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Additional Information

Determination of the bacterial microbiome of free-living amoebae isolated from wastewater by 16S rRNA amplicon-based sequencing

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Abstract

Free-living amoebae (FLA) are ubiquitous protozoa commonly found in water. FLA are well-established hosts for amoeba-resistant bacteria, most of which are pathogenic, and offer them shelter from adverse environmental conditions or water treatments. Since there is very little knowledge about the complete bacterial microbiome of FLA, in this work the bacterial microbiome of FLA isolated from wastewater both after secondary and tertiary treatments was studied by amplicon-based sequencing. FLA were detected in 87.5% and 50.0% of wastewater samples taken after secondary and tertiary disinfection treatments, respectively. The most abundant bacterial phyla were Proteobacteria, Planctomycetes, Bacteroidetes and Firmicutes, which represented 83.77% of the total bacterial FLA microbiome. The most abundant class of bacteria was Gammaproteobacteria, which contains an important number of relevant pathogenic bacteria. The bacteria of public health concern *Aeromonas*, *Arcobacter*, *Campylobacter*, *Helicobacter*, *Klebsiella*, *Legionella*, *Mycobacterium*, *Pseudomonas* and *Salmonella* were detected as part of the FLA microbiome. Although different microbial communities were identified in each sample, there is no correlation between the microbiome of FLA and the extent of wastewater treatment. To our knowledge, this is the first work in which the bacterial microbiome of FLA isolated from wastewater is studied. Obtained results indicate that FLA are hosts of potentially pathogenic bacteria in treated wastewater used for irrigation, which may pose a public health threat.

Keywords: free-living amoebae; microbiome; wastewater; pathogenic bacteria; 16S rRNA amplicon sequencing

1. Introduction

Free-living amoebae (FLA) are a heterogeneous group of protozoa which are commonly found worldwide in water and soil (Rodriguez-Zaragoza, 1994). They typically have two developmental stages: the trophozoite and the cyst. The trophozoite is the metabolically active form in which they are able to move, feed and multiply, and the cyst is the dormant form, which is metabolically inactive.

The presence of FLA in water has been extensively studied, being detected in wastewater (García et al., 2011), drinking water (Magnet et al., 2013), rivers (Magnet et al., 2013), wells (Montalbano Di Filippo et al., 2015), recreational water (Reyes-Batlle et al., 2017), bottled water (Maschio et al., 2015a), industrial water (Scheikl et al., 2014) and even in biofilms from the wastewater distribution system (Valster et al., 2009).

The relationship between FLA and bacteria is well established. It is noteworthy that FLA feed on bacteria and, at the same time, can harbour bacteria that survive and even multiply intracellularly: the so-called amoeba-resistant bacteria (ARB) (Greub and Raoult, 2004). These host-parasite relationships are highly complex and depend on FLA and bacteria virulence as well as on environmental conditions (Siddiqui and Khan, 2012). The outcome of these interactions can only benefit, either the FLA or the bacteria, or lead to the development of a symbiotic relationship between both (Siddiqui and Khan, 2012). It has been suggested that, since ARB can resist to FLA digestion, they could also be capable of resisting macrophages digestion (Greub and Raoult, 2004). Moreover, taking into account the widespread of FLA in water, FLA seem to be resistant to water treatments and therefore, they would protect internalized bacteria from adverse environmental conditions that would normally affect them, which may pose a human risk if it happens to reach humans.

It has been previously stated that ARB contain pathogenic bacteria (Thomas et al., 2010). From the initial description of FLA-*Legionella* interaction (Rowbotham, 1980), this association has

been the most extensively studied (Marciano-Cabral and Cabral, 2003). However, there are many other pathogenic bacteria which have been shown to interact with FLA, and therefore are considered as ARB, such as *Campylobacter jejuni*, *Helicobacter pylori*, *Mycobacterium avium* or *Pseudomonas aeruginosa*, among others (White et al., 2010; Bui et al., 2012; Maschio et al., 2015b; Moreno-Mesonero et al., 2019). Extensive lists of ARB can be found in Balczun and Scheid (2017) and in Thomas et al. (2010). Moreover, other microorganisms such as the protozoa *Cryptosporidium parvum*, the fungus *Cryptococcus neoformans* or the virus *Megavirus chilensis* have been also found to interact with FLA (Steenbergen et al., 2001; Gómez-Couso et al., 2007; Arslan et al., 2011).

Although there is a broad range of articles describing FLA-ARB interactions, there are very few in which the complete bacterial microbiome of FLA is studied so far. In fact, to our knowledge, there are only two previous studies in which this subject is investigated. Delafont et al. (2013) studied the bacterial microbiome of FLA isolated from drinking water samples by means of amplicon-based sequencing. Moreover, our group has recently studied the bacterial microbiome of FLA isolated from vegetables, also by means of amplicon-based sequencing (Moreno-Mesonero et al., 2020). Hence, more researches on FLA bacterial microbiome should be carried out in order to establish which bacteria tend to interact with FLA and to discover new possible ARB.

The amplicon-based sequencing approach is widely used to characterize microbial communities from environmental samples. The 16S rRNA gene is commonly used as the amplification target on which these studies focus. It is a phylogenetic marker that contains conserved regions, useful for primers design, and hypervariable regions, which allow for the differentiation and taxonomical assignation of the different bacteria (Huse et al., 2008).

Due to water scarcity, wastewater reuse for agricultural purposes is a current practice in our geographical area. Thus, the aims of this study were to characterize the bacterial microbiome of

FLA isolated from wastewater both after secondary and tertiary treatments, paying special attention to potentially pathogenic bacteria, and to determine whether the microbiome of FLA would differ depending on the extent of wastewater treatment.

2. Material and methods

2.1. Samples

A total of 16 wastewater samples were taken after secondary and UV tertiary disinfection treatments of a wastewater treatment plant (WWTP) located in Valencia province, Spain. This WWTP treats wastewater for almost 190,000 equivalent inhabitants and has a capacity of more than 40,000 m^3/day. All tertiary effluents are intended for irrigation purposes. Samples were taken in 8 sampling campaigns, each taken at a different time. Each sampling campaign included one sample after secondary treatment and one sample after tertiary disinfection treatment. A volume of 1 litre of each water sample was carefully collected by a sampling container at the WWTP. Water samples were transferred to sterilized bottles and transported to the laboratory at 4 ºC, where they were processed within two hours.

2.2. Free-living amoebae cultivation

One litre of each wastewater sample was filtered through nitrocellulose filters with 3 µm of pore size (Whatman, United Kingdom). Filters were placed upside down on Non-Nutrient Agar (NNA) plates prepared with Page's saline solution (PAS; 2.5 mM NaCl, 1 mM KH2PO_4, 0.5 mM Na_2HPO_4, 40 µm $CaCl_2 \cdot 6H_2O$ and 20 µm $MgSO_2 \cdot 7H_2O$) and were incubated at 28 ºC. After 24 hours, filters were removed and plates were kept at 28 ºC up to 30 days or until FLA growth was observed. FLA growth was observed under a phase-contrast microscope and it was confirmed when typical FLA trophozoites, characterized by the movement projecting pseudopodia, and typical cysts

were observed. Then, plates' content was recovered by adding PAS solution and scratching the agar, by using a sterile cell scraper.

2.3. Blocking of non-internalized bacterial DNA

NNA plates' content was concentrated by centrifugation (500 g for 3 min) and the sediment was resuspended in PAS solution. FLA-external bacteria were subsequently killed by treating NNA plates' content with sodium hypochlorite at a final concentration of 100 ppm for 1 hour under darkness. Sodium hypochlorite was removed washing the sample by centrifugation (500 g for 3 min). Thereafter, samples were treated with a final concentration of 50 µM of propidium monoazide (PMA), which was incubated for 10 min under darkness with occasional mixing to allow better reagent penetration (Moreno-Mesonero et al., 2016). Then, samples were exposed to blue LED light for 15 min at the photoactivation system PhAST Blue (GenIUL, Spain). Afterwards, samples were centrifuged at 14,000 rpm for 5 min, resuspended in 200 µl of phosphate saline buffer (PBS) and stored at -20 ºC until use (Agustí *et al.,* 2010).

2.4. DNA extraction and sequencing

DNA was extracted using GeneJET™ Genomic DNA Purification Kit (Thermo Scientific, Germany) following the cultured mammalian cells DNA extraction protocol but increasing from 10 to 30 min the incubation time at 56 °C for cell lysis (Moreno-Mesonero *et al.,* 2016). Final DNA was eluted in 50 µl of elution buffer.

To determine the bacterial microbiome of FLA, extracted DNA was sequenced by FISABIO Sequencing and Bioinformatics Services (Valencia, Spain). First, amplicon libraries were prepared following the 16S Metagenomic Sequencing Library Preparation (Part # 15044223 Rev. B) using the recommended set of primers, which target the 16S rDNA V3-V4 regions and amplify a single amplicon of around 460 bp (Klindworth *et al.,* 2013). The DNA libraries with dual indexes were sequenced on MiSeq (Illumina, USA) using a kit for paired-end sequencing (2 × 300 bp). A

negative control of sequencing was included. The sequencing data generated in this study can be accessed at Sequence Read Archive platform of the NCBI (reference number PRJNA635271).

2.5. Bioinformatics analysis

Illumina MiSeq-generated data were analysed using QIIME 1.9.1 (http://qiime.org; Caporaso *et al.,* 2010), applying additional scripts available in Microbiome Helper VirtualBox (Comeau *et al.,* 2017). As a first step, forward and reverse reads were merged using PEAR v0.9.19 (Zhang *et al.,* 2014). FastQC tool (Andrews, 2010) was used to confirm that reads were correctly merged. Then, sequences present in the negative control were removed. Subsequently, merged reads were filtered by length and quality score (reads with less than 200 bp or a minimum Q30 quality score over at least 90% of the bases were removed) using FASTX-Toolkit v0.0.14 (Gordon, 2009). Reads with any ambiguous bases ("N") were also filtered out. Potentially chimeric sequences were screened out using VSEARCH v1.11.1. (Rognes *et al.,* 2016). The remaining sequences were processed using the QIIME's open reference script, applying the methods SortMeRNA v2.0 (Kopylova *et al.,* 2012) and SUMACLUST v1.0.00 (Mercier *et al.,* 2013) for the reference-based and *de novo* clustering steps, respectively. Operational Taxonomic Units (OTUs) were defined at 97% genetic similarity cut-off. The SILVA v132 ribosomal database (Quast *et al.,* 2013) was used to perform the taxonomic assignation. Sequences assigned to chloroplasts and mitochondria were removed from further downstream analysis.

2.6. Analysis of the microbial community

In order to compare the samples on an equal basis, they were normalized to an equal sampling depth (13,307 reads). Prior to normalization, alpha diversity indices (Shannon, Simpson and Chao1), Good's coverage and rarefaction curves were calculated with subsampled sequencing data (13,307 reads) to reduce the effects of different sampling depths. Mann-Whitney U test was performed using R Software v 3.6.3 (R Core Team, 2020) to test the significance of diversity differences among bacterial microbiome of FLA in secondary and tertiary treatments ($p<0.05$).

Graphic representations were produced using Microsoft Excel 2016 and R Software v 3.6.3 (R Core Team, 2020). Beta diversity was determined using QIIME, calculating unweighted UniFrac distance metrics from the normalized data. Principal coordinate analysis (PCoA) was used to visualize the differences in bacterial community composition among the samples. The analysis of similarity statistics (ANOSIM) was calculated to test the significance of differences among the bacterial microbiome with regard to the treatments on which FLA were isolated ($p<0.05$).

3. Results

3.1. Free-living amoebae cultivation

A total of 11 out of the 16 (68.8%) analysed wastewater samples were positive for the presence of FLA. More specifically, 7 out of the 8 (87.5%) wastewater samples taken after secondary treatment and 4 out of the 8 (50.0%) wastewater samples taken after tertiary disinfection treatment presented FLA positive cultures. In three out of the eight samplings, FLA growth was observed after both, secondary and tertiary treatments; in four of them FLA growth was only observed after secondary treatment and in one of them FLA growth was only observed after tertiary treatment (Table 1).

3.2. Bacterial microbiome of FLA

A total of 691,316 raw reads were obtained after Illumina MiSeq sequencing. After quality filtering, chimeras screening and removal of chloroplasts and mitochondria sequences, 532,827 high-quality sequences remained, which were clustered into 11,902 OTUs. Samples were rarefied to 13,307 sequences/sample in order to make comparisons among them on an equal basis (Supplementary Table 1).

Good's coverage was calculated to estimate how representative the obtained data were of the bacterial community. Good's coverage values ranged between 92.70% and 99.75% (Supplementary Table 2), which was checked with rarefaction curves (Supplementary Figure 1). Alpha-diversity analysis was used to reflect the diversity and richness of the bacterial communities detected as part of the FLA microbiome. Alpha diversity indices of the bacterial microbiome from FLA isolated from secondary and tertiary wastewater treatments are summarized in Figure 1. A comparison between alpha diversity indices of FLA bacterial microbiome from secondary and tertiary treatments showed no significant differences ($p>0.05$). Beta diversity based on unweighted UniFrac distance metrics is represented in Figure 2, which shows no clustering according to wastewater treatment on which FLA were isolated. This was confirmed by the ANOSIM test, which confirmed that there were no significant differences ($p>0.05$) among these two groups.

The most abundant phyla of the bacterial microbiome of FLA were Proteobacteria (40.25%), Planctomycetes (17.71%), Bacteroidetes (15.02%) and Firmicutes (10.79%), which represented 83.77% of the total bacterial FLA microbiome (Figure 3). The same phyla were the most abundant in wastewater samples taken after secondary treatment, although in different proportions. In the case of wastewater samples taken after the tertiary disinfection treatment, the most abundant phyla were Proteobacteria, Planctomycetes, Bacteroidetes, and Verrucomicrobia (Supplementary Table 3).

The most abundant classes of the bacterial FLA microbiome were Gammaproteobacteria (22.31%), Planctomycetacia (16.78%), Alphaproteobacteria (15.80%), Bacteroidia (14.89%) and Bacilli (9.64%), representing 79.42% of the total classes of the bacterial microbiome. The same classes were the most abundant in wastewater samples taken after secondary treatment, although in different proportions. In the case of wastewater samples taken after the tertiary

disinfection treatment, the most abundant phyla were Gammaproteobacteria, Bacteroidia, Planctomycetacia, Alphaproteobacteria and Verrucomicrobiae (Supplementary Table 4).

The most abundant genera (>2%) belonging to the bacterial microbiome of FLA were *Bacillus* (8.91%), *Aeromonas* (8.87%), *Flavobacterium* (7.36%), *Isosphaera* (6.86%), *Sphingobium* (4.35%), unclassified Opitutaceae (2.69%), unclassified Isosphaeraceae (2.59%), unclassified Pirellulaceae (2.48%), unclassified Obscuribacterales (2.20%) and *Prosthecobacter* (2,15%), which represented 48.47% of the total population (Figure 4A, Supplementary Table 5). The most abundant bacteria of the FLA microbiome in wastewater samples taken after secondary treatment were *Bacillus* (13.99%), *Aeromonas* (13.79%), *Isosphaera* (7.67%), *Sphingobium* (6.72%), *Flavobacterium* (4.77%), unclassified Isosphaeraceae (3.10%), unclassified Obscuribacterales (2.64%) and unclassified Pirellulaceae (2.02%), which represented 54.70% of the total population (Figure 4B, Supplementary Table 6). In the case of the wastewater samples taken after tertiary disinfection treatment, the most abundant bacteria of the FLA microbiome were *Flavobacterium* (11.90%), *Isosphaera* (5.45%), unclassified Opitutaceae (4.40%), *Cellvibrio* (3.78%), unclassified Pirellulaceae (3.27%), *Prosthecobacter* (3.01%), *Legionella* (2.20%), *Herpetosiphon* (2.17%), *Fluviicola* (2.03%) and SC-I-84 species (2.02%), which represented 40.23% of the total population (Figure 4C, Supplementary Table 6). *Pseudomonas*, *Flavobacterium* and unclassified Burkholderiaceae were detected among all wastewater samples.

Bacteria of public health interest identified as part of bacterial FLA microbiome were *Aeromonas*, *Arcobacter*, *Campylobacter*, *Helicobacter*, *Klebsiella*, *Legionella*, *Mycobacterium*, *Pseudomonas* and *Salmonella* (Table 2). Sample 13 had the greatest amount of these bacteria (99.31%), followed by sample 6 (7.12%), sample 3 (6.46%), sample 2 (5.16%), and sample 1 (3.77%). The rest of the samples had <1% relative abundance of these bacteria.

4. Discussion

One of the methods used to obtain FLA cultures from environmental samples is to cultivate them into NNA plates seeded with *Escherichia coli*. Since this method does not allow performing 16S rRNA amplicon-based sequencing studies of intra-amoebic bacteria, because *E. coli* presence would alter the results, Delafont et al. (2013) seeded NNA plates with the yeast *Saccharomyces cerevisiae* so that it did not affect their analysis. However, in the present study NNA plates were not seeded with any type of microorganism so that FLA microbiota was not altered. It was still possible to isolate FLA because wastewater contains vast amounts of bacteria which serve as a food source for FLA.

In the present research, once FLA growth was observed, NNA plates' content was recovered and treated with a high concentration of sodium hypochlorite, which affects non-internalized bacteria but not FLA, nor internalized bacteria. PMA treatment was used to block the DNA of all non-internalized bacteria and free DNA. PMA is a DNA intercalating dye which binds to DNA of membrane damaged cells or free DNA, preventing its further amplification by PCR techniques (Fittipaldi et al., 2011). Afterwards, DNA was extracted, 16S rRNA amplicon-based sequencing was carried out using universal bacteria primers and a bioinformatics pipeline was followed to taxonomically assign sequenced bacteria. This way, the identified bacteria are very likely to be part of the FLA microbiome. However, since this technique is based on DNA, the detection of residual DNA of phagocytised and digested bacteria cannot be excluded, as suggested by other authors (Delafont et al., 2013; Moreno-Mesonero et al., 2020).

FLA were found in wastewater samples both after secondary and tertiary treatments (Table 1). In Spain, there are several studies in which the presence of FLA in wastewater samples has been investigated. Moreno-Mesonero et al. (2017) isolated FLA from 79.7% of wastewater samples (75.6% after secondary treatment and 87.5% after tertiary treatment). García et al. (2011) and

Moreno-Mesonero et al. (2019) isolated FLA from 100% and 66.7% of WWTP effluent samples, respectively.

In four of the samplings of the current study, FLA growth was observed after wastewater secondary treatment but FLA were not detected after UV disinfection tertiary treatment, which would indicate that, in these cases, WWTP disinfection treatment had been effective against FLA. However, in three of the samplings, FLA growth was not affected by the disinfection process, since FLA were cultured before and after it. Moreover, in one of the samplings, FLA growth was only observed after the disinfection process, thus suggesting the existence of a FLA reservoir in the tertiary disinfection treatment tank. In a previous study carried out by Magnet et al. (2013), *Acanthamoeba* spp. was detected by culture and qPCR techniques in wastewater and drinking water samples in both, raw and treated water. These results are in agreement with ours and show that the usual processes of treatment of wastewater are not always effective against FLA. Moreover, Magnet et al. (2013) detected *Acanthamoeba* spp. in treated water but not in the raw water from the same sampling, as it occurs in our study. Thus, this seems to suggest that FLA presence in water treatment plant effluents could be due, not only to their treatment resistance, but also to the fact that they can survive and grow in water treatment plant pipes, probably protected in biofilms (Thomas and Ashbolt, 2011).

The blocking of non-internalized bacterial DNA using PMA after a sodium hypochlorite at a high concentration treatment allowed for detecting only internalized bacteria into FLA. Moreover, the amplicon-based sequencing protocol along with the primers used permitted the detection of a broad range of bacteria. However, as commented above, since this technique is based on DNA amplification, in this case from inside FLA, the detection of DNA from digested bacteria cannot be excluded (Delafont et al., 2013).

To our knowledge, there are only two previous works in which the complete FLA microbiome is studied. Delafont et al. (2013) determined the microbiome of FLA isolated from drinking water

samples, and recently, our team provided the microbiome of FLA isolated from vegetables (Moreno-Mesonero et al., 2020). In both of these studies, most of the bacteria belonged to the phyla Proteobacteria and Bacteroidetes. In the current work, Proteobacteria was the highest abundant phylum and Bacteroidetes was the phylum with the third-highest abundance (Figure 3). Moreover, Proteobacteria was the most abundant phyla in WWTPs, followed by Bacteroidetes, which was the second or third most abundant phyla (Zhang et al., 2011; Liu et al., 2016). Planctomycetes phylum had the second-highest abundance in the current study and was only slightly higher than Bacteroidetes. A great part of the abundance of Planctomycetes was due to the genus *Isosphaera*, which has been previously found in a municipal WWTP (Chouari et al., 2003).

It is noteworthy that Gammaproteobacteria was the class with the highest abundance in the average of all samples, since this class contains an important number of relevant pathogenic bacteria. In several studies, in which the bacterial population was determined in WWTPs, the most common bacterial class was Betaproteobacteria (Zhang et al., 2011; Liu et al., 2016). However, in the current work, no sequence was classified into the Betaproteobacteria class. The fact that the Gammaproteobacteria class was dominant may be due to its great resistance to stress conditions, including disinfection processes (Li et al., 2017).

It is well known that FLA act as reservoirs of some pathogenic bacteria, and most of them are included in the Gammaproteobacteria class. The genera *Bacillus* and *Aeromonas* were the most abundant (Figure 4A). However, this is because the former had a relative abundance of 97.79% in sample 15 and the latter, a relative abundance of 96.15% in sample 13, which greatly increased their average relative abundance values (Supplementary Table 5). The third most abundant genus, *Flavobacterium,* was also detected by our group as part of the bacterial microbiome of FLA isolated from vegetables (Moreno-Mesonero et al., 2020). The genera that were detected among all sampling sites in Delafont et al. (2013) study (*Sphingomonas*,

Bradyrhizobium, *Afipia* and *Escherichia*), were also detected in the current study, although not in all the samples and with very low relative abundances.

Pseudomonas, *Flavobacterium* and unclassified Burkholderiaceae were detected among all the wastewater samples in the current study. Among them, only *Pseudomonas* was previously detected as part of the FLA bacterial microbiome by amplicon-based sequencing in both Delafont et al. (2013) and Moreno-Mesonero et al. (2020) studies. *Flavobacterium* was only detected in Moreno-Mesonero et al. (2020) study. Both of these genera, *Pseudomonas* and *Flavobacterium,* are waterborne bacteria, which seem to be resistant to water disinfection treatments since they have been frequently detected in the water distribution system (AWWA 2006; Bertelli et al., 2018). One of their resistant strategies could be to remain protected by FLA, as it seems to occur in the present study.

All of the bacteria of public health significance detected as part of the bacterial microbiome in our study have been previously identified as ARBs for their ability to internalize and survive within FLA, either *in vitro* and/or in environmental samples (Tezcan-Merdol et al., 2004; Bui et al., 2012; Yousuf et al., 2013; Villanueva et al., 2016; Moreno-Mesonero et al., 2017; Moreno et al., 2019; White et al., 2010; Maschio et al., 2015b). Moreover, all of them, except for *Campylobacter*, have been previously detected by the amplicon-based sequencing approach as part of the microbiome of FLA in environmental samples (Delafont et al., 2013; Moreno-Mesonero et al., 2020).

Aeromonas is known to cause a wide number of infections in both humans and animals. They are mostly associated with chronic diarrheas in children, elderly and immunocompromised individuals, acute diarrheas in immunocompetent adults and travelers' diarrhea (Batra et al., 2016). *Arcobacter* contains human pathogenic species which have been linked to enteritis, diarrhea and occasionally to bacteremia (Ferreira et al., 2017). *Campylobacter* causes disease in humans after the consumption of contaminated poultry products, raw milk or water, which

results in acute gastrointestinal illness (Young et al., 2007). Among *Helicobacter* genus, *H. pylori* is the species with the greatest relevance, since it is recognized by the WHO as a type I human carcinogen and can cause chronic gastritis, peptic ulcer, gastric lymphoma and gastric cancer (Marshall, 2002). Among the *Legionella* genus, there are human pathogenic species that are opportunistic and cause legionellosis in mainly immunocompromised individuals (Gomez-Valero et al., 2014). *Mycobacterium* genus is quite diverse, but it contains species which can cause human illnesses with great mortality and morbidity, such as mycobacteriosis, leprosy or tuberculosis. Within the *Pseudomonas* genus, *P. aeruginosa* is the most important pathogen. It is an opportunistic human pathogen that causes nosocomial infections, most of which are associated with immunocompromised hosts (Lyczak et al., 2000). Finally, *Salmonella* pathogenic species cause enteric (typhoid) fever and gastroenteritis (Miller and Pegues, 2000).

As commented above, *Aeromonas* has been found to be almost the only bacteria of the microbiome of FLA isolated from sample 13. Apart from this specific case, the rest of the pathogenic bacteria relative abundances were much lower (Table 2). It could have been of much interest to determine which species of these important genera were present in the FLA isolated from wastewater samples. However, in our study, the amplicon-based sequencing technique was not able to provide this information except for *Pseudomonas*. Among all *Pseudomonas* spp. OTUs, only two of them were assigned to *P. aeruginosa* and *Pseudomonas putida*, respectively. Therefore, to determine whether potentially pathogenic species, which moreover are normally in low abundances, are part of the bacterial microbiome of FLA, other techniques such as qPCR or FISH should be applied instead (Moreno-Mesonero et al., 2020). Nevertheless, the results obtained in this work provide an insight into the bacterial genera which are part of the FLA microbiome, in this case from FLA isolated from wastewater samples.

As indicated by the Good's coverage, the sequencing depth of the analysis had high coverage of each sample community, which was further verified by the rarefaction curves (Supplementary

Figure 1). Bacterial diversity and richness of each sample were measured by the alpha diversity indices Chao1 and Shannon and Simpson, respectively (Figure 1). There were no significant differences in FLA bacterial microbiome diversity and richness among wastewater treatments. However, although different bacterial communities associated with FLA were identified in each sample, those differences seem to be random, and, as indicated above, are not related to wastewater treatment. More samples should be analyzed in order to establish whether FLA bacterial microbiome has a relationship with any samples' characteristic or not. The extent to which two or more communities differ was measured by beta diversity. As revealed by ANOSIM, there were no significant differences in FLA bacterial microbiome, regarding the wastewater treatment from which FLA were isolated. However, a cluster of samples of both secondary and tertiary treatments clustered together, indicating that their bacterial composition was similar (Figure 2). This altogether indicates that, in our study, there is no correlation between the microbiome of FLA and the extent of wastewater treatment, thus indicating that FLA may have a preference for certain bacteria, regardless of whether or not the UV disinfection treatment was carried out.

This work provides the first study of bacterial microbiome of FLA isolated from wastewater samples. The amplicon-based sequencing technique used was useful to determine the bacterial biodiversity among FLA internalized microbiome. Among this bacterial biodiversity, bacteria of public health concern have been detected in both wastewater samples after secondary and after UV disinfection treatments, indicating that FLA are their hosts in the environment. Moreover, FLA hosting potentially pathogenic bacteria have been detected in treated water, which is frequently used for agricultural irrigation purposes in our geographical area, due to water scarcity. Thus, in case this water reaches vegetables, it may pose a public health threat. Therefore, it would be advisable to monitor the microbial quality of wastewater also taking into account the presence of FLA.

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Table legends:

Table 1: Presence of FLA in wastewater samples

Table 2: Relative abundances (%) of bacteria of public health interest detected as part of FLA microbiome.

Figure legends:

Figure 1: Boxplots of the alpha diversity indices Chao1 (A), Shannon (B) and Simpson (C). Boxes represent the interquartile range (IQR) between the first and third quartiles (25th and 75th percentiles, respectively), and the horizontal line inside the box defines the median. Whiskers represent the lowest and highest values within 1.5 times the IQR from the first and third quartiles, respectively.

Figure 2: Two-dimensional principal coordinate analysis (PCoA) plots based on unweighted UniFrac distance matrices.

Figure 3: Relative abundances (%) of the most dominant phyla of the bacterial FLA microbiome. AV: average.

Figure 4: Relative abundances (%) of the most dominant genera of the bacterial FLA microbiome in the average of all wastewater samples (A), taken after secondary treatment (B) and after tertiary disinfection treatment (C).