



**RHODES UNIVERSITY**

*Where leaders learn*

**Effect of adding a probiotic to an aquaponic system on  
plant and fish growth, water quality, and microbial  
diversity**

A thesis submitted in fulfilment of the requirements for the degree of

**DOCTOR OF PHILOSOPHY**

**(Fisheries Science)**

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by

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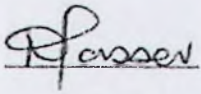
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February 2022

## DECLARATION

I, Nasser Kasozi, declare that this thesis is my original work and has never been submitted in any form for another degree at any other university. Where other sources of information have been used, acknowledgement has been made in the text and list of references given.

Signature: 

Date: 20<sup>th</sup> February 2022

## **ETHICS STATEMENT**

All the studies presented and reported in this thesis were conducted in accordance with the ethical guidelines for the use of animals in research and was approved by the Animal Research Ethics Committee (AREC) of Rhodes University, South Africa (RU-AREC references: 29102018; 2019-1145-2120 and 2020-2824-4869).

## PUBLICATIONS DERIVED FROM THIS THESIS

### Peer reviewed journal articles

**Kasozi, N., Tandlich, R., Fick, M., Kaiser, H., & Wilhelmi, B. (2019).** Iron supplementation and management in aquaponic systems: A review. *Aquaculture Reports*, 15, 100221.

**Kasozi, N., Kaiser, H., & Wilhelmi, B. (2020).** Metabarcoding analysis of bacterial communities associated with media grow bed zones in an aquaponic system. *International Journal of Microbiology*, 2020, 8884070.

**Kasozi, N., Abraham, B., Kaiser, H., & Wilhelmi, B. (2021).** The complex microbiome in aquaponics: significance of the bacterial ecosystem. *Annals of Microbiology*, 71, 1–13.

**Kasozi, N., Kaiser, H., & Wilhelmi, B. (2021).** Effect of *Bacillus* spp. on lettuce growth and root associated bacterial community in a small-scale aquaponics system. *Agronomy*, 11(5), 947.

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**Kasozi, N., Kaiser, H., & Wilhelmi, B. (2022).** Determination of phylloplane associated bacteria of lettuce from a small-scale aquaponic system via 16S rRNA gene amplicon sequence analysis. *Horticulturae*, 8(2), 151.

### Conferences

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## ABBREVIATIONS AND ACRONYMS

|                              |                                                            |
|------------------------------|------------------------------------------------------------|
| AHLs                         | <i>N</i> -Acyl homoserine lactones                         |
| Anammox                      | Anaerobic ammonium oxidation                               |
| AOA                          | Ammonia-oxidising archaea                                  |
| AOAC                         | Association of Official Analytical Chemists                |
| AOB                          | Ammonia-oxidising bacteria                                 |
| BLAST                        | Basic Local Alignment Search Tool                          |
| CCI                          | Chlorophyll content index                                  |
| Comammox                     | Complete ammonia oxidation                                 |
| DNA                          | Deoxyribonucleic acid                                      |
| DNS                          | 3,5-Dinitrosalicylic acid                                  |
| DO                           | Dissolved oxygen                                           |
| <i>E. coli</i>               | <i>Escherichia coli</i>                                    |
| EC                           | Electrical conductivity                                    |
| EFSA                         | European Food Safety Authority                             |
| FAO                          | Food and Agriculture Organization                          |
| ICP-OES                      | Inductively coupled plasma – optical emission spectrometry |
| IGF-I                        | Insulin-like growth factor I                               |
| N                            | Nitrogen                                                   |
| N <sub>2</sub>               | Nitrogen gas                                               |
| N <sub>2</sub> O             | Nitrous oxide                                              |
| NCBI                         | National Center for Biotechnology Information              |
| NGS                          | Next-generation sequencing                                 |
| NH <sub>3</sub>              | Free ammonia                                               |
| NH <sub>4</sub> <sup>+</sup> | Ammonium                                                   |
| NO                           | Nitric oxide                                               |

|                              |                                                  |
|------------------------------|--------------------------------------------------|
| NO <sub>2</sub> <sup>-</sup> | Nitrite                                          |
| NO <sub>3</sub> <sup>-</sup> | Nitrate                                          |
| NOB                          | Nitrite-oxidising bacteria                       |
| OTU                          | Operational taxonomic unit                       |
| P                            | Phosphorus                                       |
| PCR                          | Polymerase chain reaction                        |
| Pty Ltd                      | Private company limited                          |
| QIIME                        | Quantitative insights into microbial ecology     |
| RAS                          | Recirculating aquaculture systems                |
| RDP                          | Ribosomal database project                       |
| RM-ANOVA                     | Repeated measures analysis of variances          |
| rRNA                         | Ribosomal ribonucleic acid                       |
| SAIAB                        | South African Institute for Aquatic Biodiversity |
| Sobs                         | Observed species richness                        |
| spp.                         | Species                                          |
| TAN                          | Total ammonia nitrogen                           |
| TDS                          | Total dissolved solids                           |
| TNF-α                        | Tumour necrosis factor alpha                     |
| Tukey's HSD                  | Tukey's honest significant difference            |

#### **Symbols (Units)**

|                    |                    |
|--------------------|--------------------|
| <                  | less than          |
| >                  | greater than       |
| °C                 | degrees Celsius    |
| *                  | asterisk           |
| kJ g <sup>-1</sup> | kilojoule per gram |
| %                  | percentage         |

|                               |                                     |
|-------------------------------|-------------------------------------|
| $\mu\text{g}$                 | microgram                           |
| $\mu\text{L}$                 | microlitre                          |
| bp                            | base pair                           |
| $\text{CFU g}^{-1}$           | colony-forming unit per gram        |
| $\text{CFU mL}^{-1}$          | colony-forming units per millilitre |
| cm                            | centimetre                          |
| $\text{d}^{-1}$               | per day                             |
| g                             | gram                                |
| h                             | hour                                |
| $\text{kg m}^{-3}$            | kilogram per cubic metre            |
| L                             | litre                               |
| $\text{L h}^{-1}$             | litre per hour                      |
| M                             | molarity                            |
| mg                            | milligram                           |
| $\text{mg L}^{-1}$            | milligram per litre                 |
| $\text{mg mL}^{-1}$           | milligram per millilitre            |
| min                           | minute                              |
| mL                            | millilitre                          |
| mM                            | millimolar                          |
| $\text{mS cm}^{-1}$           | milliSiemens per centimetre         |
| ng                            | nanogram                            |
| $\text{ng } \mu\text{L}^{-1}$ | nanogram per microlitre             |
| nm                            | nanometre                           |
| $\beta$                       | beta                                |
| V                             | volt                                |
| w/v                           | weight by volume                    |
| $\alpha$                      | alpha                               |
| $\mu\text{g L}^{-1}$          | micrograms per litre                |

## ABSTRACT

The current conventional food production systems are neither ecologically sound, nor do they address the issues of the circular economy of decreasing waste. Aquaponics, a technology that integrates aquatic organisms and plants, provides part of the solution. As microbes are vital to the functioning of aquaponic systems, the integration of probiotics in aquaponics is a possible strategy for promoting sustainable agriculture.

Prior to the commencement of this research, there was no published data on the effects of addition of probiotics on microbial diversity in aquaponics. The aim of this research was to investigate the effects of the addition of a commercial probiotic mixture of *Bacillus subtilis* and *B. licheniformis* to a coupled aquaponic system on plant and fish growth, water quality, and bacterial diversity. Results from plant growth trials indicated that vegetative growth of lettuce was significantly higher in the *Bacillus*-treated systems than the untreated control systems. The concentrations of phosphorus, potassium, and zinc in the lettuce leaves were significantly higher in *Bacillus*-treated than control systems. There was a significant increase in the concentration of nitrate and phosphate in the *Bacillus*-treated systems which may have contributed to improved lettuce growth. Although Proteobacteria was the most dominant bacterial phylum associated with the lettuce root and leaf surfaces, 16S rRNA gene sequence analysis showed that the addition of *Bacillus* species significantly increased species richness and species evenness of the lettuce roots as compared to leaves. The alpha diversity indices showed that root-associated bacterial communities were significantly more diverse than those associated with leaves when a probiotic was added to aquaponic water. Analysis of lettuce epiphytic bacterial communities revealed that fresh lettuce leaf surfaces were inhabited by food-borne pathogens belonging to *Shigella* and *Aeromonas*.

Trials related to fish growth, digestive enzyme activities, and bacterial gut communities found that the addition of *Bacillus* stimulated the activities of intestinal amylase, protease, and

alkaline phosphatase and showed a significantly higher percentage increase in weight gain, specific growth rate, and improved feed conversion ratio than the control. The use of the commercial *Bacillus* preparation did not affect whole-body composition of *O. mossambicus* suggesting that optimum conditions for keeping this species were maintained. Water quality remained within recommended ranges for tilapia culture.

Illumina MiSeq sequencing of 16S rRNA gene amplicons showed that bacterial gut communities of *O. mossambicus* were dominated by the phylum Fusobacteria, specifically the species *Cetobacterium somerae*. Members from the *Enterobacteriaceae* family were part of the *O. mossambicus* gut microbiota, which was mostly represented by *Plesiomonas shigelloides*. Other OTUs assigned to *Aeromonas diversa*, *Edwardsiella anguillarum*, and *Klebsiella pneumoniae* were also observed, at low abundances.

A further study investigated the bacterial communities associated with the dry, root, and mineralised zones of a flood-and-drain media bed system. Bacteroidetes and Proteobacteria were the most dominant phyla across the three zones and in the effluent water. At genus level, in the effluent water samples of *Bacillus*-treated systems, *Spirosoma* was the most dominant, with a relative abundance of 71.44% of the total bacterial community compared to 1.68% of the total bacterial community from the control systems. All three *Spirosoma* OTUs had sequences closely related to the bacterium *Spirosoma aerophilum*.

In conclusion, the results from this research suggest that integrating *Bacillus* species into aquaponic systems may be considered beneficial, having positive effects on plant and fish growth, as well as enhancement of plant nutrients and the modulation of the composition of bacterial communities. Therefore, this research has contributed to our understanding of the bacterial community changes as a result of adding probiotics.

## CHAPTER 1

### General introduction and literature review

Modified from a paper published as: Kasozi, N., Abraham, B., Kaiser, H. and Wilhelmi, B. (2021). The complex microbiome in aquaponics: significance of the bacterial ecosystem. *Annals of Microbiology*, 7(1), 1–13. <https://doi.org/10.1186/s13213-020-01613-5>

#### 1.1 Aquaponics: an emerging technology

The global human population stood at 7.7 billion as of 2020 and is expected to reach 9.8 billion in 2050 (United Nations, 2019). To feed this expanding global populace by 2050, food production needs to increase by 50% (FAO, 2017). It is paramount that new food production technologies are developed that contribute not only to water usage efficiency but also to agricultural input efficiency, in order to decrease the land footprint needed for production. In this context, sustainable food production systems such as aquaponics are viewed as an important technology (Lennard and Goddek, 2019; Folorunso et al., 2020). The concept of aquaponics was pioneered in the 1970s (Naegel, 1977) as an environmentally sustainable agricultural method. Consequently, this emerging food production technology is now perceived as a possible solution towards sustainable production of fish and plants (Bosma et al., 2017).

In a broad sense, aquaponics involves several disciplines, such as, agriculture, microbiology, aquaculture, mathematics, biochemistry, engineering, and chemistry (Junge et al., 2017). It is defined as an integrated technology of growing aquatic organisms and plants in which the nutrient-rich effluent excreted by aquatic organisms undergoes bacterial transformations and is then used as a source of nutrients for the cultivated plants (Rakocy et al., 2006; Yep and Zheng, 2019). An important principle of aquaponics is the use of fish and feed wastes as nutrient sources for plant growth. Therefore, both the fish's and plant's nutritional requirements in an aquaponic system has to be fulfilled by the feed. Advantages associated with aquaponics are water use efficiency, usage of limited amount of nutrient

supplementation and the ability to produce more than two crops from one input source (Tyson et al., 2011; Wongkiew et al., 2017). Despite these benefits, aquaponics still largely remains a 'backyard activity' rather than having achieved wide scale commercialisation (Miličić et al., 2017; Palm et al., 2018). Management of microbiota to maximise the benefits nutrient cycling through the process of mineralisation is one of the main technical challenges hindering the commercialisation of aquaponics (Bartelme et al., 2018; Joyce et al., 2019). Therefore, to improve plant and fish growth, aquaponic systems require proper functioning of the microbial community. However, microbial research in aquaponic systems is limited in comparison to that of soil-based agriculture. For example, there are still information gaps regarding the integration of beneficial bacteria in aquaponics, and how these bacteria influence the water quality and microbial diversity of plants and fish.

## **1.2 Aquaponic systems design**

### **1.2.1 Coupled aquaponics**

According to Rakocy (2006), the first commercial coupled aquaponic system, a raft system that combined the production of Nile tilapia (*Oreochromis niloticus*) and lettuce (*Lactuca sativa*) was set up at the University of the Virgin Islands in the USA in 1980. This type of aquaponic system design has subsequently been adopted in several countries with modifications to meet the requirements of cultivated plant species. Coupled aquaponic systems range from small to medium scale and large-sized systems. The basic components of a coupled aquaponic system include a fish culture tank, a sedimentation unit a biological filter, a sump, and a hydroponic unit for plant growth (Palm et al., 2019). These units are connected by pipes and are arranged in a single process loop that directs nutrient-rich water generated after bacterial transformation to the plants and then back to the fish rearing tank (Palm et al., 2019). The production of tilapia species integrated with varieties of lettuce is the most common farming model practised in this type of aquaponics (Yep and Zheng, 2019).

Depending on the scale of production, a wide range of hydroponic subsystems can be used. The most common hydroponic subsystems are deep-water culture and media-filled beds (Palm et al., 2019). In deep-water systems, plants are suspended in polystyrene sheets with their roots hanging into the water. With media bed culture, the grow beds are filled with inert substrate, e.g., expanded clay, gravel or perlite, which supports plants and provides substrate for mechanical and biological filtration. Coupled systems have advantages related to the efficient utilisation of water, absorption of nutrients, and improvement of water quality conditions, such as increasing oxygen levels and lowering turbidity (Palm et al., 2019). However, balancing the specific nutrient and water quality requirements of the plants, bacteria, and fish remains a challenge, and this affects the productivity of coupled systems (Monsees et al., 2017).

### **1.2.2 Decoupled aquaponics**

Decoupled systems differ from coupled aquaponics because of the separation of the water and nutrient loops for the fish and hydroponic units. Thus, in decoupled aquaponics, water from the hydroponic unit is not redirected to the fish tanks and conditions within the hydroponic unit can be managed separately (Monsees et al., 2017; Goddek et al., 2019). Given the differing specific nutrient and environmental requirements of plants and fish, pilot studies involving decoupled systems have been initiated. In a pilot study that compared plant and fish yields in both decoupled and coupled aquaponics, Monsees et al. (2017) reported that tomato yield was 36% higher in decoupled aquaponics than in coupled aquaponics, whereas fish growth was the same in both systems. The main reasons for better performance were attributed to the independent pH regulation and re-mineralisation of bound nutrients. Although decoupled aquaponic systems provide solutions to some challenges inherent to single-loop systems, they

are more complex to manage and require high capital investment, space, and technical expertise (Monsees et al., 2017; Sallenave and Shultz, 2019).

### **1.3 Water quality requirements within aquaponic systems**

Optimisation of water quality parameters for the plants, fish, and microbes is key to a productive aquaponic system (Yildiz et al., 2017; Lennard and Goddek, 2019). To ensure bacterial processes and plant growth, while maintaining the fish welfare, it is important to maintain the correct concentrations of nutrients, pH, temperature, dissolved oxygen, and total dissolved solids (Schmautz et al., 2021). If the dissolved oxygen, total ammonia nitrogen (TAN) and nitrite concentrations within an aquaponic system are within the acceptable ranges for the fish, the plant and bacterial requirements are also met (Lennard and Goddek, 2019). By monitoring the key water quality parameters such as pH, temperature, and TAN on a regular basis, timely adjustments can be made to avoid losses in productivity.

#### **1.3.1 pH**

Water pH is one of the main physicochemical factors that regulates plant growth, fish metabolism, and microbial activity (Zou et al., 2016). However, the three main organisms (fish, plants, and bacteria) in aquaponic systems have different optimal pH ranges. Tilapia species (*O. niloticus* and *O. mossambicus*) can tolerate a pH range of 5.5 to 9.0 (El-Sayed, 2019). Most plants require a pH value of between 5.5 and 7.5 to enhance the uptake of nutrients (Somerville et al., 2014; Yildiz et al., 2017). On the other hand, nitrification in aquaponics has been reported to be most efficient at a narrow pH range from 7.5 to 8.0 (Tyson et al., 2011; Zou et al., 2016). Low pH (pH 5.2 to 6.0) negatively affects ammonia-oxidising bacteria (AOB) and nitrite-oxidising bacteria (NOB), reduces nitrification, enhances nitrous oxide (N<sub>2</sub>O) emission, and

causes stress to the fish in aquaponic systems (Ruiz et al., 2003; Sallenave, 2016; Wongkiew et al., 2018).

According to Somerville et al. (2014), the most common nitrifying bacteria found in aquaponics are *Nitrosomonas* and *Nitrobacter*, with their pH tolerance levels ranging from 7.2 to 7.8 and 7.2 to 8.2, respectively. Wongkiew et al. (2017) reported a decrease in nitrification below pH 6.0. pH also influences the distribution of bacterial populations in aquaponic systems. For example, Wongkiew et al. (2018) reported that in biofilters at a pH range of 6.8 to 7.0, *Fusobacteria* dominated over *Acidobacteria*, but this abundance was reversed in plant roots at the same pH. However, at a pH of 5.2, the relative abundance of *Acidobacteria* decreased in the root samples, which were then dominated by *Proteobacteria*. Although it is not possible to achieve an optimum pH for fish, bacteria, and plants in aquaponic systems, a compromise between nitrification, and nutrient availability can be reached in aquaponic systems by maintaining pH values within the levels of 6.5 – 7.0 (Somerville et al., 2014; Sallenave, 2016).

### 1.3.2 Water temperature

The performance of fish, plants, and bacterial growth in aquaponic systems is mainly regulated by water temperature. Water temperature can be affected by ambient air temperature, tank materials, the hydroponic system, and the insulation of components used in the construction of aquaponic systems (Somerville et al., 2014). The water temperature influences both the type of fish and the plant species that can be grown in aquaponic systems, because different species have different optimal temperature requirements. For instance, fish species such as African catfish, *Clarias gariepinus* and Mozambique tilapia, *O. mossambicus* prefer temperatures ranging from 22°C to 32°C (Somerville et al., 2014; Yildiz et al., 2017).

Water temperature affects plant growth by regulating root development, uptake of water and nutrients. The suitable temperature for greenhouse crops ranges from 18°C to 30°C (Somerville et al., 2014). However, some vegetables have specific temperature conditions. For example, higher temperatures ( $> 26^{\circ}\text{C}$ ) affect the taste and growth of lettuce through bolting (Somerville et al., 2014). In contrast, a study on red leaf lettuce showed that exposure of lettuce roots to low temperature ( $10^{\circ}\text{C}$ ) for seven days in a hydroponic system significantly reduced leaf area, stem diameter, shoot and root fresh weight compared to those exposed to  $20^{\circ}\text{C}$  for the same duration (Sakamoto and Suzuki, 2015).

Additionally, water temperature is paramount because some species of bacteria are able to dominate the nitrification process based on the changes in ambient temperature. Although the optimal growth temperature for most nitrifiers ranges between  $25^{\circ}\text{C}$  to  $30^{\circ}\text{C}$ , AOB populations increase faster than NOB within this temperature range (Tyson et al., 2011; Sallenave, 2016). An experiment conducted by Head and Oleszkiewicz (2004) on the impact of rapid temperature changes from  $20^{\circ}\text{C}$  to  $10^{\circ}\text{C}$  showed an average decrease of 58% in the nitrification rate. In addition, variation in temperature influences microbial dynamics in nitrogen removal systems (Urakawa et al., 2008; Karkman et al., 2011). Karkman et al. (2011) reported that the genera *Nitrosomonas* and *Nitrospira* were dominant at  $10^{\circ}\text{C}$ , and that *Nitrosospira* dominated at  $5^{\circ}\text{C}$ , thus, suggesting *Nitrosospira* might be more resistant to low temperature stress conditions. Fish and plant species should be matched as closely as possible for water temperature requirements.

### 1.3.3 Dissolved oxygen

Dissolved oxygen (DO) is vital to fish, plant roots and most of the microflora. Most plant roots and microflora can survive on low DO of approximately  $3\text{ mg L}^{-1}$  whereas most fish require DO above  $5\text{ mg L}^{-1}$ , especially in systems with high fish densities (Sallenave, 2016).

Therefore, if the DO concentration within an aquaponic system is set and maintained for the fish requirement, the plant and microbe requirement is also met. The DO concentration decreases in aquaponic systems due to the metabolic activity of aerobic microorganisms and the fish.

Although plants use their stems and leaves to absorb oxygen during respiration, roots also require oxygen. DO is important for plant metabolism including nitrogen uptake and assimilation, root formation and root growth (Roosta et al., 2016). Insufficient DO levels ( $< 3 \text{ mg L}^{-1}$ ) affect root respiration, which eventually decreases water and nutrient uptake (Rakocy et al., 2006). Roosta et al. (2016) evaluated the effect of different oxygen concentrations in nutrient solutions of hydroponic systems on eggplant growth. The authors reported that significantly higher shoot fresh weight of eggplant was observed at DO concentration of  $4 \text{ mg L}^{-1}$  compared to concentrations of  $1 \text{ mg L}^{-1}$ ,  $2 \text{ mg L}^{-1}$ , and  $3 \text{ mg L}^{-1}$ .

The effects of DO levels on microbial activity has not yet been reported in aquaponic systems. However, studies using synthetic wastewater have shown the activity of AOB and NOB to be sensitive to decreased DO levels, although the influence of free ammonia was more substantial on NOB inhibition at levels as low as  $0.1 \text{ mg L}^{-1} \text{ N}$  (Kim et al., 2005). In aquaponic systems with inadequate aeration and sludge build-up, anaerobic bacteria such as sulphate reducing bacteria, may dominate and produce toxic metabolites (Somerville et al., 2014).

#### **1.3.4 Electrical conductivity**

Electrical conductivity (EC) reflects the concentration of ions in an aquaponic solution (Rakocy et al., 2006; Lennard and Goddek, 2019). The major ions that influence the levels of EC are calcium, magnesium, potassium, nitrate, phosphate, and sulphate (Rakocy et al., 2006). Levels of EC in the range of  $0.3$  to  $1.1 \text{ mS cm}^{-1}$  are enough to sustain plant production as nutrients are generated continuously (Rakocy et al., 2006; Wortman, 2015). However, higher

levels of EC ( $> 2.0 \text{ mS cm}^{-1}$ ) indicate that aquaponic water is polluted, and such levels may affect fish growth. The concentrations of nutrients in aquaponic systems are influenced by various factors including, water source, feed composition, the type of buffers used, the stocking density, the growth stage of fish and plants, and the nitrification rates (Rakocy et al., 2006; Wortman, 2015). Because the nutrient concentrations of aquaponic systems have to be managed to sustain plant production, the measurement of EC to provide information about the nutrient density is vital.

### **1.3.5 Total dissolved solids**

The levels of total dissolved solids (TDS) represent the amount of organic matter, inorganic salts, and other dissolved materials in an aquaponic solution (Reyes-Yanes et al., 2020). The recommended range for TDS is 1,000 to 1,500  $\text{mg L}^{-1}$  in hydroponic solutions and a much lower range of 200 to 400  $\text{mg L}^{-1}$  in aquaponic solutions (Rakocy et al., 2006). High amounts ( $> 1,000 \text{ mg L}^{-1}$ ) of TDS can cause a toxic medium for most fish species (Reyes-Yanes et al., 2020). High feeding rates, low water exchange and insufficient plant growing areas can lead to the rapid build-up of dissolved nutrients to potentially phytotoxic levels. Because aquaponic systems harbour different microorganisms, it is possible that pathogenic microbes or aquatic pests might develop, particularly if solids accumulate (Reyes-Yanes et al., 2020). High suspended solids may also affect plant roots by adhering to the roots, resulting in nutrient uptake inefficiency (Lennard and Goddek, 2019). These suspended solids also encourage the prevalence of heterotrophic bacteria, which, if allowed to dominate aquaponic systems, may outcompete other bacterial species involved in the nitrification process.

### 1.3.6 Total ammonia nitrogen, nitrite, and nitrate

Total ammonia nitrogen (TAN) comprises two forms, namely, ammonia ( $\text{NH}_3$ ) and ammonium ( $\text{NH}_4^+$ ). The relative concentration of each of these forms in the water depends on the temperature and pH (Masser et al., 1999).  $\text{NH}_3$  is extremely toxic to fish compared to  $\text{NH}_4^+$  (Masser et al., 1999; Somerville et al., 2014). Accumulation of TAN in aquaponics may either be due to fish excretions as result of protein metabolism or feed decomposition (Wongkiew et al., 2017). Masser et al. (1999) reported that free ammonia concentrations as low as  $0.02 - 0.07 \text{ mg L}^{-1}$  affect fish growth. The toxicity of nitrite ( $\text{NO}_2^-$ ) to fish is due to its ability to enhance the production of methaemoglobin, which reduces the total oxygen carrying ability for cell respiration resulting into reduced growth (Wongkiew et al., 2017). Sallenave (2016) suggested that the desirable nitrite levels for plants, fish and bacteria activity should be maintained at less than  $1 \text{ mg L}^{-1}$ . Plants primarily utilise nitrogen in the form of nitrate ( $\text{NO}_3^-$ ) and a fully functioning aquaponic system should maintain  $\text{NO}_3^-$  concentrations in the range of 5 to  $150 \text{ mg L}^{-1}$  (Sallenave, 2016; Reyes-Yanes et al., 2020). It is recommended to add more plants to the existing grow beds when high nitrate levels are noticed (Sallenave, 2016).

### 1.4 Application of next-generation sequencing

Traditionally, the identification of bacteria has relied on the selective culturing and enumeration of presumptive isolates. Although this technique is cheap, it is limited as relatively few bacteria are cultured (Biswas and Sarkar, 2018). The application of next-generation sequencing (NGS) technology has transformed research related to the taxonomic composition in aquaponic systems (Kasozi et al., 2021a). The success of NGS technology is due to its ability to generate huge amounts of sequence data per instrument run in a rapid and cost-effective way (Bharti and Grimm, 2021). Specifically, NGS-based on 16S ribosomal RNA (rRNA) gene amplicons, has been applied to explore the bacterial community structure and diversity in

biofilter and sump samples from aquaponic systems (Schmautz et al., 2017; Eck et al., 2019). However, a large proportion of microbial communities that are distributed across the different components of aquaponic systems are still uncharacterised.

The general workflow for NGS includes sample processing, library preparation, sequencing, and data analysis. Maintenance of quality control in each stage of the process is vital to obtain quality sequence data. For instance, the suitability of DNA in terms of quality and quantity for the NGS pipeline is vital (Lucena-Aguilar et al., 2016; Han et al., 2018). The ratio of absorbance at 260 and 280 nm is used to assess DNA purity and it is considered 'pure' when the  $A_{260}/A_{280}$  ratio is within the range of 1.8 to 2.0 (Lucena-Aguilar et al., 2016; Han et al., 2018). Although nucleic acids are purified with the help of commercially available DNA extraction kits, the presence of proteins or other organic components in the eluate cannot be entirely ruled out, especially for samples obtained from the environment (Paniagua-Voirol et al., 2021). The  $A_{260}/A_{230}$  ratio is also applied as a secondary measure of DNA purity. However, this ratio is not considered to be a good measure of DNA quality due to the changes in values when a saline elution buffer is used to dissolve the DNA (Boesenberg-Smith et al., 2012).

#### **1.4.1 Next-generation sequencing based on 16S rRNA gene amplicons**

NGS technologies have enabled investigations of microbial studies in aquaponics to become more cost-effective through the generation of 16S rRNA gene sequences from microorganisms present in environmental samples without the need for intermediate culturing steps (Joyce et al., 2019). Sequence analysis of the 16S rRNA gene has been generally used to identify bacterial species of environmental importance (Janda and Abbott, 2007; Bharti and Grimm, 2021). The 16S rRNA gene encodes the prokaryotic small 30S subunit of the 70S ribosomal complex in most bacteria and archaea (Bharti and Grimm, 2021). The 16S rRNA gene sequence is approximately 1,500-base pair (bp) long and comprises nine hypervariable

regions (V1 – V9), interspaced with more conserved regions (Johnson et al., 2019; Winand et al., 2019). The characterisation of the hypervariable regions of the 16S rRNA gene forms a basis for the culture-independent classification of bacterial species (Boers et al., 2019). Although sequencing the entire 1,500-bp region provides a better resolution at species level, Clarridge (2004) stated that sequencing the first 500-bp of the 16S rRNA gene is satisfactory for identification of taxa at genus level. There are few studies from aquaponics and recirculating aquaculture systems (RAS) that have applied 16S rRNA gene based genotyping protocols to identify and catalogue bacterial profiles (Table 1.1). As indicated in Table 1.1, these studies have targeted different sub-regions of the 16S rRNA gene, which ranges from a single hypervariable region to three hypervariable regions.

Several public databases host curated catalogues of 16S rRNA gene sequences, including the Ribosomal database project (RDP) (Cole et al., 2005), GREENGENES (DeSantis et al., 2006) or SILVA (Pruesse et al., 2007). The SILVA datasets appear to be a preferred reference database in comparison to GREENGENES for classification of data from NGS approaches (López-García et al., 2018). However, accurate taxonomic identification of 16S rRNA gene data for bacterial identification depends on several factors, including the target region of the 16S rRNA gene, sequence length, and the sequencing technology. Short sequencing reads obtained from NGS protocols, especially from Illumina platforms decrease the precision for species identification (Winand et al., 2019). Another challenge is related to the choice of the nine hypervariable regions (V1 – V9) within the 16S rRNA gene, as no single hypervariable region can effectively be used to distinguish between closely related taxa (Johnson et al., 2019). However, amplicons targeting multiple regions of the 16S rRNA gene may provide a higher bacterial richness and diversity than a single hypervariable region.

**Table 1.1:** Overview of sample characterisation from aquaponics and recirculating aquaculture systems using NGS-based 16S rRNA gene amplicons.

| Samples characterised/system                                                            | Amplified Region | NGS platform   | Bioinformatics pipeline | Reference              |
|-----------------------------------------------------------------------------------------|------------------|----------------|-------------------------|------------------------|
| Biofilter and sump samples (Aquaponics)                                                 | (V1 – V3)        | Illumina MiSeq | QIIME                   | Eck et al. (2019)      |
| Biofilter, plant roots, periphyton and fish faeces (Aquaponics)                         | (V1 – V3)        | Illumina MiSeq | MGX                     | Schmautz et al. (2017) |
| Samples from surface, middle and bottom of biofilter sand (RAS system)                  | (V4 – V5)        | Illumina MiSeq | CutAdapt                | Bartelme et al. (2017) |
| Rearing tank water, solid-waste, clarifier, biofilter, and degassing tower (RAS system) | (V6)             | Illumina HiSeq | CutAdapt                | Bartelme et al. (2019) |
| Samples of rhizosphere and endosphere of halophyte species (Aquaponics)                 | (V3 – V4)        | Roche 454      | QIIME                   | Oliveira et al. (2020) |
| Water samples from fish rearing tanks (RAS system)                                      | (V4)             | Illumina MiSeq | PEAR                    | Sanchez et al. (2019)  |

Several sequencing platforms that use NGS have been developed including Illumina platform, pyrosequencing, Ion Torrent technology, and SOLiD (Sequencing by Oligonucleotide Ligation and Detection). Illumina technology is one of the most common sequencing systems, particularly with the HiSeq and MiSeq sequencers (Slatko et al., 2018). The Illumina platform generates more data at a lower cost in comparison with other platforms. The current Illumina platforms are greatly improved and the MiSeq can now produce paired-end reads up to  $2 \times 300 - 350$  bp (Bukin et al., 2019). In addition, the Illumina sequencing platforms enable rapid sequencing of large stretches of DNA bp covering entire genomes and can produce a large data output in a single sequencing run (Bukin et al., 2019). However, the analysis of 16S rRNA gene NGS data is still a challenge due to the large and complex datasets generated (Bharti and Grimm, 2021). The choice of bioinformatics algorithms and their settings

can affect the final microbiota results obtained. The two commonly used tools for statistical downstream analysis on microbial data are based on MOTHUR (Schloss et al., 2009) and Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al., 2010). For each pipeline, several steps are involved including demultiplexing, sequence alignment and clustering, chimera filtering and sequence classification (López-García et al., 2018; Bharti and Grimm, 2021). Ensuring quality control during the analysis of microbial data is key for reliable and accurate results (Bharti and Grimm, 2021).

### **1.5 Methods of cycling**

Cycling refers to a process of establishing a bacterial ecosystem in a new aquaponic system. This process can be conducted with or without the presence of fish in the system (DeLong and Losordo, 2012; Sallenave, 2016; Brailo et al., 2019). The main relevance of cycling is to manage and control the seeding of nitrifying bacteria cells in a biological filter (DeLong and Losord, 2012).

#### **1.5.1 Fish cycling**

With this approach, it is recommended to use low fish stocking densities, and low amounts of feed to prevent a rapid build-up of TAN that may be harmful to fish (DeLong and Losordo, 2012; Sallenave, 2016). As the biofilter establishes, fish density can be incrementally increased, and feeding regimes adjusted appropriately (Brailo et al., 2019). Cycling with fish can take up to 6 weeks for sufficient bacterial populations to develop (Sallenave, 2016). TAN and  $\text{NO}_2^-$  levels should be measured daily during this process. When  $\text{NO}_3^-$  is detected in the system and the TAN and  $\text{NO}_2^-$  are below  $0.5 \text{ mg L}^{-1}$ , the system is considered cycled and ready for plant cultivation (Sallenave, 2016).

### 1.5.2 Fishless cycling

This is the most frequently used approach for biofilter establishment (Sallenave, 2016; Brailo et al., 2019). In this case, ammonium products in dry powder form or concentrated liquid suspension are added to the system, which allows nitrifying bacteria to establish in response to the availability of nutrients. Chemical compounds such as ammonium chloride, ammonium nitrite or organic liquid fertilisers with  $\text{NH}_4^+$  such as SeaGro or SeaBrix are added to an aquaponic systems to achieve 'fishless cycling' at a dosage that raises the TAN levels to between 3 and 5  $\text{mg L}^{-1}$  (Sallenave, 2016). This method requires less time than cycling with fish, taking approximately three weeks for biofilter establishment (Sallenave, 2016; Brailo et al., 2019). As with the fish cycling method, as soon as TAN and  $\text{NO}_2^-$  levels drop to near zero and a measurable level of  $\text{NO}_3^-$  is reached, the system is considered fully cycled and ready for fish culture. Supplementation of  $\text{NH}_4^+$  should be stopped once the fish is added into the system.

### 1.6 Microbial distribution in aquaponic systems

Microorganisms are found in almost every compartment of an aquaponic system (Table 1.2). Fish or plant species normally introduce their own unique microbial flora into aquaponic systems, resulting in substantial changes in the microbial diversity between systems at different phases of operation (Eck et al., 2019; Schmautz et al., 2021). However, few studies have employed molecular techniques to characterise microbial composition and structure in the different parts of aquaponic systems. The limited research available on microbial characterisation has focussed on bacterial diversity associated with periphyton, plant roots, biofilter, and fish faeces (Schmautz et al., 2017). There is still a scope for more research to characterise microbial communities related to media bed zones, fish gut, rhizosphere and phyllosphere.

**Table 1.2:** Prominent taxa identified from different compartments and substrates of aquaponic systems.

| Aquaponic compartment/source/substrate | Predominant taxa (Phyla/family/order /genera)                                                                                                                                                                                                                                                        | Reference                                                              |
|----------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------|
| Sump                                   | <i>Rhizobiaceae</i> , <i>Sphingomonas</i> spp.,<br><i>Devosia</i> spp., <i>Novosphingobium</i> spp.,<br><i>Acidovorax</i> spp., <i>Ralstonia</i> spp., and<br><i>Deinococcus</i> spp.                                                                                                                | Eck et al. (2019)                                                      |
| Fish faeces                            | Fusobacteria, Bacteroidetes, Firmicutes,<br>Proteobacteria, <i>Enterobacteriales</i> ,<br><i>Cetobacterium</i> spp., <i>Plesiomonas</i> spp.                                                                                                                                                         | Schmautz et al. (2017)                                                 |
| Plant roots                            | Proteobacteria, Planctomycetes,<br>Acidobacteria, Bacteroidetes, Nitrospirae,<br>Gemmatimonadetes, <i>Methylophilales</i> ,<br><i>Nitrosomonadales</i> , and <i>Pseudomonas</i> spp.                                                                                                                 | Hu et al. (2015);<br>Schmautz et al. (2017);<br>Wongkiew et al. (2018) |
| Biofilter                              | Bacteroidetes, Verrucomicrobia,<br>Fusobacteria, Planctomycetes, Chloroflexi,<br>Nitrospirae, Proteobacteria,<br><i>Microbacteriaceae</i> , <i>Rhodobacterales</i> ,<br><i>Rhizobiales</i> , <i>Sphingomonadales</i> ,<br><i>Nitrosomonadales</i> , <i>Burkholderiales</i> ,<br><i>Rhodocyclales</i> | Eck et al. (2019);<br>Wongkiew et al. (2018)                           |
| Periphyton in fish rearing tanks       | <i>Xanthomonadales</i> , <i>Rhizobiales</i><br><i>Dokdonella</i> spp., <i>Thermomonas</i> spp.                                                                                                                                                                                                       | Schmautz et al. (2017)                                                 |
| Fish tank effluent                     | Nitrospirae, <i>Nitrobacter</i> spp.                                                                                                                                                                                                                                                                 | Wongkiew et al. (2018)                                                 |

Schmautz et al. (2017) and Eck et al. (2019) separately collected samples from several aquaponic compartments and characterised the bacteria with metabarcoding techniques. They both found that the phyla Proteobacteria and Bacteroidetes were dominant. Eck et al. (2019) indicated that Proteobacteria and Bacteroidetes accounted for 34.6% and 25.5% of the total

bacterial community, respectively, from the 22 samples collected from various aquaponic and aquaculture systems. Schmautz et al. (2017) reported that 46.7% of the total biofilter bacterial community was assigned to Proteobacteria, while Bacteroidetes accounted for 17.5% of total lettuce root bacterial community.

At genus level, the biofilter samples identified by Schmautz et al. (2017) contained a relatively high abundance of *Nitrospira* (3.9% of total community), and low abundances in the periphyton (0.14%) and lettuce roots (0.08%). The genera *Cetobacterium* and *Plesiomonas* accounted for 73.8% and 3.0% of the total bacterial community, respectively in samples from fish faeces. Similarly, the periphyton contained a relatively high abundance of *Dokdonella* and *Thermomonas*, which constituted 7.61% and 1.82% of the total bacterial community, respectively.

### 1.7 Role of microbial communities in aquaponic systems

Bacteria, archaea, and fungi constitute the aquaponics microbiome, with compositional structure being influenced by nutrient availability and variations in environmental conditions (Joyce et al., 2019). Bacteria are vital in the maintenance and functioning of an aquaponic ecosystem. Bacterial communities in aquaponics systems are involved in nitrification, organic matter decomposition, denitrification, iron cycling, as well as phosphorus mineralisation (Table 1.3). If bacterial diversity is not balanced, or operational conditions are not suitable for bacterial activity, the water quality in aquaponic systems may fluctuate, creating a harmful environment for fish and plants (Kasozi et al., 2021a). Although autotrophic and heterotrophic bacteria are likely to exist in both RAS and aquaponic systems, there are likely to be differences in species distribution between parts of the fish rearing section and the hydroponic units (Wongkiew et al., 2017).

**Table 1.3:** Major microbiological processes and contributing microorganisms in aquaponic systems.

| Microbiological process                   | Genera                                                                                                            | Reference                                                                                           |
|-------------------------------------------|-------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------|
| a) Nitrification<br>Ammonia oxidation     | <i>Nitrosomonas</i> , <i>Nitrosococcus</i> ,<br><i>Nitrospira</i> , <i>Nitrosolobus</i> ,<br><i>Nitrosovibrio</i> | Ebeling et al. (2006); Schreier et al. (2010); Rurangwa and Verdegem (2015); Schmautz et al. (2017) |
| Ammonia oxidation by archaea              | <i>Trosopumilus</i> , <i>Nitrososphaeras</i>                                                                      | Bartelme et al. (2019)                                                                              |
| Nitrite oxidation                         | <i>Nitrobacter</i> , <i>Nitrospira</i> ,<br><i>Nitrococcus</i> , <i>Nitrospina</i>                                | Ebeling et al. (2006); Schmautz et al. (2017); Wongkiew et al. (2018)                               |
| Complete ammonia oxidation (Comammox)     | <i>Nitrospira</i>                                                                                                 | Daims et al. (2015); Bartelme et al. (2019)                                                         |
| b) Denitrification                        | <i>Dokdonella</i> , <i>Thermomonas</i>                                                                            | Schmautz et al. (2017)                                                                              |
| c) Mineralisation                         | <i>Pseudomonas</i> , <i>Flavobacterium</i> ,<br><i>Sphingobacterium</i> , <i>Arcobacter</i>                       | Wongkiew et al. (2018); Eck et al. (2019)                                                           |
| d) Anaerobic ammonium oxidation (Anammox) | <i>Brocadia</i>                                                                                                   | Schmautz et al. (2017)                                                                              |
| e) Sulphate reduction                     | <i>Fusibacter</i> , <i>Bacteroides</i><br><i>Desulfovibrio</i> , <i>Dethiosulfovibrio</i>                         | Schreier et al. (2010); Somerville et al. (2014)                                                    |
| f) Organic phosphorus mineralisation      | <i>Modestobacter</i>                                                                                              | Kasozi et al. (2020)                                                                                |
| g) Iron cycling                           | <i>Acidibacter</i>                                                                                                | Kasozi et al. (2020)                                                                                |
| h) Nitrogen fixation                      | <i>Pontibacter</i> , <i>Pseudonocardia</i>                                                                        | Kasozi et al. (2020)                                                                                |

### 1.7.1 Nitrifying bacteria

Nitrifying bacteria are one of the most important organisms in an aquaponic system (Munguia-Fragozo et al., 2015). The main role of these bacteria is to transform  $\text{NH}_4^+$  and  $\text{NH}_3$  excreted by the fish or derived from organic material, into  $\text{NO}_2^-$  and then  $\text{NO}_3^-$ , the latter being relatively less toxic to fish than  $\text{NH}_3$  or  $\text{NO}_2^-$ . In aquaponics, nitrification mostly takes place in the biological filter or on substrates that allow colonisation. In systems with hydroponic media beds, the inert media (such as pumice stones, sand, gravel or expanded clay pebbles) can also provide a habitat for the bacteria and biological filtration (Somerville et al., 2014). Therefore, for a newly established aquaponic system, a fishless cycling approach is

recommended to allow the population of nitrifying bacteria to establish without water quality fluctuations affecting fish welfare (Sallenave, 2016).

Nitrification plays a vital role in regulating the availability of nitrogen in aquaponic systems. This process is conducted by three different groups of bacteria (Daims et al., 2015; Holmes et al., 2019). The AOB oxidise  $\text{NH}_4^+$  and  $\text{NH}_3$  to  $\text{NO}_2^-$  through the first stage of nitrification termed nitrification. Bacteria of the genera *Nitrosomonas*, *Nitrosococcus*, *Nitrospira*, *Nitrosolobus*, and *Nitrosovibrio* are involved in the nitrification step (Rurangwa and Verdegem, 2015). The NOB oxidise  $\text{NO}_2^-$  to  $\text{NO}_3^-$  through a process of nitrification. This second group of bacteria includes the genera *Nitrobacter*, *Nitrococcus*, *Nitrospira*, *Nitrospina*, *Nitrolancea*, and *Nitrotoga* (Shourjeh et al., 2021). The third group from the lineage II of *Nitrospira* is involved in the direct oxidation of  $\text{NH}_4^+$  to  $\text{NO}_3^-$  through a process of Comammox (Daims et al., 2015).

Although the aerobic autotrophic nitrification forms a major part towards nitrogen transformations in aquaponic systems (Munguia-Fragozo et al., 2015), anaerobic ammonium oxidation (Anammox) and heterotrophic ammonia-oxidising bacteria (HAOB) have been identified in biofilters, as well as from sediments of marine and freshwater (Holmes et al., 2019). Anammox bacteria oxidise  $\text{NH}_4^+$  using  $\text{NO}_2^-$  as an electron acceptor, and form gaseous nitrogen as an end-product, while the HAOB oxidation pathway involves  $\text{NH}_4^+$  being oxidised by ammonia monooxygenase to hydroxylamine, which is then oxidised to  $\text{NO}_2^-$  by a hydroxylamine-quinone reductase (Zou et al., 2016; Holmes et al., 2019).

### 1.7.2 Heterotrophic bacteria

Most heterotrophic bacteria are beneficial to the functioning and productivity of aquaponic systems. These bacteria are important during organic matter decomposition and mineralisation (Somerville et al., 2014). For example, heterotrophic bacterial metabolism

mineralises the solid waste, thereby providing soluble nutrients for the plants in aquaponic systems. Bacteria of the genera *Flavobacterium* and *Sphingobacterium* have been postulated to decompose organic matter in aquaponics (Eck et al., 2019; Kasozi et al., 2021a). Heterotrophic bacteria utilise organic matter such as carbohydrates, amino acids, peptides and lipids as carbon and energy sources (Munguia-Fragozo et al., 2015; Stouvenakers et al., 2019).

Although heterotrophic bacteria form a diverse group within an aquaponic system, they are mostly concentrated in areas where solid waste accumulates (Joyce et al., 2019). For instance, in the media bed, heterotrophic bacteria are predominantly distributed in the mineralised zone (Somerville et al., 2014). Heterotrophs may have a negative effect on biofiltration at high total carbon levels because their growth rates are accelerated by the dynamics of carbon to nitrogen (C/N) ratio (Rurangwa and Verdegem, 2015). The ratios of total organic carbon to  $\text{NH}_4^+$ , and dissolved oxygen in aquaponic solution, are therefore important factors in regulating the microbial ecology (Hagopian and Riley, 1998; Aoi et al., 2000).

### 1.7.3 Denitrifying bacteria

Under limited oxygen conditions, bacteria can sequentially reduce  $\text{NO}_2^-$  or  $\text{NO}_3^-$  to nitrogen gas ( $\text{N}_2$ ) via the intermediates nitric oxide (NO) and nitrous oxide ( $\text{N}_2\text{O}$ ) through the process termed denitrification (Zumft, 1997; Pajares and Bohannon, 2016). This biological process may lead to more than 50% loss of total nitrogen available in an aquaponic system (Hu et al., 2015; Zou et al., 2016). Although aerobic denitrification has been reported, most denitrifiers are facultative anaerobes that reduce nitrate in the absence of oxygen (van Rijn and Schreier, 2006). Denitrifiers can perform either complete or incomplete denitrification based on the enzymes involved. A complete denitrification pathway comprises four metalloenzymes:  $\text{NO}_3^-$  reductase,  $\text{NO}_2^-$  reductase, NO reductase, and  $\text{N}_2\text{O}$  reductase (Zumft, 1997; Lu et al.,

2014; Holmes et al., 2019). Incomplete denitrifying microorganisms lack the  $\text{NO}_2^-$  reductase enzyme, which enables complete denitrification (Holmes et al., 2019).

Denitrification is performed by a diverse group of bacteria of the genera *Achromobacter*, *Aerobacter*, *Acinetobacter*, *Bacillus*, *Brevibacterium*, *Flavobacterium*, *Pseudomonas*, *Proteus*, and *Micrococcus* (Lu et al., 2014; Holmes et al., 2019). These microorganisms use nitrate as the electron acceptor, and dissolved organic carbon as the electron donor, with denitrification rates dependent on the carbon source. Hu et al. (2015) reported nitrogen loss ( $\text{NH}_3$ ,  $\text{N}_2\text{O}$  and  $\text{N}_2$ ) of 51.2% in tomato, and 57.3% in pak choi aquaponic systems, although gaseous loss of  $\text{NH}_3$  and  $\text{N}_2\text{O}$  were negligible relative to  $\text{N}_2$  emission. The mineralised zones of gravel media beds of aquaponic systems may have pockets of anoxic sub-zones that may provide favourable conditions for the occurrence of denitrification (Kasozzi et al., 2020). However, the link between denitrification rates and the microbial ecology in aquaponic systems warrants further investigation.

#### 1.7.4 Root associated microorganisms

Root health is essential for plant health and survival, as well as to the composition of microbial populations close to the root surface (Chagas et al., 2018). The root-associated microbial community varies due to the intrinsic characteristics of the plants, including the plant genotype and developmental stage, immune system signalling and root exudation (Chagas et al., 2018). For example, a 16S rRNA gene metabarcoding study of lettuce roots by Schmautz et al. (2017) revealed the genera *Acidovorax*, *Sphingobium*, *Flavobacterium*, and *Pseudomonas* in an aquaponic system integrated with *O. niloticus*. However, the role of these bacteria on roots, including the effect of root exudates in aquaponic systems, is not yet known. Using denaturing gradient gel electrophoresis and a barcoded pyrosequencing approach, Oliveira et al. (2020) reported the genera *Denitromonas*, *Mesorhizobium*, *Colwellia*, *Dokdonella*, and

*Arcobacter* as dominant bacterial species associated with the root systems of three halophyte plants grown in aquaponics, namely, *Halimione portulacoides*, *Salicornia ramosissima*, and *Sarcocornia perennis*. However, the role of these bacteria on roots, including the effect of root exudates in aquaponic systems, is not yet known. It is known that in traditional soil-based environments, plant roots produce, accumulate and release an array of compounds, including organic acids, phenolics, nucleotides, carbohydrates, putrescine, sterols, and vitamins (Michalet et al., 2013; Chagas et al., 2018). These secreted compounds can act as both chemical attractants and repellents for microorganisms (Kawasaki et al., 2016). This could provide the plant with access to beneficial symbiotic microorganisms, and protection from potentially pathogenic bacteria. Some root-associated bacteria, for example, *Bacillus amyloliquefaciens* and *Pseudomonas fluorescens*, possess gene clusters for detoxification, as well as antibiotic and siderophore synthesis (Paulsen et al., 2005; Lareen et al. 2016). This is further supported by Schmutz et al. (2017), who reported a relative abundance of 2.2% of the genus *Pseudomonas* from root samples of aquaponic lettuce, which may confer a biocontrol benefit. Compounds which can be produced by *Pseudomonas*, and which have antibiotic activity include 2,4-diacetylphloroglucinol, oomycin A, and phenazine (Lareen et al., 2016).

#### 1.7.5 Fish and plant pathogens

A range of fish or plant pathogens can unintentionally be introduced into aquaponic systems (Joyce et al., 2019; Stouvenakers et al., 2019). Although aquaponic systems can maintain a healthy ecosystem, culture-independent approaches have identified the presence of bacterial fish pathogens of the genera *Aeromonas* and *Plesiomonas* in aquaponic water samples (Chitmanat et al., 2015). Pathogens can be introduced into aquaponic systems through water additions, equipment, fish feed, infected plants or seeds, diseased fish, as well as media grow bed aggregates (Mori and Smith, 2019; Stouvenakers et al., 2019). Preventing the introduction

and suppressing the growth of pathogens in aquaponic systems is vital because the water recirculation process provides a favourable environment for the multiplication of fish pathogens.

The microclimate of indoor systems, which is characterised by humid, warm, windless, and rain-free conditions, can be conducive for the proliferation of plant diseases if not managed properly. Although pathogens have been reported in aquaponic systems, there are limited studies on transmission mechanisms (Mori and Smith, 2019), possibly due to time required to conduct such studies and the complexity of the research (Mehle et al., 2014). Hydroponic-related literature indicates that waterborne infection occurs when the pathogen is released from a diseased plant, for example, from the roots of an infected plant. The relative abundance of pathogens in a system is dependent on the rate of pathogen shedding and their viability in solution (Mehle et al., 2014).

A review by Mori and Smith (2019) found no reports of pathogen transmission in aquaponic systems, with information on pathogen transmission in soilless systems only being available from aquaculture and hydroponic studies. Research by Mehle and Ravnkar (2012) demonstrated plant virus survival in water and suggested viral concentration-dependent transmission through the roots of plants. Other studies have highlighted the proliferation of the oomycetes (*Phytophthora* spp. and *Pythium* spp.) in aquaponic systems (Li et al., 2014b; Lee and Lee, 2015). These zoosporic organisms are adapted to warm, humid and windless conditions, which are common in greenhouse structures (Stouvenakers et al., 2019). Additionally, the ability to locate utilisable carbon sources quickly allows zoospores to spread rapidly, particularly in water recycling systems (Hong and Moorman, 2005). For example, *Pythium* spp., due to their mobile form of dispersion, spread through water recirculating systems, and cause root rot in hydroponic plants (Lee and Lee, 2015). Thus, it is important to manage fungal infections in aquaponic operations. The introduction of beneficial

microorganisms into aquaponic systems may be a possible strategy to control plant pathogens but information regarding their effectiveness against plant pathogens is limited (Stouvenakers et al., 2019).

### 1.8 Integration of probiotics in aquaponics

The FAO/WHO joint panel defined probiotics as 'live microorganisms, which when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2002). This definition has become the most recognised version (Hill et al., 2014). Probiotics encompass a wide range of bacteria, bacteriophages, microalgae, and yeast. In aquaculture, probiotics can be administered through the addition to a water medium or used as a feed supplement. *Bacillus* and other genera, such as *Lactobacillus*, *Lactococcus*, *Pseudomonas*, *Pediococcus*, are regarded as probiotics (Balcázar et al., 2006; Vine et al., 2006). Although *Bacillus* species are amongst the most frequently studied plant growth promoters (Ahmad et al., 2008), there is a paucity of information regarding their role for both fish and plant health in aquaponic systems.

The genus *Bacillus*, a member of the phylum Firmicutes is a gram-positive, endospore-forming bacteria with an important role in the sustainability of agriculture production (Radhakrishnan et al., 2017). In agriculture, *Bacillus* species are classified as extracellular plant growth-promoting rhizobacteria inhabiting the rhizosphere or the spaces between the root cortex cells (Gouda et al., 2018) and the majority are regarded as safe microorganisms (Balcázar et al., 2006). The most frequent *Bacillus* species used as probiotic strains in aquaculture are *B. subtilis*, *B. licheniformis*, *B. pumilus*, and *B. amyloliquefaciens* (Kuebutornye et al., 2019). *Bacillus* species as probiotics possess positive characteristics such as the ability to replicate rapidly and to tolerate a wide range of environmental conditions (Kuebutornye et al., 2019). Research on the application of *Bacillus* species as probiotics in

recirculating aquaculture systems has focussed mainly on their use as feed utilisation enhancers and health improvement supplements for fish, but there is a paucity of work on their use in aquaponic crop production. Therefore, there is an opportunity to investigate the application of *Bacillus* species in aquaponics. Within the context of agriculture, most of the research on *Bacillus* reflects findings derived from soil-based systems, and few from hydroponics systems (García et al., 2004; Gül et al., 2008). The only study regarding probiotics in aquaponics was reported by Cerozi and Fitzsimmons (2016), who evaluated the effect of *Bacillus* on phosphorus accumulation. Although the authors reported an increase in phosphorus concentration in aquaponic systems that received the probiotic, their study did not investigate the bacterial microbiota of the aquaponic system. Furthermore, the design of their hydroponic growing system was based on the nutrient film technique, which is different from this research, which includes a gravel media bed and deep-water culture. Palm et al. (2018) reported that the design of a hydroponic growing subsystem influences nutrient availability in an aquaponic system. Based on the literature reviewed, further investigations are needed to determine the effects of *Bacillus* on bacterial diversity and nutrient availability in coupled aquaponic systems. From research papers, mainly from traditional-based agriculture, with a few from hydroponics and RAS, the benefits of *Bacillus* are outlined in the subsections (1.8.1–1.8.4) of this work.

### **1.8.1 Phosphate solubilisation**

Phosphorus is one of the essential nutrients required by plants in adequate quantities for growth and development (Joyce et al., 2019). At cellular level, phosphorus plays an important role in energy transfer, growth of new tissues, respiration, cell division, as well as photosynthesis (Gouda et al., 2018). However, 95% to 99% of phosphorus in soil solution exists as insoluble inorganic phosphorus and cannot be utilised by plants. Phosphate solubilising microorganisms are capable of hydrolysing organic and inorganic insoluble

phosphorus compounds to soluble forms that can easily be assimilated by plants. *Bacillus* spp. converts the complex form of phosphorus into simple bioavailable forms such as dihydrogen phosphate ( $\text{H}_2\text{PO}_4^-$ ), hydrogen phosphate ( $\text{HPO}_4^{2-}$ ), and phosphate ( $\text{PO}_4^{3-}$ ), which can be absorbed by the plant roots (Gouda et al., 2018).

### 1.8.2 Plant growth promotion

In traditional agricultural systems, research has demonstrated that inoculating plants with *Bacillus* spp. can be an effective strategy to stimulate crop growth through mechanisms such as nutrient uptake or increased nutrient availability by nitrogen fixation, mineralisation of organic compounds, solubilisation of mineral nutrients, and production of phytohormones (Shafi et al., 2017; Radhakrishnan et al., 2017). In reference to the application of *Bacillus* spp., García et al. (2004) investigated the effects of *B. licheniformis* on the growth of pepper and tomato in hydroponics. The authors reported that the total weight of pepper harvested, and the number of tomato fruits produced were increased significantly by the inoculation. Thus, *B. licheniformis* has considerable colonisation and competitive benefits, and it is recommended as a biofertiliser.

### 1.8.3 Improvement of water quality

Optimal conditions and the physicochemical status of the rearing water is an essential consideration in aquaponics, as it affects all three groups of organisms (fish, plants, and bacteria). Typically, low water quality in aquaponics affects fish physiology, fish and plant growth rates and the nitrification process (Yildiz et al., 2017). Improvement in water quality has been recorded in studies involving the addition of *Bacillus* spp. These improvements include a decrease of total dissolved solid concentration, and an increase in the mineralisation of organic matter (Hlordzi et al., 2020; Jasmin et al., 2020). Other mechanisms through which

*Bacillus* species improve water quality is by ammonification, nitrification, denitrification, and nitrogen fixation (Hlirdzi et al., 2020). A study by Yu et al. (2005) reported that *Bacillus amyloliquefaciens* reduced ammonia by up to 93% after 24 h of inoculation with an initial concentration of ammonia of 200 mg L<sup>-1</sup>, at a temperature of 35°C and a pH of 7.0. Jasmin et al. (2020) reviewed various studies in which *Bacillus* species were used under a wide range of aquaculture conditions to treat wastewater from aquaculture operations, in particular from shrimp culture, and the authors reported that *Bacillus* species lowered ammonia and nitrite concentrations in several studies.

#### 1.8.4 Other benefits

The application of *Bacillus* species in aquaculture has gained attention in the maintenance of fish health (Balcázar et al., 2006). *Bacillus* species possess characteristics such as the inhibition of pathogens by competing for adhesion sites with pathogenic microbes to inhibit their growth, as well as the production of antibiotics and bacteriocins, and the inhibition of virulence gene expression (Balcázar et al., 2006). Bacterial pathogens, particularly the *Vibrio* and *Aeromonas* species, regulate the expression of virulence genes through quorum sensing, which is bacterial cell-to-cell communication with small signal molecules. Quorum sensing disruption has been suggested to be a promising strategy to control conventional and emerging infections in fish and plants. Among fish, *B. amyloliquefaciens* was found to have an inhibitory effect on pathogenic *Aeromonas hydrophila* associated with the eel, *Anguilla anguilla* (Cao et al., 2011). *Bacillus* species act as quorum-quenching bacteria which can degrade AHLs (N-acyl homoserine lactones), which are responsible for the expression of virulence factor mediated by quorum sensing in *Vibrio* (Defoirdt et al., 2011). Regarding plants, *Bacillus cereus* and *B. thuringiensis* contain the *aiiA* gene, which encodes an enzyme that catalyses the degradation of AHLs. Because *Bacillus* species can degrade AHLs, they have been used as

biocontrol agents against *Pectobacterium carotovorum*, a plant pathogen that causes soft rot and wilt in various vegetables such as Chinese cabbage, carrot, and tomato (Lim et al., 2013). Cui et al. (2019) reported that *B. amyloliquefaciens* KC-1 effectively reduced the symptoms of bacterial soft rot in Chinese cabbage, and decreased transmission of the plant pathogen *P. carotovorum* under greenhouse conditions.

Another characteristic of *Bacillus* species is its ability to produce digestive enzymes such as protease, trypsin, amylase, and lipase which potentially increase nutrient digestibility in fish (Balcázar et al., 2006; Kuebutornye et al., 2019; Maas et al., 2021). In brief, enzymes produced by *Bacillus* species are efficient at metabolising proteins, carbohydrates, and lipids. The presence of digestive enzymes and their level of activity in fish is a relative indicator of food acceptance, the digestive capacity in relation to the type of feed offered (Kuebutornye et al., 2019). Besides the production of fish digestive enzymes, *Bacillus* species can produce chitinase and  $\beta$ -1,3-glucanase. These lytic enzymes are active in degrading fungal cell walls, thereby contributing to the defence of plants against fungal pathogens (Shafi et al., 2017). In conclusion, these beneficial properties of *Bacillus* provide a compelling reason to investigate the effects of supplementing a commercial mixture of *Bacillus subtilis* and *B. licheniformis* in a coupled aquaponic system on plant and fish growth and bacterial diversity.

## 1.9 Statement of relevance

The literature reviewed indicates that the development of environmentally sustainable plant and fish production in aquaponic systems requires a complete understanding of the systems' biological components. As aquaponics is a relatively new research field with the potential to address contemporary challenges faced by traditional agriculture, a better understanding of the roles of microbial communities is important for system management.

Bacteria are an indispensable part of this ecosystem, and their presence is beneficial for the system performance. Different approaches to improving productivity in soilless systems are either unpractical on a large scale (e.g., use of composting worms) or are not environmentally friendly (e.g., application of synthetic fertilisers or indiscriminate use of pesticides). Aquaponics must rely on environmentally friendly methods to enhance productivity, or else the health of the fish and beneficial bacterial population become compromised. Considering that microbes are vital in the functioning and chemical equilibrium of aquaponic systems, integration of probiotics in aquaponics is a sustainable strategy for mitigating environmental impacts, and for promoting improved agriculture. Based on the current literature, research on the application of *Bacillus* species as a probiotic in recirculating aquaculture systems has focussed mainly on enhancing feed utilisation and health improvement supplements for aquatic animals. In addition, the benefits of *Bacillus* species have focussed primarily on soil-based crops, and no in-depth studies have been found regarding their potential role in influencing microbial diversity

With the presence of both plants and fish, aquaponic systems are complex systems, with more diverse microbial communities than traditional production systems which are specialised towards either rearing fish or the growing of plants. Understanding the relationships between the fish, plants, and bacteria in aquaponic systems is crucial for managing any changes in water quality and for enhancing system performance. To date, there is limited information regarding the effects of the addition of *Bacillus* on such a complex system. It is hypothesised that integrating a probiotic mixture of *B. subtilis* and *B. licheniformis* into aquaponic systems could influence nutrient availability, water quality, fish and plant growth, as well as the bacterial structure and composition. Ultimately, testing this hypothesis will contribute to improving our understanding of the aquaponics microbiome and provide valuable baseline data on the

bacterial community compositions of plants, fish, and gravel media beds. The research will also enhance our knowledge of the potential role of *Bacillus* species in soilless systems.

### 1.10 Aim and objectives of the study

The overall aim of this research is to investigate the effects of *Bacillus* as a probiotic on the fish and plant biomass, as well as the composition of bacterial communities in an aquaponic system. To achieve this aim, the following objectives were set:

- 1) To investigate the effect of *Bacillus* species on lettuce growth and the root associated bacterial community in a small-scale aquaponic system.
- 2) To determine the effect of *Bacillus* species on the phylloplane associated bacteria of lettuce in a small-scale aquaponic system.
- 3) To evaluate the effect of *Bacillus* species on the water quality, growth and digestive enzyme activity of Mozambique tilapia, *Oreochromis mossambicus*.
- 4) To evaluate the effect of adding a commercial mixture of *Bacillus* species to the water of an aquaponic system on the gastrointestinal microbiota of juvenile Mozambique tilapia, *O. mossambicus*.
- 5) To explore the bacterial diversity and community structure associated with the media bed zones of a small-scale aquaponic system supplemented with *Bacillus* species.

## **CHAPTER 2**

### **General materials and methods**

#### **2.1 Introduction**

This chapter explains the aquaponic design used in this research. In addition, the important steps undertaken for biofilter establishment in newly built aquaponic systems are outlined. This chapter also discusses laboratory techniques relevant to the objectives of the research. The software package used to analyse the 16S rRNA gene sequencing data is also presented.

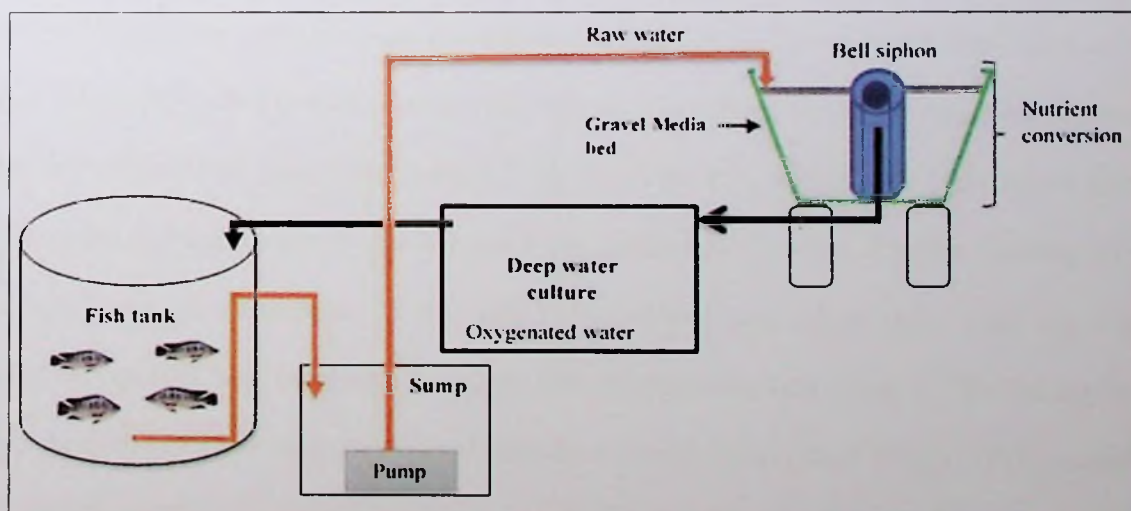
#### **2.2 System setup and operation**

Four independent (not connected to each other) aquaponic units were designed, constructed, and maintained in a controlled environment, indoor plant growth room at the Department of Biochemistry and Microbiology, Rhodes University. Due to space limitations, and control of environmental variables, four aquaponic units were maintained. The modular small-scale aquaponic design used in this research was adapted from commercial systems used by local practitioners in South Africa. Following training in modular aquaponic systems at Practical Aquaponics (Pty) Ltd., in Salem, the Principal investigator and the thesis author designed and built the small-scale aquaponic systems used in this research. These modular systems use low-tech installations, making them suitable for use in developing countries.

Each aquaponic system was one experimental unit comprising of a 100 L fish tank, a 40 L sump with a submersible pump (SOBO 6000, 85 W, Zhongshan, SOBO Electric Co., Ltd., China), a 120 L flood-and-drain media bed for biological filtration, and a 126 L deep-water hydroponic culture unit (Figure 2.1). These units were connected by pipes to form a closed water cycle.

Each gravel media bed was fitted with a bell siphon, which maintained a flood-and-drain system. Water exiting the fish tank was fed by gravity to the sump (lowest point), from where it was pumped up to the gravel media bed at  $180 \text{ L h}^{-1}$ . As the water volume increased in the gravel bed (highest point), the water drained into a deep-water hydroponic unit to achieve a water cycling rate of 4 cycles  $\text{h}^{-1}$ . From the hydroponic unit, water returned to the fish rearing tank, thus completing the cycle (Figure 2.1).

The volume of recirculating water in each aquaponic system was kept constant by adding rainwater filtered through a MilliQ Millipore ultrapure water purification system fixed with a  $0.22 \mu\text{m}$  membrane filter. Filtered water was added to compensate for the water loss by evaporation and evapotranspiration. The media grow bed units acted as both a mechanical and biological filter, by removing solids from the water that flowed from fish tanks, and by enabling the conversion of toxic organic wastes into bioavailable nutrients for the plants. Each media bed was filled with crushed granite stones of approximately 19 mm diameter. The depth and width of each media bed were 31 cm and 120 cm, respectively.



**Figure 2.1:** Schematic diagram of a single experimental aquaponic unit and directions of flow of water.

Aeration in the fish tanks and deep-water culture unit was provided by air pumps, while each fish rearing tank contained a submerged heater with a thermostat, with the aim of maintaining a water temperature range of between 25°C to 27°C, although this was not always possible. The air temperature in the plant growth room was maintained using a wall-mounted air conditioner (Samsung Electronics Co., Ltd., China) in the range of 18°C to 22°C.

Because indoor systems rely on efficient electrical lighting to produce crops, lights were controlled by a digitally timed electrical switch. Plants were grown under fluorescent lighting (Sylvania F58W/GRO Gro-Lux fluorescent tubes) with a 13 h light phase and an 11 h dark phase. After water exchange for a week, each aquaponic system was considered suitable for cycling once all the leaks were fixed, and a stable flow rate was achieved.

### **2.3 Biofilter establishment**

Before the commencement of the experiments, a 25-day fishless cycling strategy was undertaken. According to Sallenave (2016) and Brailo et al. (2019), this strategy requires less time than cycling with fish, taking approximately three weeks for biofilter establishment. To regulate pH in the first week, sodium bicarbonate ( $1.6 \text{ g } 100 \text{ L}^{-1}$ ) was added daily until a pH range of 6.5 to 7.5 was attained. During this period of cycling, the cycle rate was reduced through the gravel bed from 10 cycles  $\text{h}^{-1}$  to 4 cycles  $\text{h}^{-1}$ , which was then maintained throughout the different trials. In the second week, ammonia (SeaGro, Premier Fishing, Pty, Ltd., Cape Town, South Africa, 25 mL  $100 \text{ L}^{-1}$  of water) was added daily until the total ammonia nitrogen (TAN) reached a concentration of approximately  $3 \text{ mg } \text{L}^{-1} \text{ N}$ . During the last five days, the systems were inoculated with the commercial product FINCO (Filter start<sup>®</sup>, Hikari, Japan; supplied by KoiPetCo, Johannesburg, South Africa), which provided a source of nitrifying bacteria at an application rate of  $0.5 \text{ g } 100 \text{ L}^{-1}$  of water. The TAN and  $\text{NO}_2^-$

concentrations were monitored during this period prior to the addition of fish, until they decreased below  $0.12 \text{ mg L}^{-1}$  and  $0.1 \text{ mg L}^{-1}$ , respectively.

## 2.4 Experimental design

This research focussed on the three major life forms (plants, fish, and bacteria) which constitute an aquaponic system. This was achieved by conducting interrelated studies which investigated the effects of *Bacillus* species on water quality, lettuce and fish growth, and on the bacterial community structure and diversity. As indicated in Table 2.1, the first experiment focussed on plants (growth, nutrient concentration dynamics, root and phylloplane-associated bacteria) comprising a total of three lettuce growth cycles. The second experiment, linking to the first experiment focussed on fish growth, proximate composition, digestive enzyme activities, and fish gut microbiota (Table 2.1). The bacterial communities of the vertical zones of the media bed systems were characterised using Illumina-based 16S rRNA gene sequencing. Because water is a key medium for the above-mentioned life forms, and a major carrier of nutrients required for plant growth within an aquaponic system, water quality parameters were determined throughout the study.

Two aquaponic systems were randomly assigned to each treatment (two control units and two treatment units). The commercial product (Sanolife® PRO-W;  $5.0 \times 10^{10} \text{ CFU g}^{-1}$ , INVE Technologies, Dendermonde, Belgium) contained a mixture of *Bacillus subtilis* and *B. licheniformis*. Sanolife® PRO-W also comprises soybean meal as organic filler, calcium carbonate as free-flowing agent and silicon dioxide as anti-caking agent (Appendix 2.1). The product was dissolved before being introduced into the sump component of each of the two aquaponic systems. The mixture of *Bacillus subtilis* and *B. licheniformis* was used in this research as these two species are regarded as non-toxicogenic, biological supplements in aquaculture industry (EFSA Panel on Biological Hazards, 2021).

Lettuce was considered for this research because of its availability, low nutrient requirements and economical value in aquaponic systems. In addition, the relatively short growing period of lettuce allows multiple growth trials to be conducted. At the start of each trial, lettuce plants (Locarno cultivar) of similar sizes were planted to ensure that there were no significant differences in initial weights between treatments. The growth period for lettuce lasted for 30 days in each growth cycle. Each aquaponic unit had 16 plants with a spacing of 15 cm × 15 cm between plants, with only the roots submerged in the water using grip plant holders. The accumulated solids in the deep-water hydroponic units were removed at the end of each harvesting cycle.

**Table 2.1:** Deliverables and conditions of the trials investigating the effects of a commercial probiotic on plant and fish growth, water quality, and bacterial diversity.

| Plants           |                                                                                              |                               |                                                                      |
|------------------|----------------------------------------------------------------------------------------------|-------------------------------|----------------------------------------------------------------------|
| Dataset          | Lettuce plants in each aquaponic system                                                      | Initial fish stocking density | Deliverables                                                         |
| I                | 16                                                                                           | 3.45 kg m <sup>-3</sup>       | Lettuce growth, leaf nutrient analysis, and root-associated bacteria |
| II               | 16                                                                                           | 1.13 kg m <sup>-3</sup>       | Nutrient concentration dynamics and lettuce growth                   |
| III              | 16                                                                                           | 1.13 kg m <sup>-3</sup>       | Phylloplane associated bacteria                                      |
| Fish             |                                                                                              |                               |                                                                      |
| IV               | 16                                                                                           | 3.45 kg m <sup>-3</sup>       | Fish growth, proximate composition, and digestive enzyme activities  |
| V                | 16                                                                                           | 1.13 kg m <sup>-3</sup>       | Fish gastrointestinal microbiota                                     |
| ↓                |                                                                                              |                               |                                                                      |
| Gravel media bed |                                                                                              |                               |                                                                      |
| VI               | Bacterial communities associated with media grow bed zones (dry, root and mineralised zones) |                               |                                                                      |

Fish from the same strain and source were used throughout the fish growth trials. Mozambique tilapia (*Oreochromis mossambicus*) was considered suitable for this research as they are indigenous to South Africa. This fish species is widely cultured in the Eastern Cape,

South Africa. It can also tolerate a wide range of water quality conditions. The fish were hand-fed with a commercial floating pellet (Avi Products Pty Ltd., Cato Ridge, South Africa). Netting (20 mm mesh size) covered the fish tanks to prevent fish from jumping out of the tanks. Water chemistry and stocking density were managed to optimise the living conditions for the fish used in this research. The stocking densities between experiments was based on the initial concentrations of nitrate and phosphate measured during the process of fishless cycling.

## **2.5 Laboratory analytical procedures**

Prior to use, all laboratory glassware was washed with detergent before being soaked in a 20%  $\text{HNO}_3$  (v/v) solution for 24 h, after which the solution was discarded. This procedure was repeated three times. Subsequently, the prepared glassware was rinsed five times with deionised water and dried. All chemicals were analytical grade unless specifically stated.

### **2.5.1 Physicochemical analysis of water samples**

Physicochemical parameters, namely water temperature, pH, total dissolved solids (TDS) and electrical conductivity (EC) were measured using a multi-parameter water quality monitor probe (PHT-027, China). Water samples were taken from each of the fish tanks and deep-water culture units. The probe was calibrated every two weeks using standard solutions. After measurement of each sample, the probe was rinsed with deionized water to avoid cross contamination among different samples. The concentration of dissolved oxygen (DO) was measured using a dissolved oxygen meter (Pen-850045, Sper Scientific Ltd., Scottsdale, AZ, USA).

Nitrite ( $\text{NO}_2^-$ ) was measured using a nitrite-test kit (Sera GmbH, Heinsberg, Germany). Nitrate ( $\text{NO}_3^-$ ), total ammonia nitrogen (TAN) and phosphate ( $\text{PO}_4^{3-}$ ) were measured using Spectroquant® test kits following standard methods (Merck Pty Ltd., products; 1.14773.0001,

1.14752.0001, and 1.14848.0001, respectively) and recorded using a spectrophotometer (Merck Spectroquant® Pharo 300 spectrophotometer, Merck, Darmstadt, Germany). spreadsheet in MS Excel was created to calculate the concentration of free ammonia (NH<sub>3</sub>) in the water samples using the values of temperature and pH generated in experiments as described by Emerson et al. (1975).

### **2.5.2 Leaf mineral analysis**

Dry ashing digestion was applied during sample preparation for the determination of iron (Fe), phosphorus (P), potassium (K), magnesium (Mg), calcium (Ca), zinc (Zn), sodium (Na), and copper (Cu) in leaf samples. Lettuce leaves were harvested, weighed, and oven-dried at 72°C for 48 h to determine dry mass. This temperature was sufficient to remove the moisture from the leaves without causing thermal decomposition. After drying, the leaf material was ground through a 1 mm sieve and 1.2 g of grounded-dry leaf material from each sample were placed into a muffle furnace (Gallenkamp muffle furnace, REX-C900, England) for 4 h at 500°C. After cooling, 1 g of each sample was dissolved in 5 mL of 20% HCl. To further dissolve the residue, the solution was heated on a hot plate for 3 min at 90°C. The solution was filtered through acid-washed Whatman filter paper into a 50 mL volumetric flask. After digestion, dilutions were made to meet analytical requirements. Before determination of mineral elements with Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, 715-ES, Varian Associates, Walnut Creek, CA, USA), the instrument was calibrated by creating a calibration curve for the measurement of emission for standard solutions and the blank. The analysis was conducted at the Nelson Mandela University, InnoVenton facility, South Africa.

### 2.5.3.3 Determination of crude fat using oil solvent extraction method

Total fat was extracted from samples according to the methods of Bligh and Dyer (1959) with slight modifications by Kinsella et al. (1977). Briefly, 2 g of completely homogenised dried sample was soaked for 4 h in a solution of chloroform and methanol (2:1), filtered and emulsified with distilled water and allowed to separate until two clear layers of solution were obtained. The oil solution was concentrated with a rotary evaporator (Rotavapor® R-215, Switzerland) at 60°C in a pre-weighed 50 mL round-bottom flask. The boiling flask with extracted fat was cooled in a desiccator and weighed. An increase in weight of the flask gave the crude fat.

$$\text{Fat (\%)} = \frac{(\text{weight of flask and extracted fat} - \text{weight of empty flask}) \times 100}{\text{weight of dried sample}}$$

### 2.5.3.4 Crude protein analysis

The crude protein concentration was determined using the Kjeldahl method (nitrogen  $\times$  6.25) following three steps that included digestion, distillation, and titration. Briefly, 3 g of homogenised fish dried sample was weighed and transferred into a Kjeldahl flask where 7 g of potassium sulphate, 1.2 g of copper sulphate, and 15 mL of concentrated sulphuric acid were added. The mixture was then heated at 200°C for 4 h until a clear solution was attained. After digestion, the mixture was cooled at room temperature and diluted with 100 mL of distilled water. In the distillation unit, 50 mL of sodium hydroxide was added to the digestion tube.  $\text{NH}_3$  was captured in 50 mL of 4% boric acid solution containing 3 drops of methyl red indicator. The distillate collected in the boric acid solution was titrated with 0.1 M hydrochloric acid (HCl) until the solution had a slightly violet colour. The percentage of protein was calculated using the formula:

$$\text{Crude protein (\%)} = \frac{(\text{titre vol sample} - \text{titre vol blank}) \times 0.014 \times 0.1 \times 6.25 \times 100}{\text{weight of sample}}$$

### **2.5.3.5 Gross energy**

The gross energy content was determined using an adiabatic bomb calorimeter (Calorimeter Bomb Parr 1261; PARR Instrument, IL, USA) using benzoic acid as a calibration standard. A dried sample of 0.5 g was placed into a crucible and pressed to make a firm pellet. The crucible was then placed into a bomb vessel. A firing wire was attached between the electrodes and a loop was formed above the pellet. Prior to firing the calorimetry bomb, the thermometer reading was recorded as the initial temperature. When the bomb vessel temperature stabilised, the sample was then ignited, and the energy value ( $\text{kJ g}^{-1}$ ) was recorded.

## **2.6 Data computation**

### **2.6.1 Plant measurements and growth**

Non-destructive measurements of plant physiological activity (chlorophyll content index and maximal efficiency of PSII) were carried out on fully developed lettuce leaves at the end of each growth cycle. The chlorophyll content index (CCI) was measured using a portable chlorophyll meter (Apogee MC-100, Apogee Instruments, Inc. USA). Before assessment for chlorophyll fluorescence, the leaves were placed in the dark for 20 min. Chlorophyll fluorescence was assessed by measuring the maximum quantum yield of PSII photochemistry ( $F_v/F_m$ ) of lettuce leaves after dark adaptation using a plant efficiency analyser, Handy PEA+ (Hansatech Instruments Ltd., Norfolk, UK).

The plant growth characteristics were measured in terms of root and shoot biomass. The plant biomass was determined by measuring plant height (cm), number of leaves per plant, shoot fresh weight (g), and root length (cm). Plant height (from the base to the growing tip) was measured to the nearest mm. Absolute growth rate (AGR) and height gain (HG,  $\text{cm plant}^{-1}$ ) were calculated using the equations:

$$AGR = (M_2 - M_1) / T$$

$$HG = FH - IH$$

Whereby:

$M_2$  = average final plant mass (g plant<sup>-1</sup>)

$M_1$  = average initial plant mass (g plant<sup>-1</sup>)

T = number of days of the growth period

FH = average final height (cm plant<sup>-1</sup>)

IH = average initial height (cm plant<sup>-1</sup>)

## 2.6.2 Fish growth parameters

The production performance parameters of the fish were determined using data on the weight and feed consumed throughout the study. Percentage weight gain (WG), specific growth rate (SGR, % day<sup>-1</sup>), feed conversion ratio (FCR), protein efficiency ratio (PER), feed efficiency (FE, %), percentage survival (SR), and percentage growth increase (PGI) were calculated using the growth equations:

$$WG = 100 \times ((FW - IW) / IW)$$

$$SGR = [(\ln FW - \ln IW) / T] \times 100$$

$$FCR = DM / BMG$$

$$PER = BMG / PI$$

$$FE = 100 \times BMG / FI$$

$$SR = 100 \times (N_f / N_i)$$

$$PGI = 100 \times ((MT - MC) / MC)$$

Whereby:

FW = average final weight (g fish<sup>-1</sup>)

IW = average initial weight (g fish<sup>-1</sup>)

T = number of days of the growth period

ln FW = the natural logarithm of the mean final weight

ln IW = the natural logarithm of the mean initial weight

DM = total dry matter of feed supplied (g)

BMG = wet biomass gain (g)  
 PI = total dry weight of feed protein consumed (g)  
 FI = weight of feed fed (g)  
 N<sub>f</sub> and N<sub>i</sub> were the final number and initial number of fish per tank, respectively.  
 MT and MC were the mean measurements of the treatment and the control, respectively.

### 2.6.3 Digestive enzyme activities

Whole intestines of the fish were pooled for each system and homogenised in (1:3 w/v) cold 50 mM Tris-HCl (pH = 8). The samples were homogenised using an electric blender for 2 min. The homogenates were centrifuged at 10,000 × g for 30 min at 4°C (BOECO centrifuge U-320R, Germany) to eliminate tissue debris and lipids. The supernatant was collected and stored in small aliquots of 1.5 mL subsets and stored at -80°C until just before analysis. The activities of amylase, total alkaline protease, and alkaline phosphatase were determined and specific details for each assay are described in Chapter 5. The specific activities were calculated using the equations:

$$\begin{aligned} \text{Specific amylase activity (U mg protein}^{-1}\text{)} &= V_1 \times ((G)/ T \times V_2 \times P) \\ \text{Specific alkaline phosphatase activity (U mg protein}^{-1}\text{)} &= V_1 \times ((N)/ T \times V_2 \times P) \\ \text{Specific protease activity (U mg protein}^{-1}\text{)} &= V_1 \times ((L)/ T \times V_2 \times P) \end{aligned}$$

Whereby:  
 G = μmol of glucose liberated min<sup>-1</sup>  
 N = μmol of *P*-nitrophenol liberated min<sup>-1</sup>  
 L = μmol of L-tyrosine liberated min<sup>-1</sup>  
 V<sub>1</sub> = assay volume (mL)  
 V<sub>2</sub> = volume of enzyme (mL)  
 P = protein content of the enzyme (mg)  
 T = reaction time (min)

## 2.7 General molecular analyses

Each procedure of the molecular analyses, comprising DNA extraction, PCR amplification, and sequencing was conducted under sterile conditions. The characterisation of the bacterial diversity and community composition of samples from root and leaves of lettuce, water samples, fish gut, media gravel beds, and effluent water were conducted using the 16S rRNA gene amplicon NGS method. Different approaches were applied to concentrate DNA from these samples as described in Chapters 4, 5, 7, and 8. Total genomic DNA was extracted from roots, leaves, gravel stones, and effluent water using a ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research, Tustin, CA, USA) following the manufacturer's instructions. The genomic DNA of the gastrointestinal microbiota of *Oreochromis mossambicus* was extracted using the Quick-DNA™ DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research, Tustin, CA, USA). Both the quality and quantity of isolated DNA were assessed by measuring the absorbance at 260 and 280 nm wavelengths using a NanoDrop™ 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

Amplification of the variable region V3 – V4 of the 16S rRNA gene was performed using a universal primer set 16Sa-F (5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CAG CAG CCG CGG TAA- 3') and 16Sa-R (5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGT AAG GTT CYT CGC GT-3'). The reaction mixture for each sample contained 50 ng of template DNA, 0.3 µM of each oligonucleotide primer, 0.3 mM of dNTP mix, 1× reaction buffer, 2.5 mM of MgCl<sub>2</sub>, 1 unit of Accupol™ DNA polymerase, which was then made up to 25 µL with PCR-grade water. A control, in which nuclease-free water was added instead of DNA, was included in all samples used for PCR. All samples were amplified using a T100™ Thermal Cycler (BioRad Laboratories, Hercules, CA, USA) with the following conditions: 98°C for 5 min; 7 cycles of 98°C for 45 sec, 45°C for 30 sec, 72°C for 1 min

followed by 18 cycles of 98°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min. A final extension was done at 72°C for 5 min.

Electrophoresis was performed for 45 min at 100 V. PCR products were visualised on a 1% agarose gel under a ChemiDoc™ XRS+ (Bio-Rad, Hercules, CA, USA). The amplicon band was excised from the gel and purified using the Zymoclean™ Gel DNA recovery Kit (Zymo Research, Tustin, CA, USA) according to kit instructions. The Illumina MiSeq integrated next-generation sequencer (Illumina® Inc., USA) and a MiSeq Reagent Kit v3 (600 cycles) (Illumina®, San Diego, CA, USA) were used to sequence the prepared amplicon libraries at the Aquatic Genomics Research Platform of the South African Institute of Aquatic Biodiversity (SAIAB).

## **2.8 Bioinformatics processing and statistical analysis**

The sequence FASTQ files from the Illumina MiSeq were analysed using the MOTHUR platform (Schloss et al., 2009). Dataset curation included removal of reads shorter than 100 bases, as well as reads longer than 550 bases and those with ambiguous nucleotides. Chimeric sequences were removed using the VSEARCH command (Rognes et al., 2016) within MOTHUR. Subsequently, unique reads were checked for chimeric sequences followed by their removal from the datasets. Non-bacterial, chloroplast and mitochondrial operational taxonomic units (OTUs) were considered to be contaminating sequences and removed prior to downstream analysis. The sequence data sets were aligned against the SILVA 16S rRNA gene database release version 132, and a pairwise distance matrix was created from the curated aligned datasets in order to group sequences into Operational Taxonomic Units (OTUs) at a confidence level of 97%. Classification of the reads was done to the genus level using the Naïve Bayesian classifier algorithm against the SILVA 16S rRNA gene database with a confidence threshold of 90% to assign taxonomic identity. All OTUs were clustered at a cut-off of 0.03.

Alpha diversity metrics, including the analysis of Good's coverage, abundance-based estimators of species richness (Chao1 and Observed species richness), estimator of species richness and species evenness (Shannon and InvSimpson) were calculated based on OTUs with  $\geq 97\%$  identity using the summary single command in MOTHUR. Rarefaction curves were standardised to the sample yielding the least number of total sequences. Species identification for the dominant OTUs was performed using the NCBI Basic Local Alignment Search Tool (BLAST) command line 'blastn' (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), while heatmaps were constructed to visualise the distribution of the dominant OTUs using the Morpheus application (<https://software.broadinstitute.org/morpheus>). All other analyses were performed using the Statistica software package (TIBCO Software, Palo Alto, USA, version 13.5.0).

## CHAPTER 3

### Effect of *Bacillus* species on lettuce growth and root associated bacterial community of lettuce in a small-scale aquaponic system

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#### 3.1 Introduction

This chapter highlights the significance of integrating *Bacillus* species into aquaponic systems on plant growth, nutrient availability, and bacterial community diversity associated with roots. Regardless of the type of agricultural system, root health is essential to the growth of plants. The microorganisms associated with roots can be beneficial or harmful for the growth and health of the plants. However, little is known about the bacterial community structure and diversity associated with plants following the introduction of a probiotic into aquaponic systems.

In traditional agricultural systems, research has demonstrated that inoculating plants with plant-growth promoting bacteria (PGPB) such as *Bacillus* spp., can be an effective strategy to stimulate crop growth (Ahmad et al., 2008), however, research on the application of PGPB in aquaponics is still limited (Cerozi and Fitzsimmons, 2016; Bartelme et al., 2018). In soil-based systems, PGPB are effective substitutes for chemical inputs to meet both plant growth requirements and to reduce the impact of biotic stress (Ahmad et al., 2008). The direct promotion by PGPB entails either providing the plant with growth-promoting substances which are synthesised by the bacteria, or facilitating the uptake of certain plant nutrients from the environment. PGPB are capable of stimulating plant growth through a number of mechanisms, including nitrogen fixation (Ahmad et al., 2008), stimulation of root growth (Lugtenberg and Kamilova, 2009), organic matter mineralisation, suppression of growth or activity of disease-causing organisms (Zahir et al., 2004), improvement of plant nutrition, and increasing the bioavailability of nutrients (Martínez-Viveros et al., 2010). Bacteria beneficial to plants form

stable biofilms on the roots (Lareen et al., 2016). Various genera of bacteria, *Pseudomonas*, *Enterobacter*, *Bacillus*, *Variovorax*, *Klebsiella*, *Burkholderia*, *Azospirillum*, *Serratia* and *Azotobacter* have been reported to exhibit plant growth-promoting characteristics (Zahir et al., 2004; Ahmad et al., 2008; Nadeem et al., 2014). In addition to advancing aquaponic crop production, research into the use of PGPB in soilless-based environments has the potential to advance our understanding of root-associated microorganisms. The use of a mixture of *Bacillus subtilis* and *B. licheniformis* is an environmentally safe method to improve fish growth and water quality, however, their effects on the root microbiota of soilless plants require further investigation. Among *Bacilli*, strains of *Bacillus subtilis* are the most widely used PGPB due to their disease-reducing and antibiotic-producing capabilities when applied as seed treatments (Hayat et al., 2010). Therefore, this study investigated the effect of supplementation of a commercial product (Sanolife®PRO-W) containing a mixture of *Bacillus subtilis* and *B. licheniformis* on the growth of 'Locarno' leaf lettuce cultivar (*Lactuca sativa*) under deep-water culture, integrated with Mozambique tilapia (*Oreochromis mossambicus*) in modular coupled aquaponic systems.

## **3.2 Materials and methods**

### **3.2.1 Cultivation system**

The design of the aquaponic systems used in this study is described in Section 2.2 (Chapter 2). This study consisted of two separate 30-day experimental phases of lettuce growth and data collection. The first trial started on 08 June 2019 while the second trial commenced on 09 September 2020. In each trial, plants were grown under fluorescent lighting following lighting schedules described in Section 2.2 (Chapter 2). The air temperature in the growth room for both trials was maintained in a range of between 18°C to 22°C. The process of building

bacterial ecosystems during the setup of the aquaponic systems followed fishless cycling for 25 days is described in Section 2.3 (Chapter 2).

### 3.2.2 Fish rearing conditions

Red Mozambique tilapia (*Oreochromis mossambicus*) juveniles were obtained from the Aquaculture Innovations farm located in Grahamstown, South Africa. Initial stocking densities varied over the two trials depending on the initial concentrations of nitrates and phosphates during the fishless cycling. Although the initial stocking densities were  $3.45 \text{ kg m}^{-3}$  and  $1.13 \text{ kg m}^{-3}$  for the first and second trial, respectively, average fish weight in each tank were similar within each trial. Fish were fed a commercial floating pellet (Avi Products Pty Ltd.) containing 40% crude protein, 0.7% phosphorus, 5% crude fat, and 3% calcium at between 2% and 3% of body mass  $\text{day}^{-1}$ , distributed over three feedings (09h00, 13h00, and 16h00). Water temperature in fish rearing tanks was maintained within the target range ( $21.0^{\circ}\text{C} - 26.5^{\circ}\text{C}$ ) using thermostat heaters.

### 3.2.3 Lettuce conditions

Three-week old 'Locarno' lettuce seedlings at a four-leaf stage were transplanted. Prior to planting, each plant root was submerged, rinsed and cleaned in sterilised water. After cleaning the roots, plants were individually weighed, and then randomly planted into polystyrene floating rafts. Each aquaponic unit had 16 plants with a spacing of  $15 \text{ cm} \times 15 \text{ cm}$  between plants. Lettuce trials were only initiated when nitrate levels were above  $10 \text{ mg L}^{-1}$  and oxygen levels above  $6.0 \text{ mg L}^{-1}$  in the deep-water hydroponic unit. Aquaponics units assigned to a probiotic received 5.31 g of a commercial *Bacillus* mixture of *B. subtilis* and *B. licheniformis* twice a week following manufacturer's instructions ( $0.02 \text{ g product L}^{-1}$  of water), until the end of the experiment. Because lettuce is a short-cycle single harvest leafy

green crop, each growth cycle was conducted for 30 days. Aquaponic systems are often iron deficient due to low amounts of Fe in commercial fish feeds (Kasozi et al., 2019). Thus, Fe was added in its chelated form (6% Fe-EDDHA) at an application rate of 2 mg L<sup>-1</sup> once every two weeks. The pH was periodically adjusted with 2 g of calcium hydroxide (Ca(OH)<sub>2</sub>) added to the sump only when the pH dropped below 6.5.

### 3.2.4 Water quality and leaf mineral analysis

Water temperature, pH, total dissolved solids, and electrical conductivity were measured daily using a multi-parameter water quality probe. The concentration of dissolved oxygen was measured twice a week using a dissolved oxygen meter. Nitrite (NO<sub>2</sub><sup>-</sup>) was measured twice a week using a Sera nitrite-test kit, while nitrate (NO<sub>3</sub><sup>-</sup>), total ammonium nitrogen (TAN), and phosphate (PO<sub>4</sub><sup>3-</sup>) were measured three times a week using Spectroquant test kits as described in Section 2.5.1 (Chapter 2). The concentration of free ammonia (NH<sub>3</sub>) in the water samples from the fish rearing tanks was determined following the calculations of Emerson et al. (1975) using the temperature and pH values on the day samples were taken. Other nutrient parameters, including P, K, Mg, Ca, Zn, Na, and Cu were determined using ICP-OES. Dried leaf samples were analysed for Fe, P, K, Mg, Ca, Zn, Na, and Cu following the procedures described in Section 2.5.2 (Chapter 2).

### 3.2.5 Plant growth characteristics

The chlorophyll content index (CCI) and chlorophyll fluorescence were measured on three leaves per plant using a portable chlorophyll meter and a plant efficiency analyser, respectively as described in Section 2.6.1 (Chapter 2). A total of 16 plants from each deep-water hydroponic unit were harvested after 30 days. At the end of each crop cycle, the plants were transferred to the laboratory where the shoots were detached from the roots using a

sterilised scalpel blade. The fresh weight of each shoot and root was recorded using an electronic balance (Adam Equipment, PGL-2002, with a precision of 0.01 g). To determine the final yield, shoot fresh weight of the lettuce was considered as a vegetative growth parameter. Shoot and root samples were oven-dried at 72°C for 48 h separately to determine their dry mass. Absolute growth rate (AGR) and height gain (HG, cm plant<sup>-1</sup>) of the lettuce were calculated following the equations described in Section 2.6.1 (Chapter 2).

### 3.2.6 DNA extraction and PCR amplification

At the end of the first trial, four samples of lettuce roots were assembled by cutting one entire root from four plants from each of the four aquaponic units (total of 16 roots). The tools for cutting were held in a propane gas flame for 5 min before use and disinfected with 70% ethanol. Each sample was placed into a 250 mL bottle filled with 50 mL sterile ultrapure water and vortexed at maximum speed for 20 min. The samples from each system were pooled to be treated as one sample for further analyses. This was done to remove pseudoreplication bias. Two washing steps were conducted for each sample. To concentrate the bacteria, a 100 µL aliquot of root wash solution from each sample was filtered through 0.2 µm filters (Supor® Membrane disc filters, PALL Life Sciences, Ann Arbor, MI, USA) with a vacuum pump (Rocker Model 801, vacuum pump 167801-22, Taiwan). DNA was extracted from microbial cells associated with 0.2 µm filters using a ZymoBIOMICS™ DNA Miniprep Kit, according to the manufacturer's instructions. Purity and quantity of the extracted DNA were measured by NanoDrop 2000 spectrophotometer. The preparation of the reaction mixture and conditions for the PCR followed the protocol described in Section 2.7 (Chapter 2). The sequence datasets generated in this study were deposited in the sequence reads archive (SRA) database of the National Centre of Biotechnology Information (SRA accession: SRR11496721).

### 3.2.7 Processing of amplicon sequencing data

Raw 16S rRNA gene sequences were processed using the MOTHUR platform version 1.41.3 release (Schloss et al., 2009) following the procedure described in Section 2.8 (Chapter 2). Estimation of alpha diversity was measured using Shannon, InvSimpson, Sobs and Chao1. The analysis of the common and unique OTUs was conducted to investigate the root bacterial community from the two replicates per treatment through a Venn diagram. Only those taxa with an overall abundance of more than 0.2% (phylum level) and 0.1% at genus level were considered for statistical analysis.

### 3.2.8 Statistical analysis

Results were expressed as mean  $\pm$  standard error (SE), and treatment mean differences were analysed using Student's *t*-test for equal sample variance for all data except for the phosphate and nitrate concentrations. To test whether there was a significant difference between treatments on the phosphate and nitrate concentrations over time, a repeated measures analysis of variance (RM-ANOVA) was used. Student's *t*-test has been proposed to be more suitable for small sample sizes (de Winter, 2013), similar to those used in this study, than the use of non-parametric alternative tests. Where necessary, results from *t*-tests for unequal variances were used. An alpha error level of 5% ( $P < 0.05$ ) was used to test the null hypothesis of no differences between treatment means or, in the case of RM-ANOVA, no interaction between treatment and repeated measures (time). To avoid bias caused by pseudoreplication (Hurlbert, 1984), each aquaponic system was treated as an experimental unit, so that the values of all plants in each experimental unit were averaged before they were used for statistical analyses.

### 3.3 Results

#### 3.3.1 Water quality parameters in fish rearing tanks

In growth trial 1, water temperature fluctuated between 21.3°C and 25.6°C for the control tanks and between 21.0°C and 25.3°C for the *Bacillus*-treated tanks (Table 3.1). The changes in temperature over time were similar between the *Bacillus*-treated and control systems (RM-ANOVA,  $P = 0.91$ ). Both the change in levels over time of TDS and EC were not influenced by the addition of the *Bacillus* product (RM-ANOVA,  $P = 0.50$  and  $P = 0.41$  respectively). The levels of dissolved oxygen were maintained above 5 mg L<sup>-1</sup>. During this growth trial, nitrite levels were maintained below 1.0 mg L<sup>-1</sup>. With fish present, the average NH<sub>3</sub> concentration for the control tanks was 0.002 mg L<sup>-1</sup> while the *Bacillus*-treated tanks averaged 0.003 mg L<sup>-1</sup> (Table 3.1). The treatment did not have a significant effect on the changes of ammonia over time (RM-ANOVA,  $P = 0.56$ ). The average value of TAN for the control tanks was 0.33 mg L<sup>-1</sup> while the *Bacillus*-treated tanks averaged 0.27 mg L<sup>-1</sup> and this average was not influenced by the addition of *Bacillus* (RM-ANOVA,  $P = 0.42$ ).

In growth trial 2, water temperature varied between 26.2°C and 26.7°C for the control tanks and between 25.5°C and 26.7°C for the *Bacillus*-treated tanks (Table 3.1). The fluctuations in temperature were not influenced by *Bacillus* (RM-ANOVA,  $P = 0.28$ ). The levels of dissolved oxygen and nitrite levels were maintained above 5 mg L<sup>-1</sup> and below 1.0 mg L<sup>-1</sup>, respectively, during this trial. Average pH over time was not significantly affected by probiotic addition (RM-ANOVA,  $P = 0.55$ ). During this trial, TDS fluctuated between 330 and 471 mg L<sup>-1</sup> for the control tanks and 331 and 446 mg L<sup>-1</sup> for the *Bacillus*-treated tanks (Table 3.1). The changes in the levels of TDS and EC over time were not significantly affected by inclusion of *Bacillus* in the fish water tanks (RM-ANOVA,  $P = 0.95$ ;  $P = 0.83$ , respectively). The average value of TAN, for the control tanks was 0.14 mg L<sup>-1</sup>, while the treatment tanks averaged 0.15 mg L<sup>-1</sup> and these values were not influenced by the addition of *Bacillus* (RM-

ANOVA,  $P = 0.15$ ). The addition of *Bacillus* did not have a significant effect on the changes of  $\text{NH}_3$  over time (RM-ANOVA,  $P = 0.17$ ). At the end of the trial,  $\text{NH}_3$  in the control systems averaged  $0.0006 \text{ mg L}^{-1}$  as compared to  $0.0005 \text{ mg L}^{-1}$  in the *Bacillus*-treated systems (Table 3.1).

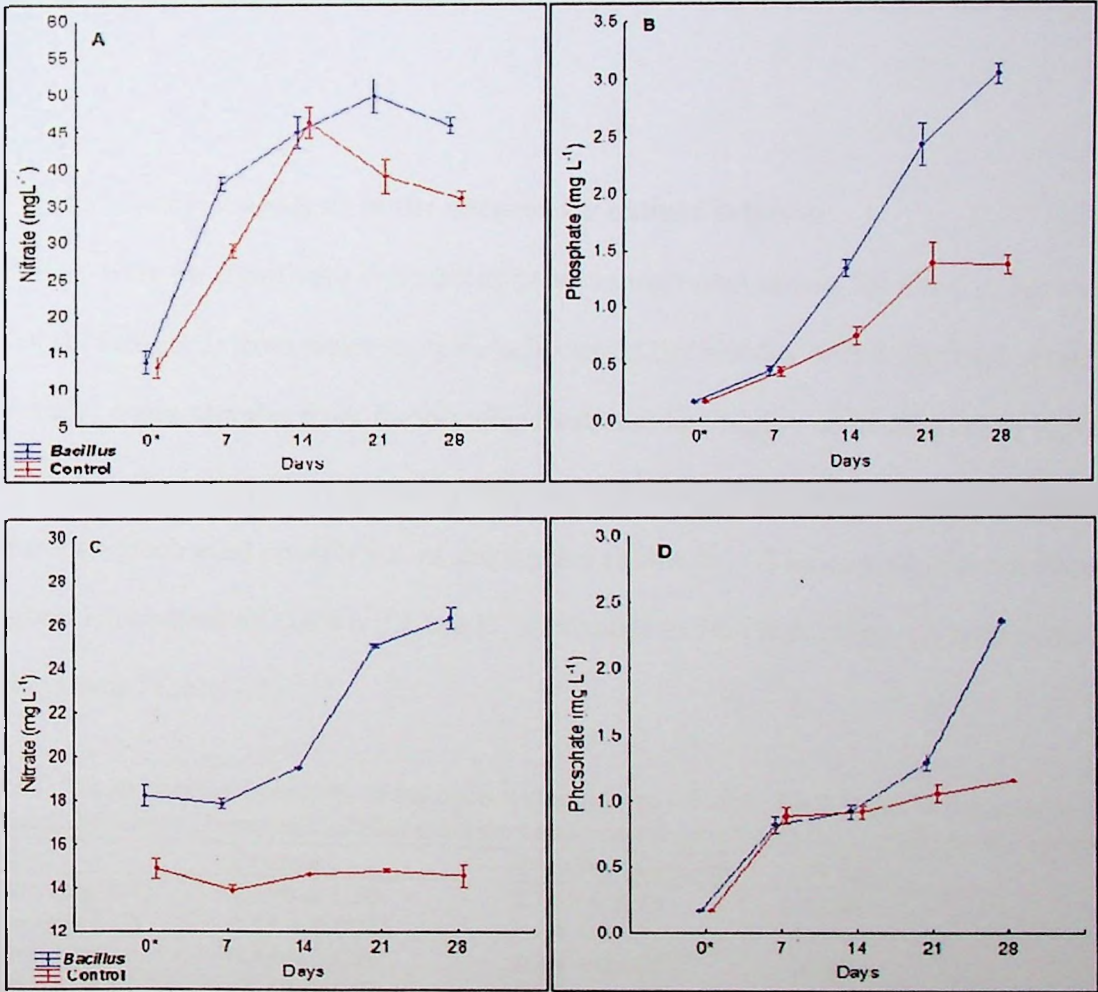
**Table 3.1:** Comparison of water quality in the control and *Bacillus*-treated systems from the fish rearing tanks. The ranges of water quality variables during the two lettuce production trials are given. Mean values followed by the range in parentheses.

| Variables                                       | Growth trial 1           |                           | Growth trial 2             |                            |
|-------------------------------------------------|--------------------------|---------------------------|----------------------------|----------------------------|
|                                                 | Control                  | <i>Bacillus</i> treatment | Control                    | <i>Bacillus</i> treatment  |
| Temperature ( $^{\circ}\text{C}$ )              | 24.46<br>(21.29 – 25.56) | 24.37<br>(21.0 – 25.33)   | 26.40<br>(26.20 – 26.74)   | 26.26<br>(25.53 – 26.73)   |
| Dissolved oxygen ( $\text{mg L}^{-1}$ )         | 6.0<br>(5.90 – 6.10)     | 6.1<br>(5.90 – 6.20)      | 5.5<br>(5.30 – 5.90)       | 5.6<br>(5.20 – 6.0)        |
| Total dissolved solids ( $\text{mg L}^{-1}$ )   | 521<br>(401 – 584)       | 452<br>(350 – 541)        | 398<br>(330 – 471)         | 370<br>(331 – 446)         |
| pH                                              | 7.15<br>(6.78 – 7.47)    | 7.23<br>(6.63 – 7.80)     | 6.80<br>(6.61 – 7.09)      | 6.72<br>(6.52 – 6.89)      |
| Electrical conductivity ( $\text{mS cm}^{-1}$ ) | 0.69<br>(0.55 – 0.84)    | 0.61<br>(0.50 – 0.71)     | 0.57<br>(0.46 – 0.78)      | 0.54<br>(0.46 – 0.72)      |
| Total ammonia nitrogen ( $\text{mg L}^{-1}$ )   | 0.33<br>(0.10 – 0.72)    | 0.27<br>(0.13 – 0.52)     | 0.14<br>(0.10 – 0.29)      | 0.15<br>(0.04 – 0.35)      |
| Free ammonia ( $\text{mg L}^{-1}$ )             | 0.002<br>(0.001 – 0.004) | 0.003<br>(0.001 – 0.007)  | 0.0006<br>(0.0003 – 0.002) | 0.0005<br>(0.0001 – 0.002) |
| Nitrite ( $\text{mg L}^{-1}$ )                  | < 0.1                    | < 0.1                     | < 0.1                      | < 0.1                      |

### 3.3.2 Nitrate and phosphate concentration dynamics in the deep-water culture solution

In growth trial 1, there was a significant increase in the concentration of nitrate and phosphate over the 30-day period in the *Bacillus*-treated systems compared to the control systems (RM-ANOVA,  $F_{4,8} = 5.5$ ,  $P = 0.019$ ;  $F_{4,8} = 26.5$ ,  $P = 0.00011$ , respectively). After approximately 21 days, the average nitrate level in the *Bacillus*-treated deep-water culture tanks

increased significantly relative to the control systems. At the end of the experiment, nitrate in the control tanks averaged 35.87 mg L<sup>-1</sup>, compared to 45.80 mg L<sup>-1</sup> in the *Bacillus* treatment (Figure 3.1A). The *Bacillus* product significantly increased the phosphate concentrations in the *Bacillus*-treated systems from day 14 to the end of the experiment. The phosphate concentration reached 1.37 mg L<sup>-1</sup> in the control, while the *Bacillus* treatment averaged 3.08 mg L<sup>-1</sup> (Figure 3.1B).



**Figure 3.1:** Nitrate and phosphate concentration changes (A-D) in deep-water culture solution in the control and *Bacillus* treatment for the two trials. Each data point is the mean  $\pm$  SE. Water samples were collected three days in each week from each system. RM-ANOVA was used to compare means for phosphate and nitrate concentrations between treatments over time (weeks). Tukey's HSD post-hoc test at  $\alpha = 0.05$  was used to compare treatment means between treatments and treatment  $\times$  time. 0\* time at planting.

In the growth trial 2, a similar trend was observed, with a significant increase in the concentration of nitrate and phosphate in the *Bacillus*-treated systems (RM-ANOVA,  $F_{4,8} = 64.2$ ,  $P < 0.00001$ ;  $F_{4,8} = 84.7$ ,  $P < 0.00001$ , respectively). The *Bacillus* product significantly increased the nitrate concentrations in *Bacillus*-treated systems from day 7 until the end of the experiment ( $P < 0.0001$ ). At the end of 30 days, nitrate in the control averaged  $14.52 \text{ mg L}^{-1}$  as compared to  $26.27 \text{ mg L}^{-1}$  in the *Bacillus* treatment (Figure 3.1C). The highest phosphate concentration in the control systems and *Bacillus* treatment was  $1.14 \text{ mg L}^{-1}$  and  $2.35 \text{ mg L}^{-1}$ , respectively (Figure 3.1D).

### 3.3.3 Water chemical analysis in the deep-water culture solution

There were no significant differences between treatment means for Ca, Cu, Fe, and Mg ( $P > 0.05$ ) (Table 3.2) from water samples collected at the last day of the first trial. At the end of the study, water samples from the *Bacillus* treatment had higher concentration of K, Na, P, and Zn than in the control. For example, *Bacillus*-treated systems accumulated approximately four times as much total phosphorus as the control (Table 3.2). The control systems recorded the highest Na content of  $116.8 \pm 1.6 \text{ mg L}^{-1}$  compared to  $98.6 \pm 1.35 \text{ mg L}^{-1}$  in the *Bacillus*-treated systems (Table 3.2).

**Table 3.2:** Water chemical analysis of the deep-water culture solution. Each value is the mean  $\pm$  SE. Significant differences between *Bacillus* treatment and control ( $P < 0.05$ ) are marked by an asterisk.

| Variables                         | Control           | <i>Bacillus</i> treatment | <i>P</i> -Value |
|-----------------------------------|-------------------|---------------------------|-----------------|
| Calcium ( $\text{mg L}^{-1}$ )    | $27.90 \pm 1.50$  | $27.58 \pm 3.35$          | 0.940           |
| Copper ( $\text{mg L}^{-1}$ )     | $0.15 \pm 0.0005$ | $0.14 \pm 0.03$           | 0.710           |
| Iron ( $\text{mg L}^{-1}$ )       | $0.14 \pm 0.002$  | $0.14 \pm 0.002$          | 0.804           |
| Potassium ( $\text{mg L}^{-1}$ )  | $18.48 \pm 0.82$  | $20.31 \pm 0.01$          | 0.004*          |
| Magnesium ( $\text{mg L}^{-1}$ )  | $16.15 \pm 0.61$  | $15.17 \pm 0.01$          | 0.251           |
| Sodium ( $\text{mg L}^{-1}$ )     | $116.8 \pm 1.60$  | $98.6 \pm 1.35$           | 0.013*          |
| Phosphorus ( $\text{mg L}^{-1}$ ) | $1.13 \pm 0.13$   | $4.26 \pm 0.26$           | 0.008*          |
| Zinc ( $\text{mg L}^{-1}$ )       | $0.03 \pm 0.002$  | $0.08 \pm 0.001$          | 0.002*          |

### 3.3.4 Lettuce growth and chlorophyll concentration

In both trials, the  $F_v/F_m$ , the mean shoot dry weight, and the mean fresh weight of the harvested shoots from the *Bacillus* treatment was significantly higher ( $P < 0.05$ ) than that of the control treatment (Table 3.3). Consistent with shoot fresh and dry weight increase, the application of probiotic bacteria significantly increased the absolute growth rate (AGR) of the lettuce (Table 3.3). In growth trial 1, shoot fresh weight at harvest was 23.8% higher ( $24.84 \pm 0.18 \text{ g plant}^{-1}$ ) in plants from the *Bacillus* treatment than in the control ( $20.07 \pm 0.02 \text{ g plant}^{-1}$ ) (Table 3.3). Corresponding dry weight of the lettuce shoot from the *Bacillus* treatment ( $0.76 \pm 0.03 \text{ g plant}^{-1}$ ) was 38.2% higher than in the control ( $0.55 \pm 0.006 \text{ g plant}^{-1}$ ). The chlorophyll fluorescence parameter  $F_v/F_m$  for the *Bacillus* treatment was 0.83 compared to 0.71 for the control.

In the second growth cycle, shoot fresh weight at harvest was 29.4% higher ( $33.08 \pm 1.36 \text{ g plant}^{-1}$ ) in plants from the *Bacillus* treatment than in the control ( $25.57 \pm 0.53 \text{ g plant}^{-1}$ ; Table 3.3). Corresponding average root fresh weight of plants from the *Bacillus*-treated systems ( $2.11 \pm 0.04 \text{ g plant}^{-1}$ ) was 27.9% higher than in the control ( $1.65 \pm 0.05 \text{ g plant}^{-1}$ ). The AGR was significantly higher in the *Bacillus* treatment than the control ( $t$ -test,  $P = 0.03$ ; Table 3.3). No significant differences were observed in CCI between the *Bacillus* treatment and the control ( $P = 0.30$ ).

**Table 3.3:** Growth of 'Locarno' lettuce cultivar in deep-water culture solution in the control and *Bacillus* treatment for two crop cycles. Significant differences between *Bacillus* treatment and control ( $P < 0.05$ ) are marked by an asterisk. Data are means  $\pm$  standard error (SE).

| Variables                                      | Growth trial 1   |                           |                 |
|------------------------------------------------|------------------|---------------------------|-----------------|
|                                                | Control          | <i>Bacillus</i> treatment | <i>P</i> -value |
| Initial plant mass (g plant <sup>-1</sup> )    | 3.50 $\pm$ 0.09  | 3.51 $\pm$ 0.005          | 0.293           |
| Shoot fresh weight (g plant <sup>-1</sup> )    | 20.07 $\pm$ 0.02 | 24.84 $\pm$ 0.18          | 0.001*          |
| Root fresh weight (g plant <sup>-1</sup> )     | 1.06 $\pm$ 0.009 | 1.58 $\pm$ 0.004          | 0.002*          |
| Height gain of plant (cm plant <sup>-1</sup> ) | 20.24 $\pm$ 0.64 | 20.92 $\pm$ 1.66          | 0.738           |
| Absolute growth rate (g day <sup>-1</sup> )    | 0.59 $\pm$ 0.0   | 0.77 $\pm$ 0.005          | 0.0008*         |
| F <sub>v</sub> /F <sub>m</sub>                 | 0.71 $\pm$ 0.01  | 0.83 $\pm$ 0.007          | 0.018*          |
| CCI                                            | 2.06 $\pm$ 0.05  | 2.20 $\pm$ 0.09           | 0.305           |
| Shoot dry weight (g plant <sup>-1</sup> )      | 0.55 $\pm$ 0.006 | 0.76 $\pm$ 0.03           | 0.002*          |
| Root dry weight (g plant <sup>-1</sup> )       | 0.09 $\pm$ 0.005 | 0.10 $\pm$ 0.004          | 0.094           |
|                                                | Growth trial 2   |                           |                 |
|                                                | Control          | <i>Bacillus</i> treatment | <i>P</i> -value |
| Initial plant mass (g plant <sup>-1</sup> )    | 4.63 $\pm$ 0.01  | 4.62 $\pm$ 0.05           | 0.698           |
| Shoot fresh weight (g plant <sup>-1</sup> )    | 25.57 $\pm$ 0.53 | 33.08 $\pm$ 1.36          | 0.036*          |
| Root fresh weight (g plant <sup>-1</sup> )     | 1.65 $\pm$ 0.05  | 2.11 $\pm$ 0.04           | 0.023*          |
| Height gain of plant (cm plant <sup>-1</sup> ) | 20.31 $\pm$ 0.14 | 29.32 $\pm$ 0.69          | 0.006*          |
| Absolute growth rate (g day <sup>-1</sup> )    | 0.76 $\pm$ 0.02  | 1.02 $\pm$ 0.05           | 0.032*          |
| F <sub>v</sub> /F <sub>m</sub>                 | 0.72 $\pm$ 0.01  | 0.82 $\pm$ 0.04           | 0.029*          |
| CCI                                            | 2.41 $\pm$ 0.004 | 2.46 $\pm$ 0.03           | 0.307           |
| Shoot dry weight (g plant <sup>-1</sup> )      | 0.85 $\pm$ 0.01  | 1.47 $\pm$ 0.031          | 0.001*          |
| Root dry weight (g plant <sup>-1</sup> )       | 0.14 $\pm$ 0.01  | 0.24 $\pm$ 0.01           | 0.024*          |

### 3.3.5 Leaf mineral composition

The average leaf K, P, and Zn contents in the *Bacillus* treatment were significantly higher than in the control (*t*-test,  $t = 5.14$ ,  $P = 0.036$ ;  $t = 11.94$ ,  $P = 0.007$ ;  $t = 5.85$ ,  $P = 0.028$ , respectively, Table 3.4). However, there were no significant differences between *Bacillus* treatment and control for the means of Ca, Mg, Na, Cu, and Fe concentrations (Table 3.4).

**Table 3.4:** Leaf mineral composition of lettuce grown under deep-water culture. Each value is the mean  $\pm$  SE. Significant differences between *Bacillus* treatment and control ( $P < 0.05$ ) are marked by an asterisk.

| Mineral composition              | Control          | <i>Bacillus</i> treatment | <i>P</i> -Value |
|----------------------------------|------------------|---------------------------|-----------------|
| Calcium (g kg <sup>-1</sup> )    | 51.40 $\pm$ 1.50 | 50.65 $\pm$ 0.69          | 0.692           |
| Potassium (g kg <sup>-1</sup> )  | 50.95 $\pm$ 0.20 | 54.87 $\pm$ 0.73          | 0.036*          |
| Magnesium (g kg <sup>-1</sup> )  | 29.86 $\pm$ 0.66 | 29.38 $\pm$ 0.26          | 0.575           |
| Sodium (g kg <sup>-1</sup> )     | 25.20 $\pm$ 0.44 | 21.21 $\pm$ 1.09          | 0.078           |
| Phosphorus (g kg <sup>-1</sup> ) | 4.83 $\pm$ 0.22  | 7.95 $\pm$ 0.12           | 0.007*          |
| Copper (mg kg <sup>-1</sup> )    | 4.96 $\pm$ 0.09  | 4.04 $\pm$ 0.51           | 0.221           |
| Iron (mg kg <sup>-1</sup> )      | 48.36 $\pm$ 1.81 | 47.99 $\pm$ 1.22          | 0.883           |
| Zinc (mg kg <sup>-1</sup> )      | 6.25 $\pm$ 0.39  | 14.75 $\pm$ 1.39          | 0.028*          |

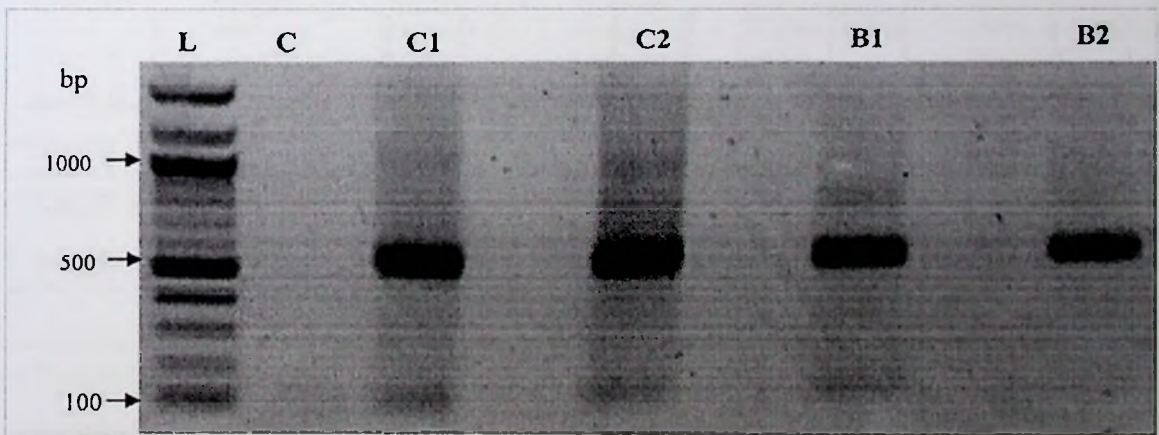
3.3.6 Evaluation of DNA quality and quantity from lettuce root samples

The purity and concentration of the DNA obtained from root samples were determined by the  $A_{260}/A_{280}$  absorbance ratio (Table 3.5). All the root samples had  $A_{260}/A_{280}$  ratios above 1.8 and slightly above 2.0 implying pure DNA (Table 3.5). After PCR amplification, all samples showed discrete bands (Figure 3.2). No fragmented DNA was observed in the lanes.

**Table 3.5:** Purity and concentration of the extracted genomic DNA from root samples obtained from the control and *Bacillus*-treated systems.

| Sample | DNA concentration (ng $\mu\text{L}^{-1}$ ) | $A_{260}/A_{280}$ | DNA integrity * |
|--------|--------------------------------------------|-------------------|-----------------|
| C1     | 2.1                                        | 2.21              | +               |
| C2     | 5.3                                        | 2.17              | ++              |
| B1     | 12.0                                       | 1.96              | ++              |
| B2     | 9.3                                        | 1.85              | ++              |

\*DNA integrity was assayed by agarose electrophoresis and indicated as good (++) or sufficient (+) based on the band clarity, density, and presence of smearing. C1 and C2 represent root samples from the control; and B1 and B2 are the root samples from systems supplemented with a *Bacillus* probiotic.



**Figure 3.2:** Agarose gel electrophoresis of the PCR products amplified from the roots of lettuce with or without *Bacillus* treatment. Lane L shows the 100-bp DNA ladder; followed by lane C (a negative control). Lanes C1 and C2 are the amplification products from root samples from the control; and lanes B1 and B2 are the root samples from systems supplemented with a *Bacillus* probiotic.

3.3.7 Taxonomic assignment of reads from root samples

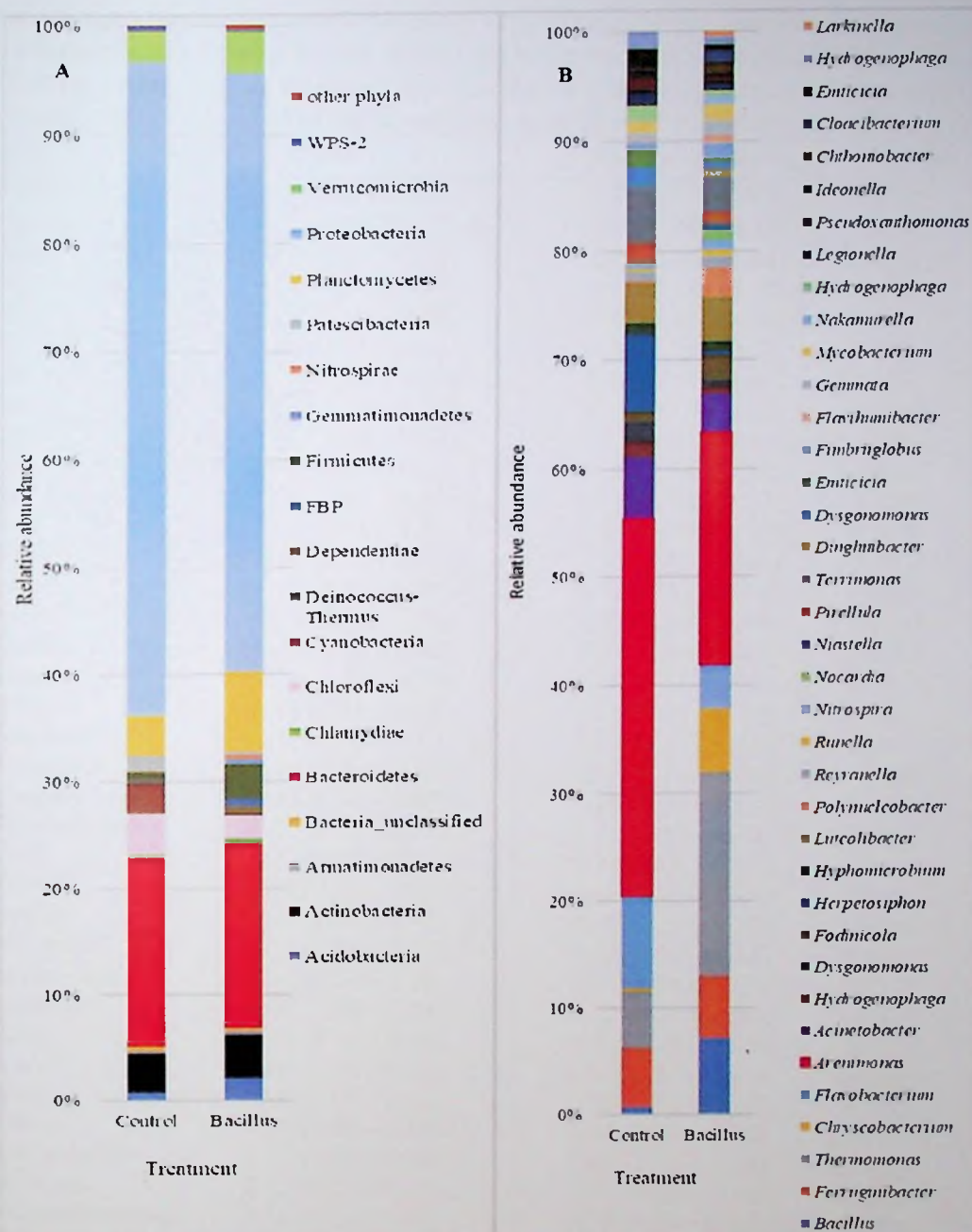
A total of 179,490 raw sequence reads from the two replicate samples were obtained, with *Bacillus*-treated systems providing a total of 76,751, while the control systems yielded a total of 102,739 raw sequence reads (Table 3.6). After removing poor-quality reads, a total of 65,906 sequences for the *Bacillus*-treated samples and 91,014 sequences for the control samples were generated. A total of 66,607 unique sequences for both *Bacillus*-treated samples and control samples were also generated. Removal of chimeras resulted in a total of 56,543 and 79,279 sequences for *Bacillus*-treated samples and control samples, respectively. The total percentage of sequences flagged as chimeric was 13.4% for all samples. After screening and filtering, a total of 47,044 and 68,125 reads were obtained for *Bacillus*-treated samples and control samples, respectively.

Table 3.6: Summary of sequencing data obtained from root samples.

| Treatment                 | Number of raw sequences | Number of sequences before chimeras | Number of sequences after chimeras | Chloroplast and Mitochondria reads | Number of reads after screening and filtering |
|---------------------------|-------------------------|-------------------------------------|------------------------------------|------------------------------------|-----------------------------------------------|
| Control                   |                         |                                     |                                    |                                    |                                               |
| Sample C1                 | 42,899                  | 37,663                              | 33,101                             | 1,547                              | 28,145                                        |
| Sample C2                 | 59,840                  | 53,351                              | 46,178                             | 2,366                              | 39,980                                        |
| <i>Bacillus</i> treatment |                         |                                     |                                    |                                    |                                               |
| Sample B1                 | 56,285                  | 47,935                              | 40,859                             | 246                                | 34,348                                        |
| Sample B2                 | 20,466                  | 17,971                              | 15,684                             | 102                                | 12,696                                        |

Based on the taxonomic assignment of the reads, the bacterial communities in the two systems differed from each other (Table 3.7). Irrespective of the treatment, the dominant phyla were Proteobacteria, Bacteroidetes, Planctomycetes, Actinobacteria, and Verrucomicrobia (Figure 3.3). The Proteobacteria phylum accounted for more than half of the total number of reads in both the control and the *Bacillus* treatment. All sequences were identified into 30

and *Luteolibacter* were abundant in the two systems, with *Flavobacterium* representing 2.78% for the control and 1.67% for the *Bacillus*-treated system, and *Luteolibacter* representing 1.18% and 1.62% of the total community, respectively. In the *Bacillus*-treated systems, the top two predominant genera were *Thermomonas* (7.7%) and *Arenimonas* (8.67%), which belong to the phylum Proteobacteria. In addition, *Bacillus* (2.90%) and *Chryseobacterium* (2.38%), belonging to the phyla Firmicutes and Bacteroidetes, respectively, were abundant. Samples from the *Bacillus*-treated systems contained relatively higher numbers of *Nitrospira* (0.37%) compared to 0.09% in the control systems.



**Figure 3.3:** (A): Bar charts representing the average relative abundance of bacterial phyla from lettuce roots in aquaponic systems in the control and *Bacillus* treatments. Phyla that represented less than 0.2% of the total reads are labelled "other phyla", which represent the phyla Elusimicrobia, Epsilonbacteraeota, Dadabacteria, Fibrobacteres, Spirochaetes, Omnitrophicaeota, Hydrogenedentes, Kiritimatiellaeota, Fibrobacteres, candidate division FCPU426, BRC1, and WS4. (B): Bar charts representing the average relative abundance of bacterial genera from lettuce roots in aquaponic systems in the control and *Bacillus* treatments.

**Table 3.8:** Relative abundance (%) of genera from lettuce roots from the control and *Bacillus*-treated systems. Mean values are followed by the range in parentheses. Means for taxonomic group abundances were compared using Student's *t*-test. Significant differences between *Bacillus* treatment and control ( $P < 0.05$ ) are marked by an asterisk. Only genera with relative abundance of more than 0.1% of the total community in at least one of the investigated samples were considered for statistical analysis.

| Genera                   | Control               | <i>Bacillus</i> treatment | <i>P</i> -Value |
|--------------------------|-----------------------|---------------------------|-----------------|
| <i>Acinetobacter</i>     | 1.78 (1.75 – 1.81)    | 1.42 (1.39 – 1.45)        | 0.012*          |
| <i>Arenimonas</i>        | 11.29 (10.71 – 11.86) | 8.67 (8.60 – 8.73)        | 0.046*          |
| <i>Bacillus</i>          | 0.24 (0.22 – 0.26)    | 2.90 (2.82 – 2.98)        | <0.001*         |
| <i>Brevifollis</i>       | 0.02 (0.01 – 0.03)    | 0.11 (0.11 – 0.12)        | 0.018*          |
| <i>Chryseobacterium</i>  | 0.08 (0.07 – 0.08)    | 2.38 (2.37 – 2.39)        | <0.001*         |
| <i>Chthoniobacter</i>    | 0.11 (0.10 – 0.12)    | 0.39 (0.36 – 0.42)        | 0.013*          |
| <i>Cloacibacterium</i>   | 0.00 (0.00 – 0.00)    | 0.49 (0.47 – 0.51)        | 0.002*          |
| <i>Defluviimonas</i>     | 0.02 (0.02 – 0.03)    | 0.12 (0.12 – 0.12)        | 0.002*          |
| <i>Dinghuibacter</i>     | 0.04 (0.04 – 0.04)    | 0.25 (0.23 – 0.26)        | 0.005*          |
| <i>Dysgonomonas</i>      | 0.60 (0.50 – 0.69)    | 0.28 (0.28 – 0.28)        | 0.083           |
| <i>Emticicia</i>         | 0.53 (0.52 – 0.54)    | 0.19 (0.16 – 0.22)        | 0.007*          |
| <i>Ferruginibacter</i>   | 1.76 (1.61 – 1.91)    | 2.31 (2.27 – 2.35)        | 0.072           |
| <i>Fimbriiglobus</i>     | 0.20 (0.19 – 0.21)    | 0.50 (0.47 – 0.52)        | 0.009*          |
| <i>Flaviumibacter</i>    | 0.04 (0.03 – 0.04)    | 0.33 (0.32 – 0.34)        | 0.002*          |
| <i>Flavobacterium</i>    | 2.78 (2.66 – 2.89)    | 1.67 (1.66 – 1.68)        | 0.011*          |
| <i>Fodinicola</i>        | 0.28 (0.26 – 0.30)    | 0.91 (0.85 – 0.97)        | 0.010*          |
| <i>Gemmata</i>           | 0.22 (0.20 – 0.24)    | 0.49 (0.46 – 0.53)        | 0.023*          |
| <i>Haloferula</i>        | 0.18 (0.16 – 0.20)    | 0.08 (0.05 – 0.12)        | 0.144           |
| <i>Herbaspirillum</i>    | 0.11 (0.09 – 0.14)    | 0.15 (0.15 – 0.16)        | 0.257           |
| <i>Herpetosiphon</i>     | 2.34 (2.30 – 2.37)    | 0.17 (0.15 – 0.20)        | <0.001*         |
| <i>Hydrogenophaga</i>    | 0.47 (0.44 – 0.49)    | 0.24 (0.21 – 0.27)        | 0.026*          |
| <i>Hypomicrobium</i>     | 0.31 (0.31 – 0.31)    | 0.37 (0.33 – 0.42)        | 0.337           |
| <i>Ideonella</i>         | 0.23 (0.22 – 0.24)    | 0.11 (0.10 – 0.12)        | 0.015*          |
| <i>Larkinella</i>        | 0.02 (0.02 – 0.03)    | 0.23 (0.23 – 0.24)        | 0.001*          |
| <i>Legionella</i>        | 0.42 (0.40 – 0.44)    | 0.30 (0.27 – 0.32)        | 0.059           |
| <i>Luteolibacter</i>     | 1.18 (1.08 – 1.27)    | 1.62 (1.57 – 1.68)        | 0.056           |
| <i>Mycobacterium</i>     | 0.37 (0.34 – 0.40)    | 0.65 (0.65 – 0.65)        | 0.012*          |
| <i>Nakamurella</i>       | 0.03 (0.02 – 0.03)    | 0.29 (0.26 – 0.32)        | 0.015*          |
| <i>Niastella</i>         | 0.02 (0.01 – 0.03)    | 0.22 (0.20 – 0.25)        | 0.012*          |
| <i>Nitrospira</i>        | 0.09 (0.06 – 0.12)    | 0.37 (0.33 – 0.40)        | 0.023*          |
| <i>Nocardia</i>          | 0.06 (0.05 – 0.07)    | 0.35 (0.34 – 0.36)        | 0.002*          |
| <i>Pirellula</i>         | 0.55 (0.51 – 0.59)    | 0.44 (0.42 – 0.46)        | 0.127           |
| <i>Polynucleobacter</i>  | 0.05 (0.04 – 0.06)    | 1.08 (1.02 – 1.14)        | 0.003*          |
| <i>Pseudomonas</i>       | 0.15 (0.14 – 0.16)    | 0.08 (0.06 – 0.10)        | 0.089           |
| <i>Pseudoxanthomonas</i> | 0.35 (0.32 – 0.38)    | 0.20 (0.19 – 0.22)        | 0.050           |
| <i>Reyranella</i>        | 0.28 (0.24 – 0.31)    | 0.42 (0.39 – 0.45)        | 0.090           |
| <i>Runella</i>           | 0.09 (0.08 – 0.10)    | 0.24 (0.23 – 0.25)        | 0.010*          |
| <i>Sphingomonas</i>      | 0.10 (0.10 – 0.10)    | 0.14 (0.13 – 0.14)        | 0.006*          |
| <i>Terrimonas</i>        | 1.61 (1.54 – 1.69)    | 1.32 (1.29 – 1.34)        | 0.062           |
| <i>Thermomonas</i>       | 1.72 (1.62 – 1.82)    | 7.70 (7.37 – 8.03)        | 0.003*          |

### 3.3.8 Alpha diversity and rarefaction

There was a significant effect of *Bacillus* species on Sobs (Observed species richness), Shannon index, and InvSimpson ( $t$ -test,  $P < 0.05$ , Table 3.9). Total species richness as calculated by Sobs and Chao1 was significantly higher in the *Bacillus*-treated systems. Samples from the *Bacillus*-treated aquaponic units contained a more diverse community as the Shannon index was significantly higher than that of the control (Table 3.9). The Shannon index yielded an average of 5.60 for the *Bacillus*-treated samples and 5.30 for the control samples, suggesting a relatively higher diversity of bacterial sequences in the *Bacillus*-treated samples. The average Good's coverage was 95.16% and 95.60% for the *Bacillus*-treated and the control systems, respectively. The subsampling of the sequences still yielded sufficient resolution of bacterial communities, as suggested by the Good's coverage index.

**Table 3.9:** Comparison of bacterial community diversity indices associated with root samples of lettuce grown in the control and *Bacillus*-treated aquaponic systems. Significant differences between *Bacillus* treatment and control ( $P < 0.05$ ) are marked by an asterisk.

| Coverage and alpha diversity indices | Treatment                      |                                | P-value |
|--------------------------------------|--------------------------------|--------------------------------|---------|
|                                      | Control                        | <i>Bacillus</i>                |         |
| Good's coverage (%)                  | 96.60<br>(95.53 – 95.66)       | 95.16<br>(95.09 – 95.23)       | 0.044*  |
| InvSimpson                           | 43.06<br>(38.75 – 47.38)       | 69.62<br>(69.51 – 69.73)       | 0.025*  |
| Sobs                                 | 1230.0<br>(1226 – 1234)        | 1349.50<br>(1348 – 1351)       | 0.001*  |
| Chao1                                | 2046.79<br>(2045.05 – 2048.52) | 2304.18<br>(2276.83 – 2331.52) | 0.011*  |
| Shannon                              | 5.30<br>(5.23 – 5.35)          | 5.60<br>(5.59 – 5.60)          | 0.037*  |

Rarefaction curves were used to compare species diversity in the two systems (Figure 3.4). The rarefaction curves of these samples did not approach the asymptote, which indicated that each system showed highly diversified bacterial communities. In this study, these curves



**Figure 3.5:** A Venn diagram showing shared and specific OTUs for the lettuce root samples obtained from the control and *Bacillus*-treated systems. OTUs are defined at the 97% sequence similarity level from replicate samples. B and C represent systems for *Bacillus* and control, respectively.

### 3.4 Discussion

Water temperature,  $\text{NH}_3$ , TAN,  $\text{NO}_2^-$ , pH and dissolved oxygen were maintained at similar levels between trials. These parameters were within acceptable levels reported for aquaponics (Somerville et al., 2014; Reyes-Yanes et al., 2020). The water temperature for the fish tanks in both the control and *Bacillus*-treated systems across the two trials ranged from 21.0°C to 26.7°C.  $\text{NH}_3$  and  $\text{NO}_2^-$  concentrations remained below 1 mg L<sup>-1</sup> during the trials. These low concentrations generally allow tilapia to be reared without negative effects to their health (Stone et al., 2004; Reyes-Yanes et al., 2020). The pH values ranged from 6.63 and 7.8 in growth trial 1, and between 6.52 and 7.09 for growth trial 2. Fish tolerate a wide range of pH but do best at levels of 6.5 – 8.5 (Tyson et al., 2011; Somerville et al., 2014). Even at the higher pH values the concentrations of free ammonia ( $\text{NH}_3$ ) remained below harmful levels for tilapia. In addition, the dissolved oxygen concentrations were above 5 mg L<sup>-1</sup> which is an acceptable level for aquaponics as recommended by Sallenave (2016). EC and TDS values

increased in both systems across the two trials over time, indicating the presence of ions released from both the fish feed and from the mineralisation of accumulated organic matter. However, levels of EC and TDS did not differ between treatments for each trial. In our study, EC levels ranged from 0.55 to 0.84 mS cm<sup>-1</sup> for trial 1, and from 0.46 to 0.78 mS cm<sup>-1</sup> for trial 2. The TDS ranged from 350 to 584 mg L<sup>-1</sup> and 330 to 471 mg L<sup>-1</sup> for trial 1 and trial 2, respectively. Both EC and TDS were within the optimum range for culturing fish (Yildiz et al., 2017; Lennard and Goddek, 2019).

In both growth trials, the levels of nitrate in the deep-water culture solution were within the tolerable limits (< 150 mg L<sup>-1</sup>) for plants in aquaponic systems. The average nitrate values in the *Bacillus*-treated systems were significantly higher than in the control systems. The increase in phosphate and nitrate concentrations in aquaponic systems treated with *Bacillus* enhanced lettuce growth. Although the aquaponic systems were inoculated with the same amount of commercial product FINCO (Filter start®, Hikari, Japan; 0.5 g 100 L<sup>-1</sup> of water) during biofilter establishment (which provided a source of nitrifying bacteria), the levels of nitrate and phosphate accumulated faster in the *Bacillus*-treated systems than in the control systems during the growth trials. It could be possible that *Bacillus* might have enhanced biological nitrification, which resulted in significant changes in nitrate levels. The different nitrate and phosphate levels observed between the trials was due to the fact that each was run independently and could be attributed to the total fish biomass at the beginning of each trial. The phosphate levels in the control systems remained relatively low in both trials. Most plants need a phosphate concentration of between 1.9 – 2.8 mg L<sup>-1</sup> for adequate growth in culture solutions (Asher and Loneragan, 1967). The systems treated with *Bacillus*, in contrast, showed significant increases in the phosphate concentrations in the deep-water culture solution. As *Bacillus* can mineralise different forms of phosphorus, it is possible that the addition of the probiotic increased organic phosphorus mineralisation in the deep-water culture solutions. It

has been reported that species belonging to *Bacillus* are able to produce phytase enzymes for the mineralisation of phytates (Rodríguez and Fraga, 1999; Martínez-Viveros et al., 2012; Hayat et al., 2010), which possibly could have contributed to increased phosphate levels. Despite variations between trials in the changes in nitrate and phosphate levels over time, the addition of the *Bacillus* product increased nitrate and phosphate levels in both trials, which further strengthens the conclusion that *Bacillus* can increase the levels of these plant nutrients. Basing on the composition of the commercial product (Sanolife® PRO-W), the increased levels of nitrate and phosphate observed in the *Bacillus*-treated systems are likely to be a result of both the microbial activity and inherent N and P contribution of the product.

In general, lettuce growth was relatively low. After 30 days in trial 1, the average final shoot weight was 24.84 and 20.07 g plant<sup>-1</sup> for the *Bacillus*-treated and the control systems, respectively, compared to 33.08 and 25.57 g plant<sup>-1</sup> in trial 2. Hernandez et al. (2020) reported higher average final lettuce fresh weight in the range of 53.42 g and 62.7 g plant<sup>-1</sup> with a crop cycle of 55 days. Our study used the same cultivar as reported by Hernandez et al. (2020), however there were variations in systems design. Additionally, a study by Cerozi and Fitzsimmons (2016) indicated that plants grown in *Bacillus*-treated systems accumulated more dry mass (4.1 g) than untreated systems (1.3 g) under nutrient film technique-based hydroponics for a period of six weeks. However, their study did not report on the fresh weight of harvested shoots. The differences in growth could be due to differences in growth duration, the scale and maturity of the systems, type of systems (hydroponics vs aquaponics), and the light intensity. Regardless, the growth trials demonstrated improved growth in the *Bacillus*-treated aquaponic systems.

The fresh weight of the harvested shoots and the shoot dry weight were significantly higher in the *Bacillus*-treated systems compared to the control systems in both crop cycles. Increased shoot weight observed in the *Bacillus*-treated systems could possibly be attributed to

increased levels of nitrate and phosphate. A study by Cerozi and Fitzsimmons (2016) showed enhanced lettuce growth and phosphorus accumulation in aquaponic systems treated with *Bacillus*. In addition, *Bacillus* species may influence factors that stimulate root growth leading to greater nutrient uptake. *Bacillus* species are also known plant growth enhancers, with an ability to protect plants against pathogens through mechanisms associated with induced systemic resistance (García et al., 2004; Martínez-Viveros et al., 2010; Niazi et al., 2014). The  $F_v/F_m$  was significantly higher in the *Bacillus*-treated systems. According to Murchie and Lawson (2013), the value of  $F_v/F_m$  for unstressed leaves is approximately 0.81–0.83. Similar values were observed in the *Bacillus*-treated systems in our study suggesting that the lettuce plants were not stressed.

At the end of trial 1, significantly higher concentrations of P, K and Zn were observed in lettuce leaves from the *Bacillus*-treated systems than the control systems. The accumulation of these mineral elements in the lettuce leaves were related to their levels in the deep-water culture units. *Bacillus* spp. possess strong growth-promoting activities such as the ability to solubilise P and K (Chen et al., 2007; Radhakrishnan et al., 2017). Through oxido-reductive systems and proton extrusion, *Bacillus* can increase the bioavailability of Zn by producing chelating ligands and secreting organic acids (Mumtaz et al., 2017), such as 2-ketogluconic acid and gluconic acid, which solubilise Zn.

With regard to the root-associated core microbiota, there was a predominance of Proteobacteria and Bacteroidetes in both the control and *Bacillus*-treated systems. Proteobacteria and Bacteroidetes are known to respond rapidly to carbon sources, and are generally considered to be r-strategists and fast-growing bacteria (Peiffer et al., 2013; Wongkiew et al., 2018). The enrichment of Proteobacteria and Bacteroidetes in aquaponics was reported by Schmautz (2017) and Eck et al. (2019), but with different relative abundances to the present study. The results from this study confirmed that these organisms predominate

in the roots. In soil-based studies, Proteobacteria and Bacteroidetes have been described as effective rhizosphere and root colonisers in several plants such as rice (Edwards et al., 2015) and wheat (Ai et al., 2015), due to their ability to utilise root exudates (Fierer et al., 2007).

Members of the bacterial phyla Acidobacteria, Verrucomicrobia, Firmicutes, and Planctomycetes, were more abundant in the *Bacillus*-treated systems than the control systems. These phyla are mostly involved in physiological functions such as carbon usage, nitrogen assimilation, metabolism of iron, antimicrobials, and abundance of transporters (Hu et al., 2015; Kielak et al., 2016). For example, Acidobacteria have a large proportion of genes encoding for transporters. A high number of different transport systems facilitates the acquisition of a broad range of substrate categories, including amino acids, peptides, siderophores, cations, or anions (Kielak et al., 2016). The phylum Verrucomicrobia has been detected in different soil, freshwater and marine environments (Cabello-Yeves et al., 2017). One of the most notable features of freshwater Verrucomicrobia is their role in polysaccharide degradation (Cardman et al., 2014; Cabello-Yeves et al., 2017). Firmicutes have been reported to utilise carbon sources and produce lactic acid, acetone, butanol, and ethanol which contribute to nutrient turnover (Gu et al., 2010). The high abundance of Planctomycetes from this study may be an indication of nitrogen cycling in the aquaponic systems. Members of the Planctomycetes are known to perform anaerobic ammonium oxidation (anammox). They oxidise  $\text{NH}_4^+$  with  $\text{NO}_2^-$  as the electron acceptor to yield  $\text{N}_2$  (Kuenen, 2008).

At genus level, there were relatively high numbers of *Arenimonas* and *Flavobacterium* enriched in the lettuce roots in both the control and *Bacillus*-treatments. The enrichment of *Arenimonas* could possibly be due to their versatile abilities to utilise root metabolites, degrade aromatic compounds and produce anti-microbial substances. Li et al. (2014a) reported the dominance of *Arenimonas* at early stages of succession in the rhizosphere, indicating that they may be copiotrophic and fast-growing bacteria, which are able to exploit a transient niche for

nutrition at the early growth stage. It has been reported that abundance of the genus *Flavobacterium* is often associated with the capacity to degrade complex organic compounds (Kolton et al., 2016).

*Thermomonas* was one of the genera that was most influenced by *Bacillus* addition to the systems. The relative abundance of this group was 1.72% and 7.70% of the total genus-defined root-associated bacteria in the control samples and *Bacillus*-treated samples, respectively. *Thermomonas* spp. are filamentous aerobic chemoorganotrophs that do not reduce nitrate to nitrite and do not usually utilise carbohydrates, suggesting the presence of several organic compounds in the aquaponics environment. Wongkiew et al. (2018) also reported dominant OTUs belonging to *Thermomonas* spp. in plant roots of aquaponic systems. *Thermomonas* has been previously demonstrated to perform denitrification. However, some bacterial species lack the critical enzymes required to reduce  $\text{NO}_3^-$  to  $\text{NO}_2^-$ ,  $\text{NO}_2^-$  to  $\text{NO}$ , or  $\text{N}_2\text{O}$  to  $\text{N}_2$  (Holmes et al., 2019). This may explain the gradual increase of  $\text{NO}_3^-$  in *Bacillus*-treated systems. Further investigations are needed to determine whether *Thermomonas* can be considered true denitrifiers.

A relatively high proportion of reads (2.9%) from the plant roots was assigned to the genus *Bacillus* and (0.08%) to *Pseudomonas* in samples from *Bacillus*-treated systems, indicating that lettuce plants could have selected a community which is able to perform inherent biocontrol on its roots. Schmutz et al. (2017) reported similar findings with lettuce plants in aquaponic systems integrated with tilapia, although with different relative abundances. However, the potential of plant root communities for inherent biocontrol of plant pathogens in aquaponics is not yet known. A large group of *Pseudomonas* spp. and *Bacillus* spp. have been reported to produce antimicrobial compounds that can inhibit growth of a wide range of pathogens (Raaijmakers et al., 2010). It has been reported that *Pseudomonas* spp. produce

phenazine-1-carboxylic acid and 2,4-diacetylphloroglucinol involved in detoxification, as well as production of antibiotics and siderophores (van Loon and Bakker, 2006; Lareen, 2016).

*Bacillus*-treated systems were characterised by a significantly higher abundance of bacteria assigned to *Chryseobacterium*, *Nitrospira*, and *Cloacibacterium*. It has been demonstrated that siderophore production by *Chryseobacterium* spp. alleviates iron starvation in tomato plants (Radzki et al., 2013). In addition, *Nitrospira* was identified as the dominant nitrite-oxidising bacteria (NOB). *Nitrospira* are generally considered K-strategists NOB favouring oligotrophic environments (Bartelme et al., 2017). The abundance of *Nitrospira* in *Bacillus*-treated systems shows a versatile metabolic network driving nitrification in biofilters of aquaponic systems. For instance, nitrite oxidising *Nitrospira* spp. possess a diverse array of metabolic pathways such as complete ammonium oxidation (Daims et al., 2015), hydrolysis of urea and cyanate to ammonia, thereby initiating nitrification (Palatinszky et al., 2015; Bartelme et al., 2017). This metabolic versatility enables *Nitrospira* to adapt to different environments characterised by low  $\text{NH}_4^+$  and  $\text{NO}_2^-$  concentrations (Palatinszky et al., 2015). Whether *Nitrospira* in aquaponic systems transform complete nitrification through these alternate pathways requires further research.

The addition of a commercial mixture of *Bacillus* spp. increased lettuce growth in two independent growth trials compared to the control systems with no supplementation. The addition of the probiotic also increased the relative levels of phosphate and nitrate, which likely to have contributed to the increased lettuce growth in the *Bacillus*-treated systems. *Bacillus* supplementation resulted in significant changes in the composition of bacterial communities associated with lettuce roots. However, microbes colonise all plant surfaces, where each plant compartment or structure has its own distinct microbiome. The next chapter (Chapter 4) considers the epiphytic bacterial community structure and how it can be modulated when a probiotic product is added to aquaponic systems.

## CHAPTER 4

### Determination of phylloplane associated bacteria of lettuce from a small-scale aquaponic system via 16S rRNA gene amplicon sequence analysis

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#### 4.1 Introduction

The phylloplane is an ecologically and economically important microbial habitat for a diverse microbial community (Vorholt, 2012). It is largely inhabited by bacteria, archaea, filamentous fungi, and yeasts (Lindow and Brandl, 2003; Williams and Marco, 2014). These microorganisms are either associated with plant surfaces as epiphytes or may reside inside tissues as endophytes (Bringel and Couée, 2015). In soil-based plants, epiphytic bacterial communities differ greatly in size among and within plants of the same species, as well as over growing seasons due to changes in solar radiation, temperature, humidity, and heterogeneous availability of nutrients (Bringel and Couée, 2015). Although leaf surfaces host a wide variety of microbes, bacteria are among the most abundant with densities averaging  $10^6$  to  $10^7$  cells  $\text{cm}^{-2}$  (Leveau, 2006). Epiphytic microorganisms may provide specific benefits to plants including increased stress tolerance (Sánchez-López et al., 2018), biogeochemical cycling of nutrients (Förnkrantz et al., 2008), protection of plants against pathogen infections (Lindow and Leveau, 2002; Rastogi et al., 2013), and stimulation of plant growth (Berlec, 2012).

Characterisation of the epiphytic bacterial communities of plants grown in aquaponic systems is also important within the context of food safety. Human pathogens such as *Escherichia coli* O157:H7, have been found to persist on both leaf surfaces and internally on soil-grown lettuce and spinach for several weeks (Erickson et al., 2010). Similarly, Shiga toxin-producing *E. coli* have been reported on the leaf surfaces of lettuce, basil, and tomato grown in indoor aquaponic and hydroponic systems (Wang et al., 2020), but they were not internalized. Weller et al. (2020) reported detectable but low levels of generic *E. coli* in water samples

collected from three out of five hydroponic systems sampled, however no *Salmonella* spp. were detected. Among the leafy greens, lettuce is the most frequently grown in aquaponic systems due to their low nutrient requirements and economical value (Yep and Zeng, 2019). While many studies have focussed on interactions between agricultural plants and microorganisms in the soil or rhizosphere (Stone et al., 2018), only a few studies have investigated the bacterial diversity on lettuce cultivated in aquaponic systems (Fox et al., 2012; Riggio et al., 2019; Wall et al., 2019; Wang et al., 2020; Weller et al., 2020). These studies focussed on *E. coli* contamination, while the phyllosphere microbiome was not studied. Because plants do not represent a homogeneous microbial habitat, understanding the spatial heterogeneity in microbial distributions across different morphological regions of plants is important for understanding the bacterial communities associated with plants (Wang et al., 2020). While the need to conduct such studies has been recognised for microbiological analyses of leaves of cultured terrestrial plants, more research on plants grown in aquaponics or hydroponic systems is needed (Wall et al., 2019).

Considering that plant leaves harbour diverse bacterial communities which play a crucial role in plant health (Lindow and Leveau, 2002), the integration of a mixture of *Bacillus subtilis* and *B. licheniformis* in aquaponic systems is desirable. *Bacillus* is one of the major plant growth-promoting probiotic bacteria which colonises different plant parts, including the phylloplane, and exerts beneficial effects on plant health (Radhakrishnan et al., 2017). Afzal et al. (2019) reviewed studies on plant growth-promoting bacteria and the authors reported that a number of studies have focussed on the mechanisms and diversity of the beneficial bacteria which colonise plant roots in soil-based environments. In an attempt to influence microbial diversity in aquaponic systems, the use of probiotics has been tested to influence water quality (Cerozi and Fitzsimmons, 2016; Hlordzi et al., 2020), to enhance the immunity against diseases in the farmed fish species (Hai, 2015), to promote plant growth (Cerozi and Fitzsimmons, 2016;

Ahmad et al., 2008), or to study the microbial diversity of root-associated bacteria in aquaponic systems (Kasozi et al., 2021b). However, these studies did not report on the potential effect of probiotics on the microbiome inhabiting the plant surface. Using Illumina-based 16S rRNA gene sequencing, the objective of this study was to characterise and compare the epiphytic bacterial communities of lettuce plants grown in aquaponic systems treated with a commercial product (Sanolife®PRO-W) containing a mixture of *Bacillus subtilis* and *B. licheniformis* with untreated systems under indoor conditions. In addition, as lettuce growth may be influenced by nutrient availability, the effect of the addition of the commercial mixture of *Bacillus* on water quality and lettuce growth was also tested.

## **4.2 Materials and methods**

### **4.2.1 Lettuce growth conditions**

Three-week-old Locarno-variety lettuce plants at the four-leaf stage were randomly seeded into the four aquaponic systems as described in Section 2.4 (Chapter 2). Prior to planting, each plant root was submerged in sterilised water for 1 min, and then rinsed and cleaned under running ultrapure water. After cleaning the roots, the plants were individually weighed and randomly planted into a polystyrene floating raft.

### **4.2.2 Chlorophyll fluorescence and vegetative growth of lettuce**

Prior to harvesting, the leaf chlorophyll content index (CCI) and chlorophyll fluorescence were measured on three leaves as described in Section 2.6.1 (Chapter 2). During harvesting, a total of 16 plants from each deep-water hydroponic unit were removed from the growing cells. The number of the mature leaves of each plant was counted. All the shoots were detached from the roots using a sterilised scalpel blade, and the individual weight of each fresh shoot and root weight was recorded. Shoot and root samples were oven-dried at 72°C for 48 h to determine

their dry mass. Absolute growth rate (AGR) of lettuce and height gain (HG, cm plant<sup>-1</sup>) were determined following the calculations described in Section 2.6.1 (Chapter 2).

#### **4.2.3 Sample collection and isolation of leaf epiphytic bacteria**

Leaf samples from lettuce plants with no obvious damage were collected from the *Bacillus*-treated and control systems 30 days after planting. In order to obtain a representative sample of the bacterial communities residing on the lettuce leaf surfaces under simulated farming conditions, leaves were not surface-sterilised or prewashed. Two leaf samples from each lettuce plant were cut using sterile scissors and six plants in each experimental unit were sampled, i.e., there were 12 leaf samples from each experimental unit. The scissors were sterilized between sampling using a flame. The leaf samples were stored in sterile zip-lock plastic bags. The samples from each system were pooled to be treated as one sample for further analyses. This was done to remove pseudoreplication bias. Epiphytic bacteria were isolated through a leaf washing technique described by Sánchez-López et al. (2018) and Sare et al. (2020). Analyses commenced within an hour after collection of samples and sterile procedures were followed. Each sample was suspended in an Erlenmeyer flask containing 150 mL sterile distilled water and kept in a rotary shaker at 150 rpm for 2 h at 20°C. Two washing steps were conducted for each sample. A 300 µL aliquot of leaf wash solution was filtered through 0.2 µm sterile filter paper (Supor® Membrane disc filters, PALL Life Sciences, Ann Arbor, MI, USA) by vacuum filtration for DNA extraction.

#### **4.2.4 DNA extraction, PCR amplification, and sequencing**

Filter papers (0.2 µm) containing bacteria were added to bead-beating tubes and processed with a ZymoBIOMICS™ DNA Miniprep Kit according to the manufacturer's protocol. The PCR conditions, DNA purification and library preparations were performed

according to the protocol described in Section 2.7 (Chapter 2). Sequencing was performed using the Illumina MiSeq platform (Illumina, San Diego, CA, USA). Raw sequences from 16S rRNA gene profiling were deposited into the National Center for Biotechnology Information (NCBI) under project accession number PRJNA623517 through the Sequence Read Archive (SRA) study accession number: SRR14373968.

The raw paired-end Illumina amplicon sequences in FASTQ format were processed with MOTHUR platform version 1.41.3 release as described in Section 2.8 (Chapter 2). Analysis of the common and unique OTUs was conducted to investigate the lettuce epiphytic bacterial community from the two replicates per treatment through a Venn diagram. Species identification for the dominant 35 OTUs was performed using the BLAST command line 'blastn'. A heatmap was constructed to visualise the distribution of the dominant OTUs using the Morpheus application.

#### **4.2.5 Statistical analysis of lettuce growth and water quality**

Lettuce growth data were expressed as mean  $\pm$  standard error of the mean (SE), and a student's *t*-test was used to compare means for lettuce growth between treatments. Student's *t*-test has been proposed to be more suitable for small sample sizes (de Winter, 2013) than non-parametric alternative tests. To test whether there was a significant difference between treatments in the change in water quality over the culture period, repeated measures analysis of variance (RM-ANOVA) was used with treatment and time as the independent variables and water quality measurements as the dependent variables. Differences between means or interactions between main effects were considered significant when the Type 1 error probability was less than 5%, i.e.,  $P < 0.05$ . All analyses were performed using the Statistica 12® software package (TIBCO Software, Palo Alto, USA, version 13.5.0).

## 4.3 Results

### 4.3.1 Vegetative growth of lettuce and chlorophyll fluorescence

The probiotic bacteria had positive effects on fresh and dry biomass of lettuce and general growth of lettuce (Table 4.1). Shoot fresh weight at harvest was 15.3% higher in plants from the *Bacillus*-treated systems than in plants from the control systems (shoot weight,  $45.03 \pm 0.06$  g and  $39.07 \pm 0.91$  g plant<sup>-1</sup>, respectively). Root fresh weight of plants in the *Bacillus* treatment ( $2.42 \pm 0.005$  g plant<sup>-1</sup>) was 21.0% higher than in the control ( $2.00 \pm 0.04$  g plant<sup>-1</sup>). Mean shoot dry weight and root dry weight from the *Bacillus*-treated systems were significantly higher than the values obtained for the control systems ( $P < 0.05$ ). The highest plant height gain was observed in the *Bacillus* treatment. Mean chlorophyll content index (CCI) values did not differ between the *Bacillus* treatment and the control ( $P = 0.26$ ). The chlorophyll fluorescence parameter  $F_v/F_m$  was higher in the *Bacillus* treatment than in the control (Table 4.1). The absolute growth rate and height gain of lettuce were significantly highest in the *Bacillus*-treated systems (Table 4.1).

**Table 4.1:** Vegetative growth and chlorophyll fluorescence of 'Locarno' lettuce cultivar grown in the control and *Bacillus*-treated aquaponic systems. Significant differences between *Bacillus* treatment and control ( $P < 0.05$ ) are marked by an asterisk. Data are means  $\pm$  standard error (SE).

| Variables                                   | <i>Bacillus</i> treatment | Control          | <i>P</i> -value |
|---------------------------------------------|---------------------------|------------------|-----------------|
| Initial plant mass (g plant <sup>-1</sup> ) | $6.18 \pm 0.02$           | $6.21 \pm 0.04$  | 0.619           |
| Shoot fresh weight (g plant <sup>-1</sup> ) | $45.03 \pm 0.06$          | $39.07 \pm 0.91$ | 0.022*          |
| Root fresh weight (g plant <sup>-1</sup> )  | $2.42 \pm 0.005$          | $2.00 \pm 0.04$  | 0.011*          |
| Height gain (cm plant <sup>-1</sup> )       | $26.66 \pm 0.48$          | $21.60 \pm 0.38$ | 0.014*          |
| Leaf number (plant <sup>-1</sup> )          | $19.60 \pm 0.22$          | $15.0 \pm 0.19$  | 0.004*          |
| Absolute growth rate (g day <sup>-1</sup> ) | $1.38 \pm 0.005$          | $1.16 \pm 0.03$  | 0.019*          |
| $F_v/F_m$                                   | $0.82 \pm 0.05$           | $0.72 \pm 0.01$  | 0.011*          |
| CCI                                         | $2.36 \pm 0.05$           | $2.25 \pm 0.05$  | 0.260           |
| Shoot dry weight (g plant <sup>-1</sup> )   | $2.07 \pm 0.07$           | $1.72 \pm 0.01$  | 0.043*          |
| Root dry weight (g plant <sup>-1</sup> )    | $0.27 \pm 0.005$          | $0.17 \pm 0.01$  | 0.016*          |

### 4.3.2 Water quality parameters in the deep-water culture growth beds

Water temperature varied between 25.0°C and 26.5°C for the deep-water culture solution for the control systems and between 24.9°C and 26.5°C for the *Bacillus*-treated deep-water culture growth beds (Table 4.2). The average temperature was not influenced by the *Bacillus* treatment (RM-ANOVA,  $P = 0.82$ ). The levels of dissolved oxygen were maintained above 6 mg L<sup>-1</sup> and treatment did not affect how the dissolved oxygen concentration changed over time. Average pH was not affected by probiotic addition (RM-ANOVA,  $P = 0.15$ ). TDS fluctuated between 734 and 931 mg L<sup>-1</sup> for the deep-water culture solution from the control systems and 661 and 839 mg L<sup>-1</sup> for the *Bacillus*-treated systems (Table 4.2). Average concentrations of TDS and EC were not significantly affected by the addition of *Bacillus* spp. (RM-ANOVA,  $P = 0.96$ ;  $P = 0.97$ , respectively). There was a significant increase in the concentration of nitrate and phosphate in the *Bacillus*-treated systems (RM-ANOVA,  $P = 0.009$ ,  $P < 0.0001$ , respectively, Table 4.2).

**Table 4.2:** Water quality in the control and *Bacillus*-treated systems from the deep-water culture growth beds. Mean values are followed by the range in parentheses.

| Variables                                         | <i>Bacillus</i><br>treatment | Control                  |
|---------------------------------------------------|------------------------------|--------------------------|
| Temperature<br>(°C)                               | 25.94<br>(24.96 – 26.50)     | 25.94<br>(25.04 – 26.50) |
| Dissolved oxygen<br>(mg L <sup>-1</sup> )         | 6.72<br>(6.53 – 6.83)        | 6.79<br>(6.67 – 6.90)    |
| Total dissolved solids<br>(mg L <sup>-1</sup> )   | 741<br>(661 – 839)           | 835<br>(734 – 931)       |
| pH                                                | 6.87<br>(6.74 – 6.97)        | 6.79<br>(6.68 – 6.90)    |
| Electrical conductivity<br>(mS cm <sup>-1</sup> ) | 1.05<br>(1.05 – 1.19)        | 1.20<br>(1.03 – 1.35)    |
| Nitrate (mg L <sup>-1</sup> )                     | 33.25<br>(25.27 – 43.60)     | 19.73<br>(14.50 – 27.37) |
| Phosphate (mg L <sup>-1</sup> )                   | 2.92<br>(2.34– 4.43)         | 1.60<br>(1.14– 2.39)     |

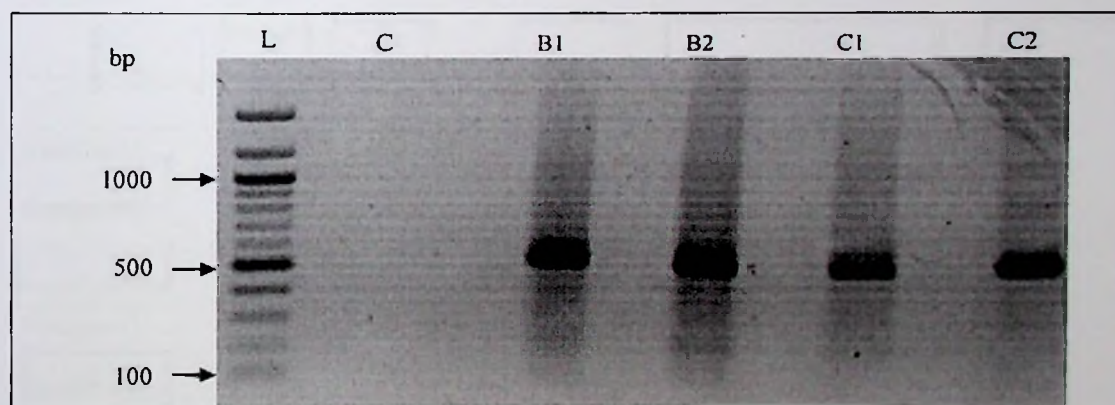
### 4.3.3 Assessment of DNA concentration and purity from leaf samples

Relative purity of the extracted DNA was good as indicated by the  $A_{260}/A_{280}$  ratio ranging from 1.81 to 1.94 (Table 4.3). The highest DNA concentrations were observed from leaf samples obtained from the *Bacillus*-treated systems. All the samples exhibited successful amplification as demonstrated by clear bands of approximately 550-bp (Figure 4.1).

**Table 4.3:** Purity and concentration of the extracted genomic DNA from leaf samples of lettuce obtained from *Bacillus*-treated and control systems.

| Sample | DNA concentration<br>(ng $\mu\text{L}^{-1}$ ) | $A_{260}/A_{280}$ | DNA integrity* |
|--------|-----------------------------------------------|-------------------|----------------|
| B1     | 42.2                                          | 1.91              | ++             |
| B2     | 30                                            | 1.94              | ++             |
| C1     | 18.5                                          | 1.81              | +              |
| C2     | 28.4                                          | 1.85              | ++             |

\*DNA integrity was assayed by agarose electrophoresis and indicated as good (++) or sufficient (+) based on the band clarity, density, and presence of smearing. B1 and B2 represent leaf samples obtained from systems supplemented with a *Bacillus* probiotic and C1 and C2 are the leaf samples from the control.



**Figure 4.1:** Agarose gel electrophoresis of the PCR products amplified from the leaf samples of lettuce with or without *Bacillus* treatment. Lane L shows the 100-bp DNA ladder; followed by a negative control. Lanes B1 and B2 are the amplification products from leaf samples obtained from systems supplemented with a *Bacillus* probiotic; and lanes C1 and C2 are the leaf samples from the control.

### 4.3.4 Sequencing summary and diversity of leaf epiphytic bacteria

A total of 20,1243 raw sequences were generated by Illumina sequencing from four samples. Leaf samples obtained from the *Bacillus*-treated systems had a total of 96,579 sequences, while those from the control systems yielded a total of 104,664 raw sequences (Table 4.4). After removing poor-quality reads, a total of 71,364 sequences for the *Bacillus*-

treated systems and 76,103 sequences for the control systems were generated. In addition, a total of 35,965 unique sequences for both *Bacillus*-treated samples and control samples were also generated. Removal of chimeras resulted in a total of 68,658 and 74,830 sequences for *Bacillus*-treated samples and control samples, respectively. The total percentage of sequences flagged as chimeric was 2.7% for all samples which was a low number of sequences to be flagged as chimeric. The non-target sequence reads accounted for 41,719 and 46,428 for the *Bacillus*-treated samples and control samples, respectively, implying that leaf surfaces are inhabited by other microbes apart from bacteria. After quality filtering and removal of chimeras and single reads, a total of 6,971 and 1,868 reads were obtained for the *Bacillus*-treated samples and control samples, respectively (Table 4.4).

**Table 4.4:** Summary of sequencing data from leaf samples.

| Treatment                 | Number of raw sequences | Number of sequences before chimeras | Number of sequences after chimeras | Eukaryotes, and unknown reads | Number of sequences after screening and filtering |
|---------------------------|-------------------------|-------------------------------------|------------------------------------|-------------------------------|---------------------------------------------------|
| <i>Bacillus</i> treatment |                         |                                     |                                    |                               |                                                   |
| Sample B1                 | 48,788                  | 35,348                              | 34,874                             | 24,544                        | 622                                               |
| Sample B2                 | 47,791                  | 36,016                              | 33,784                             | 17,175                        | 6,349                                             |
| Control                   |                         |                                     |                                    |                               |                                                   |
| Sample C1                 | 63,098                  | 41,061                              | 40,336                             | 16,544                        | 1,119                                             |
| Sample C2                 | 41,566                  | 35,042                              | 34,494                             | 29,884                        | 749                                               |

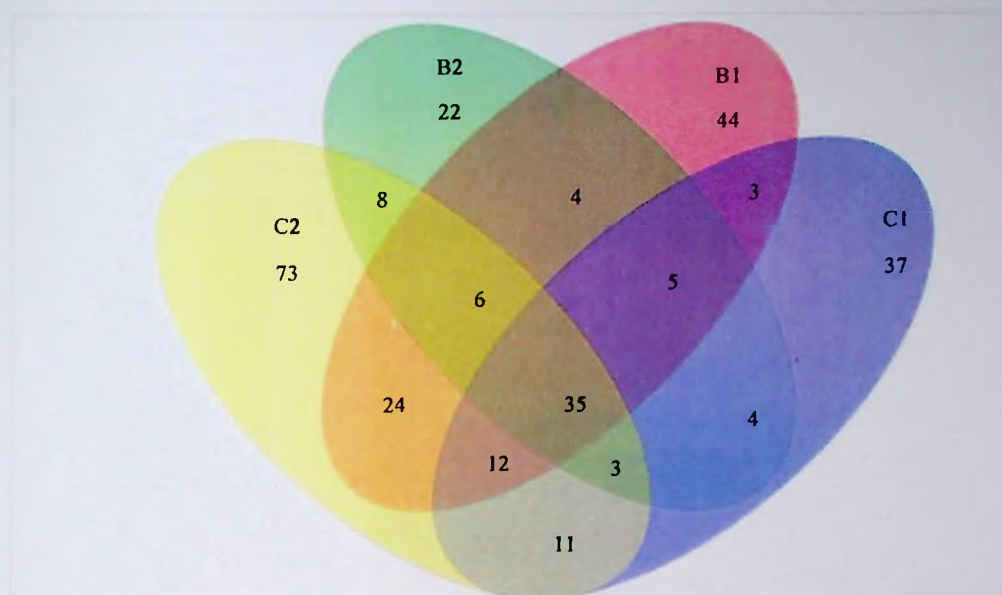
Although the results from Table 4.5 showed high variability between treatments, there was no significant effect of *Bacillus* on Invsimpson, Sobs, Chao1, and Shannon ( $P > 0.05$ ; Table 4.5). The diversity of the epiphytic bacterial communities varied widely within the *Bacillus* treatment (Table 4.5). Sequence analyses for leaf samples from both systems showed a low estimated coverage ranging from 87.62 – 92.44% and 87.29 – 90.51% from *Bacillus*-treated and control systems, respectively (Table 4.5).

**Table 4.5:** Coverage and alpha diversity of epiphytic bacteria bacterial communities associated with leaf samples of lettuce plants grown in *Bacillus*-treated and control aquaponic systems. Mean values are followed by the range in parentheses. There was no significant effect of *Bacillus* treatment on alpha diversity and coverage (*t*-test,  $P > 0.05$ ).

| Coverage and indices | Treatment                   |                             | <i>P</i> -value |
|----------------------|-----------------------------|-----------------------------|-----------------|
|                      | <i>Bacillus</i>             | Control                     |                 |
| InvSimpson           | 13.53<br>(7.95 – 19.12)     | 23.25<br>(10.34 – 36.15)    | 0.561           |
| Sobs                 | 110<br>(87 – 133)           | 141<br>(110 – 172)          | 0.506           |
| Good's coverage (%)  | 90.0<br>(87.62 – 92.44)     | 88.9<br>(87.29 – 90.51)     | 0.735           |
| Chao1                | 218.65<br>(177.08 – 260.22) | 255.41<br>(195.55 – 255.27) | 0.907           |
| Shannon              | 3.32<br>(2.94 – 3.70)       | 3.81<br>(3.23 – 4.39)       | 0.554           |

#### 4.3.5 Shared and unique operational taxonomic units

In the control systems, there were a greater number of unique OTUs, relative to those in *Bacillus*-treated systems, where 37 and 73 were found on leaf surfaces from the control systems compared to 22 and 44 in leaf samples obtained from *Bacillus*-treated systems (Figure 4.2). A total of 133 and 87 OTUs were found in leaf samples obtained from *Bacillus*-treated systems, B1 and B2 respectively, while 110 and 172 were found in leaf samples from control systems, C1 and C2. The number of OTUs shared between samples from *Bacillus*-treated systems (B1 and B2) were 50, while 61 OTUs were shared between samples from the control systems. The number of unique OTUs as shown by the Venn diagram suggests that the control samples contained the majority of OTUs in the dataset.

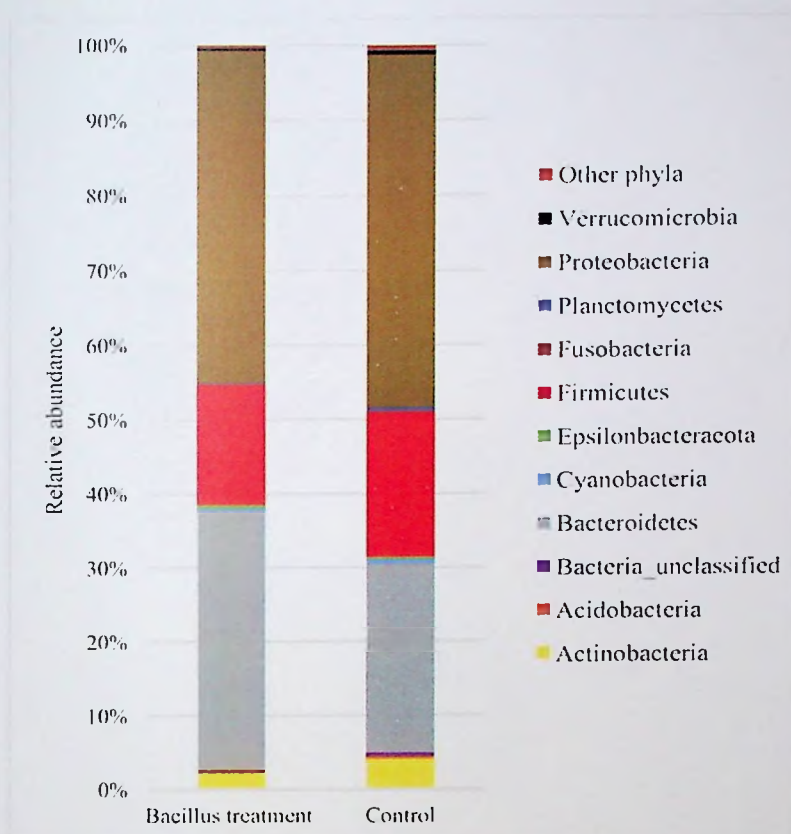


**Figure 4.2:** A Venn diagram showing shared and unique OTUs among epiphytic bacterial communities of lettuce plants grown in *Bacillus*-treated systems and control systems. B1 and B2 represent leaf samples obtained from systems supplemented with a *Bacillus* probiotic and C1 and C2 represent leaf samples from the control systems.

#### 4.3.6 Overview of bacterial taxa associated with lettuce leaf samples

All sequences were identified into 17 classified phyla, but only 10 had a relative abundance of more than 0.2% across the control and the *Bacillus*-treated systems (Figure 4.3). Across all samples, the bacterial taxa observed were largely members of the Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria phyla (Figure 4.3). In the *Bacillus*-treated systems, Proteobacteria and Bacteroidetes were the common inhabitants of lettuce epiphytes with average relative abundances of 44.33% and 35.06% of the total bacterial community, respectively. Similarly, in the control systems, the Proteobacteria and Bacteroidetes constituted 47.24% and 25.50% of the total bacterial community, respectively. Other major dominant phyla from the *Bacillus*-treated systems included Firmicutes (16.20%) and Actinobacteria (2.11%), and these phyla in the control systems constituted 19.49% and 4.13% of the total bacterial community, respectively (Figure 4.3). A small fraction (0.28 – 0.46%) of the total sequences could not be classified into any known phyla (Figure 4.3). Other phyla including

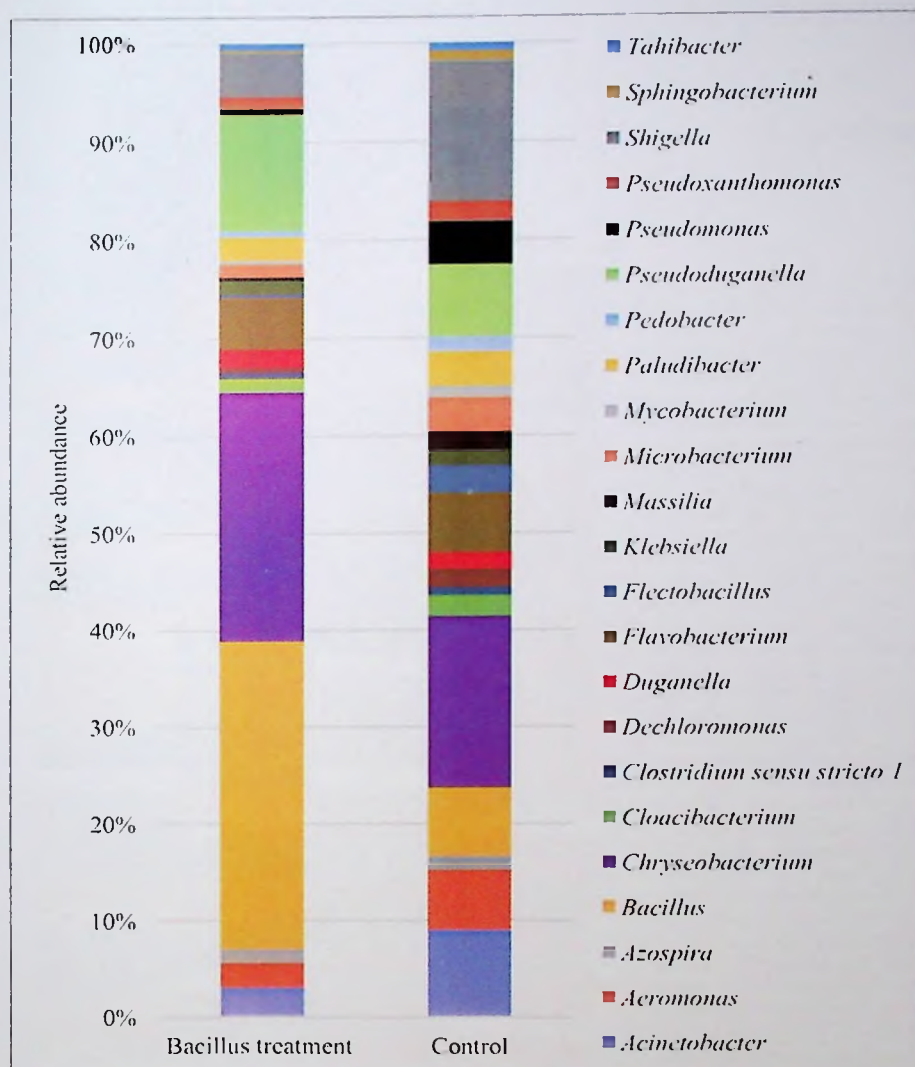
Acidobacteria, Epsilonbacteracota, Planctomycetes, Fusobacteria, Verrucomicrobia, and Cyanobacteria were present in lower abundances.



**Figure 4.3:** Mean relative abundances of taxa (at the phylum level) among leaf epiphytic bacterial communities from lettuce plants obtained from *Bacillus*-treated and control systems. Phyla that represented less than 0.2% of the total reads are labelled "other phyla", which represent the phyla Armatimonadetes, Chloroflexi, Chlamydiae, Dependientiae, Nitrospirae, Patescibacteria, Spirochaete.

A total of 23 genera were found with a relative abundance of more than 0.5% of total bacteria reads (Figure 4.4). The lettuce leaf surfaces from the *Bacillus*-treated systems were represented by *Bacillus* (27.20%), *Chryseobacterium* (21.90%), *Pseudoduganella* (10.26%), and *Flavobacterium* (4.49%). Other genera including *Shigella* (3.86%), *Aeromonas* (2.14%), *Paludibacter* (2.02%), and *Cloacibacterium* (1.14%) were also inhabitants of lettuce epiphytes. (Figure 4.4). In the control systems, the major dominant genera included *Chryseobacterium*

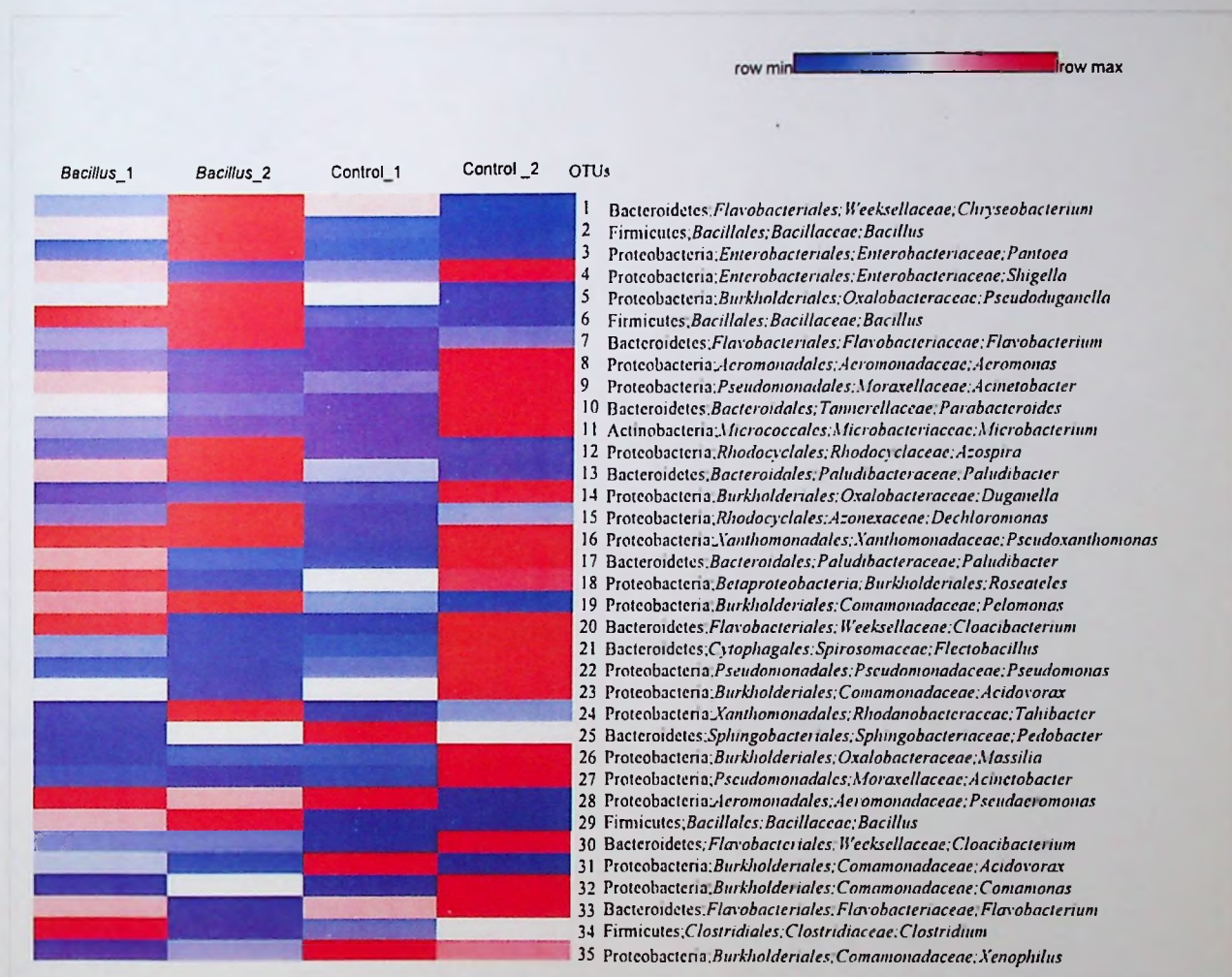
(9.13%), *Shigella* (7.38%), *Acinetobacter* (4.63%). *Pseudoduganella* (3.90%), *Bacillus* (3.67%), *Aeromonas* (3.14%), *Flavobacterium* (3.16%), and *Paludibacter* (1.80%).



**Figure 4.4:** Average relative abundance of bacterial genera among leaf epiphytic bacterial communities of lettuce plants obtained from *Bacillus*-treated and control systems. Only genera representing more than 0.5% of the total reads in at least one sample are presented.

The distribution of the top 35 dominant OTUs is exhibited in a heat map (Figure 4.5). The red colour illustrates highest abundance, and the blue colour expresses lowest abundance of species. The 35 abundant OTUs were assigned to four phyla including Proteobacteria, Bacteroidetes, Firmicutes, and Actinobacteria (Figure 4.5). The corresponding OTUs were identified to species level with a sequence similarity between 97 – 100% based on 16S rRNA

gene sequences in the NCBI database (Table 4.6). Through BLAST search (Table 4.6), the most abundant OTU overall was OTU1, assigned to the genus *Chryseobacterium* (Figure 4.5), closely related to *Chryseobacterium rhizoplanæ* (Table 4.6).



**Figure 4.5:** A heatmap showing the distribution of the top 35 dominant OTUs based on the abundance of 16S rRNA gene sequences from leaf samples of lettuce grown in *Bacillus*-treated systems and control systems. Each column in the heatmap represents a sample, and each row represents an OTU. Taxonomy (right column) indicates the phylum, order, family, and genus of each OTU. The colour code refers to sequence abundance, with high abundances (red colours) and low abundances (blue colours).

**Table 4.6:** The 35 dominant bacterial OTUs and their closest phylogenetic neighbours associated with leaf samples of lettuce plants grown in both control and *Bacillus*-treated aquaponic systems.

| Bacterial OTUs | Related species (NCBI-Blast)                              | Similarity (%) | Accession   |
|----------------|-----------------------------------------------------------|----------------|-------------|
| OTU1           | <i>Chryseobacterium rhizoplanae</i> strain JM-534         | 98.27%         | NR_134711.1 |
| OTU2           | <i>Bacillus australimaris</i> strain MCCC 1A05787         | 98.72%         | NR_148787.1 |
| OTU3           | <i>Pantoea rwandensis</i> strain LMG 26275                | 99.14%         | NR_118121.1 |
| OTU4           | <i>Shigella sonnei</i> strain CECT 4887                   | 98.28%         | NR_104826.1 |
| OTU5           | <i>Pseudoduganella eburnea</i> strain 10R 5-21            | 98.28%         | NR_159256.1 |
| OTU6           | <i>Bacillus haynesii</i> strain NRRL B-41327              | 98.50%         | NR_157609.1 |
| OTU7           | <i>Flavobacterium lindanitolerans</i> strain IP-10        | 98.26%         | NR_044208.1 |
| OTU8           | <i>Aeromonas hydrophila</i> strain DSM 30187              | 99.36%         | NR_119190.1 |
| OTU9           | <i>Acinetobacter vivianii</i> strain NIPH 2168            | 98.71%         | NR_148847.1 |
| OTU10          | <i>Parabacteroides chartae</i> strain NS31-3              | 98.92%         | NR_109439.1 |
| OTU11          | <i>Microbacterium laevaniformans</i> strain DSM 20140     | 98.72%         | NR_044935.1 |
| OTU12          | <i>Azospira oryzae</i> PS                                 | 98.72%         | NR_074103.1 |
| OTU13          | <i>Paludibacter propionigenes</i> WB4                     | 97.41%         | NR_074577.1 |
| OTU14          | <i>Duganella sacchari</i> strain Sac-22                   | 98.28%         | NR_108216.1 |
| OTU15          | <i>Dechloromonas hortensis</i> strain MA-1                | 98.72%         | NR_042819.1 |
| OTU16          | <i>Pseudoxanthomonas mexicana</i> strain NBRC 101034      | 97.43%         | NR_113973.1 |
| OTU17          | <i>Paludibacter propionigenes</i> WB4                     | 97.41%         | NR_074577.1 |
| OTU18          | <i>Roseateles depolymerans</i> strain 61A                 | 96.56%         | NR_111995.1 |
| OTU19          | <i>Pelomonas saccharophila</i> strain NBRC 103037         | 98.71%         | NR_114189.1 |
| OTU20          | <i>Cloacibacterium normanense</i> strain NRS1             | 98.92%         | NR_042187.1 |
| OTU21          | <i>Flectobacillus roseus</i> strain GFA-11                | 98.70%         | NR_116312.1 |
| OTU22          | <i>Pseudomonas plecoglossicida</i> strain NBRC 103162     | 99.36%         | NR_114226.1 |
| OTU23          | <i>Acidovorax temperans</i> strain PHL                    | 99.35%         | NR_028715.1 |
| OTU24          | <i>Tahibacter aquaticus</i> strain PYM5-11                | 97.22%         | NR_115098.1 |
| OTU25          | <i>Pedobacter glacialis</i> strain 8-24                   | 98.70%         | NR_134125.1 |
| OTU26          | <i>Massilia alkalitolerans</i> DSM 17462 strain YIM 31775 | 98.92%         | NR_043094.1 |
| OTU27          | <i>Acinetobacter lwoffii</i> strain JCM 6840              | 99.14%         | NR_113346.1 |
| OTU28          | <i>Pseud aeromonas sharmiana</i> strain GPTSA-6           | 98.28%         | NR_043470.1 |
| OTU29          | <i>Bacillus haynesii</i> strain NRRL B-41327              | 97.64%         | NR_157609.1 |
| OTU30          | <i>Cloacibacterium rupense</i> strain NBRC 104931         | 98.92%         | NR_114274.1 |
| OTU31          | <i>Acidovorax monticola</i> strain K-4-16                 | 97.63%         | NR_164911.1 |
| OTU32          | <i>Comamonas composti</i> strain YY287                    | 99.57%         | NR_044039.1 |
| OTU33          | <i>Flavobacterium notoginsengisoli</i> strain SYP-B540    | 98.92%         | NR_145940.1 |
| OTU34          | <i>Clostridium beijerinckii</i> strain JCM 1390           | 99.57%         | NR_113388.1 |
| OTU35          | <i>Xenophilus aerolatus</i> strain 5516S-2                | 99.35%         | NR_116099.1 |

#### 4.4 Discussion

Water temperature, pH, dissolved oxygen, electrical conductivity, and total dissolved solids were maintained at similar levels in deep-water culture growth beds between the *Bacillus*-treated and the control systems. These parameters were within acceptable levels reported for aquaponics (Somerville et al., 2014; Reyes-Yanes et al., 2020). An oxygen

concentration above 4 mg L<sup>-1</sup> of the root environment in the hydroponics component is important to prevent root-rot (Somerville et al., 2014). In our study, the dissolved oxygen concentrations were above 6 mg L<sup>-1</sup>, which is an acceptable level for the growth of plants and fish. Both EC and TDS were within the optimum range for plant growth. EC and TDS values increased in both systems in a similar way over time, indicating the presence of ions released from both the fish feed and from the mineralization of accumulated organic matter.

Nitrate and phosphate are dissolved forms of nitrogen and phosphorus respectively, which are essential nutrients for plant growth. In this study, systems treated with *Bacillus* showed significant increases in the nitrate and phosphate concentrations in the deep-water culture solution. The increase in nitrate and phosphate is corroborated by previous studies (Kasozi et al., 2021b). Because *Bacillus* mineralises different forms of phosphorus, the addition of the probiotic may have led to the conversion of complex forms of essential nutrients, such as phosphorus and ammonium nitrogen, to simple bioavailable forms that can be used by plant roots to improve lettuce growth.

The probiotic product had positive effects on fresh and dry biomass of lettuce. The fresh weight of the harvested shoots and roots weight were significantly higher in the *Bacillus*-treated systems compared to the control systems. Higher shoot weight could be attributed to the higher nitrate and phosphate levels in the *Bacillus*-treated systems. In this study, the chlorophyll fluorescence parameter  $F_v/F_m$  was highest in the *Bacillus*-treated systems implying that the state of the photosynthetic mechanisms of the leaves was not stressed as chlorophyll fluorescence is a good indicator of photosynthesis (Kasozi et al., 2021b).

With regards to the diversity and composition of leaf epiphytic bacteria, the  $\alpha$ -diversity of epiphytic bacterial community of the leaf samples were evaluated by Sobs, Chao1, Shannon and InvSimpson. There were no significant differences in  $\alpha$ -diversity indices between leaf samples obtained from the *Bacillus*-treated and control systems, implying that the leaf samples

had similar richness and evenness. The non-significant difference between the control and *Bacillus* treatment was probably due to the high variability of alpha diversity metric values within treatment. Mercier and Lindow (2000) reported that the epiphytic bacterial populations vary from one leaf to another, even on leaves of the same plant due to changes in the utilisable carbon and nitrogen sources on leaf surfaces. Schlechter et al. (2019) reviewed studies on factors that influence epiphytic bacterial communities and the authors reported that the heterogenic nutrient conditions and fluctuating water availability of leaves are among key properties which influence bacteria colonisation. Thus, it is hypothesised from the current study that the variability of epiphytic bacterial community within a treatment might be related to physicochemical properties of leaves.

The Good's coverage, which is an index of gene sequencing depth, was relatively low with an average coverage range of 88.9 – 90.0% for the control and *Bacillus*-treated samples. This implies that lettuce leaf surfaces were inhabited by other microbes as revealed by the sequences assigned to eukaryotes, chloroplasts and mitochondria observed during sequence analysis. Bringel and Couée (2015) reported that sequencing depth is a significant limitation for the detection of phyllosphere-specific bacterial communities. This implies that bacteria are not the only microorganisms that colonise lettuce leaves.

Generally, the leaf epiphytic bacterial communities in this study were dominated by the phylum Proteobacteria, followed by Bacteroidetes, Firmicutes and Actinobacteria. These phyla have been reported to be the major colonisers of leaf surfaces in soil-based plants (Vorholt et al., 2012). The dominance of Proteobacteria has been reported in other studies where representatives of this phylum play important roles in nitrification, nitrogen fixation, methylotrophy or anoxygenic photosynthesis (Stone et al., 2018). The genera *Chryseobacterium* (OTU1), *Bacillus* (OTU2), *Pantoea* (OTU3), *Pseudoduganella* (OTU5), *Flavobacterium* (OTU7) *Paludibacter* (OTU17) and *Cloacibacterium* (OTU30) were

dominant in leaf samples obtained from the *Bacillus*-treated systems. These formed the core leaf epiphytic microbiome. In the present study, the predominance at the genus level was driven by the high abundance of more than one OTU. For example, *Bacillus* was dominant in epiphytic samples from the *Bacillus*-treated systems due to the large number of OTU2 and OTU6.

*Chryseobacterium* was the most abundant genus that colonised the lettuce leaf surfaces from the *Bacillus*-treated systems. Kumar et al. (2021) reported that rice leaf associated *Chryseobacterium* showed antifungal activity against rice blast disease when inoculated in rice seedlings. Given that leaf *Chryseobacterium* is capable of secreting chemicals that suppress other pathogenic microbes, its ecological role in aquaponics is of particular interest.

Other dominant OTUs assigned to *Pantoea*, *Pseudoduganella*, and *Flavobacterium* were detected on the leaf samples from *Bacillus*-treated systems. Members of the genus *Pantoea* are commonly isolated from a wide range of ecological niches and have various biological roles, as plant epiphytes, as biological control agents or as plant-growth promoters (De Maayer et al., 2012; Compant, 2019; Qin et al., 2019). They can establish quorum sensing systems on leaves, which may suppress pathogens on those leaves (Pusey et al., 2011). Species in the genus *Pseudoduganella* are adapted to oligotrophic conditions and are considered to be nitrogen-fixing organisms (Baldani et al., 2014). Members of the genus *Flavobacterium* are often associated with the capacity to degrade complex organic compounds and represent a significant fraction of leaf-associated microbiomes in a broad range of plant species (Kolton et al., 2016). Other dominant OTUs associated with leaf samples from the *Bacillus*-treated systems were assigned to *Paludibacter* and *Cloacibacterium*. The genus *Paludibacter* includes species often associated with cellulose and chitin degradation (Dai et al., 2016). Members of *Cloacibacterium* can produce extracellular polymeric substances linked with important properties for the phytoremediation of toxic pollutants (Wang et al., 2018).

In this study, irrespective of the treatment, the fresh lettuce leaf surface was inhabited by members of the genus *Shigella*. The presence of the genus *Shigella*, a food-borne pathogen from leaf samples of the control systems (7.38% of the total bacterial community in the control systems compared to 3.86% in the *Bacillus*-treated systems) is a great concern because infections caused by *Shigella* species continue to be an important cause of diarrheal diseases (Kapperud et al., 1995). An infective dose of *Shigella* as low as 10 – 100 bacterial cells is sufficient to produce disease (Zaidi and Estrada-García, 2014). The comparative 16S rRNA gene sequence based on the NCBI BLAST search results indicated that this OTU had the highest sequence similarity to the *Shigella sonnei* strain CECT 4887 (98.28% sequence similarity). Although little information is available on this specific strain, *S. sonnei* has been shown to multiply in lettuce and lettuce extract (Kapperud et al., 1995) and can grow within a pH range of between 4.5 – 9.0 (Bagamboula et al., 2002).

Another potential foodborne pathogen assigned to the genus *Aeromonas* was identified from lettuce leaves from both the control and *Bacillus*-treated systems (3.14% of the total bacterial community in the control systems compared to 2.14% in the *Bacillus*-treated systems). The NCBI BLAST search results indicated that the bacterial epiphyte OTU8 from our study had a 99.36% sequence identity with the *Aeromonas hydrophila* strain DSM 30187. Related studies in aquaponics have reported the presence of *Aeromonas*. Chitmanat et al. (2015) reported the presence of *Aeromonas hydrophila*, in aquaponic systems containing hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*). In addition, Schmautz et al. (2017) reported the occurrence of *Aeromonas* (0.25% of the total bacterial community) in the faecal samples of tilapia reared in an aquaponic system which included lettuce. *A. hydrophila* is amongst the most frequently reported opportunistic pathogenic bacteria in fish (Cipriano, 1984; Lehane and Rawlin, 2000; Tekedar et al., 2015) with zoonotic potential (Cipriano, 1984; Umutoni et al., 2020) which means that a reduction of its abundance in fish systems as well as

on edible plants is beneficial. In studies from retail facilities, *Aeromonas* spp. have been identified in organic vegetables at occurrences of 7.3% (Santos et al., 2012) and 34% (McMahon and Wilson, 2011). Transfer of this pathogen into aquaponic systems to form a biofilm on lettuce surfaces can be through water, equipment, fish feed and media grow bed aggregates (Tekedar et al., 2015; Umutoni et al., 2020). Results from this study showed that lettuce leaf samples obtained from *Bacillus*-treated systems had lower relative abundances of *Shigella* and *Aeromonas* suggesting that application of the commercial product containing a mixture of *Bacillus subtilis* and *B. licheniformis* might reduce pathogen colonisation on leaf surfaces.

In summary, (Chapters 3 and 4) focussed on root and leaf-associated bacteria, as well as lettuce growth. As the plant growth in aquaponics is influenced by the dissolved nutrients excreted directly by fish, it is important to provide optimum conditions for the fish. Therefore, Chapter 5 discusses how the addition of a commercial product to the water of an experimental aquaponic system influences the growth and digestive enzyme activity of *O. mossambicus*.

## CHAPTER 5

### Effect of the addition of a probiotic mixture of two *Bacillus* species to a coupled aquaponic system on water quality, growth, and digestive enzyme activity of Mozambique tilapia, *Oreochromis mossambicus*

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#### 5.1 Introduction

One strategy to enhance productivity in aquaponic systems involves monitoring and managing environmental parameters to provide fish with optimal growth conditions. In aquaponic systems, fish and feed waste provide most of the nutrients required for plant growth. Although several fish species have been successfully cultured in aquaponic systems, tilapia are the most commonly raised aquatic species, as reported by 55% of respondents in an international survey (Love et al., 2014). Important species are Nile tilapia, *O. niloticus*, and Blue tilapia, *O. aureus* (Somerville et al., 2014), while in South Africa, 82% of survey respondents raised *O. mossambicus* in aquaponic systems (Mchunu et al., 2018). The physicochemical properties of water need to provide the microbiota, fish and plants with an environment that matches their specific requirements (Lennard and Goddek, 2019), while keeping the fish healthy to ensure a high feed conversion efficiency and fast growth (Yildiz et al., 2017; 2019).

Probiotic bacteria species that have been reported to enhance growth and improve immunity of aquaculture organisms include *Arthrobacter*, *Bacillus*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Micrococcus*, *Pediococcus*, *Aeromonas*, *Enterobacter*, *Vibrio*, *Pseudomonas* and *Roseobacter* (Gatesoupe, 1999; Irianto and Austin, 2002; Balcázar et al., 2006). *Bacillus* species produce compounds that are active against fish pathogens (Vine et al., 2006). They also survive well under a wide range of environmental conditions (Kuebutornye et al., 2019), and can play an important role by removing nitrogen from an aquaculture system.

Although *Bacillus* species are amongst the most frequently studied plant growth promoters (Ahmad et al., 2008; Cerozi and Fitzsimmons, 2016), there is a paucity of information regarding their role to fish health in aquaponic systems.

Probiotics modulated gut microbiota (Nayak, 2010) and increased humoral immunity (Kuebutornye et al. 2019) in fish. They also affected digestive enzyme activity of fish and influenced nutrient digestibility and feed utilisation. This was achieved by adding the product to a formulated diet (Apún-Molina et al., 2009; Merrifield et al., 2010; Wu et al., 2012; Han et al., 2015; Liu et al. 2017; Elsabagh et al., 2018), to the water of channel catfish ponds (Queiroz and Boyd, 1998), or by enriching live feed (Suzer et al., 2008). However, these studies were not done using aquaponic systems. Suzer et al. (2008) compared the addition of *Lactobacillus* spp. to both the diet and the water of a recirculating sea water system. The authors found that enzyme activity in gilthead sea bream, *Sparus aurata*, larvae only increased when the probiotic was added to the diet. Queiroz and Boyd (1998) added a commercial mixture of *Bacillus* species to pond water and observed an increase in catfish, *Ictalurus punctatus*, production, but this effect could not be explained, and the authors recommended further studies. The objective of this study was to evaluate how the addition of a commercial probiotic product to the water of an experimental aquaponic system influenced growth, feed conversion efficiency, proximate composition, and digestive enzyme activity in *O. mossambicus*.

## 5.2 Materials and methods

### 5.2.1 Addition of probiotics

A completely randomised design was used with two control units (no probiotics) and two treatment units, to which the preparation of *Bacillus* species was added. The aquaponic units assigned to the *Bacillus* species treatment received 5.31 g (0.02 g product L<sup>-1</sup> of system water)

twice weekly until week 10. The product was dissolved in 0.5 L of system water and introduced into the sump of the system.

### **5.2.2 Fish stocking**

Mozambique tilapia, *Oreochromis mossambicus*, juveniles were obtained from the Department of Ichthyology and Fisheries Science of Rhodes University. After acclimatisation for one week, healthy fish ( $36.4 \pm 0.06$  g fish<sup>-1</sup>; mean  $\pm$  standard error) were randomly selected and stocked in each system. Each aquaponic rearing tank contained 10 fish at a biomass of 3.64 kg m<sup>-3</sup>. Fish were fed a commercial diet (Avi Products Pty Ltd., 2 mm pellet size) twice daily at 09h30 and 16h00 at 2% of their body mass per day for 10 weeks. The diet contained 40% crude protein, 0.7% phosphorus, 5% crude fat, 3% calcium, 2.2% lysine, 2% crude fibre and 10% moisture. Uneaten feed and fish faeces were siphoned each day before feeding. The amount of feed was adjusted based on the results of each fortnightly weighing of all fish in each tank.

### **5.2.3 Water quality measurements in fish rearing tanks**

Water temperature, pH, total dissolved solids (TDS) and electrical conductivity (EC) were measured daily between 08h30 and 09h00 using a multi-parameter water quality monitor probe. The concentration of dissolved oxygen (DO) was measured thrice a week using a dissolved oxygen meter. Using a spectrophotometer, NO<sub>3</sub><sup>-</sup> and TAN levels were measured thrice a week with Spectroquant test kits as described in Section 2.5.1 (Chapter 2).

### **5.2.4 Sampling for proximate analysis and digestive enzyme activities**

Proximate analysis was determined by randomly selecting ten fish from the batch of fish that was used for the experiment at the start of the study, and three fish per tank at the end of the experiment. Two fish per tank were selected to obtain samples for the testing of digestive

enzyme activities. All fish were starved for 24 h prior to handling. To record the body weight, each fish was anaesthetised with clove oil (Lennon by Aspen Ltd., La Lucia Ridge, South Africa, 90% eugenol) that had been dissolved in 100% ethanol at a ratio of 1 mL of clove oil to 10 mL of ethanol. The clove oil concentration in the water in which the fish were kept was  $40 \text{ mg L}^{-1}$ . Fish were placed individually into a 1 L container filled with 500 mL water on a tared electronic scale (Adam Equipment, PGL-2002, accuracy 0.01 g). Fish that were euthanised for proximate analysis and tests for digestive enzyme activity were exposed to a lethal clove oil concentration of  $60 \text{ mg L}^{-1}$  for 4 min. They were then processed for proximate analysis or dissected on ice to remove the whole intestine for the enzyme activity tests.

#### **5.2.5 Growth of fish**

Percentage weight gain (WG), specific growth rate (SGR,  $\% \text{ d}^{-1}$ ), feed conversion ratio (FCR), protein efficiency ratio (PER), feed efficiency (FE,  $\%$ ), and percentage survival (SR), and were calculated using the equations outlined in Section 2.6.2 (Chapter 2).

#### **5.2.6 Proximate analysis**

Proximate analysis of whole-body fish was conducted according to the Association of Official Analytical Chemists methods (AOAC, 1997) as stated in Section 2.5.3 (Chapter 2). Briefly, whole body fish samples were oven-dried at  $105^{\circ}\text{C}$  for 12 h before finely ground. The crude protein content ( $\text{N} \times 6.25$ ) was estimated by the Kjeldahl method, using a distillation unit (Gerhardt Vapodest 400, Gerhardt GmbH, Königswinter, Germany). Ash was determined by combusting samples in a muffle furnace at  $550^{\circ}\text{C}$  for 6 h. Gross energy was determined using a bomb calorimeter (Adiabatic Calorimeter Bomb Parr 1261; Parr Instrument Company, IL, U.S.A). Total fat was extracted according to the methods of Bligh and Dyer (1959) with slight modifications by Kinsella et al. (1977).

### 5.2.7 Enzyme activity assays

Preparation of enzyme extracts followed the procedures stated in Section 2.6.3 (Chapter 2). All assays were carried out in triplicate with Epoch microplate spectrophotometer (BioTek® Instruments, Inc, Winooski, VT, USA) using Falcon flat-bottom 96-well microplates.

Amylase activity was determined following modified methods adapted from Bernfeld (1955) and Namulawa et al. (2013), by estimating the reducing sugar produced from a 3,5-dinitrosalicylic acid (DNS) substrate. The reaction mixture consisted of 300  $\mu\text{L}$  of 1.33% (w/v) of potato starch dissolved in 50 mM Tris-HCl buffer, pH 8, and 100  $\mu\text{L}$  of homogenate. The reaction mixture was incubated at 37°C for 30 min after which it was centrifuged for 5 min at  $13,000 \times g$ . The supernatant (150  $\mu\text{L}$ ) of each reaction mixture was added to 300  $\mu\text{L}$  of DNS, followed by heating to 100 °C for 8 min. The mixture was then cooled on ice for 5 min, 250  $\mu\text{L}$  from each aliquot was transferred to a 96-well microplate and absorbance was read at 540 nm. A glucose standard curve over a concentration range of 0.1 to 1.0 mg mL<sup>-1</sup> was prepared from a 10 mg mL<sup>-1</sup> stock solution. Blanks without substrate, and substrate and enzyme controls were run simultaneously with the reaction mixture. One unit (U) of enzymatic activity was defined as the amount of enzyme able to produce 1 mg of glucose min<sup>-1</sup> mg protein<sup>-1</sup>.

Total alkaline protease activity of the crude extract of the whole gut was assayed following the methods by Walter (1984) and Moyano et al. (1996), with casein as a substrate. Protease activity was measured through the hydrolysis of 0.66% (w/v) casein in 50 mM Tris-HCl buffer at pH 8. Triplicate samples (50  $\mu\text{L}$ ) of crude intestine extract were mixed with 150  $\mu\text{L}$  of casein substrate at 37°C. Following incubation for 30 min, the reaction was stopped by the addition of 200  $\mu\text{L}$  of 10% (w/v) trichloroacetic acid (TCA) and the mixture was centrifuged at  $8,000 \times g$  for 5 min. An aliquot of 10  $\mu\text{L}$  from the supernatant was mixed with 50  $\mu\text{L}$  of 2 M sodium carbonate, 20  $\mu\text{L}$  of Folin & Ciocalteu's phenol reagent and 180  $\mu\text{L}$  of

water and covered with foil until it was incubated at 37°C for 3 min. Protease enzyme activity was measured as a change in absorbance at 765 nm using an Epoch microplate spectrophotometer. For the blank sample, the reaction was assayed as described above, but the enzyme extract was replaced by the sample buffer. Enzyme control and substrate control were also performed. An L-tyrosine standard curve was prepared using 10 mM stock solution. L-tyrosine was used as a standard, and one unit of enzyme activity was expressed as 1  $\mu\text{mol}$  of tyrosine  $\text{min}^{-1} \text{mg protein}^{-1}$ .

To determine the alkaline phosphatase (AP) activity, a *p*-nitrophenyl phosphate (pNPP) substrate was used (Bessey et al., 1946). A *p*-nitrophenol standard curve ranging from 0.001 to 0.5  $\mu\text{mol mL}^{-1}$  was prepared from a 1  $\mu\text{mol mL}^{-1}$  stock solution. Standards solutions of 250  $\mu\text{L}$  were added to 250  $\mu\text{L}$  of 2 M sodium carbonate. Each standard mixture (250  $\mu\text{L}$ ) was added into a 96-well microplate and the absorbance was read at 405 nm. For the AP activity, triplicate samples (50  $\mu\text{L}$ ) of crude whole intestine extract were mixed at 25°C with 195  $\mu\text{L}$  of buffer (50 mM Tris-HCl, pH 8) and 5  $\mu\text{L}$  of 100 mM *p*-nitrophenyl phosphate substrate. Following incubation for 10 min, 250  $\mu\text{L}$  of 2 M sodium carbonate was added to stop the reaction. The absorbance of 250  $\mu\text{L}$  of each sample was read at 405 nm using Epoch microplate spectrophotometer. For the blank sample, the reaction was assayed as described above, except that the enzyme extract was replaced by the sample buffer.

#### 5.2.8 Total protein

The total protein content of the homogenate was determined using the Bradford assay (Bradford, 1976). A bovine serum albumin (BSA) standard curve over a concentration range of 0.05 to 0.5  $\text{mg mL}^{-1}$ , was prepared from a 10  $\text{mg mL}^{-1}$  stock solution. Bradford reagent (230  $\mu\text{L}$ ) was mixed with 25  $\mu\text{L}$  of the homogenate. The reaction mixture was incubated at 25°C for 10 min before the absorbance was measured at 595 nm using an Epoch microplate

spectrophotometer. For the blank samples, the reaction was assayed as described above except that the homogenate was replaced by distilled water.

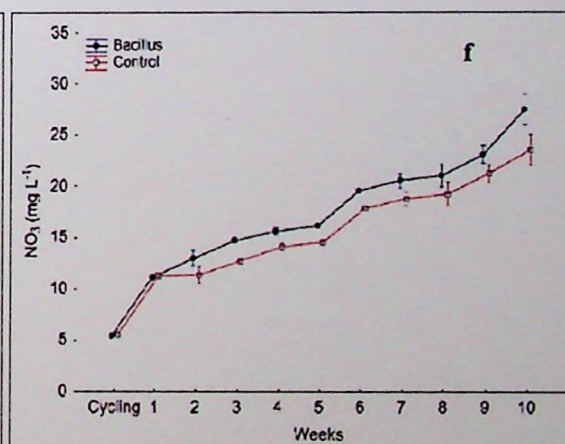
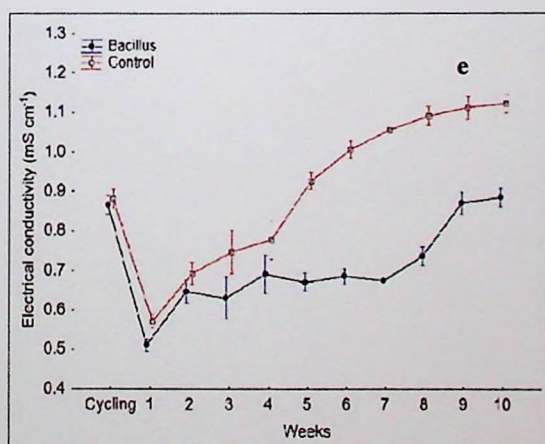
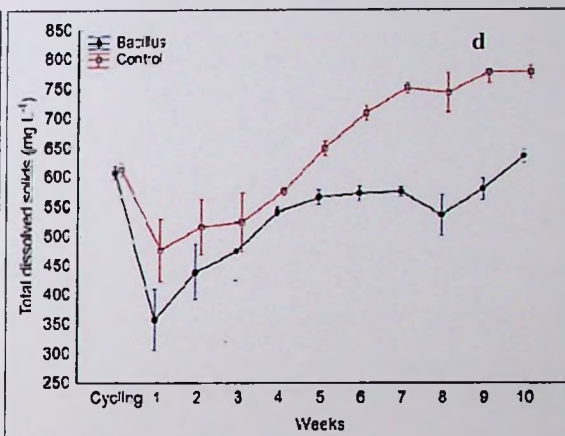
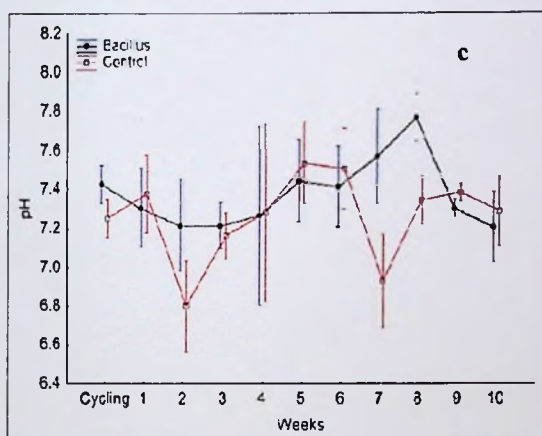
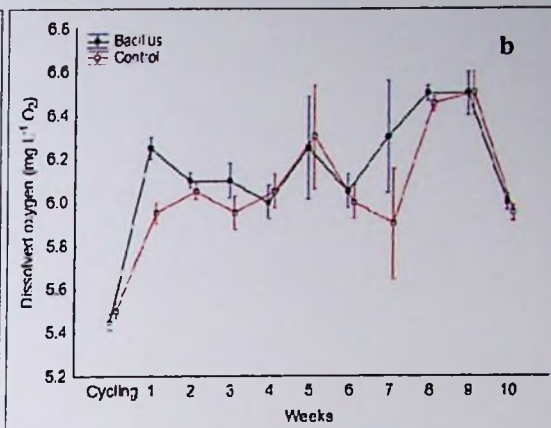
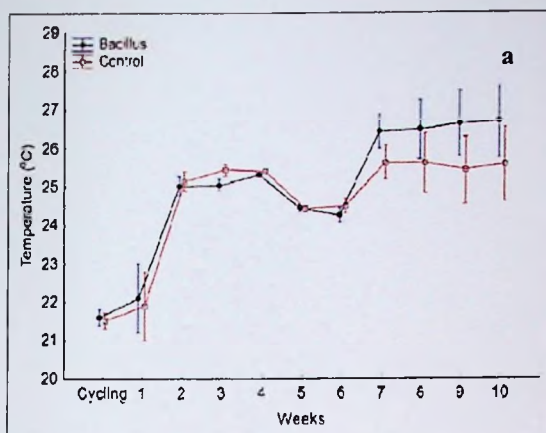
### 5.2.9 Data analysis

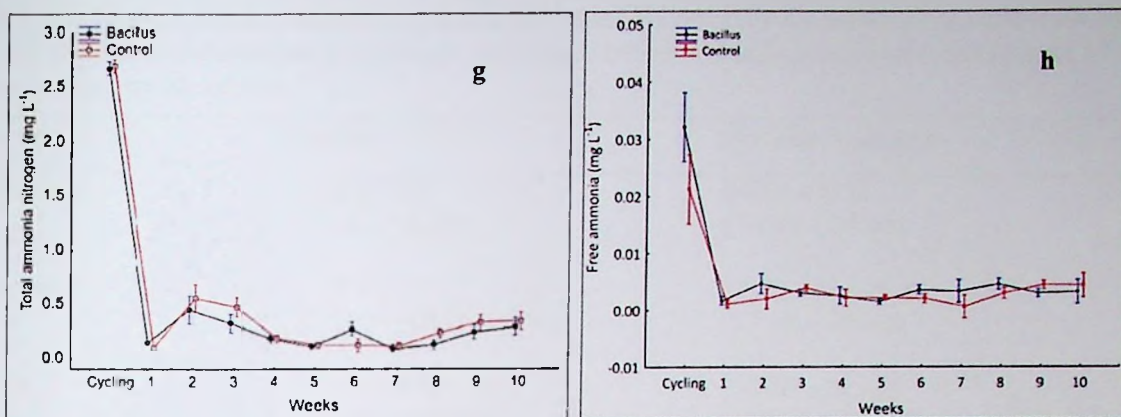
To avoid bias due to pseudoreplication, values of individual fish per tank were averaged prior to analysis. There was a low variability within treatments for growth data. On average, values within treatments differed by only 0.4% from each other and there was equality of variances (Levene's test). Therefore, Student's *t*-test was used to compare means for growth and proximate whole-body composition between treatments. As there were no significant differences between treatments in proximate composition at the end of the experiment, final data were pooled and compared to the respective average value at the start of the trial with a one-sample *t*-test. To test whether there was a significant difference between treatments in the change in water quality over the 10-week period, repeated measures analysis of variance RM-ANOVA was used. When there was a significant interaction between main effects, Tukey's HSD post-hoc test (Tukey, 1960) was used to compare treatment means at all times. RM-ANOVA was used as it allows testing for changes in treatment means as a function of time. It was also considered suitable in this case as this test can increase statistical power (Guo et al., 2013). Differences between means were considered significant when the Type 1 error probability was less than 5%, i.e.,  $P < 0.05$ . All analyses were performed using the Statistica software package (TIBCO Software, Palo Alto, USA, version 13.5.0).

## 5.3 Results

### 5.3.1 Water quality parameters

Water temperature ranged from 21.5°C to 26.7°C (Figure 5.1a). In both treatments, temperature changed in a similar way over time (RM-ANOVA, temperature  $\times$  treatment interaction,  $P = 0.841$ ). Treatment did not affect how the dissolved oxygen concentration changed over time (RM-ANOVA, dissolved oxygen  $\times$  treatment interaction,  $P = 0.703$ ; Figure 5.1b). There was no difference in average pH between treatments, and time, and there was no interaction between treatment and time (RM-ANOVA,  $P = 0.25$ ,  $P = 0.59$ , and  $P = 0.47$ , respectively; Figure 5.1c). The addition of probiotics resulted in a slower increase of both the concentration of total dissolved solids and electrical conductivity values (Figure 5.1d and 5.1e, respectively) (RM-ANOVA, interaction term,  $P = 0.031$  and  $P < 0.0001$ , respectively). In both treatments the concentration of nitrate increased over time (RM-ANOVA,  $P < 0.00001$ ) in a similar manner (RM-ANOVA, interaction term,  $P = 0.38$ ; Figure 5.1f). There was no effect of treatment on average TAN values (RM-ANOVA,  $P = 0.655$ ) and the values changed in a similar manner over time when fish were present (RM-ANOVA, TAN  $\times$  treatment interaction,  $P = 0.703$ ; Figure 5.1g). The average TAN value of the pooled data from both treatments was 0.238 mg L<sup>-1</sup> between weeks 1 to 10. Similarly, there was no difference in average free NH<sub>3</sub> values between treatments, and time, and there was no interaction between treatment and time (RM-ANOVA, NH<sub>3</sub>  $\times$  treatment interaction,  $P = 0.34$ , Figure 5.1h).





**Figure 5.1:** Changes in temperature (a), dissolved oxygen (b), pH (c), total dissolved oxygen (d), electrical conductivity (e), nitrate (f), total ammonia nitrogen (g), and free ammonia (h) in aquaponic systems during fishless cycling and during a 10-week growth period during which juvenile tilapia, *Oreochromis mossambicus*, were fed a commercial diet at 2% of body mass day<sup>-1</sup>. In the *Bacillus* treatment, a commercial probiotic was added twice a week. Significant treatment  $\times$  time interactions (RM-ANOVA) were found for TDS and EC values.

### 5.3.2 Fish growth

There was a significant effect of *Bacillus* treatment on weight gain (*t*-test, Table 5.1) during the 10-week growth period. Average fish weight and weight gain in the *Bacillus* treatment was 4.13% and 7.1% higher, respectively, than in the control (Table 5.1). The PER value from the *Bacillus*-treated systems was higher than in the control, while no significant difference was observed between the treatment and the control for average FE and FCR values (*t*-test, Table 5.1). Survival rate averaged 80% in both the *Bacillus* treatment and the control.

**Table 5.1:** Growth and feed utilisation of *O. mossambicus* from the control and *Bacillus*-treated systems at the end of a 10-week feeding experiment. Mean values are followed by the standard error of the mean and range is given in parentheses. Significant differences between *Bacillus* treatment and control ( $P < 0.05$ ) are marked by an asterisk.

| Parameter                  | Control                            | <i>Bacillus</i> treatment           |
|----------------------------|------------------------------------|-------------------------------------|
| IW (g fish <sup>-1</sup> ) | 36.39 ± 0.01<br>(36.40 – 36.38)    | 36.56 ± 0.11<br>(36.45 – 36.68)     |
| FW (g fish <sup>-1</sup> ) | 74.67 ± 0.17<br>(74.50 – 74.84)    | 77.76 ± 0.58*<br>(77.18 – 78.30)    |
| WG (%)                     | 105.19 ± 0.53<br>(104.66 – 105.73) | 112.67 ± 0.92*<br>(111.75 – 113.59) |
| PER                        | 5.84 ± 0.16<br>(5.80 – 5.87)       | 6.41 ± 0.07*<br>(6.32 – 6.49)       |
| SGR (% day <sup>-1</sup> ) | 1.03 ± 0.005<br>(1.02 – 1.03)      | 1.08 ± 0.005<br>(1.07 – 1.08)       |
| FE (%)                     | 56.13 ± 0.75<br>(55.18–58.15)      | 56.66 ± 1.30<br>(53.39 –58.87)      |
| FCR                        | 1.78 ± 0.04<br>(1.69 –1.87)        | 1.76 ± 0.05<br>(1.71– 1.80)         |

### 5.3.3 Proximate composition

At the end of the experiment, there were no significant differences in the average values between the *Bacillus* treatment and the control for moisture content ( $t = 0.24$ ,  $P = 0.829$ ). Treatment had no effect on average values (% dry matter) of crude protein ( $t = 1.64$ ,  $P = 0.242$ ), ash ( $t = 2.01$ ,  $P = 0.182$ ) and crude lipid ( $t = 0.45$ ,  $P = 0.692$ ) nor on the gross energy level ( $t = 2.86$ ,  $P = 0.103$ ; Table 5.2). At the end of the study, both lipid and ash content (% dry matter) were higher than at the start of the experiment (one-sample  $t$ -test,  $P = 0.020$ ; and  $P = 0.033$ , respectively, Table 5.2).

**Table 5.2:** Whole-body composition (percentage of dry matter) of *Oreochromis mossambicus* in the control and the *Bacillus* treatment at the end of a 10-week feeding experiment. Values are means  $\pm$  standard errors. The column on the right shows the results from a one-sample *t*-test to compare the pooled average of treatment and control at the end of the study to the average value at the start of the trial. Significant differences between *Bacillus* treatment and control ( $P < 0.05$ ) are marked by an asterisk.

| Composition (% dry matter)         | Treatment        |                  |                 |                        |                 |
|------------------------------------|------------------|------------------|-----------------|------------------------|-----------------|
|                                    | Control          | <i>Bacillus</i>  | <i>P</i> -value | Average value at start | <i>P</i> -value |
| Moisture (%)                       | 76.52 $\pm$ 0.56 | 76.26 $\pm$ 0.91 | 0.829           | 77.50                  | 0.074           |
| Crude protein (%)                  | 55.04 $\pm$ 0.03 | 58.10 $\pm$ 1.86 | 0.242           | 54.03                  | 0.345           |
| Ash (%)                            | 4.71 $\pm$ 0.02  | 4.79 $\pm$ 0.05  | 0.182           | 3.54                   | 0.020*          |
| Crude lipid (%)                    | 4.05 $\pm$ 0.52  | 4.38 $\pm$ 0.51  | 0.692           | 1.04                   | 0.033*          |
| Gross energy (kJ g <sup>-1</sup> ) | 19.75 $\pm$ 0.55 | 21.43 $\pm$ 0.19 | 0.103           | 20.57                  | 0.985           |

#### 5.3.4 Digestive enzyme activity

*Bacillus* treatment significantly increased the average activity values of amylase, protease, and alkaline phosphatase in the intestines of the fish (*t*-test,  $P < 0.05$ , Table 5.3). Alkaline phosphatase showed the lowest mean activity levels, while protease showed the highest activity values (Table 5.3).

**Table 5.3:** Specific activities of amylase, protease, and alkaline phosphatase in the whole intestine of Mozambique tilapia cultured in the control and the *Bacillus*-treated aquaponic systems at the end of the 10-week experiment. Significant differences between *Bacillus* treatment and control ( $P < 0.05$ ) are marked by an asterisk.

| Digestive enzyme     | Specific activity (U mg protein <sup>-1</sup> ) |                           |                 |
|----------------------|-------------------------------------------------|---------------------------|-----------------|
|                      | Control                                         | <i>Bacillus</i> treatment | <i>P</i> -value |
| Amylase              | 0.80 $\pm$ 0.05                                 | 1.46 $\pm$ 0.11           | 0.031*          |
| Protease             | 287.25 $\pm$ 4.23                               | 327.62 $\pm$ 1.69         | 0.012*          |
| Alkaline phosphatase | 0.16 $\pm$ 0.02                                 | 0.67 $\pm$ 0.06           | 0.018*          |

## 5.4 Discussion

The addition of a probiotic did not affect the average values of pH, TAN, DO,  $\text{NH}_3$ , and  $\text{NO}_3^-$ , which were all within acceptable levels for the rearing of *O. mossambicus* (El-Sayed, 2019). *O. mossambicus* tolerate a pH range of 5.5 to 9.0, a temperature range of 8°C to 32°C with optimal values between 28°C and 30°C, DO concentrations of more than 3 mg L<sup>-1</sup> and TAN concentrations below 3 mg L<sup>-1</sup> (El-Sayed, 2019). In addition, although nutrient-rich water recirculated between the fish tanks and the hydroponics units,  $\text{NO}_3^-$  concentrations of 10 – 27.5 mg L<sup>-1</sup> were low and acceptable for the rearing of tilapia but sufficient for plant growth (Somerville et al., 2014). Matias et al. (2002) tested the effect of commercial microbial products on water quality. Adding *B. subtilis* (10<sup>8</sup> CFU mL<sup>-1</sup>) to the rearing water maintained the concentration of nitrite, ammonia and nitrate ions to within acceptable levels for shrimp (*Penaeus monodon*) culture. Jasmin et al. (2020) reviewed studies, in which *Bacillus* species were used under a wide range of conditions to treat aquaculture wastewater, in particular from shrimp culture, and the authors reported that *Bacillus* species could reduce ammonia and nitrite concentrations. In addition, in the present study,  $\text{NH}_3$  values remained low in both the treatment and control tanks, possibly due to the uptake of nitrogenous compounds by plants, as well as the fishless cycling. Sallenave (2016) suggested a low risk of high TAN values in closed systems after fishless cycling, but the effects of this method on TAN concentrations with fish were not reported. Results from the present experiment indicate that  $\text{NH}_3$  levels could be kept at a low level after fishless cycling.

The faster increase in the TDS and EC levels in the control systems might be due to the accumulation of ions which were not absorbed by plants in the control systems. The TDS levels in the control systems, however, were not high enough to influence nitrification, as there were no differences in pH and ammonia levels between treatments. In aquaponic systems, ions can accumulate as a result of mineralisation of organic matter and from uneaten or undigested fish

feed (Yildiz et al., 2017). In the present study, growth expressed as final average weight and percentage weight gain and protein efficiency ratio differed between the *Bacillus* treatment and control. However, differences in waste excretion by the fish may not solely explain the effect of treatment on the change in concentration of TDS and EC values over time. It is possible that increased nutrient uptake of plants due to the plant-promoting actions of the probiotic may have contributed to these differences in water quality. For example, plants grown in *Bacillus*-treated systems accumulated approximately four times as much dry mass and twice as much phosphorus than plants in control systems (Cerozi and Fitzsimmons, 2016). *Bacillus* spp. can also improve water quality by converting organic matter to carbon dioxide (Dalmin et al., 2001; Balcázar et al., 2006). Although the commercial product contributes nutrients that may contribute to the water quality, the levels of N and P that dissolved in the water and in the solids were not investigated in this study.

In addition to its effects on some water quality variables, *Bacillus* affected fish growth and digestive enzyme activity. Percentage weight gain in the *Bacillus* treatment was higher than in the control. The average PER in the *Bacillus* treatment was also higher than in the control. This might be related to the ability of *Bacillus* species to activate protease responsible for protein hydrolysis (Adorian et al., 2019). Although FCR values were lower than values frequently found on farms, they are acceptable for experimental conditions with commercial diets. Fish tend to optimise their digestion in order to utilise feed nutrients efficiently, which then increases feed conversion efficiency, especially under conditions of controlled feeding (Zoccarato et al., 1994; Van Ham et al., 2003). A significant effect of *Bacillus* on weight gain was also found in other studies on the growth of Nile tilapia, *O. niloticus*, in which probiotics were used (Ridha and Azad, 2012; Adeoye et al., 2016; Liu et al., 2017; Elsabagh et al., 2018), but in these studies, probiotics were added to the fish feed rather than to the system water as was done in the present experiment. Addo et al. (2017) reported that *Bacillus* can also suppress

pathogenic bacteria in the gut, thereby increasing disease resistance and improving fish growth. A review of studies in which farmed fish were fed food supplemented with *Bacillus* species showed that these fish had improved growth and better immunity and disease resistance (Boyd et al., 2020). In aquaponic systems, in which a range of probiotic bacteria were used as microbiological control agents, *Bacillus* species improved the control of fish diseases, but more research is needed to study both the taxonomy of the bacteria and the way probiotics are being used (Folorunso et al., 2020).

In the present study, the use of the commercial *Bacillus* preparation did not affect whole-body composition of *O. mossambicus*, suggesting that optimum conditions for keeping this species were maintained. Environmental conditions contribute to variations in the proximate composition of fish (Shearer, 1994). Merrifield et al. (2010) reported that carcass composition of rainbow trout was not affected by dietary *B. licheniformis* and *B. subtilis* after 10 weeks of feeding. However, in this study in both treatments crude lipid and ash content had increased by the end of the experiment. Shearer (1994) reported that lipid content of cultured fish increased with fish size, while the relative increase in protein content was low. The significant increase in ash content over time may be attributed to increased skeletal mass and scale mineralisation, which increases as fish grow (Breck, 2014). These findings, combined with the high survival in both treatments, suggest that the environmental conditions under which the fish were kept were acceptable for this species.

The *Bacillus* treatment increased the activities of amylase, protease, and alkaline phosphatase. The higher activities observed in the *Bacillus* treatment may be either due to enzymes synthesised by the bacteria involved in the digestion processes, or it was possible that *Bacillus* stimulated the production of endogenous enzymes in the fish. Increased intestinal amylase and protease activities may facilitate the digestion and utilisation of carbohydrates and proteins, respectively. This may have contributed to the higher weight gain of the tilapia in the

*Bacillus* treatment. In addition, alkaline phosphatase activity is important for maintaining gastrointestinal health, as this enzyme limits inflammation caused by microbes (Lallès, 2019). Digestive enzyme activity has been shown to be affected by temperature (Munilla-Morán and Saborido-Rey, 1996; Apún-Molina et al., 2009), pH (Solovyev et al., 2015) and dissolved oxygen levels (Lallès, 2019). However, these variables did not differ between treatments in the present study. It is therefore suggested that *Bacillus* in the intestine may have enhanced digestive enzyme activity and growth. Enhancement of digestive enzyme activity by *Bacillus* was also reported by Silva et al. (2015) who added probiotics to a diet fed to Nile tilapia, *O. niloticus*.

In conclusion, the addition of a commercial mixture of *Bacillus subtilis* and *B. licheniformis* increased weight gain in *O. mossambicus* and reduced EC and TDS levels in the fish rearing water. The use of *Bacillus* as a probiotic may therefore be considered beneficial. Because fish in aquaponics have an intimate interaction with their surrounding water and, in turn, with the microorganisms that co-exist there, it is possible that bacteria present in the aquaponic systems may influence the composition of the gut microbiota in fish. However, the possible link between probiotics and the intestinal microbiome has received comparatively little attention in cultured aquatic species from aquaponic systems. To address this knowledge gap, Chapter 6 describes how the gastrointestinal microbiota of *O. mossambicus* is modulated by the addition of *Bacillus* species in an aquaponic system.

## CHAPTER 6

### Effect of adding *Bacillus* species to the water of a coupled aquaponic system on the gastrointestinal microbiota of Mozambique tilapia, *Oreochromis mossambicus*

#### 6.1 Introduction

The growth and welfare of fish and the quality of plants in aquaponic systems rely on the composition of the system's microbiota (Joyce et al., 2019). For example, plant growth depends on the availability of nutrients that are largely supplied in the excretions from the fish and through their microbial transformations into nutrients (Lennard and Goddek, 2019). The growth of fish depends on water quality, which is controlled through the uptake of nutrients by the plants and the microbial activity within the system's media, to which bacteria can attach (Kasozi et al., 2021a). In addition, the composition of fish gut microbiota, which can be influenced by the microbial composition of the complex ecology of aquaponic systems, affects health and growth of the fish (Shade and Handelsman, 2012). Bacteria contribute to vital physiological functions of the fish, such as digestion, metabolism, reproduction, development and immune responses (Nayak, 2010; Yukgehnash et al., 2020).

Fish have unique and complex intestinal assemblages of microorganisms, comprising of bacteria, archaea, viruses, yeasts, and protists (Egerton et al., 2018; Johny et al., 2021). The microbial colonisation, establishment, and diversity of the fish gut is influenced by genetics, the developmental stages of the fish, water quality and nutritional factors (Nayak, 2010; Sullam et al., 2012; Butt and Volkoff, 2019; Gallo et al., 2020). Among the microorganisms inhabiting the fish gut microbiome, bacteria are the most dominant (Gallo et al., 2020). They are also important for the supply of nutrients and secretion of compounds which can prevent colonisation by pathogens (Tachibana et al., 2021; Steiner et al., 2021). For example, the genera *Vibrio*, *Edwardsiella*, *Aeromonas*, and *Shewanella* have been detected in fish gut (Yukgehnash et al., 2020), while the *Cetobacterium* species may be indicators of fish health (Maas et al.,

2021; Tachibana et al., 2021). Therefore, knowledge of the bacterial composition of the fish gastrointestinal tract (GIT) can contribute to fish production in aquaponic systems. As beneficial bacterial communities constitute only a small percentage of the intestinal microbiota (Llewellyn et al., 2014), manipulating the fish gut microbiome may be beneficial for fish health and growth. The addition of probiotics in aquaculture systems can improve fish growth and mitigate fish diseases (Egerton et al., 2018; Tachibana et al., 2021). The ability of a probiotic strain to effectively modulate the intestinal microbiota depends on the viability of the bacteria during processing and storage, growth stage of the fish, and mode and duration of administration (Merrifield et al., 2010; Giatsis et al., 2016; Haygood and Jha, 2018). It is therefore necessary to test how the addition of probiotics in aquaponics water may influence the fish gut microbiome. There are a number of candidate probiotics, but only a few have been tested in aquaponic systems. For example, a commercial mixture of *Bacillus* species increased the phosphate and nitrate concentrations in the water resulting in faster lettuce growth (Kasozi et al., 2021b).

Over the past few decades, most of the studies on fish gut microbiota were carried out by conventional culture-dependent methods using general and selective agar media (de Bruijn et al., 2018; Yukgehnash et al., 2020). Because only a small number of the viable intestinal bacteria are culturable in this way, 16S rRNA gene sequencing has been applied to characterise the composition of fish microbiota (Yukgehnash et al., 2020; Parata et al., 2021). Using Illumina MiSeq sequencing of the 16S rRNA gene, the aim of this study was to evaluate the effect of adding a commercial mixture of *Bacillus subtilis* and *B. licheniformis* to the water of an aquaponic system on the gastrointestinal microbiota of juvenile *O. mossambicus*.

## 6.2 Materials and methods

### 6.2.1 Experimental fish and feeding conditions

Red Mozambique tilapia, *Oreochromis mossambicus*, juveniles (average body weight  $4.74 \pm 0.01$  g fish<sup>-1</sup>) were obtained from Aquaculture Innovations, Grahamstown, South Africa. A total of 24 fish were randomly selected and stocked into each tank of the aquaponic system, and the stocking density at the beginning of the experiment was 1.13 kg m<sup>-3</sup>. Fish were fed a commercial floating pellet (Avi Products Pty Ltd., Cato Ridge, South Africa) containing 40% crude protein, 0.7% phosphorus, 5% crude fat and 3.0% calcium at 3.0% of their estimated body mass day<sup>-1</sup>. Body mass and weight increase were estimated based on monthly records of average fish weight per tank and feed conversion ratio. The feed was distributed over three feedings (09h00, 13h00 and 16h00) for 90 days. The aquaponic units assigned to the *Bacillus* treatment received 5.31 g (0.02 g product L<sup>-1</sup> of system water) twice weekly following manufacturer's instructions until day 90. In addition, water temperature, pH, TDS, and EC were measured daily between 08h30 and 09h00 using a multi-parameter water quality monitor probe. The concentrations of TAN and NO<sub>2</sub><sup>-</sup> were determined using Spectroquant test kits as described in Section 2.5.1 (Chapter 2), while the concentration of NH<sub>3</sub> was determined following the calculations by Emerson et al. (1975) using the values of temperature and pH of the day on which the water samples were taken.

### 6.2.2 Fish growth performance

At the end of the 90-day rearing period, fish were collected from each tank to determine the final body weight of each individual using an electronic balance (Adam Equipment, PGL-2002, with a precision of 0.01 g). Percentage weight gain (WG), specific growth rate (SGR, % d<sup>-1</sup>), feed conversion ratio (FCR), feed efficiency (FE, %), and percentage survival (SR) were calculated following equations described in Section 2.6.2 (Chapter 2).

### 6.2.3 Weighing of fish and sampling for gut bacterial community

Random samples of 10 fish per tank were weighed monthly. At the end of the trial, all fish in each tank were counted and weighed to calculate survival rate and growth indices, respectively. To record the body mass, each fish was anaesthetised with clove oil (Lennon by Aspen Ltd., La Lucia Ridge, South Africa, 90% eugenol) which had been dissolved in 100% ethanol at 1 mL of clove oil and 9 mL of ethanol. The clove oil concentration in the water, in which the fish were kept was 40 mg L<sup>-1</sup>. Prior to dissection, the fish that were sampled for gut bacterial community analysis were euthanised with a clove oil concentration of 60 mg L<sup>-1</sup> for 4 min using water bath immersion. Five fish per tank were randomly selected to obtain samples for the analysis of the intestinal bacterial community. The intestinal samples were pooled as the tank was considered the experimental unit. The external surface of each fish was wiped with 70% ethanol to avoid contaminating the intestinal content with the microflora from the external body surface. The abdomen of each fish was then opened at the ventral midline and the gastrointestinal tract was aseptically removed from the abdominal cavity. All tools and dissecting surfaces were disinfected with 70% ethanol between dissections. The gastrointestinal samples were flash frozen in liquid nitrogen and stored individually in sterile tubes at -80°C until they were thawed before NGS analysis. A 200 mg sample of the fish gut content was taken from each tube and placed into a ZR Bashingbead™ lysis tube (2.0 mm) and vortexed for 5 min at maximum speed.

### 6.2.4 Genomic DNA extraction, PCR amplification, and sequence analysis

Genomic DNA was extracted from gut samples using the Quick-DNA™ DNA Fecal/Soil Microbe Miniprep Kit (Zymo Research, Tustin, CA, USA) following manufacturer's recommendations. The process of PCR amplification was conducted following the protocol described in Section 2.7 (Chapter 2). Raw sequences from 16S rRNA gene were deposited into

the National Center for Biotechnology Information (NCBI) under project accession number: PRJNA623517 through the Sequence Read Archive (SRA) study accession number: SRR14793676.

The raw sequences generated from MiSeq analysis of amplicon libraries targeting the 16S rRNA gene were processed using the MOTHUR platform version 1.41.3 release (Schloss et al., 2009), as described in Section 2.8 (Chapter 2). The taxonomic classification was performed down to genus level. The analysis of the common and unique OTUs was conducted to investigate the gastrointestinal bacterial community from the replicates of each treatment through a Venn diagram. Species identification for the dominant 50 OTUs was performed using the BLAST command line. A heatmap was constructed to visualise the distribution of the dominant OTUs.

### 6.2.5 Data analysis

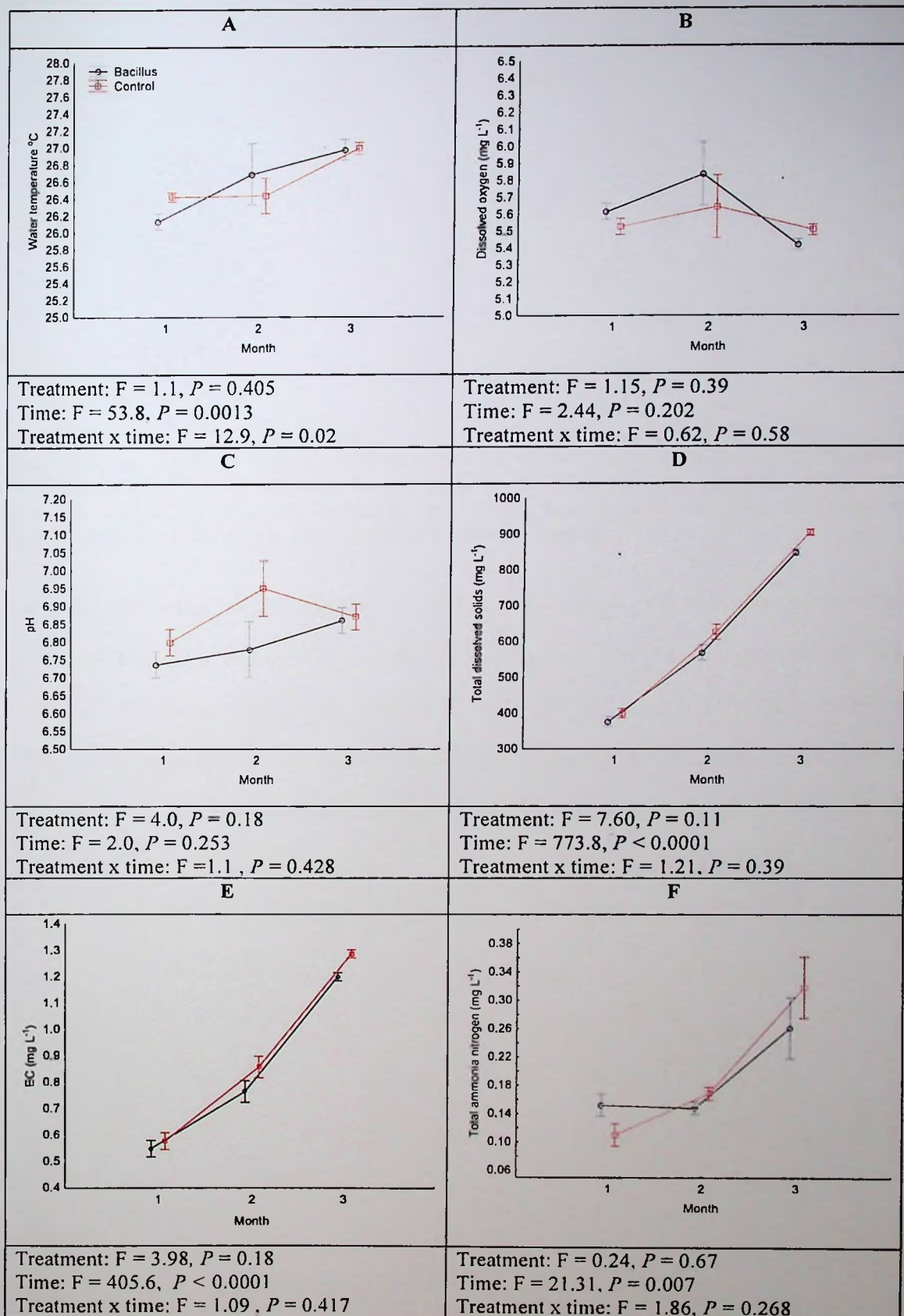
To avoid bias due to pseudoreplication, values of individual fish per tank were averaged prior to statistical analysis. As fish were weighed monthly, water quality data were averaged for each of the three months. To test whether there was a significant difference between treatments in the monthly change in water quality over the 90-day period, repeated measures analysis of variance (RM-ANOVA) was applied to monthly averages of fish weights per tank. Average values for FE and FCR values at the end of the trial were compared using Student's *t*-test, which has been suggested to be suitable for small sample sizes (de Winter, 2013). The same test was used for gut bacterial community and diversity indices. Fish survival rates (%) were log-transformed before analysis and compared between treatments using Student's *t*-test for equal variances. Differences between means were considered significant when the Type 1 error probability was less than 5%, i.e.,  $P < 0.05$ . All analyses were performed using the Statistica 12® software package (TIBCO Software, Palo Alto, USA, version 13.5.0).

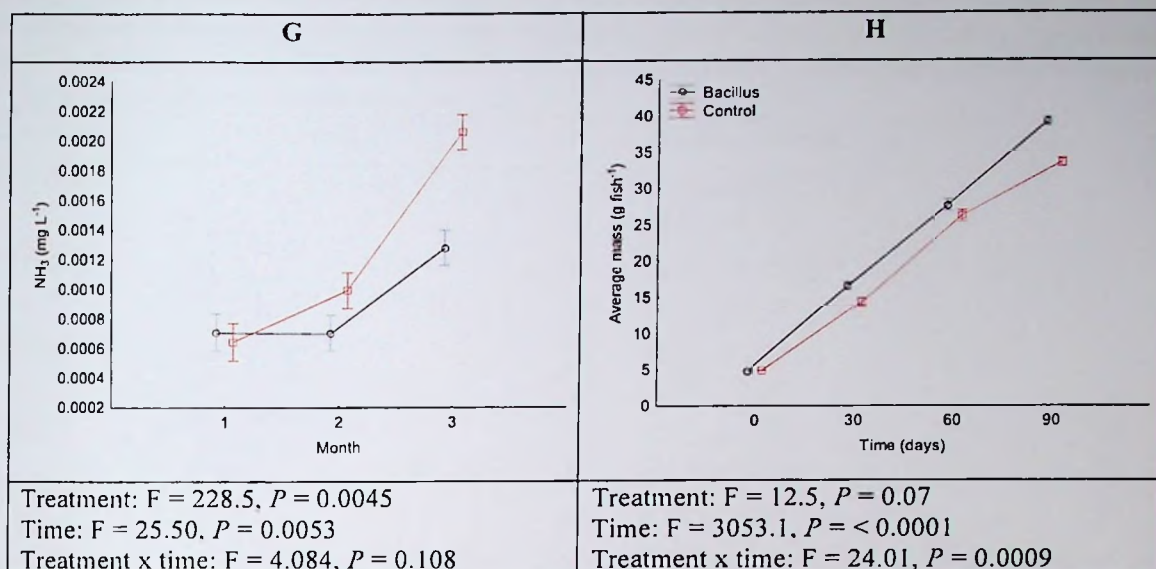
## 6.3 Results

### 6.3.1 Water quality parameters in the rearing tanks and fish growth

Water temperature fluctuated between 25.9°C and 27.2°C in the *Bacillus*-treated tanks and the control tanks, respectively, and there was no difference between treatment means. Even though there was a significant interaction between treatment and month, the largest mean difference between treatment and control was only 0.2°C (Figure 6.1A). The level of dissolved oxygen was maintained above 5 mg L<sup>-1</sup> in both treatments with no change over time and no significant interaction between treatment and time (Figure 6.1B). The average pH-values were similar between the *Bacillus*-treated and control systems (Figure 6.1C). Both the levels of TDS and EC increased over time, but the increase was not influenced by the addition of the *Bacillus* product (Figure 6.1D and 6.1E, respectively). The average value of TAN of both treatments was 0.195 mg L<sup>-1</sup>, with a similar increase in both treatments (Figure 6.1F). *Bacillus* addition reduced the average NH<sub>3</sub> values compared to the control treatment with an average of 0.0008 mg L<sup>-1</sup> in the *Bacillus* treatment and 0.001 mg L<sup>-1</sup> in the control systems (Figure 6.1G). All monthly means and statistics (repeated measures analysis of variance) are shown in Figure 6.1. Nitrite levels were below 1 mg L<sup>-1</sup> throughout the experiment (not shown in graph).

There was a significant interaction between treatment and time for the increase in fish biomass (Figure 6.1H), which resulted in fish from the *Bacillus* treatment having a significantly higher biomass at the end of the study (39.2 g fish<sup>-1</sup> with a range of 38.5 – 39.8 g fish<sup>-1</sup>) than the fish from the control systems (33.4 g fish<sup>-1</sup> with a range of 33.0 – 33.87 g fish<sup>-1</sup>).





**Figure 6.1:** Water quality parameters; temperature, dissolved oxygen, pH, total dissolved solids, electrical conductivity, total ammonia nitrogen, ammonia (A – G), and change of average fish biomass over time (H) in the control and *Bacillus*-treated systems. Results are based on repeated measures analysis of variance. Values are means and 95% confidence intervals.

The addition of *Bacillus* significantly increased the weight gain of the fish over the 90-day trial (Table 6.1). The specific growth rate was higher in the *Bacillus* treatment ( $2.35 \pm 0.02\% \text{ d}^{-1}$ ) than in the control treatment ( $2.17 \pm 0.02\% \text{ d}^{-1}$ ) ( $t$ -test,  $P < 0.05$ ; Table 6.1). The average FCR value in fish from the *Bacillus* treatment was 10.8% better, and specific growth rate was 8.3% higher than in the control fish (Table 6.1). Survival rate was above 90% in both treatments (Table 6.1).

**Table 6.1:** Growth performance and feed utilisation of *O. mossambicus* from the control and *Bacillus*-treated systems at the end of a 90-day feeding experiment. Mean values are followed by the standard error of the mean and range is given in parentheses. Significant differences between *Bacillus* treatment and control ( $P < 0.05$ ) are marked by an asterisk.

| Parameter                  | Control                         | <i>Bacillus</i> treatment            |
|----------------------------|---------------------------------|--------------------------------------|
| IW (g fish <sup>-1</sup> ) | 4.74 ± 0.03<br>(4.71 – 4.77)    | 4.75 ± 0.02<br>(4.73– 4.76)          |
| WG (%)                     | 605.0 ± 5.00<br>(600.0 – 610.0) | 725.36 ± 11.62*<br>(706.92 – 731.73) |
| SGR (% d <sup>-1</sup> )   | 2.17 ± 0.02<br>(2.15 – 2.19)    | 2.35 ± 0.02*<br>(2.33 – 2.36)        |
| FE (%)                     | 68.94 ± 0.43<br>(68.51– 69.36)  | 77.20 ± 0.90*<br>(76.31 – 78.1)      |
| FCR                        | 1.44 ± 0.005<br>(1.44 – 1.45)   | 1.30 ± 0.02*<br>(1.28– 1.31)         |
| SR (%)                     | 95.83 ± 4.16<br>(91.65–100)     | 97.91 ± 2.09<br>(95.83–100)          |

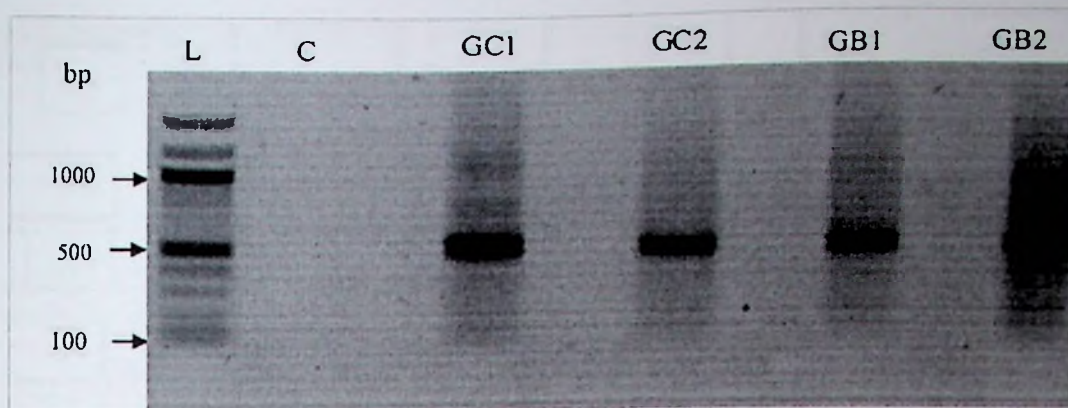
### 6.3.2 DNA quantity and quality extracted from fish gut samples

The DNA extracted from gut samples of *O. mossambicus* ranged from 74.1 ng  $\mu\text{L}^{-1}$  to 93.9 ng  $\mu\text{L}^{-1}$  (Table 6.2). The relative purity of DNA as assessed by the ratio of  $A_{260}/A_{280}$ , was within the optimal range of 1.88 to 1.93 (Table 6.2). The samples exhibited PCR amplification as demonstrated by clear bands of approximately 550-bp (Figure 6.2).

**Table 6.2:** Purity and concentration of genomic DNA extracted from the intestinal microflora of *O. mossambicus* with or without *Bacillus* treatment.

| Sample | DNA concentration (ng $\mu\text{L}^{-1}$ ) | $A_{260}/A_{280}$ | DNA integrity* |
|--------|--------------------------------------------|-------------------|----------------|
| GC1    | 81.4                                       | 1.92              | ++             |
| GC2    | 93.9                                       | 1.88              | ++             |
| GB1    | 92.5                                       | 1.86              | ++             |
| GB2    | 74.1                                       | 1.93              | ++             |

\*DNA integrity was assayed by agarose electrophoresis and indicated as good (++) based on the band clarity, density and presence of smearing. GC1 and GC2 represent gut samples obtained from control systems, and samples GB1 and GB2 are the gut samples from the *Bacillus*-treated systems.



**Figure 6.2:** Agarose gel electrophoresis of the PCR products amplified from the gastrointestinal microflora of *O. mossambicus* with or without *Bacillus* treatment. Lane L shows the 100-bp DNA ladder; followed by a negative control. Lanes GC1 and GC2 are the amplification products from gut samples obtained from the control systems; and lanes GB1 and GB2 are the gut samples from the *Bacillus*-treated systems.

### 6.3.3 Sequencing summary and diversity of gut bacterial community profiles

A total of 235,699 raw reads were obtained from high-throughput sequencing of the V3 – V4 regions of the 16S rRNA gene (118,209 and 117,490 reads for the gastrointestinal contents from the control and *Bacillus*-treated systems, respectively, Table 6.3). After removing poor-quality reads, there were 88,869 sequences for the control samples and 85,617 sequences for the *Bacillus*-treated samples. Removal of chimeras resulted in 80,418 and 81,486 sequences for the control and *Bacillus*-treated samples, respectively. The percentage of sequences flagged as chimeric was 7.21%. Non-bacterial, chloroplast, and mitochondrial operational taxonomic units (OTUs) accounted for a total of 18 and 7 sequences for the control and *Bacillus*-treated samples, respectively (Table 6.3). After quality filtering and removal of chimeras and single reads, 52,222 and 44,714 reads were obtained for the control and *Bacillus*-treated samples, respectively (Table 6.3).

**Table 6.3:** Summary of sequencing data for fish gut samples.

| Treatment                 | Number of raw sequences | Number of sequences before chimeras | Number of sequences after chimeras | Chloroplast and Mitochondria reads | Number of after screening and filtering |
|---------------------------|-------------------------|-------------------------------------|------------------------------------|------------------------------------|-----------------------------------------|
| Control                   |                         |                                     |                                    |                                    |                                         |
| Sample GC1                | 52,222                  | 43,516                              | 37,091                             | 14                                 | 31,626                                  |
| Sample GC2                | 65,987                  | 45,353                              | 43,327                             | 4                                  | 20,596                                  |
| <i>Bacillus</i> treatment |                         |                                     |                                    |                                    |                                         |
| Sample GB1                | 57,419                  | 40,742                              | 39,405                             | 2                                  | 21,206                                  |
| Sample GB2                | 60,071                  | 44,875                              | 42,081                             | 5                                  | 23,508                                  |

Bacterial community diversity was measured by both the Shannon index and InvSimpson. There were no significant differences in averages between the control and the *Bacillus* treatment for Sobs (observed species richness), Chao1 (community richness), Good's coverage (%) and InvSimpson ( $P > 0.05$ ). However, the addition of *Bacillus* significantly decreased the average values of the Shannon index ( $P < 0.05$ ; Table 6.4). All gut samples had a Good's coverage of  $\geq 98\%$  indicating that the sequencing depth was sufficient (Table 6.4).

**Table 6.4:** Coverage and alpha diversity of bacterial communities in the gut of Mozambique tilapia from the control and *Bacillus*-treated aquaponic systems. Mean values are followed by range values given in parentheses. Significant differences between *Bacillus* treatment and control ( $P < 0.05$ ) are marked by an asterisk.

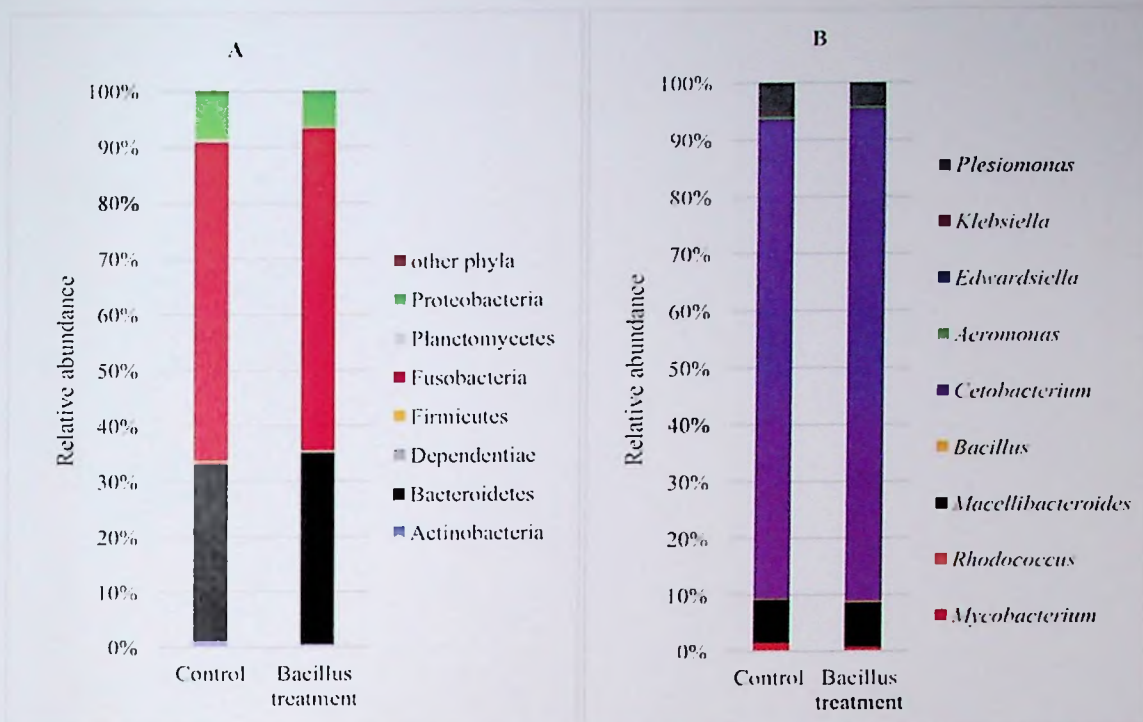
| Coverage and indices | Treatment                 |                             | P-value |
|----------------------|---------------------------|-----------------------------|---------|
|                      | Control                   | <i>Bacillus</i>             |         |
| InvSimpson           | 4.86<br>(4.48 – 5.24)     | 4.40<br>(4.0 – 4.80)        | 0.499   |
| Sobs                 | 334<br>(279 – 389)        | 273.5<br>(246 – 301)        | 0.429   |
| Good's coverage (%)  | 99.07<br>(98.9 – 99.28)   | 99.22<br>(99.13 – 99.31)    | 0.490   |
| Chao1                | 809.05<br>(697.7 – 920.4) | 703.51<br>(586.34 – 820.68) | 0.581   |
| Shannon              | 2.28<br>(2.25 – 2.31)     | 2.08<br>(2.05 – 2.11)       | 0.043*  |

### 6.3.4 Taxonomic composition of microbial community in the fish gut

The classified sequences were associated with 13 phyla, of which seven had an average relative abundance of more than 0.2% (Figure 6.3A). At the phylum level, the gut microbiota was dominated by Fusobacteria, followed by Bacteroidetes, and Proteobacteria in both the control and the *Bacillus*-treated systems. Irrespective of the treatment, Fusobacteria accounted for more than 55% of the total bacterial community, with relative abundance of 57.0% from the control, and 57.8% from the fish in the *Bacillus*-treated systems. Phylum Bacteroidetes was more abundant in the gastrointestinal samples obtained from the *Bacillus*-treated systems (34.41%) than the control systems (31.83%) (Figure 6.3A). The average relative abundance of Proteobacteria was higher in fish from the control systems (8.40%) than the *Bacillus*-treated samples (6.42%). A small fraction (0.01 – 0.04 %) of the total sequences for the samples could not be classified into any known phyla.

At the genus level, *Cetobacterium* constituted more than 50% of the total bacterial community in fish from the control (56.91%) and *Bacillus*-treated systems (57.72%). In contrast, a small percentage of the genus *Bacillus* (0.12% of the total community) was found in both the gastrointestinal tract of control fish and fish from the *Bacillus* treatment. Samples were also dominated by *Macellibacteroides*, which constituted 5.14% of the total fish bacterial community from the control systems and 5.26% from the *Bacillus*-treated systems.

Among the known pathogenic bacteria, *Plesiomonas* accounted for 3.61% of the total bacterial community from the fish in the control systems and 2.51% from the *Bacillus*-treated fish (Figure 6.3B). Furthermore, only small percentages of *Aeromonas* (0.30% in fish from the control systems and 0.27% in fish from the *Bacillus*-treated systems), *Klebsiella* (0.25%, from the control systems and 0.14% from *Bacillus*-treated system) and *Edwardsiella* (0.18% from the control systems and 0.12% from *Bacillus*-treated systems, Figure 6.3B) were recorded.



**Figure 6.3:** (A): Mean relative abundances of taxa (at the phylum level) of gut microbiota of *O. mossambicus* obtained from *Bacillus*-treated and control systems. Phyla that represented less than 0.2% of the total reads are labelled "other phyla", which represent the phyla Chloroflexi, Chlamydiae, Cyanobacteria, Verrucomicrobia, Patescibacteria and Spirochaetes. (B): Average relative abundance of bacterial genera. Only genera representing more than 0.1% of the total reads are shown.

The in-depth bacterial composition analysis detected 50 abundant OTUs (Figure 6.4). The 50 dominant OTUs were assigned mainly to the families *Fusobacteriaceae*, *Bacteroidaceae*, and *Enterobacteriaceae* (Figure 6.4). Through BLAST search (Table 6.5), the most abundant OTU overall was OTU1 assigned to genus *Cetobacterium* (Figure 6.4). All the thirteen OTUs (OTUs 1, 3, 4, 10, 12, 17, 19, 23, 25, 37, 38, 45, and 46) observed in fish gut from both the control and *Bacillus*-treated systems shared high sequence similarity with the bacterium *Cetobacterium somerae* (Table 6.5). Four OTUs (OTUs 6, 21, 40, and 48) assigned to the genus *Plesiomonas* were closely related to *Plesiomonas shigelloides*. Sequences assigned to *Klebsiella* (OTU13) and *Aeromonas* (OTU22), were closely related to *Klebsiella pneumoniae* and *Aeromonas diversa*, respectively (Table 6.5). OUT28 assigned to the genus *Edwardsiella* was closely related to *E. anguillarum* (Table 6.5).



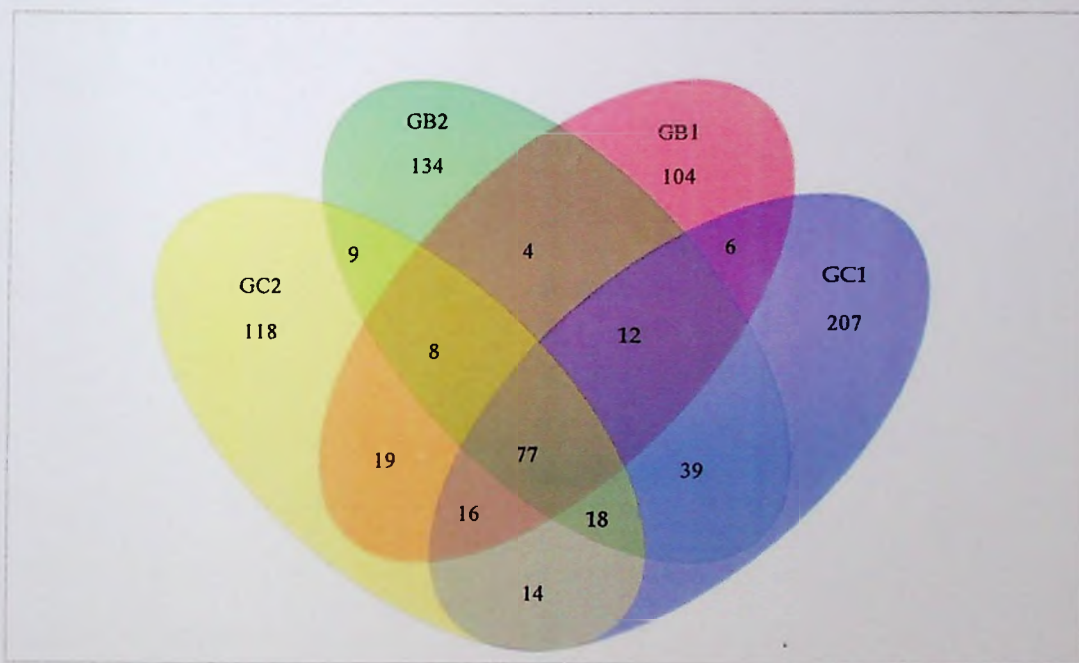
**Figure 6.4:** A heatmap showing the distribution of the top 50 dominant OTUs based on the abundance of 16S rRNA gene sequences from gut samples of *O. mossambicus* cultured in the control and *Bacillus*-treated systems of coupled aquaponics. Each column in the heatmap represents a sample (GC1 = control 1; GC2 = Control 2; GB1 = *Bacillus* treatment 1; GB2 = *Bacillus* treatment 2) and each row represents an OTU. Taxonomy (right column) indicates the phylum, order, family, and genus of each OTU. The colour code refers to sequence abundance, with high abundances (red colours) and low abundances (blue colours).

**Table 6.5:** The 50 dominant bacterial OTUs and their closest relatives from gut samples obtained from *O. mossambicus* cultured in the control and *Bacillus*-treated systems of coupled aquaponics.

| Bacterial OTUs | Related species (NCBI-Blast)                       | Similarity (%) | Accession   |
|----------------|----------------------------------------------------|----------------|-------------|
| OTU1           | <i>Cetobacterium somerae</i> strain WAL 14325      | 98.05          | NR_025533.1 |
| OTU2           | <i>Bacteroides stercoris</i> ATCC 43183            | 92.01          | NR_027196.1 |
| OTU3           | <i>Cetobacterium somerae</i> strain WAL 14325      | 99.33          | NR_025533.1 |
| OTU4           | <i>Cetobacterium somerae</i> strain WAL 14325      | 98.28          | NR_025533.1 |
| OTU5           | <i>Macellibacteroides fermentans</i> strain LIND7H | 94.61          | NR_117913.1 |
| OTU6           | <i>Plesiomonas shigelloides</i> strain NCIMB 9242  | 98.71          | NR_044827.1 |
| OTU7           | <i>Macellibacteroides fermentans</i> strain LIND7H | 93.52          | NR_117913.1 |
| OTU8           | <i>Pseudaeromonas sharmiana</i> strain GPTSA-6     | 98.71          | NR_043470.1 |
| OTU9           | <i>Citrobacter freundii</i> strain JCM 1657        | 98.28          | NR_113340.1 |
| OTU10          | <i>Cetobacterium somerae</i> strain WAL 14325      | 98.05          | NR_025533.1 |
| OTU11          | <i>Bacteroides stercoris</i> ATCC 43183            | 92.46          | NR_027196.1 |
| OTU12          | <i>Cetobacterium somerae</i> strain WAL 14325      | 97.84          | NR_025533.1 |
| OTU13          | <i>Klebsiella pneumoniae</i> strain ATCC 13883     | 98.71          | NR_119278.1 |
| OTU14          | <i>Bacteroides stercoris</i> ATCC 43183            | 91.14          | NR_027196.1 |
| OTU15          | <i>Mycobacteroides abscessus</i>                   | 98.72          | NR_074427.1 |
| OTU16          | <i>Bacteroides stercoris</i> ATCC 43183            | 91.58          | NR_027196.1 |
| OTU17          | <i>Cetobacterium somerae</i> strain WAL 14325      | 97.40          | NR_025533.1 |
| OTU18          | <i>Fusobacterium mortiferum</i> strain DSM 19809   | 96.10          | NR_117734.1 |
| OTU19          | <i>Cetobacterium somerae</i> strain WAL 14325      | 98.92          | NR_025533.1 |
| OTU20          | <i>Macellibacteroides fermentans</i> strain LIND7H | 93.52          | NR_117913.1 |
| OTU21          | <i>Plesiomonas shigelloides</i> strain NCIMB 9242  | 98.07          | NR_044827.1 |
| OTU22          | <i>Aeromonas diversa</i> strain 2478-85            | 98.50          | NR_117303.1 |
| OTU23          | <i>Cetobacterium somerae</i> strain WAL 14325      | 98.27          | NR_025533.1 |
| OTU24          | <i>Bacteroides rodentium</i> JCM 16496             | 92.01          | NR_113072.1 |
| OTU25          | <i>Cetobacterium somerae</i> strain WAL 14325      | 97.40          | NR_025533.1 |
| OTU26          | <i>Rhodococcus degradans</i> strain CCM 4446       | 99.14          | NR_145886.1 |
| OTU27          | <i>Edwardsiella anguillarum</i> strain ET080813    | 98.50          | NR_136429.1 |
| OTU28          | <i>Bacteroides rodentium</i> JCM 16496             | 92.01          | NR_113072.1 |
| OTU29          | <i>Fimbriiglobus ruber</i> strain SP5              | 87.98          | NR_148575.1 |
| OTU30          | <i>Entomomonas moraniae</i> strain QZS0            | 88.89          | NR_170488.1 |
| OTU31          | <i>Pseudaeromonas sharmiana</i> strain GPTSA-6     | 98.71          | NR_043470.1 |
| OTU32          | <i>Shigella sonnei</i> strain CECT 4887            | 98.28          | NR_104826.1 |
| OTU33          | <i>Dyella solisilvae</i> strain DHG54              | 83.54          | NR_171454.1 |
| OTU34          | <i>Crenobacter luteus</i> strain YIM 78141         | 95.05          | NR_163602.1 |
| OTU35          | <i>Bacteroides rodentium</i> JCM 16496             | 92.22          | NR_113072.1 |
| OTU36          | <i>Bacillus pumilus</i> strain ATCC 7061           | 98.50          | NR_043242.1 |
| OTU37          | <i>Cetobacterium somerae</i> strain WAL 14325      | 98.05          | NR_025533.1 |
| OTU38          | <i>Cetobacterium somerae</i> strain WAL 14325      | 98.05          | NR_025533.1 |
| OTU39          | <i>Parachlamydia acanthamoebae</i> strain Bn9      | 88.70          | NR_026357.1 |
| OTU40          | <i>Plesiomonas shigelloides</i> strain NCIMB 9242  | 97.21          | NR_044827.1 |
| OTU41          | <i>Bacteroides stercoris</i> ATCC 43183            | 90.73          | NR_027196.1 |
| OTU42          | <i>Grimontia hollisae</i> strain 75-80             | 96.78          | NR_025574.1 |
| OTU43          | <i>Legionella tunisiensis</i> strain LegM          | 83.86          | NR_109416.1 |
| OTU44          | <i>Macellibacteroides fermentans</i> strain LIND7H | 92.24          | NR_117913.1 |
| OTU45          | <i>Cetobacterium somerae</i> strain WAL 14325      | 98.27          | NR_025533.1 |
| OTU46          | <i>Cetobacterium somerae</i> strain WAL 14325      | 97.62          | NR_025533.1 |
| OTU47          | <i>Mycobacterium alsense</i> strain TB 1906        | 99.15          | NR_147779.2 |
| OTU48          | <i>Plesiomonas shigelloides</i> strain NCIMB 9242  | 97.64          | NR_044827.1 |
| OTU49          | <i>Bacteroides stercoris</i> ATCC 43183            | 91.79          | NR_027196.1 |
| OTU50          | <i>Paraclostridium benzoelyticum</i> strain JC272  | 98.06          | NR_148815.1 |

### 6.3.5 Shared and unique operational taxonomic units

The Venn diagram showed that the samples of *O. mossambicus* from both the control and *Bacillus*-treated systems shared relatively high numbers of OTUs, while each had unique OTUs, irrespective of the treatment (Figure 6.5). A total of 246 and 301 OTUs were found in gut samples of fish from the *Bacillus* treatment, GB1 and GB2 respectively, while 389 and 279 were found in the control fish, GC1 and GC2 (Figure 6.5). Fish gut samples from the control systems had a greater number of unique OTUs (207 and 118) than those in fish from the *Bacillus* treatment (104 and 134).



**Figure 6.5:** A Venn diagram showing shared and unique OTUs among gut bacterial communities of *O. mossambicus* cultured in *Bacillus*-treated and control systems. GB1 and GB2 represent gut samples obtained from systems supplemented with *Bacillus* probiotic and GC1 and GC2 represent gut samples from the control systems.

## 6.4 Discussion

The addition of the probiotic had no effect on average pH, TAN, DO, TDS, EC and  $\text{NO}_2^-$ , which were all kept at acceptable levels for tilapia culture (Reyes-Yanes et al., 2020). The concentration of  $\text{NH}_3$  was lower in the *Bacillus* treatment than in the control. As  $\text{NH}_3$  is more toxic to fish than  $\text{NH}_4^+$ , it is hypothesised that the addition of *Bacillus* improved this important aspect of water quality. In both treatments, the concentration of  $\text{NO}_2^-$  was below  $1 \text{ mg L}^{-1}$ , a concentration at which tilapia can be reared without negative effects on their health (Somerville et al., 2014). TDS and EC values increased in a similar way over time in both systems indicating the presence of ions released from both the fish feed and from the mineralisation of accumulated organic matter. For aquaponic systems, the optimum range of EC for fish has been suggested to be  $0.1 - 2.0 \text{ mS cm}^{-1}$  (Reyes-Yanes et al., 2020). In the present study, EC levels ranged from  $0.45 - 1.42 \text{ mS cm}^{-1}$  in the control, and from  $0.47 - 1.34 \text{ mS cm}^{-1}$  for the *Bacillus*-treatment. Zhou et al. (2010) added a mixture of *Bacillus subtilis* B10 and *B. coagulans* B16 to the water of a rearing system for Nile tilapia and did not find a significant effect on water quality, while the results from this study suggest that the concentration of  $\text{NH}_3$  can be reduced using a probiotic mixture of *Bacillus*. The reduction of  $\text{NH}_3$  levels observed in *Bacillus*-treated systems may be due to the ability of *Bacillus* to mineralise nitrogenous wastes through nitrification (Hlondzi et al., 2020).

The addition of the probiotic increased fish growth and feed efficiency values and improved feed conversion ratio. Faster growth may be due to the nutrient profile of the *Bacillus* product and the ability of *Bacillus* to colonise the fish gut resulting in the production of organic acids and the activation of digestive enzymes (Silva, et al., 2015; Elsabagh et al. 2018), a hypothesis that should be tested in future studies. Nayak (2010) reviewed the effects of probiotics on immunity in fish and suggested that both colonisation and the source of the probiotics play a role in increasing growth and enhancing immunity. Addo et al. (2017)

reported that *Bacillus* can suppress pathogenic bacteria in the intestine, thereby increasing disease resistance and growth. *Bacillus* addition increased weight gain in Nile tilapia (Ridha and Azad, 2012; Adeoye et al., 2016; Elsabagh et al., 2018). As these authors did not study the microbiome, results from the present experiment contribute to our understanding of the effects of *Bacillus* added to the water on the microbiome in the intestinal tract of *O. mossambicus* by adding to a growing data base of microbiome data in fish.

In this study, four indices, namely Sobs, Chao1, Shannon, and InvSimpson were used to evaluate the alpha-diversity of the gut bacterial community. Sobs and Chao1 are abundance-based estimators of species richness, while Shannon and InvSimpson are used to describe the species richness and species evenness (Kim et al., 2017). The mean Shannon index was significantly lower in fish gut from the *Bacillus*-treated systems than those from the control systems. Similar observations were reported by Niu et al. (2019) with a multi-strain probiotic supplemented diet (comprising of a mixture of *B. licheniformis*, *B. amyloliquefaciens*, *B. subtilis*, *Lactobacillus brevis*, *L. plantarum*, and *Saccharomyces cerevisiae*) for juvenile olive flounder. The authors reported a significant decrease in Shannon index based-gut bacterial diversity, without affecting the Chao1 index-based species richness. As Shannon diversity places a greater weight on species richness than species evenness (Kim et al., 2017), it is possible that the addition of *Bacillus* species into the water of an aquaponic system is likely to have more impact on the number of species present (richness) than the uniformity of abundance of each species (evenness).

In general, the addition of the probiotic did not have a strong effect on the core gut microbiome of *O. mossambicus* reared in an aquaponic system, as similar ranges of relative abundances were observed in fish from the control and the *Bacillus* treatment. In the present study, regardless of the treatment, OTUs assigned to the phyla Fusobacteria, Bacteroidetes and Proteobacteria formed the core microbiome in the gastrointestinal tract of *O. mossambicus*.

The majority of bacterial OTUs were assigned to the phylum Fusobacteria, representing on average of more than 55% of all sequences. Fusobacteria have been identified as dominant members of the gut microbiota of freshwater fish species (Larsen et al., 2014; Maas et al., 2021). Members of the phylum Fusobacteria that colonise the gut have been reported to synthesise butyrate (Bennett and Eley, 1993) and vitamins (Roeselers et al., 2011), which could have positive effects on fish health. Other abundant phyla in the gut of *O. mossambicus* were assigned to Bacteroidetes and Proteobacteria.

The gastrointestinal microbiota of *O. mossambicus* was dominated by the genus *Cetobacterium* that belongs to the Fusobacteria. This genus has been identified as a component of the microbiota of other freshwater fish species, for example, *O. niloticus* (Tsuchiya et al., 2008) and *Cyprinus carpio* (Van Kessel et al., 2011) and it produces vitamin B<sub>12</sub> (Tsuchiya et al., 2008; Wu et al., 2010). In the present study, all *Cetobacterium* sequences belonged to *Cetobacterium somerae*. This species also has been reported to produce antimicrobial peptides that allow it to inhibit other microbes, thereby occupying most niches in the gastrointestinal tract of fish (Sugita et al. 1996; Larsen et al. 2014; Maas et al., 2021). Thus, the abundance of *C. somerae* observed in the present study could confirm the competitive potential of this taxon (Ghanbari et al., 2015).

Another group of dominant OTUs could be assigned to the genus *Bacteroides* with sequences closely related to *Bacteroides stercoris*. This species is obligatory anaerobic, with a wide range of metabolic capabilities regarding carbohydrate utilisation and anaerobic respiration (Maas et al., 2021). The gastrointestinal tract was also enriched with *Macellibacteroides*. Members of this genus are closely related to *M. fermentans*. This species can decompose cellulose and hemicellulose-derived sugars, providing nutrients for the development and growth of the host (Borsodi et al., 2017; Haygood and Jha, 2018). Saha et al. (2006) isolated and characterised cellulose-degrading bacteria originating from the gut of the

omnivorous *Oreochromis mossambicus* and herbivorous grass carp (*Ctenopharyngodon idella*) using a selective carboxymethyl-cellulose-agar (CMC-agar) medium. OTUs assigned to the genus *Bacillus* contributed to only 0.12% of the total bacterial community in the gut of *O. mossambicus* from the *Bacillus* treatment. This finding illustrates the challenge of successfully colonising the fish gut with a probiotic strain. The persistence of a probiotic in the gut may depend on the developmental state of the animal, fish species, dosage, and the duration of supplementation (Giatsis et al., 2016). Similarly, Giatsis et al. (2016) observed low persistence of *B. subtilis* in the guts of Nile tilapia larvae which were exposed to the probiotic seven days after the probiotic was administered to the aquarium water.

Although members from the family *Enterobacteriaceae* are part of the fish microbiota, some can cause infection in humans (Duarte et al., 2014). Results from the present study showed that the family *Enterobacteriaceae* in the gut of *O. mossambicus* was dominated by the genus *Plesiomonas* with sequences closely related to *P. shigelloides*. This species has been considered to be a 'tropical' or 'subtropical' bacterium due to the number of isolations in the digestive tract of fishes in countries within these areas (Duarte et al., 2014). In the current study, lower abundances of family *Enterobacteriaceae* represented by the genera *Aeromonas*, *Edwardsiella*, and *Klebsiella* were observed in the gut of *O. mossambicus* with sequences closely related to *Aeromonas diversa*, *Edwardsiella anguillarum* and *Klebsiella pneumoniae*, respectively. Although there were low relative abundances of these genera which contain potential fish pathogens, fish appeared to be healthy, and survival was high in both treatments. Thus, it can be assumed that the relative abundance of these genera might have been below the threshold for disease establishment. Detection of virulence factors should be performed in future studies to confirm the pathogenic potential of these species.

Overall, the present findings indicate that some of the predominant genera observed could represent common members of the gut microbiota of tilapia juveniles or fish in general

obtained from aquaponics, suggesting that these taxa are involved in major metabolic functions in the fish gut of this species when kept in an aquaponic system. The study provided information on opportunistic pathogens associated with *O. mossambicus*, which may assist in the early detection of diseases by designing species-specific DNA probes. While the previous chapters focussed on the characterisation of the bacterial communities associated with plant roots, plant leaves, and this chapter focuses on the fish gut, the media bed described in this thesis provides a habitat for bacteria, as well as being a filter system for particulate suspended matter. As the design of a media bed creates micro-ecosystems, Chapter 7 describes the diversity of bacterial communities in the vertical zones of flood-and-drain media beds from experimental aquaponic systems which had been operating for a year. The metabarcoding analysis presented in Chapter 7 also provides insight into the bacterial community of effluent water samples from *Bacillus*-treated and control systems.

## CHAPTER 7

### **Bacterial diversity and community structure associated with media bed zones of a small-scale aquaponic system supplemented with *Bacillus* species**

#### **7.1 Introduction**

Although aquaponic gravel media beds are the habitat of a complex bacterial ecosystem, the current understanding of the effect of probiotics on the bacterial community composition in a gravel media bed of a coupled aquaponic system is limited. Somerville et al. (2014) suggested that microbial communities in media bed zones contribute to the functioning of aquaponics through nitrification, nutrient conversion, regulation of organic matter decomposition, and mineralisation of nutrients. However, factors such as design, period of operation, and configuration of aquaponic systems might affect the distribution of bacterial communities in different zones of media-based aquaponic systems (Palm et al., 2018; Kasozi et al., 2021a).

Media beds are designed to either flood and drain, or to operate as constant-flow systems (Lennard and Leonard, 2005). Flood-and-drain cycles periodically expose the hydroponic media bed to atmospheric air, which can lead to aerobic conditions in parts of the media bed (Lennard and Leonard, 2005). Consequently, there are distinct vertically differentiated zones in the media beds of flood-and-drain systems (Somerville et al., 2014). Flood-and-drain aquaponic systems provide a high surface area habitat for beneficial bacteria, as well as being a filter system for particulate suspended matter. They also provide stability for root growth, and plants with extensive root systems typically adapt well to this environment (Maucieri et al., 2018).

An international survey revealed that 86% of aquaponic farmers used media-based systems, while 35% of respondents used a combination of media bed and a raft growing technique (Love et al., 2014). Mchunu et al. (2018) reported that 96% of survey respondents in

South Africa adopted media-based aquaponics, with the flood-and-drain media bed culture being the most frequently used design. There is a paucity of research on microbial diversity in aquaponic systems, particularly on the interactions between microbiota across vertical zones of the media bed, which can function as micro-ecosystems. The media bed provides a niche for diverse populations of micro-and macro-organisms. Thus, knowledge of bacterial diversity and spatial distribution of microbes in a flood-and-drain media bed will contribute to our understanding of microbial community dynamics particularly when probiotics are applied in aquaponics. This fundamental knowledge should have benefits for applied research that focusses on enhancing water quality and the growth and health of fish and plants.

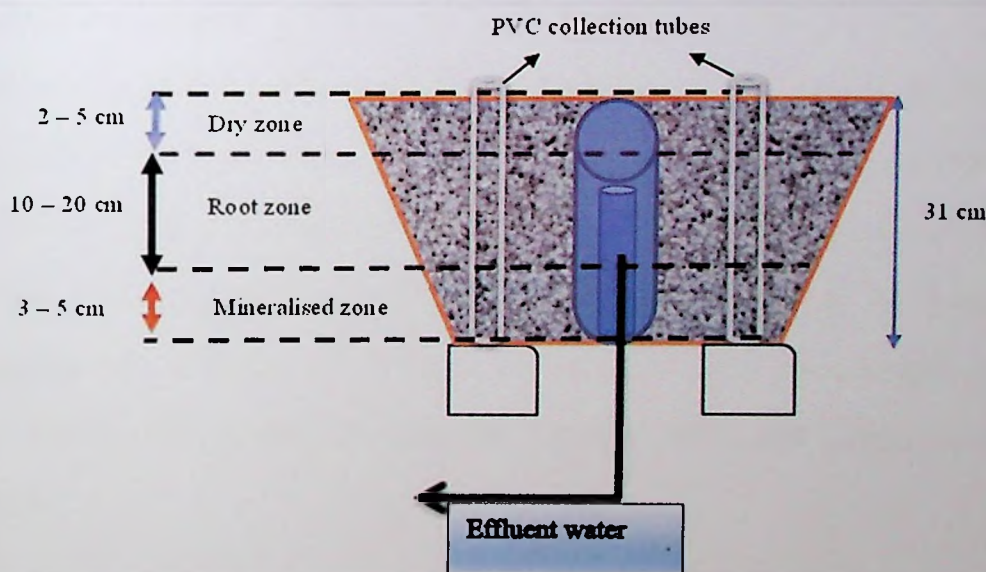
For practical reasons, such as the demand on time and computational and monetary constraints, environmental microbiologists have reported findings related to microbial diversity using limited numbers of experimental units (Lennon, 2011; Myrold et al., 2014). To contribute to the database that has been generated in studies, in which data from one point in time and from one aquaponic system were generated (Schmutz et al., 2017; Eck et al., 2019; Kasozi et al., 2021a). The aim of this study was to characterise and compare the bacterial communities of vertical zones of media bed systems treated with a commercial product (Sanolife®PRO-W;  $5 \times 10^{10}$  CFU g<sup>-1</sup>) containing a mixture of *Bacillus subtilis* and *B. licheniformis* with untreated systems under indoor conditions using Illumina-based 16S rRNA gene sequencing.

## 7.2 Materials and methods

### 7.2.1 Sediment and water sampling

Each media bed of the four aquaponic systems as described in Section 2.2 (Chapter 2) was embedded with two parallel PVC collection tubes of similar sizes and shapes (length 33 cm; internal diameter 50 mm). As indicated in Figure 7.1, these tubes were positioned at the front, and the back position of the media bed. To obtain an insight into the bacterial community

profiles, gravel stone samples were collected from each of the PVC collection tubes. Two tubes containing gravel stones from each media bed were considered for this study, i.e., there were four tubes from each of the control and *Bacillus* treatment. The gravel stone samples for each zone were transferred to separate sterile plastic bags. A total of 500 g each of gravel stones from the top dry zone (2 – 5 cm depth), root zone (10 – 20 cm depth), and the bottom mineralised zone (3 – 5 cm depth) were collected from four media bed systems (two control units and two treatment units) which had been operating for a period of one year. In addition, 500 mL of water was collected in sterile Pyrex bottles from each grow bed through the outlet pipe (Figure 7.1). To obtain a more accurate representation of the overall bacterial population, the samples from each zone were pooled to be treated as one sample for further analyses. Sample collection, processing and DNA extraction was done on the same day.



**Figure 7.1:** A schematic indicating the three zones of flood-and-drain media bed as used in the study.

### 7.2.2 DNA extraction and 16S rRNA amplicon sequencing

The pooled gravel samples for each zone were transferred to a 250 mL sterile beaker filled with 150 mL sterile ultrapure water. After vigorous stirring for 10 min, 100 mL of water

from each beaker was filtered through 0.2  $\mu\text{m}$  filters (Supor® Membrane disc filters, PALL Life Sciences, USA) with a vacuum pump (Rocker Model 801, vacuum pump 167801-22, Taiwan). This procedure was repeated three times for each sample. Water samples were also filtered through 0.2  $\mu\text{m}$  filters with the vacuum pump. The filters corresponding to different samples were transferred to ZR Bashing Bead™ lysis tubes comprising of 0.5 mm beads. DNA was extracted from microbial cells associated with filter materials using a ZymoBIOMICS™ DNA Miniprep Kit, USA, according to the manufacturer's instructions. The PCR conditions, DNA amplification and library preparations were performed following the protocol described in Section 2.7 (Chapter 2). Raw sequencing data were deposited in the Sequence Read Archive (SRA) of NCBI under Accession Number: SRR15880333.

### 7.2.3 Sequencing data analysis

The sequence FASTQ files from the Illumina MiSeq were analysed using the MOTHUR platform version 1.41.3 release (Schloss et al., 2009) following the protocol described in Section 2.8 (Chapter 2). Species identification for the dominant 50 OTUs was performed using the NCBI Basic Local Alignment Search Tool (BLAST). Bar graphs with the dominant taxonomic groups, (i.e., at least 1% of relative abundance within a given sample) were generated to identify the relative abundance of different phyla and genera in each microecosystem.

## 7.3 Results

### 7.3.1 Evaluation of DNA purity and concentration

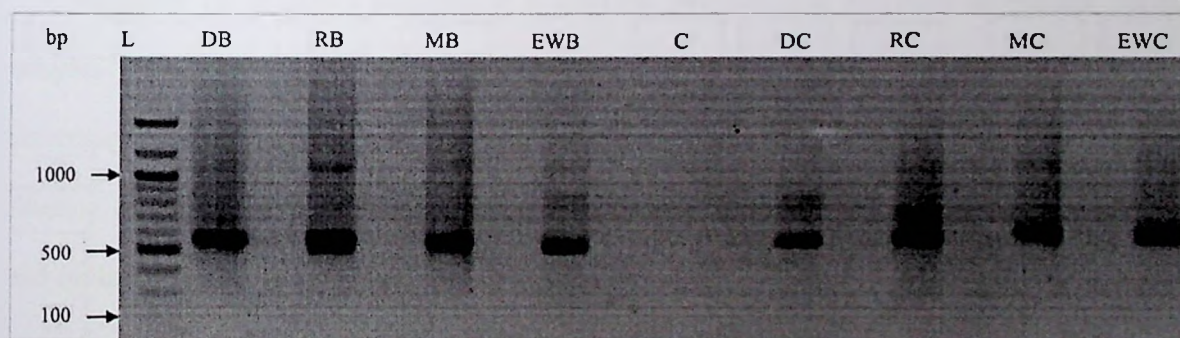
DNA concentration from samples were above 6  $\text{ng } \mu\text{L}^{-1}$ , with the highest concentration of 25.7  $\text{ng } \mu\text{L}^{-1}$  observed in the root zone of the control systems (Table 7.1). The samples from the top zone had the lowest DNA concentration (Table 7.1). The  $A_{260}/A_{280}$  ratio obtained from

samples methods ranged from 1.7 – 2.0 (Table 7.1) implying ‘clean DNA’. As indicated in Figure 7.2, the PCR amplification was sufficient to produce clear bands.

**Table 7.1:** Purity and concentration of the extracted genomic DNA extracted from gravel stone samples and effluent water collected from *Bacillus*-treated and control systems.

| and effluent water collected from <i>Bacillus</i> -treated and control systems. |                                            |                   |                |
|---------------------------------------------------------------------------------|--------------------------------------------|-------------------|----------------|
| Sample                                                                          | <i>Bacillus</i> treatment                  |                   |                |
|                                                                                 | DNA concentration<br>ng $\mu\text{L}^{-1}$ | $A_{260}/A_{280}$ | DNA integrity* |
| Dry zone (DB)                                                                   | 8.5                                        | 1.91              | ++             |
| Root zone (RB)                                                                  | 11.7                                       | 2.06              | ++             |
| Mineralised zone (MB)                                                           | 11.4                                       | 1.82              | ++             |
| Effluent water (EWB)                                                            | 12.0                                       | 1.72              | ++             |
|                                                                                 | Control                                    |                   |                |
| Dry zone (DC)                                                                   | 6.4                                        | 1.82              | +              |
| Root zone (RC)                                                                  | 25.7                                       | 1.90              | ++             |
| Mineralised zone (MC)                                                           | 19.2                                       | 1.76              | ++             |
| Effluent water (EWC)                                                            | 24.4                                       | 1.81              | ++             |

\*DNA integrity was assayed by agarose electrophoresis and indicated as good (+) or sufficient (++) based on the band clarity, density and presence of smearing.



**Figure 7.2:** Agarose gel electrophoresis of the PCR products amplified from the samples collected from different zones of flood-and-drain media bed systems. Lane L shows the 100-bp ladder. Lanes DB, RB, MB, and EWB are the amplification products from dry zone, root zone, mineralised zone, and effluent water for *Bacillus*-treated systems, respectively. Lane DC, RC, MC, and EWC are the amplification products from dry zone, root zone, mineralised zone, and effluent water for untreated systems, respectively. Lane C is the negative control.

### 7.3.2 Taxonomic assignment of reads

High-throughput sequencing of the 16S rRNA gene generated a total of 418,631 raw sequences from eight samples (Table 7.2). The samples from the *Bacillus*-treated systems yielded a total of 208,627, while those from the control systems had 210,004 raw sequences

(Table 7.2). After removing poor-quality reads, a total of 166,970 and 167,462 sequences were obtained from samples collected from the *Bacillus*-treated and control systems, respectively.

**Table 7.2:** Summary of sequencing data for the samples collected from vertical zones and effluent water from *Bacillus*-treated and control systems.

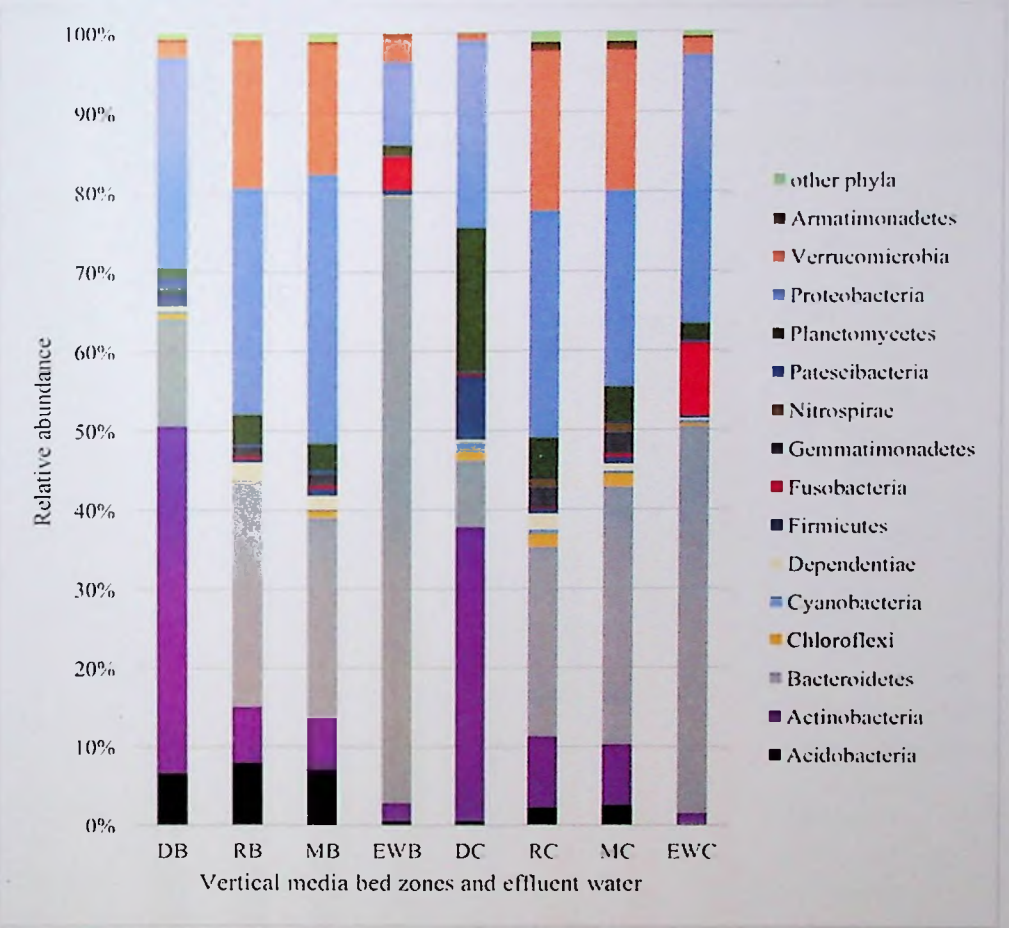
| Sample                    | Number of raw sequences | Number of sequences before chimeras | Number of sequences after chimeras | Chloroplast, mitochondria sequences | Number of sequences after screening and filtering |
|---------------------------|-------------------------|-------------------------------------|------------------------------------|-------------------------------------|---------------------------------------------------|
| <i>Bacillus</i> treatment |                         |                                     |                                    |                                     |                                                   |
| Top zone                  | 54,925                  | 40,635                              | 35,230                             | 100                                 | 20,666                                            |
| Root zone                 | 43,881                  | 36,473                              | 31,455                             | 11                                  | 26,432                                            |
| Mineralised zone          | 59,504                  | 46,676                              | 40,472                             | 185                                 | 25,572                                            |
| Effluent water            | 50,317                  | 43,186                              | 37,388                             | 36                                  | 32,444                                            |
| Control                   |                         |                                     |                                    |                                     |                                                   |
| Top zone                  | 44,162                  | 36,256                              | 31,728                             | 258                                 | 22,122                                            |
| Root zone                 | 46,038                  | 35,277                              | 28,919                             | 28                                  | 19,198                                            |
| Mineralised zone          | 66,030                  | 52,620                              | 43,923                             | 154                                 | 29,050                                            |
| Effluent water            | 53,774                  | 43,309                              | 36,910                             | 90                                  | 27,307                                            |

Removal of chimeras resulted in a total of 144,545 and 141,480 sequences for the samples collected from the *Bacillus*-treated and control systems, respectively. The total percentage of sequences flagged as chimeric was 14.5% for all samples. After screening and filtering, a total of 105,114 and 97,677 sequences were retained for the *Bacillus*-treated samples and control samples, respectively.

### 7.3.3 Predominant phyla and genera

Although the taxonomic composition of the bacterial community was similar between zones and effluent water, the frequency distribution of bacterial phyla differed between the three zones. All sequences were identified into 34 phyla, but only 15 had a relative abundance of more than 1.0% (Figure 7.3). Irrespective of the treatment, the major phylum groups were Bacteroidetes, Proteobacteria, Actinobacteria, and Verrucomicrobia (Figure 7.3). The dry zone samples were mainly composed of Actinobacteria representing 43.72% and 36.94% of the total bacterial community from the *Bacillus*-treated and control systems, respectively (Figure 7.3).

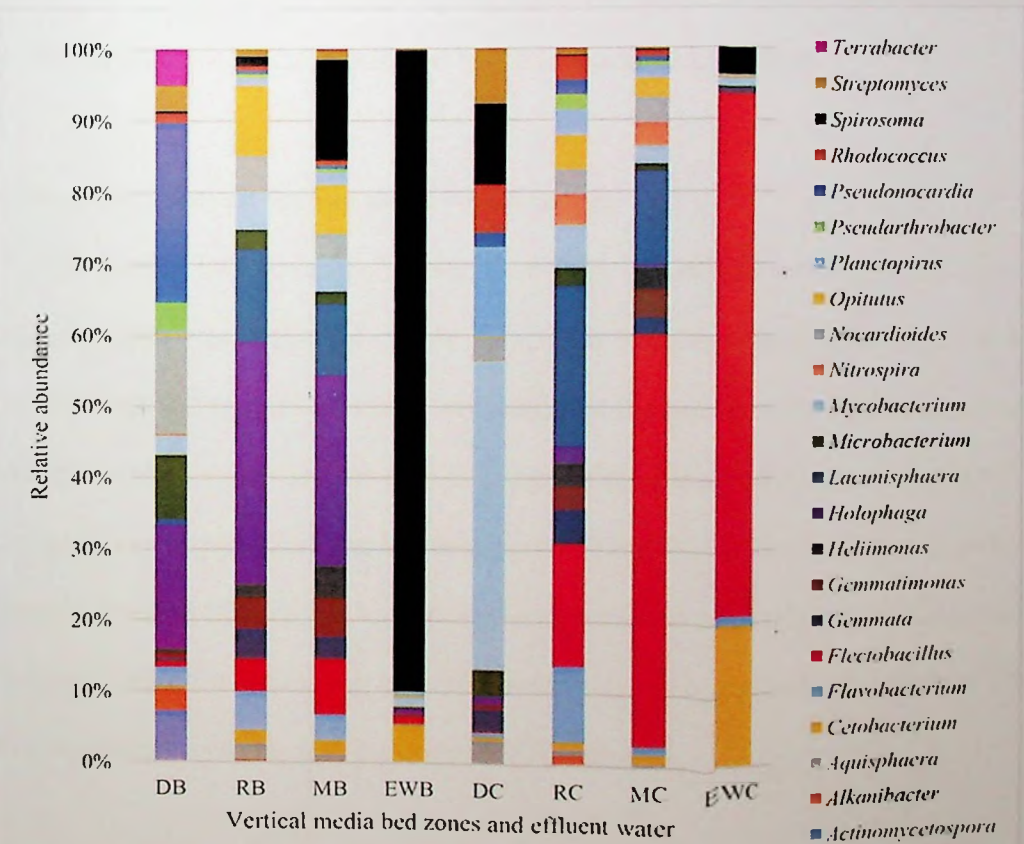
The root zone was mostly comprised of Proteobacteria representing 28.40% and 28.38% of the total bacterial community from *Bacillus*-treated and control systems, respectively. The mineralised zone from the *Bacillus*-treated systems was dominated by the phylum Proteobacteria representing 33.78%, while the control samples from the same zone were dominated by Bacteroidetes (32.44%).



**Figure 7.3:** Classification of reads from dry zone, root zone, mineralised zone, and effluent water from the media bed at phylum level indicated as a percentage of the total bacterial community. Phyla that were represented in all zones below 1.0% of total community are labelled "other phyla", which represent the phyla Hydrogenedentes, Omnitrophicaeota, Chlamydiae, Spirochaetes, Margulisbacteria, candidate division FBP and FCPU426. *Bacillus*-treated systems: DB = Dry zone; RB = Root zone; MB = Mineralised zone; EWB = Effluent water. Control systems: DC = Dry zone; RC = Root zone; MC = Mineralised zone, and EWC = Effluent water.

Effluent water from the *Bacillus*-treated systems was mainly characterised by Bacteroidetes (76.36%), Proteobacteria (10.30%), and Fusobacteria (4.11%). In the control systems, effluent water was dominated by Bacteroidetes (48.81%), Proteobacteria (33.53%) and Fusobacteria (9.14%). Other major phyla, including Verrucomicrobia (18.60%) and Acidobacteria (7.99%), were also identified in root zone samples from the *Bacillus*-treated systems. The phyla Verrucomicrobia and Acidobacteria accounted for 20.0% and 2.28% of the total bacterial community in the root zone of the control samples, respectively (Figure 7.3).

Only 23 genera were dominant with a relative abundance of more than 1% of total bacteria reads (Figure 7.4).



**Figure 7.4:** Distribution of different genera in the three media bed zones and effluent water. Only genera with relative abundance of  $\geq 1\%$  of the total community in at least one of the investigated samples are reported. *Bacillus*-treated systems: DB = Dry zone; RB = Root zone; MB = Mineralised zone; EB = Effluent water. Control systems: DC = Dry zone; RC = Root zone; MC = Mineralised zone, and EW = Effluent water.

The three media bed zones from the *Bacillus*-treated systems were dominated by different genera, including *Pseudonocardia* (9.21%), *Holophaga* (6.38%), *Nocardioides* (5.05%), and *Microbacterium* (3.31%) for the dry zone; *Holophaga* (6.73%), *Lacunisphaera* (2.55%), and *Opitutus* (1.93%) for the root zone; *Holophaga* (5.54%), *Spirosoma* (2.92%), and *Lacunisphaera* (2.07%) for the mineralised zone (Figure 7.3). The effluent water was dominated by *Spirosoma* (71.44%).

In contrast, samples from the control systems were dominated by *Mycobacterium* (18.08%), *Planctopirus* (5.25%), and *Spirosoma* (4.74%) for the dry zone; *Lacunisphaera* (5.06%), *Flectobacillus* (3.87%), and *Flavobacterium* (2.40%) for the root zone; *Flectobacillus* (18.60%), *Lacunisphaera* (4.22%), and *Nocardioides* (1.06%) for the mineralised zone (Figure 7.4). The effluent water was dominated by *Flectobacillus* (33.96%).

#### 7.3.4 Diversity analysis for bacterial communities

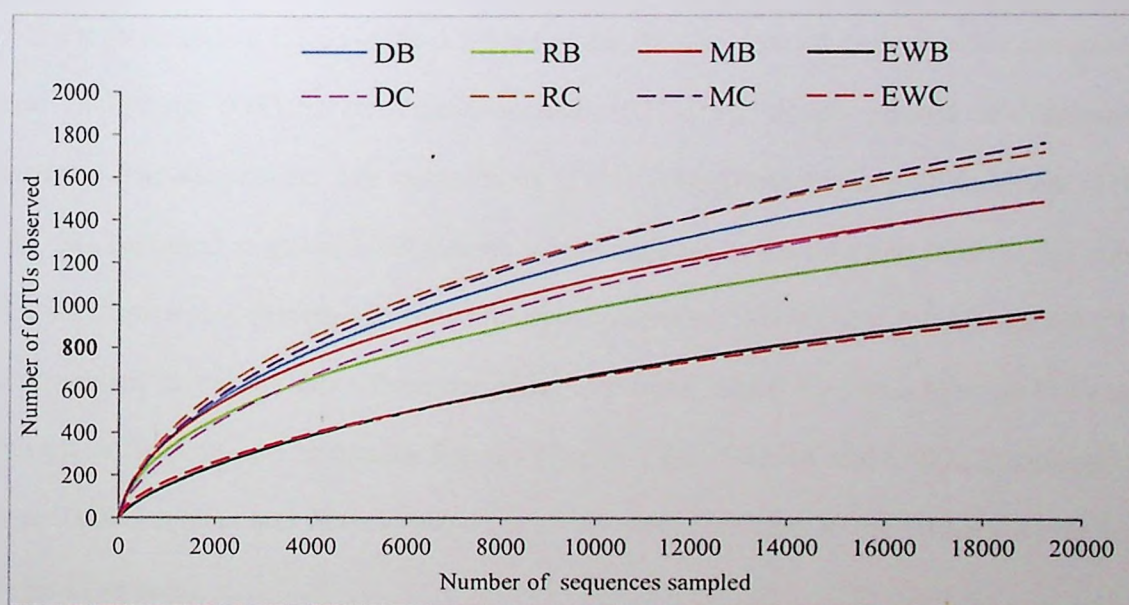
Each sample had coverage greater than 95% of Good's coverage, indicating that the sequencing depth was sufficient (Table 7.3). The Shannon index conducted for estimating species richness and species evenness indicated that the top, root, and mineralised zones from both treatments had values greater than 4.5, suggesting a relatively higher diversity of bacterial sequences than samples from the effluent water (Table 7.3). The community richness (total number of observed OTUs) was highest in the mineralised zone of the control systems. Chao1 analysis conducted for estimating bacterial community richness indicated that the mineralised zone of the control systems had the highest OTU richness.

**Table 7.3:** Richness and diversity indices of bacterial communities for aquaponic samples collected from three media bed zones and effluent water.

| Zone/source      | <i>Bacillus</i> treatment |      |            |         |               |
|------------------|---------------------------|------|------------|---------|---------------|
|                  | Good's coverage (%)       | Sobs | Invsimpson | Chao1   | Shannon index |
| Dry zone         | 96.48                     | 1619 | 65.56      | 2608.02 | 5.59          |
| Root zone        | 97.14                     | 1305 | 71.32      | 2248.59 | 5.42          |
| Mineralised zone | 96.79                     | 1484 | 99.69      | 2402.05 | 5.68          |
| Effluent water   | 97.27                     | 974  | 4.03       | 1922.62 | 2.74          |
| Control          |                           |      |            |         |               |
| Dry zone         | 96.46                     | 1488 | 22.79      | 2451.10 | 4.80          |
| Root zone        | 96.42                     | 1715 | 126.16     | 2680.74 | 5.89          |
| Mineralised zone | 95.84                     | 1758 | 42.75      | 3053.94 | 5.54          |
| Effluent water   | 97.39                     | 946  | 8.88       | 1873.78 | 3.66          |

### 7.3.5 OTU compositional analysis

Rarefaction curves were used to compare species richness in the three zones and effluent water (Figure 7.5).



**Figure 7.5:** Rarefaction analysis of samples from the three vertical zones and effluent water obtained from *Bacillus*-treated and control systems. Rarefaction curves of OTUs clustered at 97% sequence identity among samples. *Bacillus*-treated systems: DB = Dry zone; RB = Root zone; MB = Mineralised zone; EBC = Effluent water. Control systems: DC = Dry zone; RC = Root zone; MC = Mineralised zone, and EWC = Effluent water.

The curves did not approach the asymptote, which indicated that each sample showed highly diversified bacterial communities, and further sequencing would likely have generated

more OTUs in each sample. The curves indicated that OTUs were highest in samples from the mineralised zone, and lowest in the effluent water of the control systems (Figure 7.5).

The most dominant 50 OTUs based on the abundance of 16S rRNA gene sequences are represented in a heat map (Figure 7.6). The 50 most abundant OTUs were assigned mostly to three phyla, Bacteroidetes, Proteobacteria, Actinobacteria, and Verrucomicrobia. Through BLAST search (Table 7.4), the most abundant OTU overall was revealed to be OTU1, assigned to genus *Spirosoma* (Figure 7.6), closely related to *Spirosoma aerophilum* (Table 7.4). *Spirosoma* was also the most abundant OTU from the effluent water of *Bacillus*-treated systems with all the OTUs (1, 3, and 42), closely related to *S. aerophilum*. Sequences closely related to the *Flectobacillus rhizosphaerae* (OTU2) was abundant in effluent water from the control systems (Figure 7.6).

The most abundant OTUs in the dry zone of the *Bacillus*-treated systems were assigned to genera *Holophaga* (OTU5) and *Pseudonocardia* (OTU13), closely related to *Holophaga foetida* and *Pseudonocardia soli*, respectively (Table 7.4). Another group of dominant OTUs (8, 17, 38), assigned to genus *Limisphaera*, were dominant in the root and mineralised zones of the *Bacillus*-treated systems. In contrast, *Mycobacterium* (OTU4), and *Minicystis* (OTU19) were abundant in the dry zone from the control systems, while *Flectobacillus* (OTU6) was dominant in the root and mineralised zones (Figure 7.6). OTU14 and OTU22, assigned to genera *Undibacterium* and *Pelomonas*, respectively were dominant in the effluent water from the control systems.



**Figure 7.6:** A heatmap showing the 50 dominant OTUs from the vertical zones of the grow bed and effluent water based on the abundance of 16S rRNA gene sequences. The colours are proportional to the increasing percent relative OTU abundance (from blue: lower, to red: higher) within each sample. Taxonomy (right column) indicates the phylum, order, family, and genus of each OTU. Each column in the heatmap represents a sample *Bacillus*-treated systems: DB = Dry zone; RB = Root zone; MB = Mineralised zone; EWB = Effluent water. Control systems: DC = Dry zone; RC = Root zone; MC = Mineralised zone; EWC = Effluent water.

**Table 7.4:** The 50 dominant bacterial OTUs and their closest relatives associated with samples collected from the three vertically arranged zones and effluent water of a flood-and-drain media bed system.

| Bacterial OTU | Related species (NCBI-Blast)                             | Similarity (%) | Accession   |
|---------------|----------------------------------------------------------|----------------|-------------|
| OTU1          | <i>Spirosoma aerophilum</i> strain 5516J-17              | 98.69          | NR_149283.1 |
| OTU2          | <i>Flectobacillus rhizosphaerae</i> strain JC289         | 98.49          | NR_137382.1 |
| OTU3          | <i>Spirosoma aerophilum</i> strain 5516J-17              | 97.84          | NR_149283.1 |
| OTU4          | <i>Mycobacteroides immunogenum</i> strain BH29           | 98.93          | NR_104876.1 |
| OTU5          | <i>Holophaga foetida</i> strain TMBS4                    | 94.02          | NR_036891.1 |
| OTU6          | <i>Flectobacillus major</i> strain ATCC 29496            | 93.97          | NR_044736.2 |
| OTU7          | <i>Minicystis rosea</i> strain SBNa008                   | 90.63          | NR_134090.1 |
| OTU8          | <i>Limisphaera ngatamarikiensis</i> strain NGM72.4       | 87.42          | NR_134756.1 |
| OTU9          | <i>Chryseolinea soli</i> strain KIS68-18                 | 91.16          | NR_165708.1 |
| OTU10         | <i>Rhodanobacter spathiphylli</i> B39                    | 99.13          | NR_042434.1 |
| OTU11         | <i>Cetobacterium somerae</i> strain WAL 14325            | 98.27          | NR_025533.1 |
| OTU12         | <i>Undibacterium squillarum</i> strain CMJ-15            | 96.36          | NR_134224.1 |
| OTU13         | <i>Pseudonocardia soli</i> strain NW8-21                 | 98.93          | NR_163618.1 |
| OTU14         | <i>Rhodanobacter spathiphylli</i> B39                    | 98.50          | NR_042434.1 |
| OTU15         | <i>Roseateles depolymerans</i> strain 61A                | 96.99          | NR_111995.1 |
| OTU16         | <i>Limisphaera ngatamarikiensis</i> strain NGM72.4       | 86.30          | NR_134756.1 |
| OTU17         | <i>Limisphaera ngatamarikiensis</i> strain NGM72.4       | 87.47          | NR_134756.1 |
| OTU18         | <i>Bacteroides rodentium</i> JCM 16496                   | 92.22          | NR_113072.1 |
| OTU19         | <i>Polynucleobacter sinensis</i> strain MWH-HuW1         | 98.28          | NR_151920.1 |
| OTU20         | <i>Pelomonas saccharophila</i> strain NBRC 103037        | 98.49          | NR_114189.1 |
| OTU21         | <i>Paucibacter toxinivorans</i> strain 2C20              | 98.06          | NR_042941.1 |
| OTU22         | <i>Chryseolinea soli</i> strain KIS68-18                 | 92.01          | NR_165708.1 |
| OTU23         | <i>Chryseolinea soli</i> strain KIS68-18                 | 91.79          | NR_165708.1 |
| OTU24         | <i>Chryseolinea soli</i> strain KIS68-18                 | 91.58          | NR_165708.1 |
| OTU25         | <i>Lacunisphaera anatis</i> strain IG31                  | 98.50          | NR_146377.1 |
| OTU26         | <i>Mycolicibacter heraklionensis</i> strain NCTC 13432   | 98.72          | NR_117431.1 |
| OTU27         | <i>Limisphaera ngatamarikiensis</i> strain NGM72.4       | 88.46          | NR_134756.1 |
| OTU28         | <i>Microbacterium ginsengiterrae</i> strain DCY37        | 98.50          | NR_116483.1 |
| OTU29         | <i>Flectobacillus rhizosphaerae</i> strain JC28          | 94.83          | NR_137382.1 |
| OTU30         | <i>Piscinibacter aquaticus</i> strain IMCC1728           | 98.28          | NR_043921.1 |
| OTU31         | <i>Nocardioides agrisoli</i> strain djl-8                | 97.23          | NR_158069.1 |
| OTU32         | <i>Cellvibrio zantedeschiae</i> strain TPY-10            | 98.71          | NR_158055.1 |
| OTU33         | <i>Providencia vermicola</i> strain OPI                  | 93.78          | NR_042415.1 |
| OTU34         | <i>Terrimicrobium saccharophilum</i> strain NM-5         | 87.26          | NR_133878.1 |
| OTU35         | <i>Rhodococcus degradans</i> strain CCM 4446             | 99.36          | NR_145886.1 |
| OTU36         | <i>Paeniglutamicibacter kerguelensis</i> strain KGN15    | 98.93          | NR_029027.1 |
| OTU37         | <i>Ohtaekwangia kribbensis</i> strain 10AO               | 87.39          | NR_117436.1 |
| OTU38         | <i>Limisphaera ngatamarikiensis</i> strain NGM72.4       | 89.34          | NR_134756.1 |
| OTU39         | <i>Heliomonas saccharivorans</i> strain L2-4             | 97.20          | NR_135701.1 |
| OTU40         | <i>Ohtaekwangia kribbensis</i> strain 10AO               | 87.53          | NR_117436.1 |
| OTU41         | <i>Cetobacterium somerae</i> strain WAL 14325            | 98.27          | NR_025533.1 |
| OTU42         | <i>Spirosoma aerophilum</i> strain 5516J-17              | 97.62          | NR_149283.1 |
| OTU43         | <i>Mycolicibacterium sediminis</i> strain YIM M13028     | 99.15          | NR_109733.1 |
| OTU44         | <i>Lacunisphaera limnophila</i> strain IG16b             | 98.07          | NR_146349.1 |
| OTU45         | <i>Carboxylicivirga taeanensis</i> strain MEBiC 08903    | 87.37          | NR_133715.1 |
| OTU46         | <i>Opitutus terrae</i> PB90-1                            | 95.29          | NR_074978.1 |
| OTU47         | <i>Flavobacterium columnare</i> NBRC 100251 = ATCC 23463 | 97.83          | NR_113884.1 |
| OTU48         | <i>Streptomyces bryophytorum</i> strain NEAU-HZ10        | 98.29          | NR_146707.1 |
| OTU49         | <i>Chitinimonas koreensis</i> strain R2A43-10            | 95.70          | NR_043677.1 |
| OTU50         | <i>Solitalea koreensis</i> strain R2A36-4                | 86.45          | NR_044568.1 |

## 7.4 Discussion

In this study, Bacteroidetes and Proteobacteria were the most dominant phyla across the grow beds and in the effluent water. Our previous study also demonstrated the dominance of Proteobacteria and Bacteroidetes in an aquaponic gravel media bed of a mature commercial system (Kasozi et al., 2020). These phyla are known to respond rapidly to carbon sources, and are considered to be r-strategists, as they are fast-growing bacteria (Peiffer et al., 2013; Wongkiew et al., 2018) and mostly responsible for nitrogen cycling in aquaponic systems (Schmautz et al., 2017; Wongkiew et al., 2018). In this study, Bacteroidetes constituted 76.36% of the total bacterial community in the effluent water of the *Bacillus*-treated systems. The predominance of Bacteroidetes in *Bacillus*-treated systems may be attributed to their ability to rapidly exploit bio-available organic matter. A high proportion of reads was also assigned to the phylum Actinobacteria (43.72% of the total community) in the dry zone of *Bacillus*-treated systems. This phylum is involved in the decomposition of organic materials (Barka et al., 2016; Lewin et al., 2016) such as cellulose and chitin, thereby playing a crucial role in organic matter turnover and carbon cycling (Zhang et al., 2019; Eisenlord and Zak, 2010).

The top 2 – 5 cm of the media grow bed was defined as the dry zone. This zone functions as a light barrier by preventing light from reaching the nutrient-rich water in the root zone (Kasozi et al., 2020). In this study, the most predominant OTUs in the dry zone from the *Bacillus*-treated systems were members of the genera *Pseudonocardia*, *Holophaga*, and *Nocardioides*, while *Mycobacterium* dominated the control systems. Members of the genus *Pseudonocardia* play an important role in the degradation of polysaccharides (xylan) through the production of xylanase (Wang et al., 2017) and have been reported to be prominent in nutrient-limited environments (Chen et al., 2019). The low moisture content and limited nutrients characterised by the dry zone might have contributed to the dominance of these types of bacterial communities observed in this zone. Similarly, both *Holophaga* and *Nocardioides*

exhibit the capacity to degrade organic matter and are consumers of organic materials (Kielak et al., 2016; Wang et al., 2017). Wang et al. (2017) observed an increase in the abundance of *Nocardioides* in healthy soils, which implies that they are not harmful microorganisms. Consequently, the abundance of *Nocardioides* may suggest that the *Bacillus*-treated systems can be classified as 'healthy'. The dominance of the genus *Mycobacterium* in the control systems (18.08% of the total bacterial community in the control systems compared to 0.97% in the *Bacillus*-treated systems) is of concern, as this group includes opportunistic human pathogens which inhabit a diverse range of environments, including soil and water (Tsao et al., 2019).

The optimal depth for the root zone ranges from 10 to 20 cm (Somerville et al., 2014), and this is where most of the biological activity occurs (Kasozi et al., 2020). Irrespective of the treatment, the samples from the root zone reported in the present study contained high abundances of *Holophaga*, *Flavobacterium*, *Opitutus*, and *Lacunisphaera*. The genus *Holophaga* degrades polysaccharides and plays an active role in plant biomass decomposition (Wang et al., 2017). The genera *Opitutus* and *Lacunisphaera* belong to the phylum Verrucomicrobia, and are reported to degrade complex organic compounds (Rast et al., 2017). Under natural conditions, bacteria of the genus *Flavobacterium* degrade and mineralise organic substrates (such as carbohydrates, amino acids and proteins) (Kolton et al., 2016). Thus, the dominance of these genera in this zone signifies degradation processes of organic matter and mineralisation process in the flood-and-drain media beds.

The mineralised zone with an optimal depth range of 3 to 5 cm is responsible for the slow release of readily available nutrients in the aquaponic systems (Kasozi et al., 2020). In the *Bacillus*-treated systems, *Spirosoma* and *Holophaga* were the most abundant OTUs in the mineralised zone, while in the control systems, members of *Flectobacillus* were dominant. Because members of *Spirosoma* and *Holophaga* are capable of degrading complex organic

materials (Kielak et al., 2016; Wang et al., 2017), their high abundance in the mineralised zone might be helpful in the nutrient conversion and mineralisation of nutrients, vital for the functioning and health of aquaponic systems. *Flectobacillus* is reported to increase the availability of potassium in soils through solubilisation (Yadav and Sidhu, 2016). Potassium levels in aquaponics can be supplemented with potassium hydroxide, while in soil-based agriculture, increasing levels of potassium are lost to crop removal, leaching, runoff, and erosion (Yadav and Sidhu, 2016). The high relative abundance of *Flectobacillus* in the root zone of the control systems warrants further investigation. Similar to those of the root zone, the samples from the mineralised zone from both the *Bacillus*-treated and control systems were dominated with OTUs assigned to *Lacunisphaera* and *Opitutus*. Members of these genera are important players in the mineralisation of organic matter (Rast et al., 2017).

At genus level, in the effluent water samples of *Bacillus*-treated systems, *Spirosoma* was the most dominant with a relative abundance of 71.44% of the total bacterial community compared to 1.68% of the total bacterial community from the control systems. The three *Spirosoma* OTUs had sequences closely related to the bacterium *Spirosoma aerophilum*. Members of the genus *Spirosoma* are considered to be phosphate solubilising bacteria (Li et al., 2017). The presence of *Spirosoma* is important because phosphate is one of main nutrients required for plant growth in aquaponic systems (Kasozzi et al., 2021b). The effluent water samples from the control systems were dominated by *Flectobacillus* (33.96% of the total bacterial community compared to 0.92% from *Bacillus*-treated system). Although this genus is known to inhabit freshwater ecosystems, little is known about its ecology (Meneghine et al., 2017). The reasons for *Flectobacillus* being more abundant in water samples of the control systems than *Bacillus*-treated systems need further investigation.

In summary, this study provided insights into the diversity of the bacterial communities associated with aquaponic gravel media beds when the aquaponic systems are supplemented

with *Bacillus* species. The study provided an overview of the structure and taxonomic composition of bacterial communities and the roles they could play in the zones of a gravel media bed. The study clearly showed that each zone was dominated by a specific bacterial group, which may be linked to the bacterial functions in that niche environment. Results from this study showed that supplementation of a commercial mixture of *Bacillus* species into an aquaponic system resulted in the dominance of *Spirosoma* (phosphate solubilising bacteria) in the effluent water used by plants in the deep-water hydroponic unit. This is particularly vital because phosphorus is one of the limiting nutrients in aquaponics. Based on these results and those of our previous study (Kasozi et al., 2021b), it can be assumed that the gradual increase in phosphate reported in aquaponic systems supplemented with commercial mixture of *B. subtilis* and *B. licheniformis* is attributed mainly to microbial activity rather than to the inherent phosphate contribution of the product.

The final chapter (Chapter 8) gives a synopsis of the major findings over the course of this research. It also discusses the focus areas for future research.

## CHAPTER 8

### General discussion, conclusions, and future perspectives

#### 8.1 General discussion

At the outset of this research, most previous studies had focussed on beneficial microorganisms in soil-based environments. Other studies in RAS focussed on the use of traditional microbiological methods in determining the bacterial composition of fish and water. Therefore, no in-depth studies have been found in relation to the effect of probiotics on plant and fish growth, water quality, and microbial diversity in aquaponic systems. This lack of research currently limits the scope and productivity of such systems. Considering the promotion of novel approaches for sustainable production of food, this research demonstrated that the addition of a commercial mixture of *Bacillus* species had positive effects on plant and fish growth, nutrient levels, and modified the composition of bacterial communities in the aquaponic systems.

The addition of a commercial mixture of *Bacillus* species increased lettuce and fish growth across different trials compared to control systems with no supplementation (Chapters 3, 4, and 5). It is possible that the improved growth of lettuce plants grown in *Bacillus*-treated systems was due to increased concentrations of nitrate and phosphate, as demonstrated by the phosphate and nitrate dynamics analysis. Phosphate is an essential element for plant growth, and plays an important role in photosynthesis, respiration, and the regulation of enzymes in plants (Somerville et al., 2014). Radhakrishnan et al. (2017) reviewed studies in soil-based environments and reported that *Bacillus* spp. convert the complex forms of essential nutrients, such as P and N, to simple available forms that can be used by plants. In addition, significantly higher levels of P, K, and Zn were observed in lettuce leaves when *Bacillus* was added to the water of the aquaponic systems, implying that a commercial mixture of two *Bacillus* species not only enhanced growth of roots and shoots, but also regulated nutrient uptake. Levels of N

and P in the probiotic and in the fish feed, and the efficiency of the mineralisation process by the bacteria may influence the concentrations of nitrate and phosphate in an aquaponic system. The composition of the probiotic indicates that there are nutrients present in the *Bacillus* mixture, such as N and P. Although these nutrients might have been added into *Bacillus*-treated systems by the probiotic hence contributing to the overall P and N budget, we were not able to determine how much of the P and N in the water that resulted from mineralisation from either the feed or the product. Also, the amount of P and N in the sediment and nutrient efficiency by plants were not determined. Thus, the bioavailability of these nutrients is currently poorly understood. Nonetheless, it is possible that the beneficial effects of the *Bacillus* mixture were due to the combination of both the bacterial activity and the inherent nutrient contribution of the product with the former likely to be major source of the positive effects observed in systems treated with the probiotic. This is supported by the observation that the phosphate and nitrate levels did not increase in the same way in different trials.

The addition of the *Bacillus* species not only increased the weight gain of the fish, but also stimulated the activities of amylase, protease, and alkaline phosphatase in the guts of *O. mossambicus* (Chapter 5). These enzymes are responsible for the hydrolysis of the major components of the diet such as carbohydrates and proteins (Adorian et al., 2019). The evaluation of the presence of digestive enzymes and their level of activities, can be used as a relative indicator of food acceptance (Ridha and Azad, 2012). The improved fish growth observed in this study might be due to the ability of *Bacillus* to colonise the fish gut resulting into activation of digestive enzymes. Probiotic bacteria can also exert their beneficial effects in fish through upregulation of growth-related genes. For example, El-Kady et al. (2021) observed that a commercial probiotic (containing a mixture of *Bacillus*, *Pediococcus*, and *Enterococcus*) when administrated as a water additive in open concrete ponds, showed significantly higher expression levels of growth-regulated genes (TNF- $\alpha$  and IGF-I) in the

intestine, liver, and spleen of Nile tilapia than in from the control. Thus, the enhanced growth of *O. mossambicus* observed in the *Bacillus*-treated systems may be attributed to growth regulation and improved nutrient digestibility. Furthermore, supplementation of *Bacillus* did not affect the average values of pH, TAN, and DO, which were all at acceptable levels for the rearing of *O. mossambicus* (El-Sayed, 2019).

Another key dimension of this research was the application of NGS of 16S rRNA gene amplicons to investigate the microbial consortium in aquaponics. The findings from this research indicate that the microenvironments of aquaponic systems harbour distinct bacterial communities. Although Proteobacteria was the most dominant bacterial phylum associated with plant root and leaf samples, comparative 16S rRNA gene sequence analysis showed that addition of *Bacillus* species significantly increased species richness and species evenness of the plant roots as compared to the leaves. The alpha diversity indices showed that root associated bacterial communities were significantly more diverse than those associated with leaves when a probiotic was added to the aquaponic water. Radhakrishnan et al. (2017) reported that *Bacillus* spp. associate with roots and develop biofilms that stimulate root exudation thereby modulating different bacterial communities. Other than supporting microbial proliferation, the root-derived exudates are also responsible for the formation of distinct microbial assemblages (Dong et al., 2019). Thus, the highest bacterial diversity associated with the root samples in this study might be attributed to the primary site of interaction between plants and nutrient-rich water used in aquaponic systems. Additionally, the Good's coverage, which is an index of gene sequencing depth, was higher in the root samples (average range of 95.16% to 95.60%) than in the leaf samples (average range of 88.9% to 90%), suggesting that the lettuce leaf surfaces may be inhabited by distinct microbiota that might reduce bacterial gene sequencing depth (Bringel and Couée, 2015; Stone et al., 2018). In comparison to the *Bacillus*-treated systems, the leaf surfaces of lettuce from the control systems had higher

relative abundances of food-borne pathogens belonging to genera *Shigella* and *Aeromonas*, suggesting that application of the probiotic product may decrease pathogen colonisation on leaf surfaces.

This research revealed that members from the *Enterobacteriaceae* family were part of the *O. mossambicus* gut microbiota (Chapter 6). Results from this research showed that the family *Enterobacteriaceae* was mostly represented by *Plesiomonas shigelloides*. Other OTUs assigned to *Aeromonas diversa*, *Edwardsiella anguillarum*, and *Klebsiella pneumoniae* were also observed at low abundances. Although there were low relative abundances of these genera, which contain potential fish pathogens, fish appeared to be healthy, and survival was high in both the *Bacillus*-treated and control systems (Chapter 6). It is assumed that relative abundances of these genera were below the threshold value for disease establishment.

Regarding the bacterial community of different zones of a flood-and-drain media bed system (Chapter 7), the research demonstrated that gravel media beds are the habitat of a complex bacterial ecosystem. The vertical zones in a flood-and-drain media bed of a coupled aquaponic system influenced the bacterial community structure. Specifically, the samples from the dry, root, and mineralised zones harboured more diverse bacterial communities than effluent water samples. The research highlighted that each zone was dominated by a specific bacterial group which may be linked to the bacterial functions in that niche environment. Effluent water samples from the *Bacillus*-treated systems were dominated by the genus *Spirosoma*. Because phosphorus is one of the major essential macronutrients for plant growth and development (Hayat, 2016), the dominance of *Spirosoma* is therefore important in *Bacillus*-treated systems.

In summary, this research has improved our understanding towards the three overarching questions: 1) 'Who' is present in aquaponics? 2) 'What' could bacteria be doing? 3) 'What' parameters does *Bacillus* influence in aquaponics?

## 8.2 General conclusion and future perspectives

This research has demonstrated that addition of a commercial mixture of two *Bacillus* species into aquaponic systems contributed to bioavailability of phosphate and nitrates and the modulation of water quality parameters, which are key processes in enhancing the productivity of aquaponic systems. The commercial product used in this research contains mainly soybean meal as an organic filler and calcium carbonate as anti-caking agent. Because these fillers may contribute to the inherent nutrient contribution of the product, we recommend that in future studies when applying a probiotic mixture into aquaponic systems, researchers should perform an additional control treatment. For instance, using a control with the fillers but with no *Bacillus*. This control could be made up using the appropriate ingredients, or through using inactivated probiotic. Thus, more controls should be included in future studies to verify whether the observed trend of nitrate and phosphate levels and other water quality parameters in the *Bacillus*-treated systems are maintained. This is important as the focus has moved from traditional small-scale aquaponics to large commercial-scale farming applications where nutrient management is key.

From this research, it can be concluded that the application of NGS metabarcoding based on 16S rRNA gene amplicons holds great promise in aquaponics microbiology. As part of this research, this technique has provided answers to long-standing questions such as what dominant bacterial species colonise root and leaf surfaces, fish gut, the media gravel bed and effluent water in an aquaponic system, and how are these different bacteria are contributing to the ecosystem. Although 16S rRNA gene sequencing was useful in regard to bacterial classification, it was observed that this method had limitations related to low phylogenetic power at the species level, and poor discriminatory power for some genera. Further research is required to expand current findings in this research area, by combining multiple 'omics' technologies such as metagenomics and metatranscriptomics to study the diversity of

microbiota in aquaponics. Further studies such as these, will enable the identification of gene transcripts, proteins, or metabolites, providing a more detailed insight into the genes and functions expressed in the aquaponics microbiome.

Based on the findings from this research, there seems to be a potential risk of pathogen contamination of plant surfaces in aquaponic systems. However, further investigation is needed to examine whether the human food-borne pathogens can internalise aquaponically-grown fresh fruit and vegetables. Addressing this knowledge gap will allow for the development of suitable approaches for surface decontamination and sanitation procedures. To keep plant pathogens in check, it will also be beneficial to apply high-throughput pyrosequencing techniques to understand the fungal community structure and function on plants surfaces.

To conclude, this research has demonstrated that the use of *Bacillus* as a probiotic is beneficial in aquaponic systems. If the application of probiotics is adopted and upscaled among aquaponics practitioners, sustainable production of plants and fish is likely to be improved.

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## Appendices

### Appendix 2.1 Composition of Sanolife® PRO-W

| Composition                                          | Content                                  |
|------------------------------------------------------|------------------------------------------|
| Proximate composition                                |                                          |
| Crude protein                                        | 48.0%                                    |
| Crude fat                                            | 1.0%                                     |
| Crude ash                                            | 15.3%                                    |
| Crude fibre                                          | 3.5%                                     |
| Dry matter                                           | 95.0%                                    |
| Major ingredients                                    |                                          |
| Calcium carbonate                                    | < 8.0%                                   |
| Soybean meal                                         | 85.0%                                    |
| <i>Bacillus subtilis</i> and <i>B. licheniformis</i> | $5.0 \times 10^{10}$ CFU g <sup>-1</sup> |

(Source: INVE Aquaculture, Belgium)

The strains of *Bacillus subtilis* and *B. licheniformis* in Sanolife – PRO-W® are essential for improved fish growth and digestive enzymatic activity, and maintenance of water quality. The nutritional profile of Sanolife® PRO-W indicates that crude protein and crude ash accounts for 48% and 15.3%, respectively while soybean meal forms a major part of the probiotic mixture. Basing on the information provided by INVE Aquaculture, there is a possibility that Sanolife® PRO-W contributed to the total N and P in the aquaponic systems. However, we don't know how much of the N and P in the probiotic mixture contributed to growth of plants, what was utilised by bacteria, or freely suspended or accumulated in the solids.