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Comparison of Two Commercial Recirculated Aquacultural Systems and Their Microbial Potential in Plant Disease Suppression

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Abstract

Background: Aquaponics are food production systems advocated for food security and health. Their sustainability from a nutritional and plant health perspective is, however, a significant challenge. Recirculated aquaculture systems (RAS) form a major part of aquaponic systems, but knowledge about their potential to benefit plant growth and plant health is limited. The current study tested if the diversity and function of microbial communities in two commercial RAS were specific to the fish species used (Tilapia or Clarias) and sampling site (fish tanks and wastewaters), and whether they confer benefits to plants and have *invitro* antagonistic potential towards plant pathogens.

Results: Microbial diversity and composition was found to be dependent on fish species and sample site. The Tilapia RAS hosted higher bacterial diversity than the Clarias RAS; but the latter hosted higher fungal diversity. Both Tilapia and Clarias RAS hosted bacterial and fungal communities that promoted plant growth, inhibited plant pathogens and encouraged biodegradation. The production of extracellular enzymes, related to nutrient availability and pathogen control, by bacterial strains isolated from the Tilapia and Clarias systems, makes them a promising tool in aquaponics and in their system design.

Conclusions: This study explored the microbial potential of the commercial RAS with either Tilapia or Clarias as a tool to benefit the aquaponic system with respect to plant growth promotion and control of plant diseases.

Keywords: Aquaponics, Tilapia, Clarias, Bacteroidetes, Proteobacteria, Actinobacteria, extracellular enzymes, *Pseudomonas flourescens*, *Pseudomonas veronii*, plant growth promotion, *In vitro* antagonistic, Plant pathogens

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Background

Largescale challenges such as climate change, increased world population, limited availability of natural resources, and the spread of pandemics pose risks to food security [1]. Based on the United Nations sustainable development goals, the need to shift to sustainable food production systems to face these challenges is of great importance [2, 3]. Aquaculture is one of the largest food industries worldwide with respect to the production of animal proteins. The future expansion of this industry will focus primarily on land-based recirculated aquaculture systems (RAS). The environmental profiles of RAS are characterized by their ability to reduce water consumption, allow for better control of rearing conditions, and to reduce significantly the release of nutrients (organic matter, nitrogen and phosphorous) into lakes, rivers and the sea [4]. However, the accumulation of nitrates harmful to fish and the environment in RAS remains a challenge [5].

Integrating RAS and hydroponic systems to build so-called aquaponic systems for plant cultivation has been promoted as a solution to deal with the nitrate challenge, since the nitrate content can be reduced by plant uptake. This integration makes the aquaponic system an ideal candidate as a future food production system with a robust environmental profile and the potential to enhance food security. However, the sustainability of aquaponic systems still needs further improvement. Aquaponics are complex systems and we still lack knowledge concerning the ideal plant nutrient balance in relation to the amount and type of fish feed, system design, and plant and fish diseases [6]. These areas are mainly related to conditions in RAS. For example, the lack or low availability of some essential elements in fish water, that are required for plant growth - such as phosphorous and iron - remains a challenge and limits the productive efficiency of aquaponic systems [7]. These nutrients are therefore currently maintained at required levels by the addition of extra phosphorus and iron to the system. In addition, stabilization of the RAS element of the aquaponic system, with respect to water quality parameters such as temperature and pH, is crucial in order to achieve a balance between fish, plant and bacterial requirements [8, 9] and thereby a good plant growth.

From a plant pathology perspective, the aquaponic system is further challenged by a need for harmless and sustainable solutions for controlling levels of fish and plant pathogens [6]. Root diseases caused by fungal and oomycete pathogens such as Pythium spp., Phytophthora spp., Fusarium spp., Verticillium spp., or bacterial pathogens such as Ralstonia and Xanthomonas, are commonly found in aquatic environments such as hydroponic systems and consequently occur in aquaponic systems [10-13]. Control of these pathogens in hydroponic systems by using biological and non-biological tools is widely investigated and has been covered by other literature [14-17]. The ability for naturally occurring microbial communities to suppress pathogens in hydroponic systems has also been considered in different studies [18, 19]. However, the potential suppressive effect of natural microbial communities in aquaponic systems continues to require further research and investigation. This important area of research needs to deal with the complexity of the various components and aspects of the aquaponic system - from water quality and fish feed in the RAS system, through the biofilter component to the hydroponic unit - in and through which disease organisms may enter and grow to excess if not controlled. Restrictions governing pesticides to control plant diseases or antibiotics for the fish diseases emphasize our need to provide solutions that enhance the sustainability of aquaponic systems.

The presence of microbial communities can have a suppressive effect in the system against plant pathogens. For example, through different modes of action including competition, production of secondary metabolites such as antibiotics and extracellular enzymes, induced resistance and growth-promoting effects in plants, microorganisms can have various beneficial roles in

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maintaining a healthy environment [20]. In aquaponic systems, the role of microbial communities in nutrient recycling [21] and promoting plant growth [22] has been confirmed. Other studies have also shown that rhizobacteria inhabiting the root environments of aquaponic systems have antagonistic potential against pathogens such as *Pseudomonas* and *Flavobacterium* [23].

The RAS is a significant unit in the aquaponic system and has received much attention as a microbial habitat and a tool against fish diseases [24]. However, more research into the role of microbes in RAS is needed in order to deepen our understanding of how they might be optimized for plant growth and against plant diseases. Targeting those RAS factors that promote the anti-pathogenic potential and improve plant growth through the action of naturally occurring microbial communities is thus of great concern. With this in mind, the objective of the current study was to investigate microbial diversity in a commercial RAS (with no aquaponic connection) and elucidate its potential to promote plant growth and act against plant pathogens. We examined how fish species and sampling site in the system, namely the water tank compared with the fish biosolids or wastewaters, affected the results. We also investigated the microbial communities' function regarding the production of extracellular enzymes, which are known for their mode of action against plant pathogens as well as towards nutrient solubility. Our hypotheses are: (i) that a RAS (without any aquaponic connection) hosts microbial communities beneficial to plants and antagonistic to pathogens, and that these communities develop in ways that are characteristically specific to fish species used and site sampled in the system; and (ii) that the production of extracellular enzymes and the antagonistic potential to control plant pathogens in vitro are fish species-specific and site-specific.

Results

RAS conditions

Conditions at the sample collection time in the two RAS systems with different fish species were similar regarding water quality parameters such as pH, temperature, conductivity, and ammonium and nitrate content (Table 1). The two fish species differed in their combined weight per tank. Tilapia being lower than Clarias. Both fish species were fed with the same commercial feed (https://www.skretting.com/en/) but with a slight difference in the feed composition and with different daily amount. Tilapia were fed 5 times per day and Clarias 18 times per day. At the sampling occasion, the weight of Tilapia was around 50 kg and 160 kg for the Clarias.

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Table 1. Growth conditions in each recirculated aquaculture system (RAS) populated with either Tilapia or Clarias

Cultivation factors	RAS with Tilapia	RAS with Clarias
Temperature	21 ^{0}C	20.9 ^{0}C
pH	7.7	8.5
Conductivity**	130 mS cm^{-1}	180 mS cm^{-1}
Ammonium content	2.1 mg / liter	1.8 mg / liter
Nitrate content	280 mg / liter	188 mg / liter
Nitrite content	0,59 mg / liter	0,18 mg / liter
Fish weight	40-600g per tank	100-4000g per tank
Feed composition	37% protein + 10% fat	44% protein + 12% fat
Daily amount	5 times	18 times

Microbial amount

Microbial enumeration on selective media indicated more general bacterial flora and Pseudomonas fluorescens in samples collected from the Tilapia RAS system than the Clarias RAS system (Figure 1). With respect to sampling site, we found no significant differences in the amount of general bacterial flora and *P. fluorescens* between the samples collected from water tanks and waste samples in either Tilapia or Clarias RAS. However, there were differences in the amount of general fungal flora. The highest amount of the general fungal flora was found in samples collected from Clarias wastes than in Clarias water tank or any samples from the Tilapia RAS.

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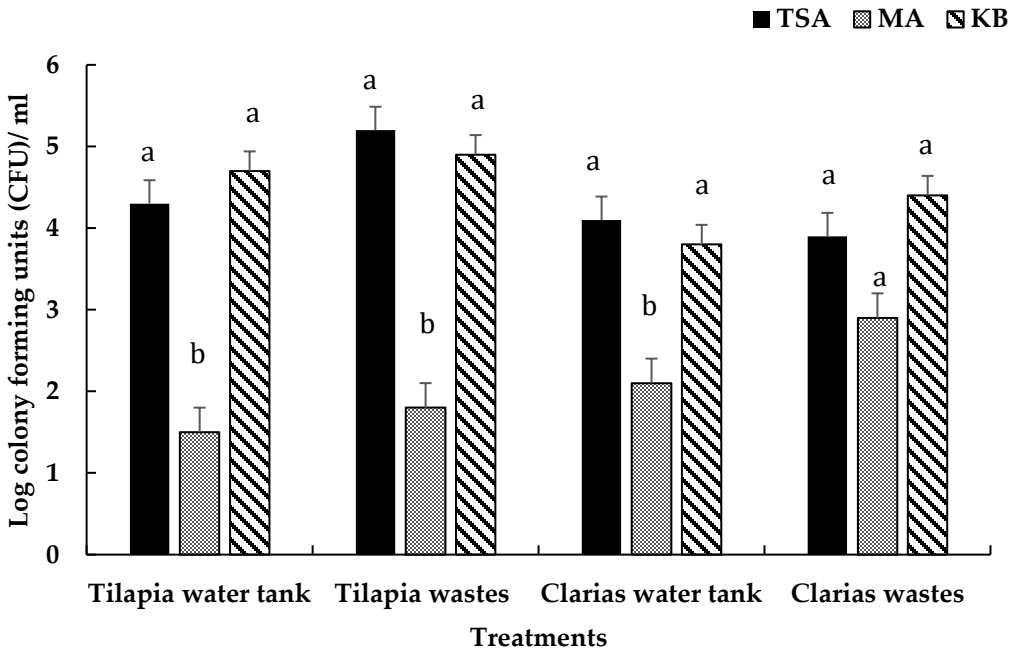

Figure 1. Microbial colonies isolated from water and waste samples from Tilapia and Clarias RAS and enumerated on 0.1% Tryptic soya agar (TSA) complemented with cycloheximide (100 µL mL^{-1}) for enumeration of the general bacterial flora; 0.5% malt extract agar (MA) for enumeration of the general fungal flora; and on King Agar B (KB) with cycloheximide (100 µg mL^{-1}) for enumeration of fluorescent pseudomonads. Letters above the bars indicate the significant differences between the treatments.

Diversity of the microbial communities

A total of 4,558 bacteria OTUs and 405 fungal OTUs were generated after processing the Ilumina MiSeq sequencing data. The total read counts for the bacterial dataset were 647,232 and 530,351 for the fungal dataset. Alpha diversity of bacterial communities was significantly higher in Tilapia water (Shannon, $p = 0.021$) compared to Clarias water samples (Figure 2a). In relation to the Shannon indices, Clarias water tank samples returned the lowest alpha diversity. Furthermore, similarities in alpha diversity were seen between water and waste samples of Tilapia and Clarias and between Tilapia samples and Clarias waste samples. The Chao1 indices also revealed a significant increase (Chao1, $p = 0.012$) in alpha diversity in samples collected from Tilapia wastes compared with Clarias water tank. Clarias water tank showed the lowest alpha diversity with respect to Chao1 indices (Figure 2b). Similarities in the Chao1 indices were also shown within Tilapia samples and between Tilapia samples and Clarias wastes.

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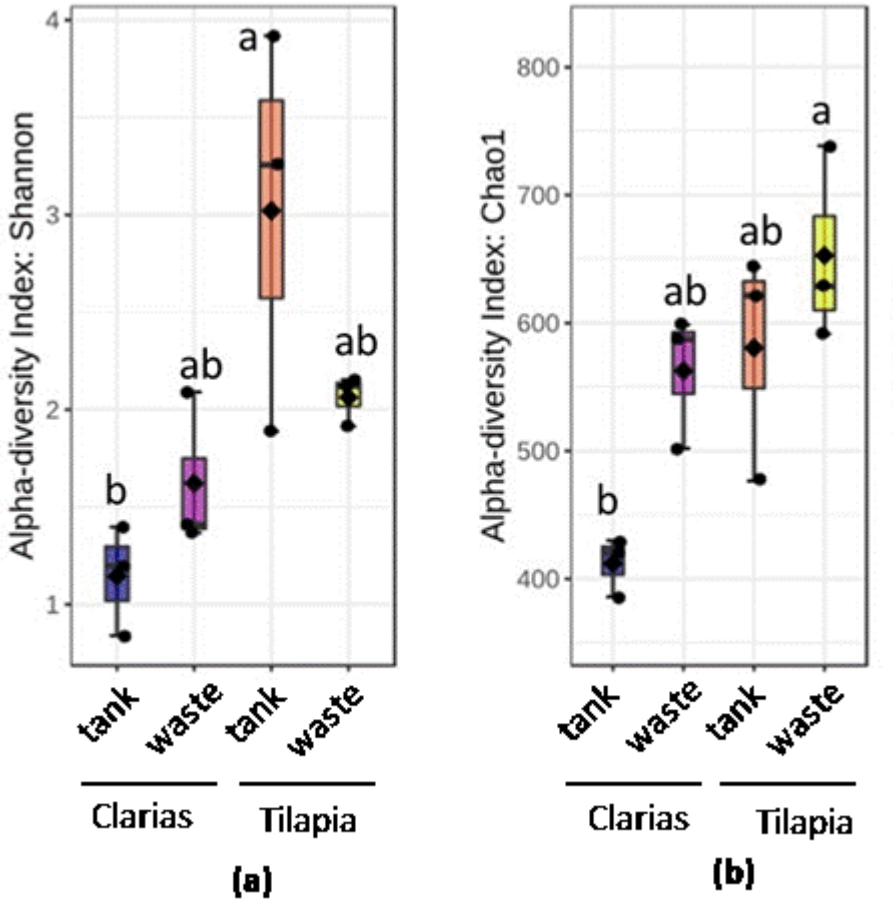

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208 **Figure 2**. Alpha diversity of bacterial communities in the fish water tank and the waste water sampling
209 sites of the Tilapia and Clarias RAS as judged by the diversity indices (a) Shannon and (b) Chao1. Letters
210 above the boxplots indicate the significant differences between the treatments.

211 With respect to the amount of *Pseudomonas*, we found no significant differences in the log-
212 transformed count of *Pseudomonas*. The amount of *Pseudomonas* in the Tilapia RAS was similar
213 to that of the Clarias RAS (Figure 3). The samples collected from water tanks and wastes samples
214 were also similar.

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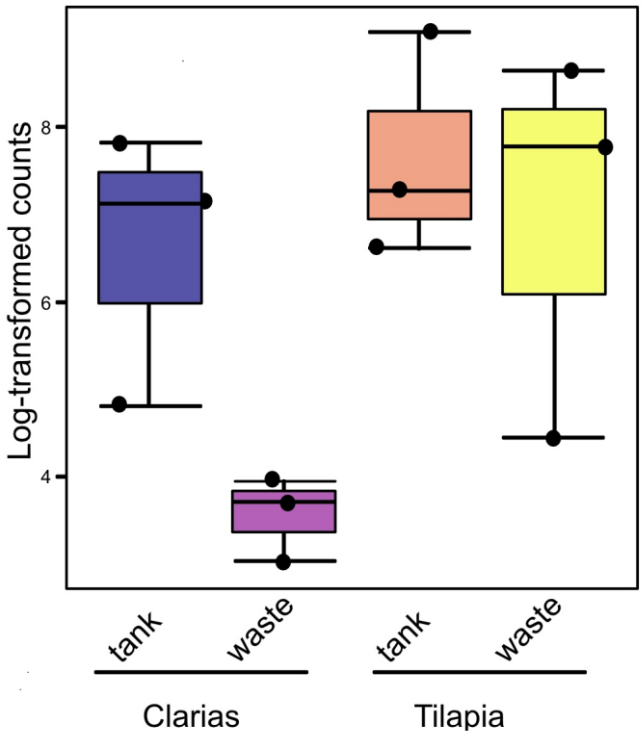

Figure 3. Log-transformed counts of *Pseudomonas* communities in the water tank and wastewater site from the Tilapia and Clarias RAS. Letters above the boxplots indicate the significant differences between the treatments.

However, although we found no significant differences in fungal community alpha diversity between the treatments in terms of the Shannon indices ($p = 0.211$) (Figure 4a), there were significant differences evident between the treatments in terms of the Chao1 indices ($p = 0.008$). There was higher fungal diversity in the Clarias waste compared to Tilapia water tank, which had the lowest fungal diversity (Figure 4b). On the hand, similarities, based on Chao 1 indices, were indicated within Tilapia and Clarias sites samples and between Tilapia samples and Clarias wastes.

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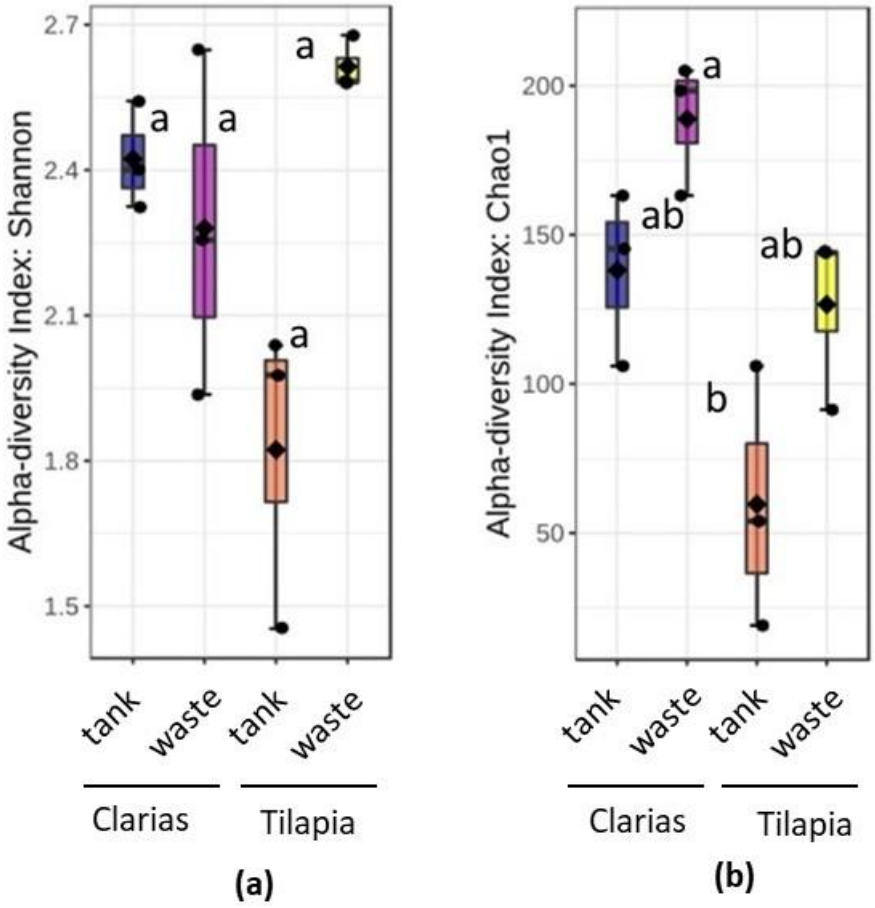

Figure 4. Alpha diversity of fungal communities from the water tank and the fish waste sample sites in the recirculated aquaculture systems populated with two different fish species -Tilapia or Clarias, as judged by the diversity indices (a) Shannon and (b) Chao1. Letters above the boxplots indicate the significant differences between the treatments.

Dendrogram analyses demonstrated that the bacterial and fungal communities also differed between the Tilapia and Clarias cultivation systems (Figure 5). The RAS populated with Tilapia indicated an apparent difference in the bacterial communities between the samples collected from the water tank and the waste (Figure 5a). However, these samples also shared similarities in their bacterial communities. Tilapia water tank and waste samples also differed in their fungal communities (Figure 5b). By contrast, there was no clear difference between samples collected from Clarias systems regarding either their bacterial or fungal communities.

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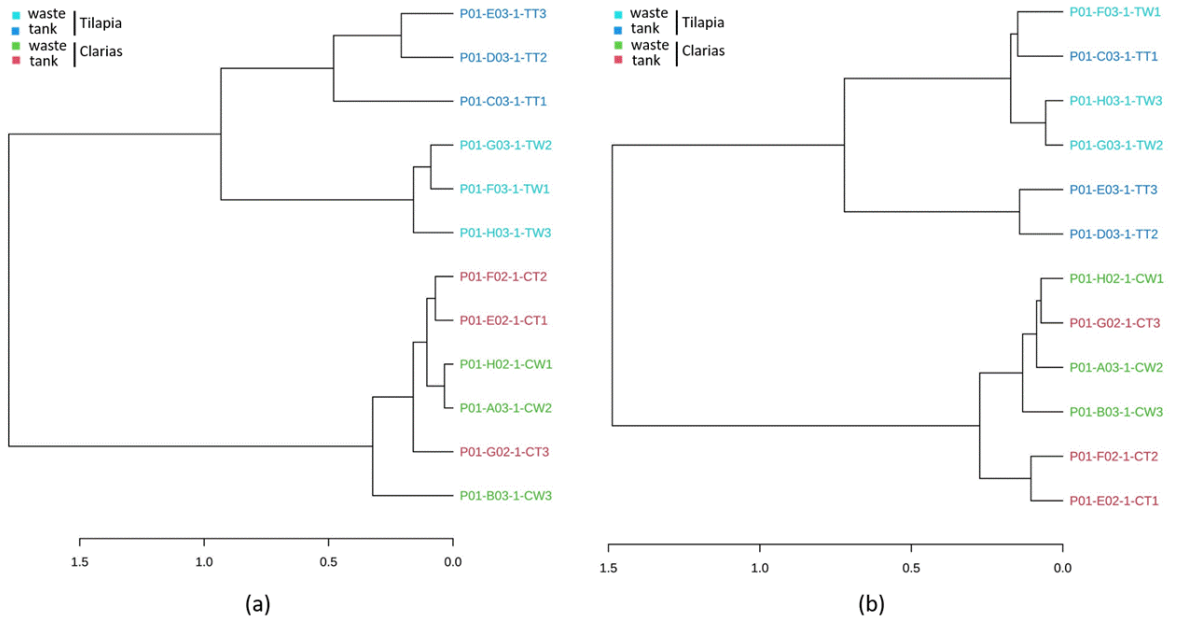

Figure 5. The dendrogram analyses of (a) bacterial and (b) fungal communities in samples collected from the RAS populated with either Tilapia or Clarias. The samples were collected from the Tilapia water tank (TT), Tilapia waste (TW), Clarias water tank (CT), and Clarias waste (CW).

The beta diversity metrics clearly distinguished the samples of the bacterial (Figure 6) and fungal (Figure 7) communities with respect to the fish species cultivated in the system and the sampling site. The first axis differentiated between the fish species (Figure 6a) and the second differentiated between the sampling sites in the system (Figure 6b). Clear differentiation with respect to the fish types and the bacterial communities could be shown. The differentiation with respect to the sampling site was more evident for samples from the Tilapia RAS than the Clarias RAS. No differences were found concerning the sampling sites in the Clarias system. Similar observations were evident regarding the fungal communities (Figure 7).

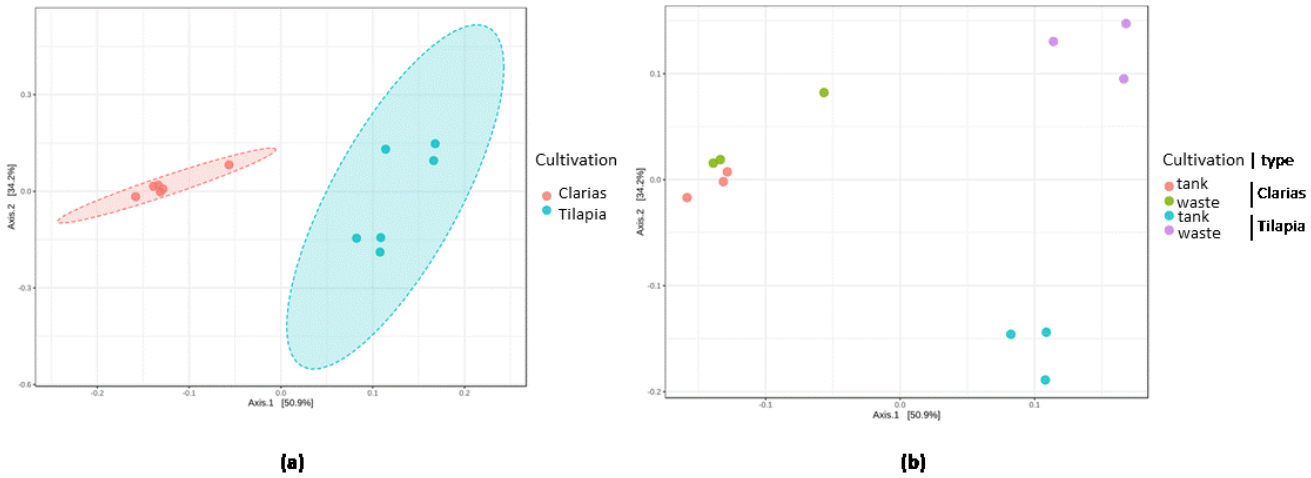

Figure 6. PCoA plots on beta diversity of the bacterial communities distinguished by (a) the cultivation system (ANOSIM; $R = 0.92$ and $p < 0.003$) or (b) sampling site (ANOSIM; $R = 0.81$ and $p < 0.001$) in the system using Tilapia or Clarias as fish species.

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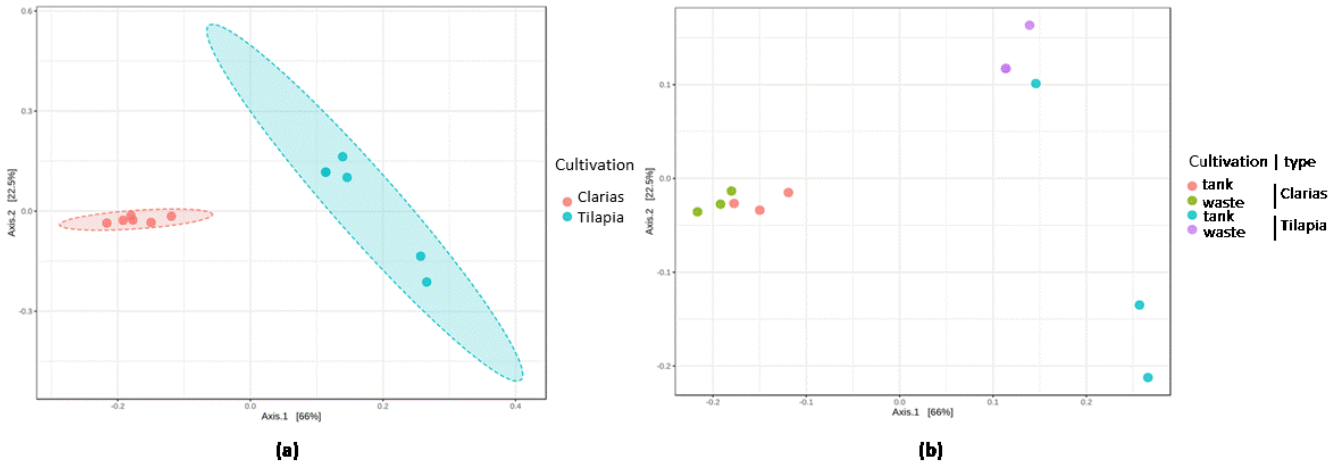

Figure 7. PCoA plots on beta diversity of the fungal communities distinguished by (a) the cultivation system (ANOSIM; $R = 0.05$ and $p < 0.003$) or (b) sampling site (ANOSIM; $R = 0.81$ and $p < 0.001$) in the system using Tilapia or Clarias as fish species.

Analyses of the relative abundance of the bacterial communities showed that the phylum *Fusobacteria* was significantly abundant (50%) in the samples collected from the Tilapia wastes, followed by the phyla *Bacteroidetes* (30%) and *Proteobacteria* (20%) (Figure 8). Tilapia water tank samples were dominated by the phylum *Proteobacteria* (60%) followed by *Bacteroidetes* (20%), *Fusobacteria* and *Actinobacteria*. (10%). *Actinobacteria* and *Proteobacteria* dominated samples collected from Clarias systems. The dominance of *Actinobacteria* was higher in the water tank (75%) than the waste samples (60%). *Bacteroidetes* and *Fusobacteria* were more dominant in Clarias wastes than Clarias water tank samples. However, compared with the Clarias RAS, *Actinobacteria* in the Tilapia system were either less abundant or completely absent.

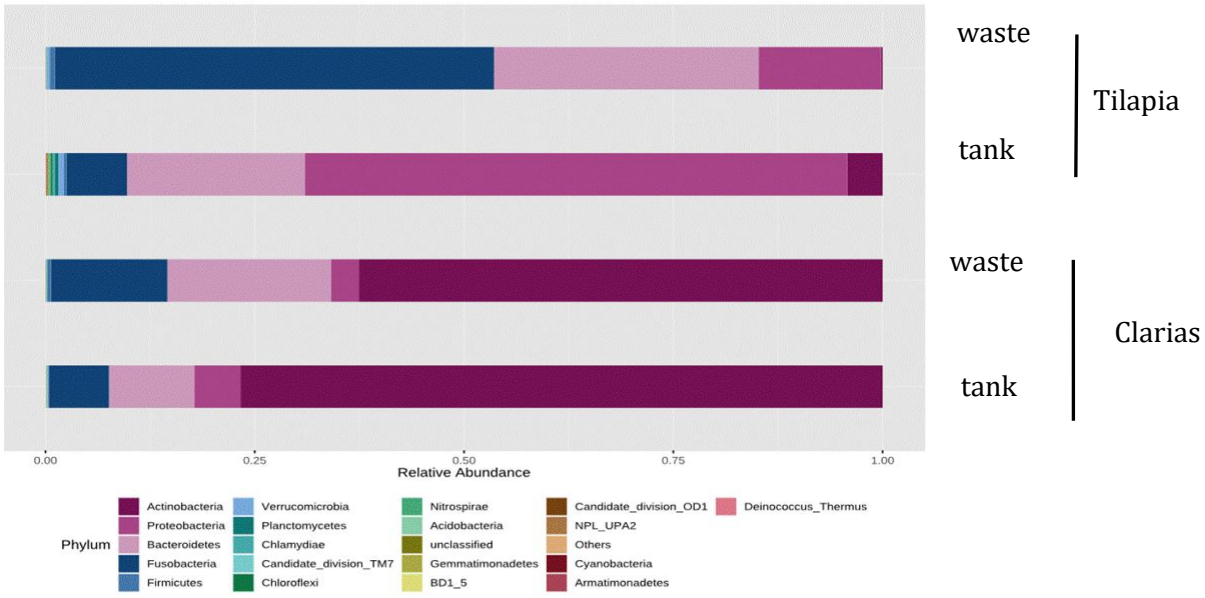

Figure 8. Relative abundance in (%) of dominant bacterial communities at phylum level in water tank, or fish wastes samples collected from RAS populated with Tilapia or Clarias.

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The bacterial genera *Microbacterium*, *Cetobacterium* and *Chrysobacterium* dominated the samples collected from the Clarias system (Figure 9). The genus *Microbacterium* dominated both water and waste samples. Tilapia waste samples were dominated by the genera *Cetobacterium* and *Flavobacterium*, followed by *Chryseobacterium*, *Pseudomonas* and *Bacteroides.* The samples from the Tilapia water tank included more genera and were dominated by *Janthinobacterium*, *Flavobacterium*, *Chryseobacterium*, *Pseudomonas*, *Pseudorhodobacter*, *Bacteroides*, *Sorangium* and *Hydrotalea*.

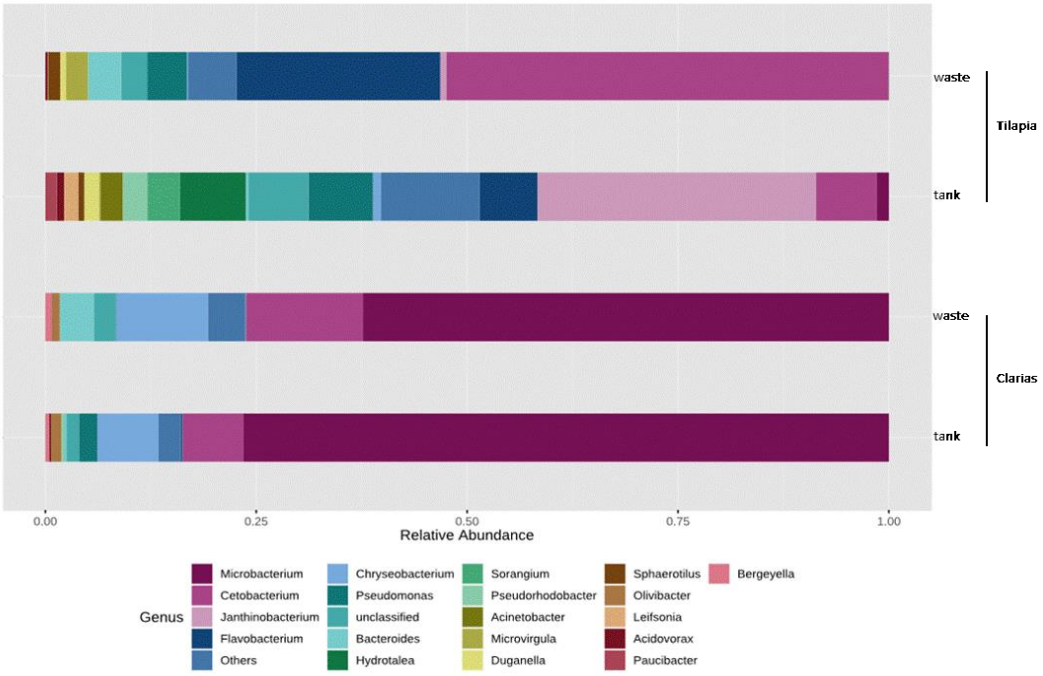

Figure 9. Relative abundance (%) of dominant bacterial communities at the genus level in water tank, or fish waste samples collected from RAS populated with either Tilapia or Clarias as fish species.

As indicated by the heatmap, the genera *Sorangium*, *Pseudomonas*, *Pseudorhodobacter*, *Acinetobacter*, *Simplicispira*, *Rhodococcus*, *Pedobacter*, *Rhodobacter*, *Undibacterium*, *Hydrogenobacter*, *Phenyloacterium*, *Alpinimonas*, *Acidovorax*, *Paucibacter*, *Legionella*, *Leisona*, *Duganella* were notably present in Tilapia tank water (Figure 10). Tilapia wastes included the genera *Janthinobacterium*, *Cetobacterium*, *Pseudomonas*, *Aremonas*, *Arcobacterium*, *Clostridium*, *Paludibacter*, *Sulfurospirium*, *Dechloromonas*, *Arcobacter*, *Propionivibrio*, *Limnohabitans*, *Paludibacter* and *Macellibacteroides*. The genera *Chryseobacterium*, *Cetobacterium*, *Simplicispiria*, *Thermomonas*, *Rhodanobacter*, *Ottowia*, *Bergeyella*, *Prevotella* and *Clostridium* were present in Clarias wastes. The genera *Chryseobacterium*, *Microbacterium*, *Macellibacteroides* and *Azospirillum* were predominant in Clarias water tank samples.

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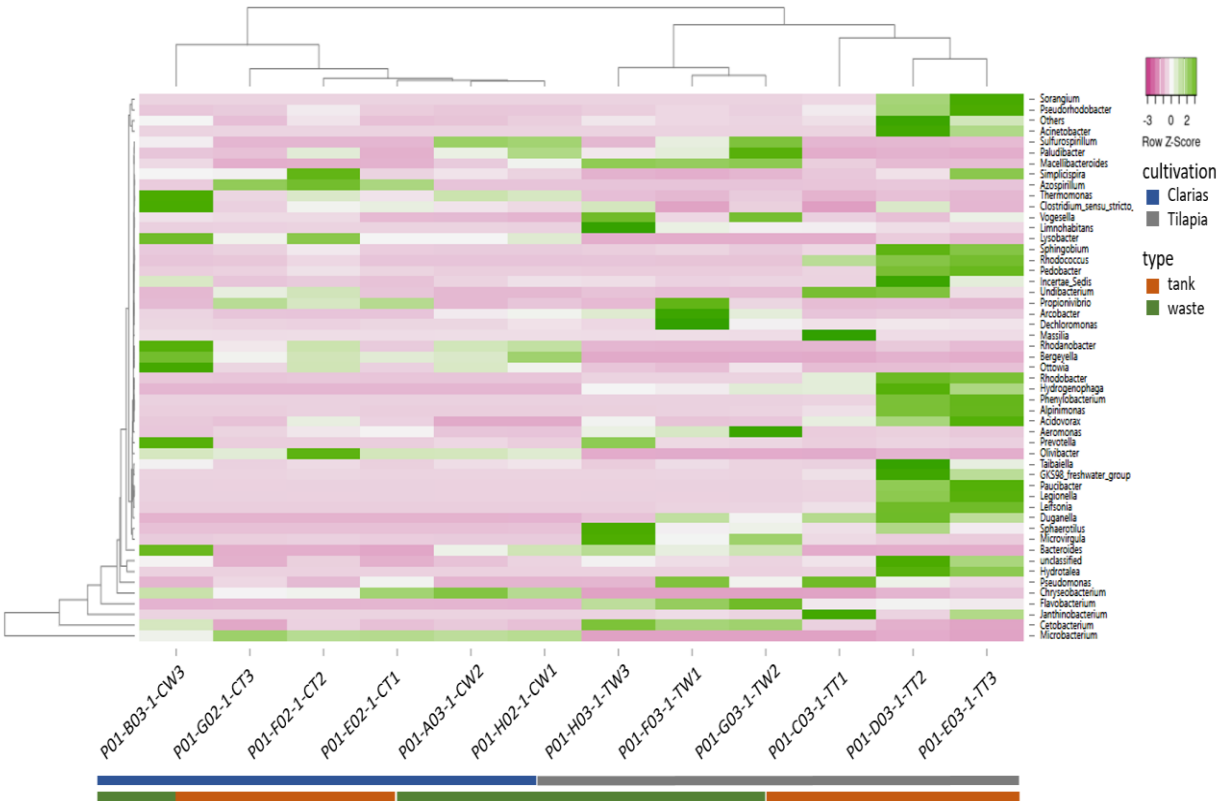

Figure 10. The heatmap shows the abundance of dominant bacterial communities at the genus level in water tank and fish waste samples collected from the Tilapia water tank (TT), Tilapia waste (TW), Clarias water tank (CT), and Clarias waste (CW).

Analyses of the relative abundance of the fungal communities also revealed differences in the dominant communities depending on the fish species and the sampling site (Figure 11). The majority (> 70%) of the fungal communities were unclassified in the samples collected from the Tilapia cultivation system, both from water tanks and Tilapia waste. However, the phylum *Ascomycetes* was well represented in samples from the Tilapia water tanks compared with the samples from the Tilapia wastes, in which the phylum Basidiomycetes was more abundant.

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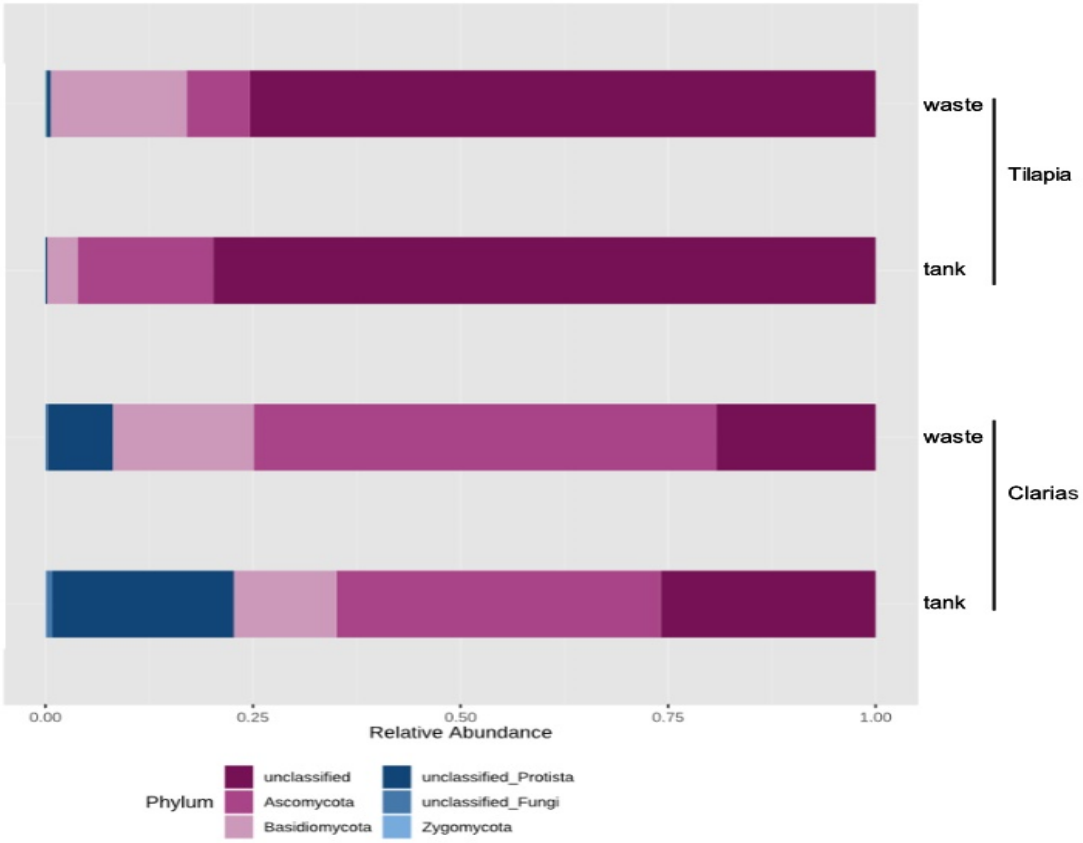

303

304 **Figure 11**. Relative abundance (%) of dominant fungal communities at phylum level in samples collected
305 from water tank and fish waste sites in RAS populated with Tilapia or Clarias.

306 By contrast, samples collected from Clarias water tanks and wastes had fewer unclassified
307 fungal communities than the Tilapia samples. However, the relative abundance of the fungal
308 communities differs between samples collected from Clarias water tanks and wastes. The waste
309 samples were highly dominated by the phyla *Ascomycetes*, *Basidiomycetes* and unclassified phyla
310 within the kingdom Protista. The water samples in Clarias system contained *Basidiomycetes* and
311 *Ascomycetes* but were dominated by an unclassified phylum within the kingdom *Protista* (Figure
312 11).
313 The unclassified fungi also dominated at the genus level (70%) in Tilapia and Clarias RAS
314 (Figure 12). *Trichosporon* spp. dominated (25%) in Clarias water and wastes and in Tilapia wastes.
315 Unclassified genera within the kingdom *Protista* were only found in the Clarias system. The genus
316 *Yarrowia* was exclusively found in a Tilapia water tank, while the genus *Trichomonascus* was only
317 detected in the Clarias system.

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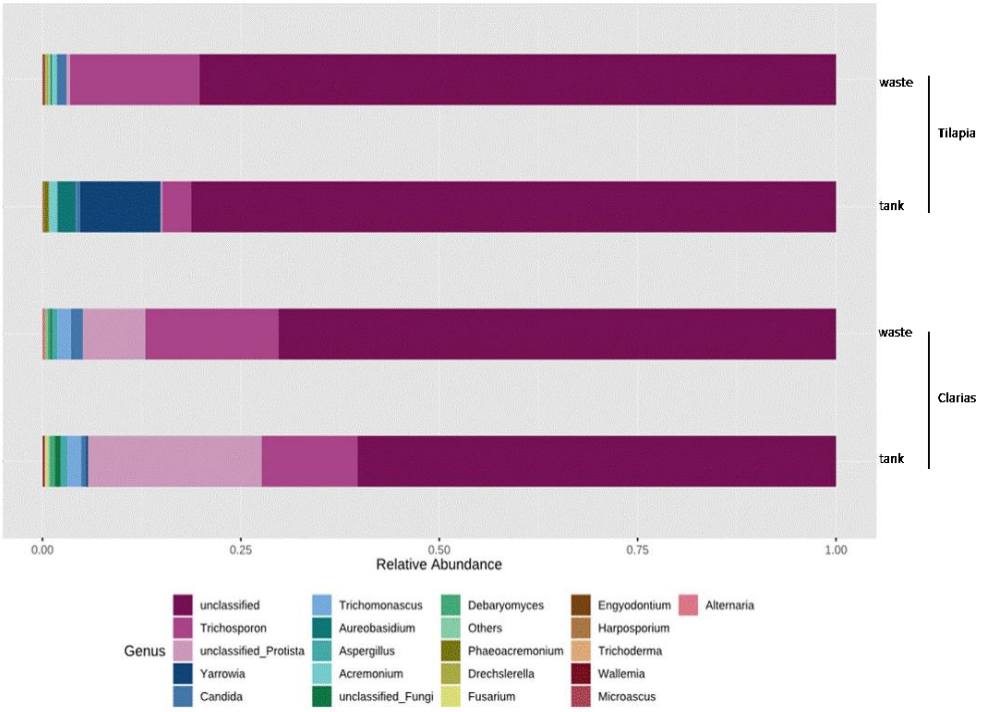

Figure 12. Relative abundance (%) of dominant fungal communities at the genus level in samples collected from water tank and fish wastes in RAS populated with Tilapia or Clarias.

The fungal communities' heatmap showed that the phylum Basidiomycetes in Clarias water tank was populated by the genera *Trichosporon*, *Trichomanoascus*, *Phodotonula*, *Malassezia*, *Mortierella*, *Ascochyta*, *Mucor*, *Cladosporium*, *Wallemia*, *Trichoderma*, *Fusarium* and *Deharyomyces* (Figur 13). The genera *Aspergillus*, *Debaryomyces*, *Wallemia*, *Sterigmatomyces, Exobasidium*, *Trichosporon* and *Pleospora* were found in Clarias wastes. The Tilapia waste contained more genus profiles than Tilapia water tank samples and was dominated by the genera *Candida*, *Trichosporon*, *Bettsia*, *Sporobolomyces*, *Penicillium*, *Entomocariticium*, *Cryptococcus*, *Preussia*, *Macrophomina*, *Sterigmatomyces*, *Microascuss* and *Enyodonium*. The Tilapia water tank was dominated by the genera *Yarrowia*, *Acremonium*, *Harposporium*, *Aureobasidium* and *Phaeoacremonium*.

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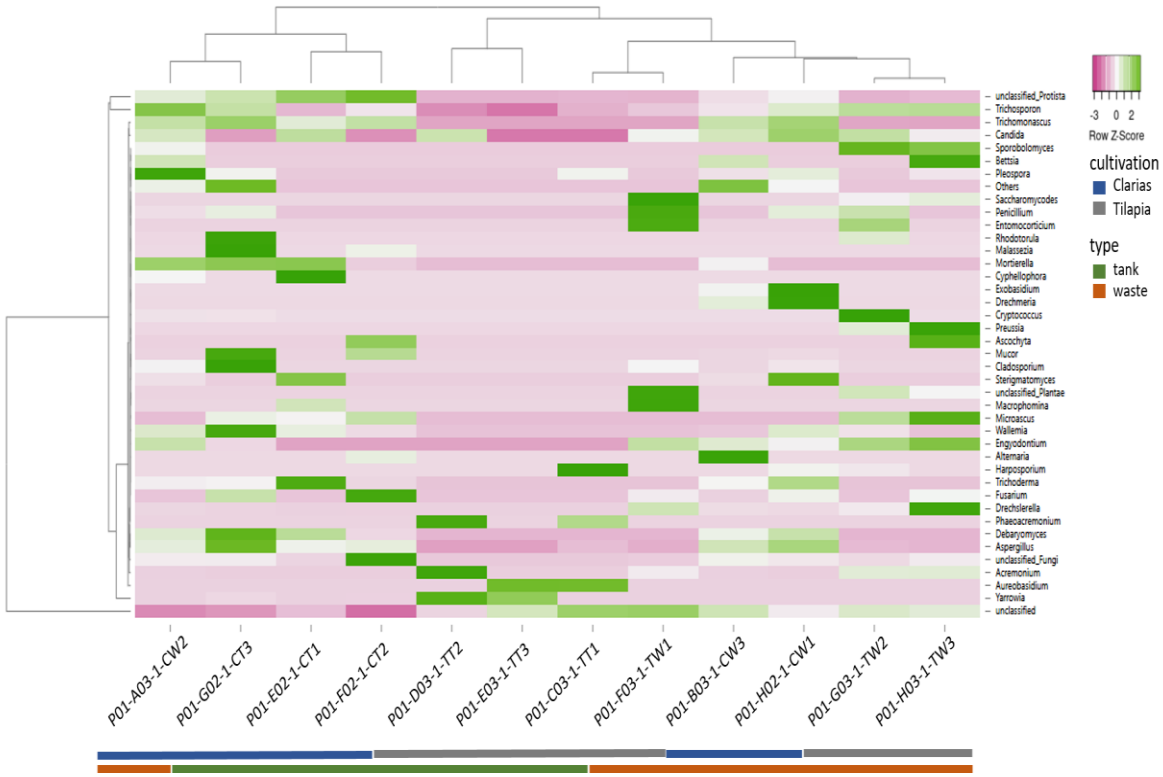

Figure 13. The heatmap presents the abundance of dominant fungal communities at the species level in samples collected from water tanks and fish wastes in RAS populated with Tilapia or Clarias.

Identification of pure cultures and their enzyme activity potential

In total, nine isolates were collected and identified as described in Table 2. *Pseudomonas fluorescens* was the most dominant isolate identified in Tilapia water tanks and wastes and Clarias wastes. The strains *P. veronii* and *Variovorax paradoxus* were identified in Tilapia wastes. *Variovorax* spp. were also identified in Clarias wastes.

With respect to the enzyme activities, cellulase activity was indicated in some of the isolated strains from both Tilapias and Clarias waste samples (Table 2). The bacterial strains identified from the Clarias wastes were actively producing protease and phosphatase. Amylase activities were positively indicated by all the strains isolated from Tilapia wastes, although not all of these strains indicated positive activities with respect to protease and phosphatase. Strains isolated from Tilapia tanks indicated positive protease and phosphatase activities and one of these strains showed amylase activity. Further, siderophore production was actively indicated by most of the strains isolated from Clarias wastes and by some of the strains isolated from Tilapia water tank and Tilapia wastes.

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Table 2. Identification and enzyme activities of the pure bacterial isolates isolated from samples collected from water tanks and wastes in RAS populated with Tilapia or Clarias. (+) indicates positive and (-) indicates negative enzyme activities.

Number of the isolate	Identification	Collection site	Amylase activity	Protease activity	Phosphatase activity	Cellulase activity	Siderophore production
1	*Pseudomonas fluorescens*	Tilapia water tank	+	+	+	-	+
2	*Pseudomonas fluorescens*	Tilapia water tank	-	+	+	-	-
3	*Pseudomonas fluorescens*	Tilapia wastes	+	-	+	+	-
4	*Pseudomonas veronii*	Tilapia wastes	+	-	+	+	+
5	*Variovorax paradoxus*	Tilapia wastes	+	-	-	-	-
6	*Pseudomonas fluorescens*	Clarias water tank	+	+	+	+	+
7	*Pseudomonas fluorescens*	Clarias wastes	-	+	+	+	+
8	*Pseudomonas fluorescens*	Clarias wastes	-	+	+	-	+
9	*Variovorax* spp.	Clarias wastes	-	-	-	-	-

In vitro antagonistic activity of bacterial isolates against plant pathogens

In vitro antagonistic tests against the plant pathogens, *P. cactorum* (Pc) and *Verticillium dahliae* (Vd) indicated inhibition activities by the isolated strains against both Pc and Vd (Figure 14). Isolate number 6 from the Clarias water tank showed better antagonistic potential against Pc and

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Vd than the other isolates. However, the antagonistic activity of number 6 was higher against Vd compared with Pc. Apart from number 6, isolate numbers 3 and 4 from Tilapia wastes indicated higher antagonistic activities against Pc and Vd than all other isolates. Isolate number 2 from Tilapia water tank and number 8 from Clarias wastes had higher antagonistic activities against Vd than Pc. By contrast, isolate number 5 from Tilapia wastes was more antagonistic to Pc than Vd.

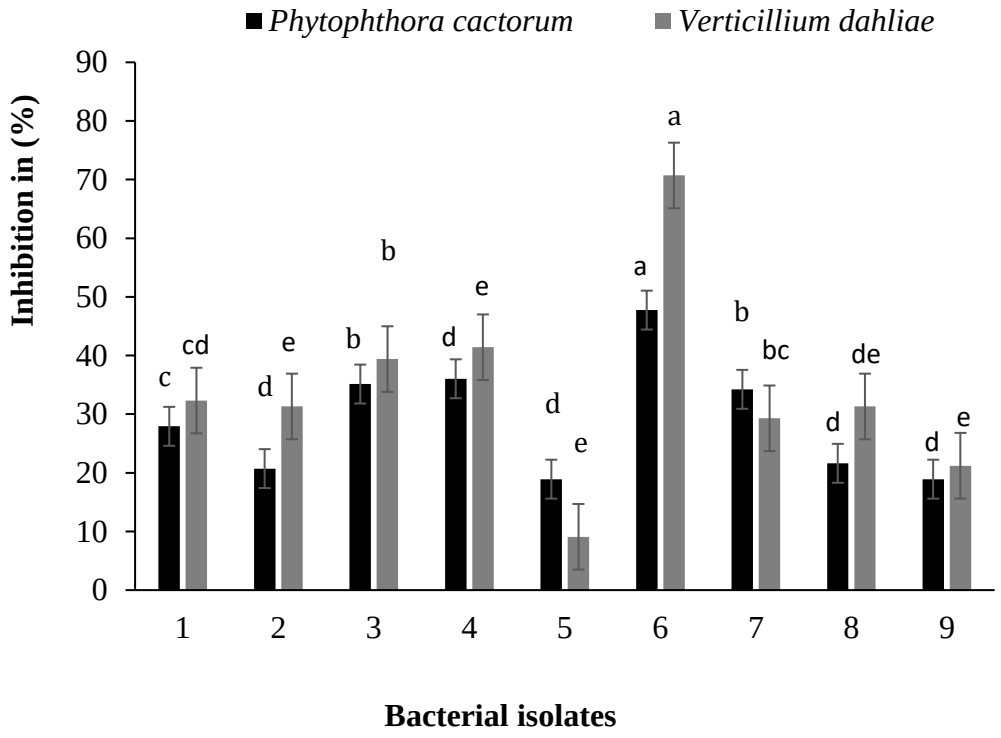

Figure 14. Inhibition (%) of radial growth of the plant pathogens *P. cactorum* and *V. dahliae* by the bacterial isolates 1-2 from Tilapia water tanks, isolates 3-5 from Tilapia wastes, isolates 6 from Clarias water tanks and isolates 7-9 from Clarias wastes.

Detached leaf assay

Two of the best performing biocontrol bacteria isolates (number 4 and 6) were tested for their biocontrol potential against *P. cactorum* on strawberry leaves. In the current study, strawberry leaves (Fragaria × ananassa cv. Sonata) were used in a detached leafy assay (DLA). The strawberry leaves inoculated with Pc started showing symptoms of infection four days after inoculation, while the leaves inoculated with sterile distilled water remained symptomless, confirming the viability of the zoospore suspension and no prior pathogen presence on the leaflets. Furthermore, the treatment with isolate number 6, combined with Pc showed potent disease inhibition. However, the bacterial isolate number 4 combined with Pc was less effective in controlling disease progression (Figure 15).

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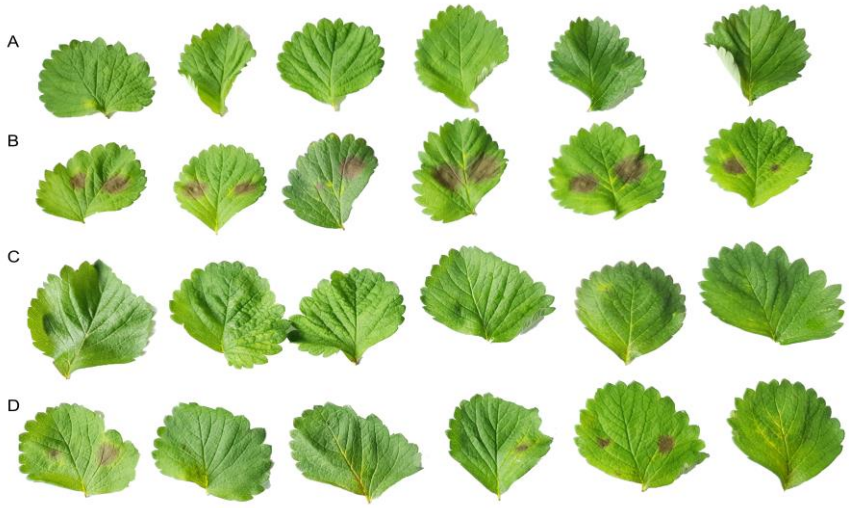

Figure 15. Infection of detached leaflets of strawberry cv. Sonata by *P. cactorum* isolate RV4 with and without potential antagonistic bacterial isolates.Treatments from top to bottom rows: **A**: water only*;* **B**: *P. cactorum*; **C**: *P. cactorum* combined with bacteria isolate no. 6 (isolated from Clarias wastes); **D**: *P. cactorum* combined with bacteria isolate no.4 (isolated fromTilapia wastes). Wild type RV4 formed large disease lesions on treatment B. Treatment C, with bacteria 6, and treatment D with bacteria 4 exhibited no or minimal disease. Leaves were photographed four days after inoculation.

Discussion

Our study has provided novel insights into the diversity of microbial communities in commercial RAS, their beneficial effects on plants, and their antagonistic potential against plant pathogens. We found that microbial diversity varied depending on the fish species populating the system and from where in the system the samples were taken. The shift in microbial composition as affected by the fish species was supported by the beta diversity analyses, which indicated significant differences and unique composition of the bacterial (Figure 6) and the fungal (Figure 7) communities between the Tilapia RAS and Clarias RAS. Fish species in the system also had significant effect in the water tank environment, which with Tilapia was richer with bacteria (Figure 2), while with Clarias it hosted more fungi than bacteria (Figure 4). These findings are in line with other studies [25, 26] indicating how fish species develop their own characteristic microbial communities. The results also agree with [26], who found higher fungal diversity in a Clarias than in a Tilapia system.

For both Tilapia and Clarias systems, sampling site had no significant effect on the evenness and richness of neither bacterial communities (Figure 2) nor fungal communities (Figure 4). By contrast, the site effect was evident in the composition of the bacterial (Figure 6) and the fungal (Figure 7) communities in the Tilapia RAS. Moreover, the dendrogram analyses (Figure 5) clearly distinguished between the bacterial and fungal communities based on fish species indicating that Tilapia bacterial and fungal communities differed from the Clarias. Differences based on sampling sites in the Tilapia RAS were also evident and could be related to the microbial community´s composition. Microbial diversity is also a function of water quality and its chemical properties such as pH, temperature, feed and gut microbiome as well as nutrient content [27-29]. In the current study, both fish species experienced closely similar cultivation conditions except for their density and weight. Tilapia biomass per tank was lower than that of Clarias (Table 1). This might have influenced condition of the water tanks in the current study. Previous studies indicated the effect of fish gut on the fish waste microbiome [27]. However, this could not be indicated in the current

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study since the microbial diversity in the waste was similar to the one in the water tank of both species.

The Tilapia RAS was dominated by bacteria and contained the bacterial phyla *Fusobacteria*, *Proteobacteria* and *Bacteroidetes* (Figure 8). These phyla were present in different relative abundances in the water tank and the waste environment primarily due to the dominance of *Proteobacteria* in the former and of *Fusobacteria* in the latter (Figure 8). The identified phyla are common in fresh water systems and have been previously indicated in Tilapia systems [27]. Our results are also in line with another study that has shown the dominance of *Proteobacteria* in biofilm in an aquaculture system [30]. *Proteobacteria* are characterized as r-strategists with an important function in nutrient recycling [31]. Their higher abundance in the Tilapia water tank might be due to the nutrient conditions in that environment. The high dominance of *Fusobacteria* in the waste of Tilapia is in accord with another study that has shown the relation of *Fusobacteria* to the microbiota in the gut where nutrient breakdown occurs [27]. Our results revealed that *Actinobacteria* was the most dominant phylum in the RAS environment with Clarias species (Figure 8). *Actinobacteria* have received much recent attention for their potential role as probiotic bacteria in marine and fresh water aquaculture [32, 33], further stressing the importance of the antimicrobial activities of these beneficial bacteria for fish and plant health.

Furthermore, microbial patterns in RAS with and without an aquaponic connection have been shown to be rich with bacteria that have the potential to promote plant growth [22]; while others have demonstrated that a Tilapia rearing tank can also host bacterial genera with a similar potential [34]. Our results are in line with these two studies [22, 34] and highlight the potential of the Clarias system to host, both in its tank water and in its waste water, bacterial genera with plant growth promoting characters such as *Microbacterium* and *Cetobacterium* (Figure 9), which are common rhizobacteria known to have beneficial effects on plant growth [22]. The Tilapia system in our study, and in contrast to the Clarias system, was richer in the genera *Pseudomonas*, *Cetobacterium*, *Flavobacterium*, *Sorangium*, *Pseudorhodobacter* and *Bacteroides*, which play a prominent role in promoting plant growth and protecting plants against root pathogens [22]. In addition, the water tank of Tilapia water (Figure 10) hosted genera of great importance in biodegradation and alleviating abiotic stress such as *Simplicispira*, *Rhodobacter* and *Acidovorax* [35, 36], conditions of high relevance in aquaponic cultivation systems. The identified genera in Tilapia wastes (Figure 10) indicate the shift in bacterial composition between the waste site and the water tank. The genera such as *Clostridium*, *Chryseobacterium*, *Janthinobacterium*, *Cetobacterium* and *Pseudomonas* identified in the waste environment also possess plant growth-promoting characters [22]. Moreover, the genera *Propionivibrio*, *Sulfurospirium* and *Dechloromonas* are involved in nutrient breakdown, or the removal of certain compounds, such as phosphorous, also by *Propionivibrio* [37]. The waste site also included genera involved in biodegredation and abiotic stress such as *Limnohabitans* and *Paludibacter* [38]. However, the occurrence of *Legionella* genera in the water tank and of *Aremonas* genera in Tilapia wastes is an area that requires further investigation. These genera are opportunistic pathogens occurring in aquatic environments and are of concern in terms of food safety and human health [39]. On a more positive note, the efficiency of *P. fluorescens* in controlling the pathogen *Aremonas* has been confirmed [24]. The genus of *Pseudomonas* is presented in both Tilapia and Clarias RAS (Figure 3). The bacterial species *P. fluorescens* is a rhizobacteria in the genus Pseudomonas, which has been identified in our samples from both RAS (Table 2). Optimizing conditions to favor beneficial microbes in a RAS and in the root environment in aquaponic systems might thus be used as a strategy to control fish diseases caused by pathogens in such aquaponic systems.

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With regard to the fungal communities in our sampled RAS, we found that they included genera with known important roles as promoters of plant growth and antagonists against plant pathogens. These genera include *Cladosporium*, *Mortierella* [40] and *Trichoderma* [41] in Clarias water, *Pleospora* [42] in Clarias wastes; and *Acremonium* [43] in Tilapia water and *Penicillium* [40] in Tilapia waste samples (Figures 12 and 13). However, these systems also contained genera with pathogenic potential concerning human and plant health, such as *Fusarium* [43], *Candida* and *Cryptococcus*, which pose a more significant challenge that needs further investigation. In addition, further investigations on fungal primers are also needed and important in order to enable the identification of those fungi in the current study that were unclassifiable beyond the level of phylum and genus.

The strains of bacteria isolated from the Tilapia system demonstrated biocontrol potential against plant pathogens hinting at their having a potential for an applied role in aquaponic systems. The only isolated bacterial strain that was positively identified and present in all samples *was P. fluorescens*. However, the log transformation of the *Pseudomonas* data in our study indicated the presence of these genera in all the investigated samples (Figure 3) and thereby strengthens our findings concerning the isolated strains. Others less ubiquitous taxa were *P. veronii* and *Vaiovorax paradoxus* in Tilapia wastes, and *Variovoras* spp. in Clarias wastes (Table 2). *Pseudomonas* spp. are essential microorganisms that can produce antimicrobial substances that control plant pathogens and promote plant growth [18, 20]. *P. veronii* is a species with bioremediation potential and is found in soil and water environments [44]. *Vaiovorax paradoxus* and *Variovoras* spp. are also known to have the ability to promote plant growth [45].

Enzyme production is one of the modes of action used by microorganisms to combat pathogen attack [20]. In aquaculture, the microbial communities of probiotic nature such as *Actinobacteria, Proteobacteria* and *Bacteroidetes* use different enzyme activities such as protease, chitinase, glucanase, amylase, cellulase, and phosphatase to break down different nutritional components in their substrates [46]. Our results in the current study indicate the production of protease, phosphatase, cellulase and siderophore by most of the strains isolated from the Tilapia system (Table 2). *P. veronii* (number 4) isolated from Tilapia wastes exhibited an ability to produce all of the above-mentioned enzymes. *P. fluorescens* isolates from Clarias water tanks (number 6) and Tilapia wastes (number 3) were also able to produce most of the enzymes. *Pseudomonas* spp. isolated from both RAS systems are well-known for siderophore production in plant roots that improves the availability of iron by aiding its uptake by plant roots [47]. Thus, the isolates in our study can be good candidates for use in aquaponic systems to increase the availability of phosphorous and iron for plant growth.

In vitro investigations into the antagonistic abilities of the isolated strains highlighted differences in their inhibitory effects on the growth of pathogens. *P. fluorescens* (number 6), *P. fluorescens* (number 3) and *P. veronii* (number 4) suppressed the growth of the plant root pathogen, *Phytophthora cactorum* (Pc) more than the other strains (Figure 14). This difference might be attributed to the production of cellulase by these strains, which not only breaks down a component in the feed, but can also break down cell walls of Pc. However, an in vivo antagonistic effect is needed in aquaponics in order for these results to have practical application. Our detached leaf assay provides strong evidence of the antagonistic potential of *P. veronii* (number 4) and *P. fluorescens* (number 6) against Pc (Figure 15).

Conclusions

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The current study has contributed new knowledge concerning the role of microbial tools in commercially based recirculated aquaculture systems (RAS) as promoters of plant growth and suppressors of disease. This knowledge strengthens the potential application of RAS as a part of aquaponic systems, which currently face challenges regarding plant nutrients and pathogens. The assemblages of microbial taxa at the level of phyla and genera both in Tilapia and Clarias RAS suggest the suitability of these systems to be used in aquaponic cultivation. However, in terms of promoting plant growth, plant protection and biodegradation, the characteristics of the richness and composition of the microbial communities in the Tilapia system make it the better choice for application in aquaponic systems. The commercial Clarias RAS could also be applied in aquaponic systems, but principally from a probiotics perspective due to the dominance of *Actinobacteria* in this system. *Pseudomonas* spp. from both Tilapia tank water and waste samples, and Clarias wastes, are good candidates with the potential to produce extracellular enzymes that enhance nutrient uptake. Although the results suggest considerable potential for using microbial communities to manage and control certain aspects of aquaponic systems, our findings need to be strengthened with in vivo studies to explore further the inhibition of plant pathogens and positive effects on plant nutrition. Risks arising from the presence of pathogens also need further investigation. However, our results can still be used as a foundation for the design of aquaponic systems populated with either Tilapia or Clarias. Still, these results need further investigations considering the microbial patterns in the RAS of Tilapia and Clarias in relation to the abiotic and biotic factors in the system.

Materials & Methods

Sample collection

Two commercial warm and fresh water RAS populated with Tilapia (*Oreochromis niloticus*) or Clarias (*Clarias gariepinus*) as the fish species and no aquaponic connection were used as the experimental units. The RAS of Tilapia consisted of a water tank of 70 m^3 and of 150 m^3 of the Clarias system. A filtration unite is connected the system including a big biofilter and a Degas column for the removal of nitrogen and carbon dioxide and addition of oxygen to the system. Three independent replicates (each 5L water samples) were collected at two different sites, viz. from the fish water tanks and from the wastewater where the fish faeces were accumulated. Both RAS with each respective fish type were similarly sampled. The collection site was considered as a treatment. Thus, in total, three fish water samples (biological replicates) and three wastewater samples (biological replicates) were randomly collected from the Tilapia and Clarias systems respectively. The samples were then transferred to the laboratory for subsequent microbial analyses.

Viable count and microbial enumeration

The viable count method was used to quantify the microbiota in each treatment. Dilution series and enumeration on selective agar media were applied [48]. From each replicate, 1 mL was serially diluted in 0.85% NaCl. From this dilution stock, 200 µL aliquots were spread, in triplicate, on the following media: i) 0.1% Tryptic soya agar (TSA, DIFCO 0369-17-6) complemented with cycloheximide (100 µL mL-1) to enumerate the general bacterial flora; ii) 0.5% malt extract agar (MA, DIFCO 0186-17-7) to enumerate the general fungal flora; and iii) King Agar B (KB) with cycloheximide (100 µg mL-1) to enumerate the fluorescent pseudomonads. The MA plates were incubated at room temperature for seven days and the TSA and KB plates were incubated for 24 hours at 25 °C.

Microbial community analyses

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Samples preparation

The total microbiome analyses started by sterile filtration of 1L of the collected samples through 0.2 µm filters using bottle-top vacuum filtration systems, PES (WVR- Sweden, 514-0332). The filtration unit filter was then transferred to a 50 mL tube, washed with 50 mL sterile autoclaved water, followed by vigorous vortexing for 2 min. The collected material was then centrifuged at 5000 rpm for 10 min and the pellets were stored at -80 °C.

DNA extraction

The DNA extraction was performed using Enzymo DNA preparation kit (D 4300, Sigma Aldrich) following the manufacturer's recommendations.

Illumina sequencing

The bacterial and fungal communities were sequenced with an Illumina MiSeq (2 × 300 bp) at LGC Genomics GmbH (Berlin, Germany) [49] using Illumina bcl2fastq 2.17.1.14 software. The bacterial 16S ribosomal gene was targeted using the forward primer 341F (5′-CCTACGGGNGGCWGCAG-3′) and the reverse primer 785R (5′-GACTACHVGGGTATCTAATCC-3′). The fungal forward primer ITS7F (5′-GTG ART CAT CGA ATCTTTG GTT G-3′) and the reverse primer (5′-TCC TCC GCT TAT TGA TAT GC-3′) were used to target the ITS region for fungal assessment. Data pre-processing and OTU picking from amplicons were performed using MOTHUR pipelines (version 1.35.1). Reads with a final length of <100 bases were discarded and primer, barcode sequences as well as chimeras were removed. For taxonomical classification, alignment against 16S Mothur-Silva SEED r119 reference was performed and sequences from other domains of life were removed. Assignment of OUTs was performed at the 97% identity level using the cluster split method. The fast Tree method was used to generate the phylogenetic trees for 16S and ITS, respectively. The sequenced data were submitted to Genbank database under the bio project submission number SUB8558843

Microbial activities

Bacterial pure cultures

For each treatment, two single colonies from the TSA plates and two from the KB plates were selected and transferred to be grown on broth media of tryptic soya broth (TSB) and King B broth (KBB), respectively. One loopful of culture was inoculated into 15 mL of the broth media and incubated at 25 °C with shaking (140 rpm) for 24 h. Bacterial DNA was extracted using the Quick-DNA Bacterial Microprep Kit according to the manufacturer's recommendations (Zymo Research, USA). The DNA yield and integrity was assessed using a NanoDrop Micro Photometer (NanoDrop Technologies, UK), and agarose gel electrophoresis, respectively. The 16s rRNA region of all bacterial isolates was PCR amplified individually with the universal primer pairs, 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3') [50]. PCRs were performed using ten ng of DNA with the following temperature parameters: initial denaturation step at 94 °C for 3 minutes, followed by 35 cycles at 94 °C for 45 s, 50 °C for 30 s, and 72 °C for 30 s, followed by a final extension step of 72 °C for 5 min. The PCR products were purified using the Qiagen QIAquick PCR Purification Kit (Qiagen, UK). Sanger sequencing for species identification was carried out at the GATC biotech AG sequencing facility (Germany) using 27F and 907R primers. DNA star software was used (DNASTAR, USA) to analyze and edit nucleotide sequences obtained from the sequencing platform manually. Resulting sequences with 16s region were searched for matching hits against the National Center for Biotechnology Information (NCBI) GenBank non-redundant nucleotide database (BLASTn; [51]). Search hits to

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598 sequences from records in the database were evaluated for coverage and identity and the best
599 matched NCBI accession was recorded.
600
601 ***Enzyme activities***
602 Assessment of the functional characters of the isolates was performed with respect to enzymatic
603 activities. The isolated bacterial colonies were screened for the production of amylase, protease,
604 phosphatase and cellulase on functional media using a plate assay [52]. The minimal medium (M9,
605 Minimal Salts (VWR- Sweden) was used as a base medium. For amylase activity, the isolates were
606 spotted on M9 amended with 1% (w/v) starch. After incubation for 24 - 72 hours at 20 + 2 0C, the
607 plates were flooded with Lugol iodine; the appearance of a zone of clearance towards the dark
608 background is a positive indication of amylase production.
609 For the protease activity, M9 plates amended with skimmed milk (20 mL L-1) were used
610 and incubated for 24 - 72 hours at 20 + 2 0C. The formation of a halo zone around the colonies
611 indicated a positive protease production. The same indication assessment and incubation conditions
612 were used for cellulase production. The M9 plates amended with carboxymethyl cellulose were
613 used and after incubation, they were flooded with Congo Red solution (0.2% w/v) for 30 min and
614 washed with 1 M NaCl solution.
615 For the phosphatase activity, Tryptose phosphate agar supplemented with Methyl Green
616 (0.05 mg mL-1) was used. The plates were incubated for 5 days at 20 + 2 0C; the development of
617 green color indicated positive phosphatase activity. The siderophore production was also assessed
618 using Chromazurol S agar plates spotted with bacterial isolates and incubated for three days at 20
619 + 2 0C. The positive result was indicated by the formation of an orange halo around the colonies.
620
621 ***In vitro antagonistic assay***
622 The bacterial strains isolated were screened for antagonistic potential towards the oomycete/fungal
623 pathogens *Phytophthora cactorum* and *Verticillium dahliae*. The assay was performed on cornmeal
624 agar (CMA) plate agar plates with the mycelial plug (1×1cm) of the test pathogen placed in the
625 centre followed by streaking test bacterial isolate three cm apart on either side of the pathogen plug
626 and monitored for ten days at 20 °C. Controls constitute only the test pathogens. The experiments
627 were repeated thrice with three replicates for each independent experiments. The radial growth of
628 the pathogen growth towards test bacteria was measured and the Growth Inhibition Percentage
629 (GIP) was calculated according to the following formula:
630 $(\%) = (RC - RT)/ \text{RC} \times 100$
631 where RC constitutes radial growth of the pathogen in the control plate (cm) and RT is the radial
632 growth of the pathogen (cm) in the treated plate [53].
633
634 ***Detached leaf assay (DLA)***
635 The antagonistic potential of bacterial isolates towards the strawberry pathogen *P. cactorum* was
636 tested in a detached leaf assay. The best performing bacterial isolates with the highest antagonistic
637 activity from the *in vitro* studies were selected for the DLA assay. The bacteria were cultured
638 overnight on Tryptic Soy Broth media at 28 °C and pelleted. The pellet was washed and
639 resuspended in distilled water, followed by density measurement and adjustment to OD 0.1 at 600
640 nm.
641 For zoospore production, *P. cactorum* isolate RV4 was cultured as described [54]. For
642 sporangia formation, the agar plugs from the outer edges of freshly growing mycelium colonies
643 were subjected to dark conditions on V8 media for three days followed by treatment with
644 autoclaved soil extract solution under light conditions for 48 hrs. The sporangial suspensions

obtained were subjected to cold treatment at 8°C to release the zoospores over an average 2–3 h period depending on the zoospore's release efficiency. The zoospore inoculum was collected and counted using a haemocytometer to adjust inoculum to 20 000 zoospores per mL.

Young strawberry leaflets (cv. Sonata) grown in controlled conditions were used to assess disease development. The leaves were placed adaxial side facing up on moist paper in clear plastic containers and inoculated with a mixture of zoospore and bacterial suspension with approximately 20000 zoospores and OD = 0.1 bacterial suspension in a 20 µl droplet on either side of the leaf midrib. The controls constituted either sterile water or pathogen only. The plastic containers were tightly sealed and placed at 20 °C with 16 h photoperiod. Pathogenicity phenotypes were photographed and assessed at 4 days post-inoculation (DPI) using the image-processing program ImageJ. Each independent experiment was evaluated with six replicates and the experiments were repeated thrice.

Statistical analyses

All statistical analyses were performed within Microbiome Analyst v1.0 [55]. OTUs were pre-filtered before conducting any statistical analysis by retaining those present in at least two samples. The data were normalized using the total sum scaling method (counts per million normalization) to address variability in sampling depth. Alpha diversity profiling was conducted using Shannon and Chao1 diversity indices and statistical significance testing using analysis of variance (ANOVA). To examine differences between the different groups in community compositions, beta diversity was calculated using unweighted Unifrac Distance and statistical comparisons performed with ANalysis of SIMilarities (ANOSIM). Patterns of sample dissimilarity were visualized using Principal Coordinate Analysis (PCoA) plots. In addition, hierarchical clustering and heatmaps were constructed using the Ward clustering algorithm and Euclidean distance measure to examine the robustness of sample clustering and relative abundances. The results from the microbial enumeration were log-transformed to meet the assumptions of homogeneity and normality. The significance effect of treatment was tested by ANOVA followed by Tukey's multiple comparison test ($p < 0.05$) using Minitab v16. To explore how top taxa differed between the different cultivation/waste types, classical univariate statistical comparisons were inferred using t-test/ANOVA method with an adjusted p-value cutoff of 0.05.

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