Økt dødelighet tidlig i produksjonen kan få store økonomiske konsekvenser fordi hele produksjonskjeden blir berørt. Tilapia-farmene vi besøkte i feltstudien vår er fortsatt under utvikling med begrensede ressurser og økonomisk tilførsel, som produserer billig fisk til det lokale markedet.

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Attachments

Appendix I: Questionnaire for tilapia farmers

Appendix II: API 20 E reading table

Appendix III: API 20 NE reading table

Appendix IV: DNeasy Blood & Tissue Kit Quick- Start Protocol

Appendix V: Results of PCR calculations

Appendix VI: Maldi-Tof results

Appendix VII: Sequencing results

Appendix VIII: Gene sequences from 16S rRNA sequencing

Appendix I. Questionnaire for tilapia farmers

About:

1. What is your name?
2. How long have you been working here?
3. Do you have any experience from other fishfarms?
4. What are your responsibilities and tasks on this production site?
5. How many times per day do you supervise the fish?

About the farm:

6. What is the name of this production site?
7. How long has this production site been producing fish?
8. How many workers are there on this site?
9. How much is produced here per year/each season?

About the production:

10. How long does the fish stay in for example the hapas, before being moved?
    a. Is it decided by the fish age or weight?
11. How long are each production cycle?
12. Do you produce multiple generations of fish at the same time?
    a. If yes: Are these generations kept together or separately?
13. What is the limit for how many fish that are allowed to be put in the same pond/hapas?
14. Do you collect fish eggs?
    a. If yes: How? Do you disinfect these eggs?
15. Which fish breed do you produce?
16. Do you vaccinate the fish?
17. What kind of food do you use to feed the fish?
18. How do you separate the broodstock from the fry?
19. Where do you get the broodstock from?
20. Which type of water source do you use?
21. Do you treat the water in any way before using it?
    a. If yes: how do you treat the water?
22. Can you regulate water parameters in the hapas?

About biosecurity, disease and treatment:

23. What do you do when it comes to biosecurity?
24. How far is it between each production site at Lake Kariba?
25. If disease emerges, do you often notice it in multiple hapases at the same time?
26. Is there any specific time of the year that diseases are more prevalent?
27. Do you have any specific problems at the production site now?
    a. If yes: have you noticed similar problems in earlier production batches?
28. Have you had any problems with fish disease earlier?
29. What do you do when the fish becomes sick?
30. Do you have any kind of control system where you write down mortality, appetite, disease indicators?
    a. If yes: How do you do it? And what indicators?
31. How do you document the treatment?
32. How do you calculate how much antibiotics to use?
33. What kind of antibiotics do you use?
34. Do you cooperate with other localities when it comes to disease control and treatment?
    a. If yes: How do you do it?

About transport:

35. How do you transport the fry to it's next location?
36. When you move fish from one site to another, how do you do it?
37. What water parameters is controlled during transport?

Other:

38. Do you have anything you register that say something about animal welfare?
    a. If yes: what? How do you register it?
39. How are the conditions at the different facilities at Lake Kariba?

Appendix II. API 20 E reading table

api® 20 E 07584D - GB - 2002/10

WASTE DISPOSAL

It is the responsibility of each laboratory to handle waste and effluents produced according to their type and degree of hazardousness and to treat and dispose of them (or have them treated and disposed of) in accordance with any applicable regulations.

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READING TABLE

TESTS	ACTIVE INGREDIENTS	QTY (mg/cup.)	REACTIONS/ENZYMES	RESULTS	
				NEGATIVE	POSITIVE
ONPG	2-nitrophenyl-ßD-galactopyranoside	0.223	ß-galactosidase (Ortho NitroPhenyl-ßD-Galactopyranosidase)	colorless	yellow (1)
ADH	L-arginine	1.9	Arginine DiHydrolase	yellow	red / orange (2)
LDC	L-lysine	1.9	Lysine DeCarboxylase	yellow	red / orange (2)
ODC	L-ornithine	1.9	Ornithine DeCarboxylase	yellow	red / orange (2)
CIT	trisodium citrate	0.756	CITrate utilization	pale green / yellow	blue-green / blue (3)
H_2S	sodium thiosulfate	0.075	H_2S production	colorless / greyish	black deposit / thin line
URE	urea	0.76	UREase	yellow	red / orange (2)
TDA	L-tryptophane	0.38	Tryptophane DeAminase	TDA / immediate: yellow	TDA / immediate: reddish brown
IND	L-tryptophane	0.19	INDole production	JAMES / immediate: colorless pale green / yellow	JAMES / immediate: pink
VP	sodium pyruvate	1.9	acetoin production (Voges Proskauer)	VP 1 + VP 2 / 10 min: colorless	VP 1 + VP 2 / 10 min: pink / red (5)
GEL	Gelatin (bovine origin)	0.6	GELatinase	no diffusion	diffusion of black pigment
GLU	D-glucose	1.9	fermentation / oxidation (GLUcose) (4)	blue / blue-green	yellow / greyish yellow
MAN	D-mannitol	1.9	fermentation / oxidation (MANnitol) (4)	blue / blue-green	yellow
INO	inositol	1.9	fermentation / oxidation (INOsitol) (4)	blue / blue-green	yellow
SOR	D-sorbitol	1.9	fermentation / oxidation (SORbitol) (4)	blue / blue-green	yellow
RHA	L-rhamnose	1.9	fermentation / oxidation (RHAmnose) (4)	blue / blue-green	yellow
SAC	D-sucrose	1.9	fermentation / oxidation (SACcharose) (4)	blue / blue-green	yellow
MEL	D-melibiose	1.9	fermentation / oxidation (MELibiose) (4)	blue / blue-green	yellow
AMY	amygdalin	0.57	fermentation / oxidation (AMYgdalin) (4)	blue / blue-green	yellow
ARA	L-arabinose	1.9	fermentation / oxidation (ARAbinose) (4)	blue / blue-green	yellow
OX	(see oxidase test package insert)		cytochrome-OXidase	(see oxidase test package insert)	

(1) A very pale yellow should also be considered positive.
(2) An orange color after 36-48 hours incubation must be considered negative.
(3) Reading made in the cupule (aerobic).
(4) Fermentation begins in the lower portion of the tubes, oxidation begins in the cupule.
(5) A slightly pink color after 10 minutes should be considered negative.
• The quantities indicated may be adjusted depending on the titer of the raw materials used.
• Certain cupules contain products of animal origin, notably peptones.

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Appendix III. API 20 NE reading table

api® 20 NE 07615H - GB - 2003/10

READING TABLE

TESTS	ACTIVE INGREDIENTS	QTY (mg/cup.)	REACTIONS/ENZYMES	RESULTS	
				NEGATIVE	POSITIVE
NO3	potassium nitrate	0.136	reduction of nitrates to nitrites	NIT 1 + NIT 2 / 5 min: colorless	pink-red
			reduction of nitrates to nitrogen	Zn / 5 min: pink	colorless
TRP	L-tryptophane	0.2	indole production (TRyptoPhane)	JAMES / immediate: colorless / pale green / yellow	pink
GLU	D-glucose	1.92	fermentation (GLUcose)	blue to green	yellow
ADH	L-arginine	1.92	Arginine DiHydrolase	yellow	orange / pink / red
URE	urea	0.76	UREase	yellow	orange / pink / red
ESC	esculin ferric citrate	0.56 0.072	hydrolysis (β-glucosidase) (ESCulin)	yellow	grey / brown / black
GEL	gelatin (bovine origin)	0.6	hydrolysis (protease) (GELatin)	no pigment diffusion	diffusion of black pigment
PNPG	4-nitrophenyl-βD-galactopyranoside	0.22	β-galactosidase (Para-NitroPhenyl-ßD-Galactopyranosidase)	colorless	yellow
GLU	D-glucose	1.56	assimilation (GLUcose)	transparent	opaque
ARA	L-arabinose	1.4	assimilation (ARAbinose)	transparent	opaque
MNE	D-mannose	1.4	assimilation (ManNosE)	transparent	opaque
MAN	D-mannitol	1.36	assimilation (MANnitol)	transparent	opaque
NAG	N-acetyl-glucosamine	1.28	assimilation (N-Acetyl-Glucosamine)	transparent	opaque
MAL	D-maltose	1.4	assimilation (MALtose)	transparent	opaque
GNT	potassium gluconate	1.84	assimilation (potassium GlucoNate)	transparent	opaque
CAP	capric acid	0.78	assimilation (CAPric acid)	transparent	opaque
ADI	adipic acid	1.12	assimilation (ADIpic acid)	transparent	opaque
MLT	malic acid	1.56	assimilation (MaLaTe)	transparent	opaque
CIT	trisodium citrate	2.28	assimilation (trisodium CITrate)	transparent	opaque
PAC	phenylacetic acid	0.8	assimilation (PhenylACetic acid)	transparent	opaque
OX	(see oxidase test package insert)	-	cytochrome oxidase	(see oxidase test package insert)	

• The quantities indicated may be adjusted depending on the titer of the raw materials used.
• Certain cupules contain products of animal origin, notably peptones.

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Appendix IV. DNeasy Blood & Tissue Kit Quick- Start Protocol

Quick-Start Protocol April 2016

DNeasy® Blood & Tissue Kit

The DNeasy Blood & Tissue Kit (cat. nos. 69504 and 69506) can be stored at room temperature (15–25°C) for up to 1 year if not otherwise stated on label.

Further information

- *DNeasy Blood & Tissue Handbook*: **www.qiagen.com/HB-2061**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Notes before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- Redissolve any precipitates in Buffer AL and Buffer ATL.
- Add ethanol to Buffer AW1 and Buffer AW2 concentrates.
- Equilibrate frozen tissue or cell pellets to room temperature.
- Preheat an incubator to 56°C.
- Refer to the handbook for pretreatment of fixed tissue, insect, bacterial or other material.

1a. **Tissue**: Cut tissue (≤10 mg spleen or ≤25 mg other tissue) into small pieces, and place in a 1.5 ml microcentrifuge tube. For rodent tails, use 1 (rat) or 2 (mouse) 0.4–0.6 cm lengths of tail. Add 180 µl Buffer ATL. Add 20 µl proteinase K, mix by vortexing and incubate at 56°C until completely lysed. Vortex occasionally during incubation. Vortex 15 s directly before proceeding to step 2.

1b. **Nonnucleated blood**: Pipet 20 µl proteinase K into a 1.5 ml or 2 ml microcentrifuge tube. Add 50–100 µl anticoagulant-treated blood. Adjust volume to 220 µl with PBS. Proceed to step 2.

Sample to Insight

QIAGEN

1c. **Nucleated blood**: Pipet 20 µl proteinase K into a 1.5 ml or 2 ml microcentrifuge tube. Add 5–10 µl anticoagulant-treated blood. Adjust volume to 220 µl with PBS. Proceed to step 2.

1d. **Cultured cells**: Centrifuge a maximum of 5 x 10^6 cells for 5 min at 300 x *g* (190 rpm). Resuspend in 200 µl PBS. Add 20 µl proteinase K. Proceed to step 2.

2. Add 200 µl Buffer AL. Mix thoroughly by vortexing. Incubate blood samples at 56°C for 10 min.
3. Add 200 µl ethanol (96–100%). Mix thoroughly by vortexing.
4. Pipet the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at ≥6000 x *g* (8000 rpm) for 1 min. Discard the flow-through and collection tube.
5. Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW1. Centrifuge for 1 min at ≥6000 x *g*. Discard the flow-through and collection tube.
6. Place the spin column in a new 2 ml collection tube, add 500 µl Buffer AW2 and centrifuge for 3 min at 20,000 x *g* (14,000 rpm). Discard the flow-through and collection tube.
7. Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.
8. Elute the DNA by adding 200 µl Buffer AE to the center of the spin column membrane. Incubate for 1 min at room temperature (15–25°C). Centrifuge for 1 min at ≥6000 x *g*.
9. **Optional**: Repeat step 8 for increased DNA yield.

Scan QR code for handbook.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual.

Appendix V. Results of PCR calculations

Number	Sample	Concentration (ng/µL)	µL of DNA sample (100/(ng/µL))	µL of sterile water (10-µL of sample)
1	3A	10,86	9,2	0,8
2	30B	63,73	1,6	8,4
3	34A	12,69	7,9	2,1
4	24A	40,44	2,5	7,5
5	18A	87,03	1,1	8,9
6	3C	42,79	2,3	7,7
7	W2-A	216,66	0,5	9,5
8	25B	167,05	0,6	9,4
9	9B	117,63	0,9	9,1
10	38A	10,42	9,6	0,4
11	21A	6,84	14,6	-4,6
12	30D	51,39	1,9	8,1
13	27A	152,58	0,7	9,3
14	25D	39,18	2,6	7,4
15	35C	38,40	2,6	7,4
16	2A	37,35	2,7	7,3
17	28C	186,48	0,5	9,5
18	23A	32,33	3,1	6,9
19	4A	5,47	18,3	-8,3
20	14B-2	3,99	25,1	-15,1
21	1C	5,17	19,3	-9,3
22	40A	57,77	1,7	8,3

Appendix VI. Maldi-Tof results

Number	Well number	Sample name	Result	Confidence level
1	E1	3A	*Exiguobacterium acetylicum*	99,9 %
2	E2	30B	*Exiguobacterium acetylicum*	99,9 %
3	E3	34A	*Brevibacillus* spp.	98,9 %
4	E4	24A	*Exiguobacterium acetylicum*	99,9 %
5	F1	18A	*Exiguobacterium acetylicum*	99,9 %
6	F2	3C	*Exiguobacterium acetylicum*	99,9 %
7	F3	W2-A	*Exiguobacterium aurantiacum*	99,9 %
8	F4	25B	*Exiguobacterium acetylicum*	99,9 %
9	G1	9B	None	
10	G2	38A	*Bacillus idriensis*	99,9 %
11	G3	21A	None	
12	G4	30D	*Exiguobacterium acetylicum*	99,9 %
13	H1	27A	*Aeromonas veronii/Aeromonas sobria*	50 %
14	H2	25D	None	
15	H3	35C	None	
16	H4	2A	None	
17	I1	28C	*Aeromonas sobria/Aeromonas veronii/Aeromonas jandaei*	33,3 %
18	I2	23A	*Exiguobacterium acetylicum*	99,9 %
19	I3	4A	None	
20	I4	14B-2	None	
21	J1	1C	None	
22	J2	40A	None	

Appendix VII. Sequencing results

Number	Sample	BLAST result	Number of base pairs
1	3A	*Exiguobacterium acetylicum*	996
2	30B	*Exoguobacterium indicum*	1021
3	34A	*Macrococcus equipercicus*	1024
4	24A	*Exiguobacterium acetylicum*	1011
5	18A	*Exiguobacterium acetylicum*	1026
6	3C	*Exiguobacterium acetylicum*	1107
7*			-
8	25B	*Exiguobacterium acetylicum*	1137
9	9B	*Exiguobacterium acetylicum*	1021
10	38A	*Bacillus indicus*	1021
11	21A	*Exiguobacterium acetylicum*	1029
12	30D	*Exiguobacterium acetylicum*	1007
13	27A	*Aeromonas veronii*	999
14	25D	*Exiguobacterium indicum*	1026
15	35C	*Fictibacillus nanhaiensis*	960
16	2A	*Fictibacillus* sp.	1017
17	28C	*Aeromonas jandaei*	997
18	23A	*Exiguobacterium indicum*	1021
19	4A	*Fictibacillus* sp.	1024
20	14B-2	*Bacilllus indicus*	1023
21	1C	*Staphylococcus epidermis*	1013
22	40A	*Planomicrobium* sp.	963

* Sample 7: No result.

Appendix VIII. Gene sequences from 16S rRNA sequencing

Explanation of tables

Sample number	Sample name	Bacteria species	Number of base pairs	Primer
DNA sequence				

1	3A	*Exiguobacterium acetylicum*	996	27F

TAAACGGAACTCTTCGGAGGGAAGGCAGTGGAATGAGCGGCGGACGGGTGAGTAACACGTAAGGAACCTGCCT
CAAGGATTGGGATAACTCCGAGAAATCGGAGCTAATACCGGATAGTTCAACGGACCGCATGGTCCGCTGATGA
AAGGCGCTCCGGCGTCACCTTGAGATGGCCTTGCGGTGCATTAGCTAGTTGGTGGGGTAACGGCCCACCAAGG
CGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA
GGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGATGAAGGTTTTC
GGATCGTAAAACTCTGTTGTAAGGGAAGAACACGTACGAGAGGAAATGCTCGTACCTTGACGGTACCTTACGA
GAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGG
CGTAAAGCGCGCGCAGGCGGCCTTTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGCCATTGGAA
ACTGGAAGGCTTGAGTACAGAAGAGAAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGG
AACACCAGTGGCGAAGGCGACTCTTTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGA
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGGGGGTTTCCGCCCCTCAGTGCTGA
AGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGACCC
GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAACTCTTGACATCCCATTGA
CCGCTTGAGAGATCAAGTTTTCCCTTCGGGGACAA

2	30B	*Exiguobacterium indicum*	1021	27F

TTTTCCGAGCGCAGGAAACTGACGGAACTCTTCGGAGGGAAGGCAGCGGAATGAGCGGCGGACGGGTGAGTAA
CACGTAAGGAACCTGCCTCAAGGATTGGGATAACTCCGAGAAATCGGAGCTAATACCGGATAGTTCAACGGAC
CGCATGGTCCGCTGATGAAAGGCGCTCCGGCGTCACCTTGAGATGGCCTTGCGGTGCATTAGCTAGTTGGTGG
GGTAACGGCCCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCG
TGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTAAGGGAAGAACACGTACGAGAGGGAATGCTCGTAC
CTTGACGGTACCTTACGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCG
TTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGCCTTTTAAGTCTGATGTGAAAGCCCCCGGCTCAAC
CGGGGAGGGCCATTGGAAACTGGAAGGCTTGAGTACAGAAGAGAAGAGTGGAATTCCACGTGTAGCGGTGAAA
TGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTTTGGTCTGTAACTGACGCTGAGGCGCGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGGGGGTT
TCCGCCCCTCAGTGCTGAAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAA
AGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAA
CTCTTGACATCCCATTGACCGCTTGAGAGATCAAGTTTTCCCTTCGGGGACAATGGTGAC

3	34A	*Macrococcus equipercicus*	1024	27F

ACAGACAGGGAGTGCTTTGCACTCCTTGAAGTCAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTACCTAT
AAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAATATGTTTCACCTCATGGTGAAACAGTGAAA
GACGGTTTCGGCTGTCACTTATAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG
CGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA
GGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTC
GGATCGTAAAACTCTGTTGTAAGGGAAGAACAAGTACGTTAGTAACTGAACGTACCTTGACGGTACCTTACCA
GAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGG
CGTAAAGCGCGCGTAGGCGGTCTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAA
ACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGG
AACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAACTGACGCTGAGGTGCGAAAGCGTGGGGATCAAACAGGA
TTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTGGGGGGTTTCCGCCCCTCAGTGCTGC
AGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGACCC
GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTTGACATCCTCTGAC
AACTCTGGAGACAGAGCGTTCCCCTTCGGGGGACAGAATGACAGGTGGTGCATGGTTGTCGTC

4	24A	*Exiguobacterium acetylicum*	1011	27F

TCGAAGCGCAGGAAACTGACGGAACTCTTCGGAGGGAAGGCAGCGGAATGAGCGGCGGACGGGTGAGTAACAC
GTAAGGAACCTGCCTCAAGGATTGGGATAACTCCGAGAAATCGGAGCTAATACCGGATAGTTCAACGGACCGC
ATGGTCCGCTGATGAAAGGCGCTTCGGCGTCACCTTGAGATGGCCTTGCGGTGCATTAGCTAGTTGGTGAGGT
AACGGCTCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCC
CAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGA
GTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTAAGGGAAGAACACGTACGAGAGGAAATGCTCGTACCTT
GACGGTACCTTACGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTG
TCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGCCTTTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGG
GGAGGGCCATTGGAAACTGGAAGGCTTGAGTACAGAAGAGAAGAGTGGAATTCCACGTGTAGCGGTGAAATGC
GTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTTTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGT
GGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGGGGGTTTCC
GCCCCTCAGTGCTGAAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGG
AATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAACTC
TTGACATCCCATTGACCGCTTGAGAGATCAAGTTTTCCCTTCGGGGACAA

5	18A	*Exiguobacterium acetylicum*	1026	27F

ACTGACGGAACTCTTTCGGAGGGAAGGCAGCGGAATGAGCGGCGGACGGGTGAGTAACACGTAAGGAACCTGC
CTCAAGGATTGGGATAACTCCGAGAAATCGGAGCTAATACCGGATAGTTCAACGGACCGCATGGTCCGCTGAT
GAAAGGCGCTTCGGCGTCACCTTGAGATGGCCTTGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAA
GGCGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGG
GAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGATGAAGGTTT
TCGGATCGTAAAACTCTGTTGTAAGGGAAGAACACGTACGAGAGGGAATGCTCGTACCTTGACGGTACCTTAC
GAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTG
GGCGTAAAGCGCGCGCAGGCGGCCTTTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGCCATTGG
AAACTGGAAGGCTTGAGTACAGAAGAGAAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGA
GGAACACCAGTGGCGAAGGCGACTCTTTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAG
GATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGGGGGTTTCCGCCCCTCAGTGCT
GAAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGAC
CCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAACTCTTGACATCCCATT
GACCGCTTGAGAGATCAAGTTTTCCCTTCGGGGACAATGGTGACAGGTGGTGCATGGTTGTCGTC

6	3C	*Exiguobacterium acetylicum*	1107	1492R

TTTGGCTCCTTGCGGGTTACCTCACCGGCTTCGGGTGTTGCAAACTCTCGTGGTGTGACGGGCGGTGTGTACA
AGACCCGGGAACGTATTCACCGCAGTATGCTGACCTGCGATTACTAGCGATTCCGACTTCATGCAGGCGAGTT
GCAGCCTGCAATCCGAACTGGGAACGGCTTTATGGGATTGGCTCCACCTCGCGGTCTCGCTGCCCTTTGTACC
GTCCATTGTAGCACGTGTGTAGCCCAACTCATAAGGGGCATGATGATTTGACGTCATCCCCACCTTCCTCCGG
TTTGTCACCGGCAGTCTCCCTAGAGTGCCCAACTCAATGCTGGCAACTAAGGATAGGGGTTGCGCTCGTTGCG
GGACTTAACCCAACATCTCACGACACGAGCTGACGACAACCATGCACCACCTGTCACCATTGTCCCCGAAGGG
AAAACTTGATCTCTCAAGCGGTCAATGGGATGTCAAGAGTTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAAC
CACATGCTCCACCGCTTGTGCGGGTCCCCGTCAATTCCTTTGAGTTTCAGCCTTGCGGCCGTACTCCCCAGGC
GGAGTGCTTAATGCGTTAGCTTCAGCACTGAGGGGCGGAAACCCCCCAACACCTAGCACTCATCGTTTACGGC
GTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCGCCTCAGCGTCAGTTACAGACCAAAGA
GTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTACACGTGGAATTCCACTCTTCTCT
TCTGTACTCAAGCCTTCCAGTTTCCAATGGCCCTCCCCGGTTGAGCCGGGGGCTTTCACATCAGACTTAAAAG
GCCGCCTGCGCGCGCTTTACGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGCTGGC
ACGTAGTTAGCCGTGGCTTTCTCGTAAGGTACCGTCAAGGTACGAGCATTACCTCTCGTACGTGTTCTTCCCT
TACAACAGAGTTTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCATCAGACTTTCGTCCATTG

7	W2-A	No result		

8	25B	*Exiguobacterium acetylicum*	1137	27F

TGCTAATACATGCAAGTCGAGCGCAGGAAACTGACGGAACTCTTCGGAGGGAAGGCAGTGGAATGAGCGGCGG
ACGGGTGAGTAACACGTAAGGAACCTGCCTCAAGGATTGGGATAACTCCGAGAAATCGGAGCTAATACCGGAT
AGTTCAACGGACCGCATGGTCCGCTGATGAAAGGCGCTTCGGCGTCACCTTGAGATGGCCTTGCGGTGCATTA
GCTAGTTGGTGGGGTAACGGCCCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGG
AGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTAAGGGAAGAACACGTACGAGAGG
GAATGCTCGTACCTTGACGGTACCTTACGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGT
AGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGCCTTTTAAGTCTGATGTGAAAGC
CCCCGGCTCAACCGGGGAGGGCCATTGGAAACTGGAAGGCTTGAGTACAGAAGAGAAGAGTGGAATTCCACGT

GTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTTTGGTCTGTAACTGACGCT
GAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGG
TGTTGGGGGGTTTCCGCCCCTCAGTGCTGAAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAG
GCTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA
GAACCTTACCAACTCTTGACATCCCATTGACCGCTTGAGAGATCAAGTTTTCCCTTCGGGGACAATGGTGACA
GGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATC
CTTAGTTGCCAGCATTCAGTTGGGCACC

9	9B	*Exiguobacterium acetylicum*	1021	27F

TCCCAGCGCAGGAAAGCTGGACGGAACTCTTCGGAGGGAAGGCAGTGGAATGAGCGGCGGACGGGTGAGTAAC
ACGTAAGGAACCTGCCTCAAGGATTGGGATAACTCCGAGAAATCGGAGCTAATACCGGATAGTTCAACGGACC
GCATGGTCCGCTGATGAAAGGCGCTTCGGCGTCACCTTGAGATGGCCTTGCGGTGCATTAGCTAGTTGGTGGG
GTAACGGCTCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGG
CCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGT
GAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTAAGGGAAGAACACGTACGAGAGGAAATGCTCGTACC
TTGACGGTACCTTACGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT
TGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGCCTTTTAAGTCTGATGTGAAAGCCCCCGGCTCAACC
GGGGAGGGCCATTGGAAACTGGAAGGCTTGAGTACAGAAGAGAAGAGTGGAATTCCACGTGTAGCGGTGAAAT
GCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTTTGGTCTGTAACTGACGCTGAGGCGCGAAAGC
GTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGGGGGTTT
CCGCCCCTCAGTGCTGAAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAA
GGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAC
TCTTGACATCCCATTGACCGCTTGAGAGATCAAGTTTTCCCTTCGGGGACAATGGTGACA

10	38A	*Bacillus indicus*	1021	27F

TCGAGCGGACCTCTTCGGAGATCAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTAAGACTGGGA
TAACTCCGGGAAACCGGAGCTAATACCGGATACTATGTCAAACCGCATGGTTTGACATTCAAAGACGGTTTCG
GCTGTCACTTACAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATGCG
TAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTA
GGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAA
ACTCTGTTGTCAGGGAAGAACAAGTGCCGGAGTAACTGCCGGCACCTTGACGGTACCTGACCAGAAAGCCACG
GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGC
GCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAAAC
TTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTG
GCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCC
TGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAACGCA
TTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGG
TGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGCCACTTCTAGAG
ATAGGAAGGTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCC

11	21A	*Exiguobacterium acetylicum*	1029	27F

CTGCCTATACATGCAAGTCGAGCGCAGGAAACTGACGGAACTCTTCGGAGGGAAGGCAGCGGAATGAGCGGCG
GACGGGTGAGTAACACGTAAGGAACCTGCCTCAAGGATTGGGATAACTCCGAGAAATCGGAGCTAATACCGGA
TAGTTCAACGGACCGCATGGTCCGCTGATGAAAGGCGCTTCGGCGTCACCTTGAGATGGCCTTGCGGTGCATT
AGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATG
GAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTAAGGGAAGAACACGTACGAGAG
GGAATGCTCGTACCTTGACGGTACCTTACGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACG
TAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGCCTTTTAAGTCTGATGTGAAAG
CCCCCGGCTCAACCGGGGAGGGCCATTGGAAACTGGAAGGCTTGAGTACAGAAGAGAAGAGTGGAATTCCACG
TGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTTTGGTCTGTAACTGACGC
TGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAG
GTGTTGGGGGGTTTCCGCCCCTCAGTGCTGAAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAA
GGCTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGA
AGAACCTTACCAACTCTTGACATCCCATTGACCGCTTGAGAGATCAAGTTTTCCCTTCGGGGACAATG

12	30D	*Exiguobacterium acetylicum*	1007	27F

CCTGGCTATACATGCAAGTCGAGCGCAGGAAACTGACGGAACTCTTCGGAGGGAAGGCAGCGGAATGAGCGGC
GGACGGGTGAGTAACACGTAAGGAACCTGCCTCAAGGATTGGGATAACTCCGAGAAATCGGAGCTAATACCGG
ATAGTTCAACGGACCGCATGGTCCGCTGATGAAAGGCGCTTCGGCGTCACCTTGAGATGGCCTTGCGGTGCAT
TAGCTAGTTGGTGGGGTAACGGCCCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGAT

GGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTAAGGGAAGAACACGTACGAGA
GGTAATGCTCGTACCTTGACGGTACCTTACGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATAC
GTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGCCTTTTAAGTCTGATGTGAAA
GCCCCCGGCTCAACCGGGGAGGGCCATTGGAAACTGGAAGGCTTGAGTACAGAAGAGAAGAGTGGAATTCCAC
GTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTTTGGTCTGTAACTGACG
CTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTA
GGTGTTGGGGGGTTTCCGCCCCTCAGTGCTGAAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCA
AGGCTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG
AAGAACCTTACCAACTCTTGACATCCCATTGACCGCTTGAGAGATC

13	27A	*Aeromonas veronii*	999	27F

AATCGAGCGGCAGCGGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCCTGGGG
ATCTGCCCAGTCGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATACGCCCTACGGGGGAAAGCAGG
GGACCTTCGGGCCTTGCGCGATTGGATGAACCCAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAG
GCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTT
CGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGGTAGCTAATAACTGCCAGCTGTGACGTTACTCGCAG
AAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGG
GCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAA
AACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAG
GAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGG
AGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCC
GCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCTGGAA
TCCTGCAGAGATGCGGGAGTGCCTTCGGGAATCAGAAA

14	25D	*Exiguobacterium indicum*	1026	27F

TCATGCAAGTCGAGCGCAGGAAACTGACGGAACTCTTCGGAGGGAAGGCAGCGGAATGAGCGGCGGACGGGTG
AGTAACACGTAAGGAACCTGCCTCAAGGATTGGGATAACTCCGAGAAATCGGAGCTAATACCGGATAGTTCAT
CGGACCGCATGGTCCGTTGATGAAAGGCGCTCCGGCGTCACCTTGAGATGGCCTTGCGGTGCATTAGCTAGTT
GGTGGGGTAACGGCCCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACG
CCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTAAGGGAAGAACACGTACGAGAGGAAATGCT
CGTACCTTGACGGTACCTTACGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGC
AAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGCCTTTTAAGTCTGATGTGAAAGCCCCCGGC
TCAACCGGGGAGGGCCATTGGAAACTGGAAGGCTTGAGTACAGAAGAGAAGAGTGGAATTCCACGTGTAGCGG
TGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTTTGGTCTGTAACTGACGCTGAGGCGC
GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGG
GGGTTTCCGCCCCTCAGTGCTGAAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAA
CTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTT
ACCAACTCTTGACATCCCATTGACCGCTTGAGAGATCAAGTTTTCCCTTCGGGGACAATGGTGAC

15	35C	*Fictibacillus nanhaiensis*	960	27F

AATGGATGAGGAGCTTTGCTCCTCTGATTTAGCGGCGGACGGGTGAGTAACACGTGGGTAATCTGCCTGTAAG
ACGGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATAAGAGAAGAAGCATTTCTTCTTTTTGAAAGTC
GGTTTCGGCTGACACTTACAGATGAGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGA
CGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGC
AGCAGTAGGGAATCTTCGGCAATGGGCGAAAGCCTGACCGAGCAACGCCGCGTGAGCGATGAAGGCCTTCGGG
TCGTAAAGCTCTGTTGTTAGAGAAGAACAAGTACGAGAGTAACTGCTCGTACCTTGACGGTACCTAACCAGAA
AGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGT
AAAGCGCGCGCAGGCGGTCTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACT
GGGAGACTTGAGTGCAGGAGAGAAAAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAAC
ACCAGTGGCGAAGGCGGCTTTTTGGCCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGGGGGTTCCACCCTCAGTGCTGAAGTTA
ACACATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACA
AGCAGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTC

16	2A	*Fictibacillus* sp.	1017	27F

TATACTGCAAGTCGAGCGAATGATGAGGAGCTTGCTCCTCTGATTTAGCGGCGGACGGGTGAGTAACACGTGG
GTAATCTGCCTGTAAGACGGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATAAGAGAAGAAGCATTT
CTTCTTTTTGAAAGTCGGTTTCGGCTGACACTTACAGATGAGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAA
CGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCA

GACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGCGAAAGCCTGACCGAGCAACGCCGCGTGAGC
GATGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTTAGAGAAGAACAAGTACGAGAGTAACTGCTCGTACCTTGA
CGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATC
CGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGG
AGGGTCATTGGAAACTGGGAGACTTGAGTGCAGGAGAGAAAAGTGGAATTCCACGTGTAGCGGTGAAATGCGT
AGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTTTGGCCTGTAACTGACGCTGAGGCGCGAAAGCGTGG
GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGGGGGTTCCACC
CTCAGTGCTGAAGTTAACACATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATT
GACGGGGGCCCGCACAAGCAGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGA
CATCCTTTGACCACTCTAGAGATAGAGCTTTCCCCTTCGGGGGACAAAGTGACAGG

17	28C	*Aeromonas jandaei*	997	27F

TTGTTGTCGGCAGCGGGAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGTAATGCCTGGGGAT
CTGCCCAGTCGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATACGCCCTACGGGGGAAAGCAGGGG
ACCTTCGGGCCTTGCGCGATTGGATGAACCCAGGTGGGATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGC
GACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAG
GCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCG
GGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTCAGTAGCTAATATCTGCTGGCTGTGACGTTACTCGCAGAA
GAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGC
GTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAA
CTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGA
ATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAG
CTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGC
ACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCTGGAATC
CTGCAGAGATGCGGGAGTGCCTTCGGGAATCAGAAC

18	23A	*Exiguobacterium indicum*	1021	27F

CATGCAAGTCGAGCGCAGGAAACTGACGGAACTCTTCGGAGGGAAGGCAGCGGAATGAGCGGCGGACGGGTGA
GTAACACGTAAGGAACCTGCCTCAAGGATTGGGATAACTCCGAGAAATCGGAGCTAATACCGGATAGTTCAAC
GGACCGCATGGTCCGCTGATGAAAGGCGCTCCGGCGTCACCTTGAGATGGCCTTGCGGTGCATTAGCTAGTTG
GTGGGGTAACGGCCCACCAAGGCGACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGA
CACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGC
CGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTAAGGGAAGAACACGTACGAGAGGGAATGCTC
GTACCTTGACGGTACCTTACGAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCA
AGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGCCTTTTAAGTCTGATGTGAAAGCCCCCGGCT
CAACCGGGGAGGGCCATTGGAAACTGGAAGGCTTGAGTACAGAAGAGAAGAGTGGAATTCCACGTGTAGCGGT
GAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTTTGGTCTGTAACTGACGCTGAGGCGCG
AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGGG
GGTTTCCGCCCCTCAGTGCTGAAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAAC
TCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTA
CCAACTCTTGACATCCCATTGACCGCTTGAGAGATCAAGTTTTCCCTTCGGGGACAATGG

19	4A	*Fictibacillus* sp.	1024	27F

TCCTATACTGCAAGTCGAGCGAATGATGAGGAGCTTGCTCCTCTGATTTAGCGGCGGACGGGTGAGTAACACG
TGGGTAATCTGCCTGTAAGACGGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAATAAGAGAAGAAGCA
TTTCTTCTTTTTGAAAGTCGGTTTCGGCTGACACTTACAGATGAGCCCGCGGCGCATTAGCTAGTTGGTGAGG
TAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGC
CCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGGCGAAAGCCTGACCGAGCAACGCCGCGTG
AGCGATGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTTAGAGAAGAACAAGTACGAGAGTAACTGCTCGTACCT
TGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTT
ATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCG
TGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGGAGAGAAAAGTGGAATTCCACGTGTAGCGGTGAAATG
CGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTTTGGCCTGTAACTGACGCTGAGGCGCGAAAGCG
TGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTGGGGGGTTCC
ACCCTCAGTGCTGAAGTTAACACATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGA
ATTGACGGGGGCCCGCACAAGCAGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCT
TGACATCCTTTGACCACTCTAGAGATAGAGCTTTCCCCTTCGGGGGACAAAGTGACAGGTGGT

20	14B-2	*Bacilllus indicus*	1023	27F

TAATGCAAGTCGAGCGGACCTCTTCGGAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTGTA
AGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATACTATGTCAAACCGCATGGTTTGACATTCAAAG

ACGGTTTCGGCTGTCACTTACAGATGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGC
GACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAG
GCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCG
GATCGTAAAACTCTGTTGTCAGGGAAGAACAAGTGCCGGAGTAACTGCCGGCGCCTTGACGGTACCTGACCAG
AAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGC
GTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAA
CTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGA
ACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCA
GCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGCCA
CTTCTAGAGATAGAAGGTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATGGGTGTCGTC

21	1C	*Staphylococcus epidermis*	1013	27F

TTACTGCAAGTCGAGCGAACAGACGAGGAGCTTGCTCCTCTGACGTTAGCGGCGGACGGGTGAGTAACACGTG
GATAACCTACCTATAAGACTGGGATAACTTCGGGAAACCGGAGCTAATACCGGATAATATATTGAACCGCATG
GTTCAATAGTGAAAGACGGTTTTGCTGTCACTTATAGATGGATCCGCGCCGCATTAGCTAGTTGGTAAGGTAA
CGGCTTACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGACACGGTCCA
GACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGT
GATGAAGGTCTTCGGATCGTAAAACTCTGTTATTAGGGAAGAACAAATGTGTAAGTAACTATGCACGTCTTGA
CGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTATC
CGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGG
AGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGC
AGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGATGTGCGAAAGCGTGG
GGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGC
CCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAA
TTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAAATCTT
GACATCCTCTGACCCCTCTAGAGATAGAGTTTTCCCCTTCGGGGGACAGAAT

22	40A	*Planomicrobium* sp.	963	27F

TTGGGAAGCTTGCTCCATTGGGTTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCCTGCAGATCGG
GATAACTCCGGGAAACCGGTGCTAATACCGAATAGTTTTTTGCCCCTCCTGGGGCGAAACGGAAAGACGGTTT
CGGCTGTCACTGCAGGATGGGCCCGCGGCGCATTAGCTAGTTGGTGGGGTAATGGCCCACCAAGGCGACGATG
CGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG
TAGGGAATCTTCCGCAATGGACGCAAGTCTGACGGAGCAACGCCGCGTGAGTGACGAAGGTTTTCGGATCGTA
AAACTCTGTTGTGAGGGAAGAACAAGTACCAAGTAACTACTGGTACCTTGACGGTACCTCACCAGAAAGCCAC
GGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCG
CGCGCAGGCGGTCCCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGGGA
CTTGAGTGCAGAAGAGGAAAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGT
GGCGAAGGCGACTTTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGC
ATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCG
GTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCGCTGACCGCCTTAG
AGA

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