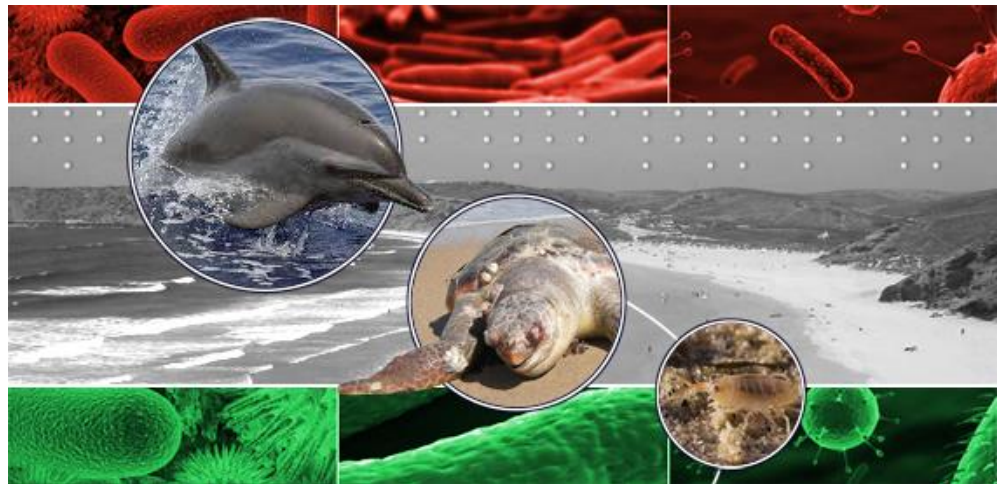
PhD Program in Ethology, Ecology, Anthropology and Biosystematics

(XXVIII Cycle)

The unseen bacterial world - Investigations on the bacterial microbiota of marine and coastal ecosystems

PhD of
Khaled Farag A. Abdelrhman



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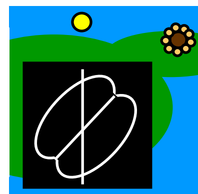
(2015)



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COORDINATORE Prof. Alberto Ugolini

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DIPARTIMENTO DI BIOLOGIA

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Declaration

Whilst registered as a candidate for the PhD degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are my own and have not been submitted for any other academic award.

November, 2015
Abdelrhman Khaled Farag A

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Thanks to my family , to my children Nora, Mayar, Manar and Farag

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Abstract

Biodiversity is a term that indicates the variety of life forms existing on Earth in different ecosystems. When refer to biodiversity, people mostly consider organisms from the Domain Eucarya, as animals, plants and fungi. The unseen world of microorganisms, despite of being the most abundant, and including organisms which play pivotal roles on biosphere functionality, has only recently being recognized as a source of biodiversity. Indeed, prokaryotic microorganisms have important roles (beneficial or harmful) in every colonized environment, from soil, to rocks, to water and to the host-associated environments. In particular, host-associated prokaryotes (mainly Bacteria) have stirred the attention of several investigators, due to their profound impact on the physiology, health status and growth of their host (e.g. plant and animals). Cultivation of microorganisms has only allowed defining no more than 2% of the total microorganism's biodiversity. The recent cultivation-independent techniques, based on the analysis of DNA directly extracted from the microbial community, have disclosed the huge microbial diversity present, especially with the recent advance in the development of metagenomic approaches.

Among the still poorly characterized environments are coastal ecosystems which, tough constituting a large fraction of terrestrial environments, with important processes taking place, have received little attention in relation to the microbial biodiversity.

The overall aims of this thesis have been related to shedding light on the microbial biodiversity of coastal ecosystems focusing on both free-living and on host associated microbial communities. Free-living communities were investigated as supralittoral sandy sediments microbiota, while host-associated communities were related to supralittoral detritivors (talitrid amphipods) and sea turtles.

Obtained results demonstrated for the first time the effect of environmental factors on sandy beaches microbiota and showed the impact of foraging behavior on amphipod guts microbiota. Finally, we reported for the first time the gut microbiota composition of the sea turtle *Caretta caretta*.

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Abbreviations

amoA	ammonia monooxygenase subunit A
ANI	Average Nucleotide Identity
ANOVA	ANalysis Of Variance
C	Conserved region of 16Sr RNA
CCA	Canonical-Correlation Analysis
Cond	Conductivity
df	Degree of freedom
dS/m	DeciSiemens per meter
dsrA	dissimilatory sulfite reductase alpha subunit
GH48	Glycosyl Hydrolase family48 genes
IJSB	International Joournal of Systematic Bacteriology
LOI	Lose –On.Ignition
P	Calculated probability
MCL	Maximum Concentration Level
µL	Microliters
NGS	Next-Generation Sequencing
OTU	Operational Taxonomic Unit
PAST	PAleontological STatistics
PCR	Ploymerase Chain Reaction
PSC	Phylogenetic Species Concept
qPCR	quantitative polymerase chain reaction

TN	Total Nitrogen
T-RFLP	Terminal Restriction Fragment Length Polymorphism
T-RFs	Terminal Restriction Fragments
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
USC	Universal Species Concept
V	Varilable region of 16Sr RNA

CHAPTER I

INTRODUCTION

Chapter 1. Introduction

1.1 Background - The microbiota and the microbiome.

The two Kingdoms classification recognized the Animal and the Plant kingdoms. then with Robert Whittaker`s five kingdom taxonomic classification of the biota. Microorganisms were initially placed in the Plant Kingdom, since then the term "Flora" was used to define and assemblage (a community) of microorganisms. Until the bacteria were removed from the plant kingdom the term "Flora" has now been changed to "Microbiota" (the microbial inhabitants of a certain location).

For **microbiota** we define the assemblage of microorganisms present in a defined environment. This bacterial census is now established using molecular methods relying on the analysis of 16S rRNA gene sequences amplified from a given environment. In the last years, a new term has been introduced, the **microbiome**, which refers to the entire habitat, including the microorganisms, their genomes (i.e., genes) and the surrounding environmental conditions. This definition is based on that of "biome", the biotic and abiotic factors of a given environments. (Jacques Ravel. 2013(Rosenberg & Zilber-Rosenberg, 2013), (Figure1.1).

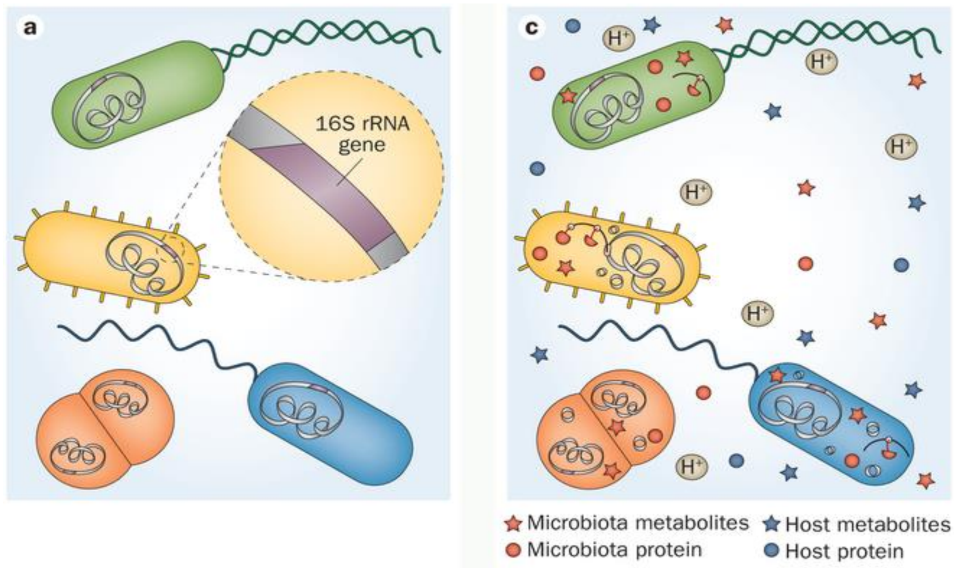

Figure 0.1 Definition of microbiota and microbioma (Samantha A. Whiteside 2015). Each image represents the same population; however, different approaches to define the population provide different information. a | Microbiota: 16S rRNA surveys are used to taxonomically identify the microorganisms in the environment b | Microbiome: the genes and genomes of the microbiota, as well as the products of the microbiota and the host environment. Abbreviation: rRNA, ribosomal RNA.

1.1.1 Species concepts in prokaryotes

The concept of biological diversity implies consensus on the discrete nature of independent species and on the mechanisms that generate speciation. The recognition of differences and similarities among the discrete features of microorganisms is more challenging and less well understood than for large multicellular organisms (Ogunseitan, 2008). Species have been and still represent a controversial issue for biologist of all disciplines. Particularly critical is the prokaryotes species concept, and several definitions have been proposed.

The original species concepts was based on morphological traits, improved later (Rossello-Mora & Amann, 2001), with the development of sequencing technology. The 1990s brought DNA, RNA, and protein sequencing to the fore, and they soon were adapted for use in phylogenetic analysis, as bacterial and archaeal species are defined on the basis of phenotypic properties and whole-genome DNA-DNA hybridization. Each species must have unique phenotypic properties and exhibit more than 70% DNA hybridization among strains. More recently, Konstantinidis and Tiedje (2005) suggested another measure, average nucleotide identity (ANI) as determined with shared orthologous genes. An ANI value of 95% corresponds roughly to traditionally define bacterial species (or the 70% DNA hybridization value).

Then, the highly conserved 16S rRNA gene became the primary macromolecule for phylogeny because of its fidelity in deducing the relatedness of Bacteria and Archaea at both high and low taxonomic levels. In particular, the operative definition of species considered a taxonomic unit threshold of 97% 16S rRNA gene sequence similarity. Strains having 16S rRNA gene sequence similarity less than 97% are considered separate species .. (Staley, 2009). (Figure 1.1.2).

However, whatever species definition is adopted, as Gevers et al. (2006) lament, “any effort to produce a robust species definition is hindered by the lack of a solid theoretical basis explaining the effect of biological processes on *cohesion* within and divergence between species.”

In an attempt to amend the phylogenetic species concept James T. Staley (2009) has included genomic analyses, and referred to as the phylogenomic species concept (PSC). The PCS suggests that genomes provide taxonomists not only with extensive phylogenetic information but also with other genomic information, such as synteny, as well as hybridization and gene expression analyses that enable further comparison among different strains. The strengths of the PSC are that it implies the evolutionary history of an organism through sequence and genomic analyses of its macromolecules, it is practical to apply, the sequences are archival, and the sequence information can be readily distributed and shared with others. Perhaps most importantly, because it can be

applied not only to microorganisms, but to all other organisms, it has been recommended as a Universal Species Concept (USC). As Bacteriologists already use PSC to identify clusters of strains of Bacteria and Archaea (Staley, 2009)..(Doolittle & Zhaxybayeva, 2009)

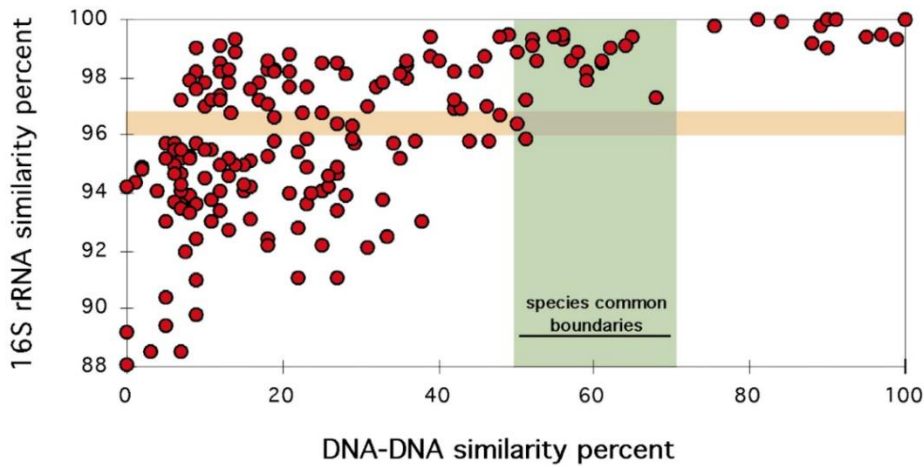

Figure 1.1.2 Comparison of DNA^DNA and 16S rRNA similarities.
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1.1.3 Diversity of prokaryotes

Biodiversity is essential for the health of our planet. Humans have long been fascinated by the extraordinary diversity of life on Earth. Not only is the sheer diversity of living creatures intriguing, but there are also striking patterns in their distribution over space and time. However, most of what we know about the origin, maintenance and distribution of biodiversity stems from research on plants and animals. Although there may be millions of prokaryotic species, researcher are only beginning to investigate patterns in their diversity and the forces that govern these patterns (Ward *et al.*, 1998; Tunlid, 1999).

The prokaryotes are by far the most abundant and the most diverse organisms, both metabolically and phylogenetically (Figure 1.1.3). Many of the most abundant prokaryotes in nature have not yet been brought into culture. Indeed, the first evidence that not all bacteria from a given environment will grow on laboratory media came from microscopy and was given the name "The Great Plate Count Anomaly". The magnitude of the anomaly varied by environment but could reach several orders of magnitude (Staley and Konopka 1985). Then, later on, with the advent of PCR-based methods and especially with 16S rRNA gene amplification and sequencing, it became clear that a large panoply of strains and taxa were not isolated on plates, but they existed as living prokaryotic cells in the so-called "unculturable state" (Stewart 2012).

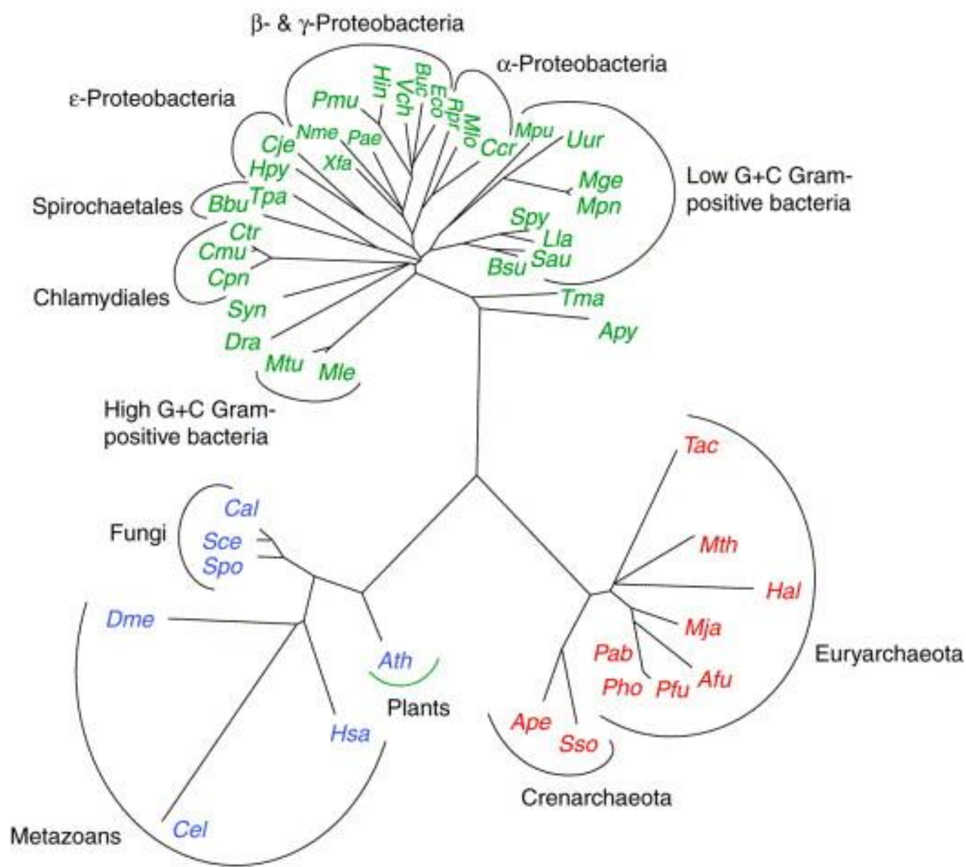


Figure 1.1.3 Evolutionary tree showing the large phylogenetic diversity of prokaryotic diversity. Bacteria are colored green, eukaryotes blue, and archaea red. Tree based on small subunit rRNA genes (16S and 18S). From Korbel *et al.*, *2002*

The exploration of this hidden prokaryotic diversity led to the discovery of entire new phyla, whose member has been never observed on plates (Figure 1.1.4).

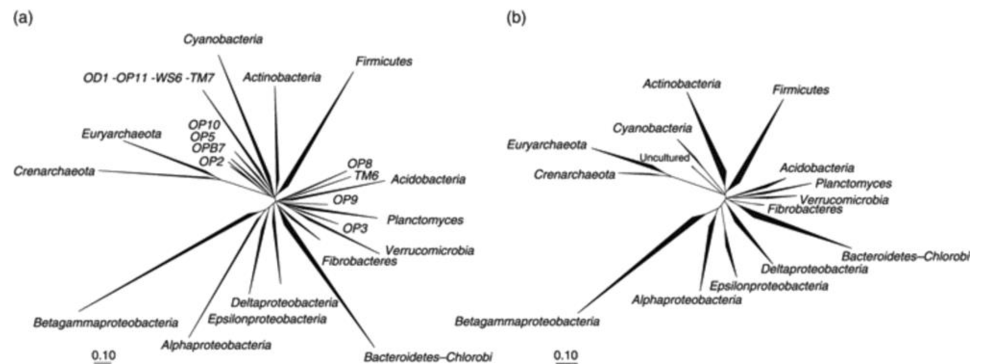


Figure 1.1.4 Phylogenetic relationship between selected prokaryotic groups represented by 16S rRNA gene sequences of total (culturable and uncultured) bacteria (a) and of culturable-type strains only (b). From Rocha *et al*., 2009

Despite the ecological importance of bacteria, past practical and theoretical constraints have limited the ability to document patterns of bacterial diversity and to understand the processes that determine these patterns (M. Claire Horner-Devine *et al.*, 2004). However, the development of high-throughput sequencing technologies, on the basis of small–subunit rRNA genes characterization of whole communities and present-day metagenomic approaches have transformed our capacity to investigate the composition and dynamics of the microbial communities that populate diverse habitats (Waldor *et al.*, 2015); (Curtis *et al.*, 2002); (Oren, 2004). In particular the use of massive sequencing technologies has allowed the microbiome composition to be described both in taxonomic and functional terms (with functional genes characterization).

1.2 The hologenome and host microbe-interactions

The tight interaction between microbes and a vast variety of ecosystems, especially with animals and plant has a long evolutionary history (Stilling *et al.*, 2014). Microbes colonize the internal and external surfaces of multicellular eukaryotes that come in contact with the external environment (leave, vessels, skin, gut, etc..). Such tight interaction is so highly relevant for host physiology that in several cases microbes and their host cannot be considered as independent units, but as a whole, the Holobiont (Bordenstein & Theis, 2015).

Recent metagenomic analyses revealed that the amount of the genetic information harbored by human microbiota exceed by ten folds that of its host. This led to prioritize the importance of microbial activities and genes, and the concept of "Hologenome" was introduced as a way to consider the host genome and microbiome (acting as one unique biological entity. This concept can enhance our vision of the role and interaction between microbes and hosts they associated with. Host and its microbiome can be transmitted across generations (even if not vertically), and thus propagate the unique properties and the holobiont and of the host the species (Rosenberg & Zilber-Rosenberg, 2013).

Figue 1.2.1 summarize the milestones towards a new vision for the central importance of symbiotic interactions as being fundamental to all aspects of animal biology (Gilbert *et al.*, 2015)**.**

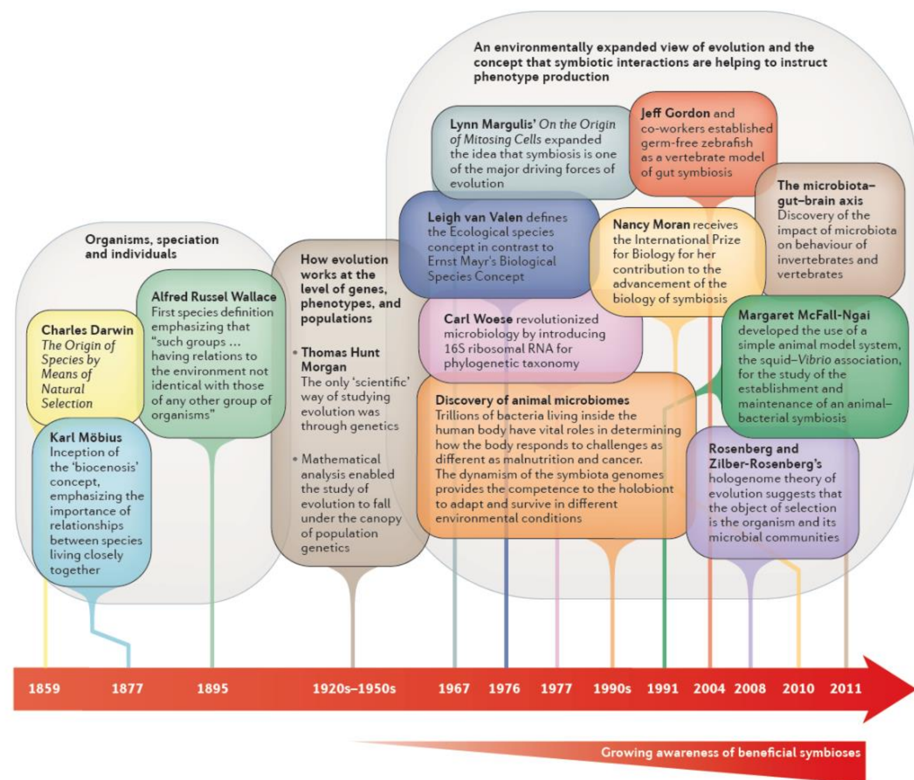


Figure 1.2.1 Milestones towards a new vision for the central importance of symbiotic interactions as being fundamental to all aspects of animal biology. This vision is especially important in evolution, if phenotype is seen to be a product of the animal genome, the symbiont (and their genomes), and the abiotic environment. From (Gilbert *et al.*, 2015).

1.2.1 Bacteria and bacterial communities in animal's gut

Immediately after birth, and having their first contact with their environment animals acquire bacteria and other microorganisms which associate with the whole external (e.g. skin) and internal (e.g. digestive trac) animal body surface (Kostic *et al.*, 2103).

The bacteria inhabiting the gastrointestinal tract of animals enhance their host metabolic potential, and helping their host to survive in different environment. Allowing herbivorous animals to digest cellulose providing variety of cellulolytic enzymes, gut bacteria often promote nutritional provisioning and nitrogen recycling for their hosts (Sullam *et al.*, 2012; Nelson *et al.*, 2013). This microbiota impacts almost every biological aspect of the

animal, from growth, to health status, to even behavior (Newell & Douglas, 2014).

1.2.2 Role of bacterial communities in animal's trophism and health

In host –microbiota relationship most commensal and pathogenic bacteria show predilection for certain hosts tissue and cells for growth. Usually due to properties of both host and the bacterium, specific bacteria colonize specific tissues by one of two mechanisms.

- Tropism is the bacterial selectivity for certain specific tissue which explained that the host provide essential nutrients and suitable environment and growth factors, oxygen level, pH, and temperature.
- Specific adherence is another mechanism believed to be due to bacterial adhesins with different receptors binding properties allowing bacteria to colonize a specific sites or tissue in a specific mode that involves complementary chemical interactions between the two surfaces (figure 1.2.2).
- Bacterial surface adhesions and host cell receptors. Bacterial component that provide adhesions are a molecular parts of their capsules, fimbraie or cell walls..

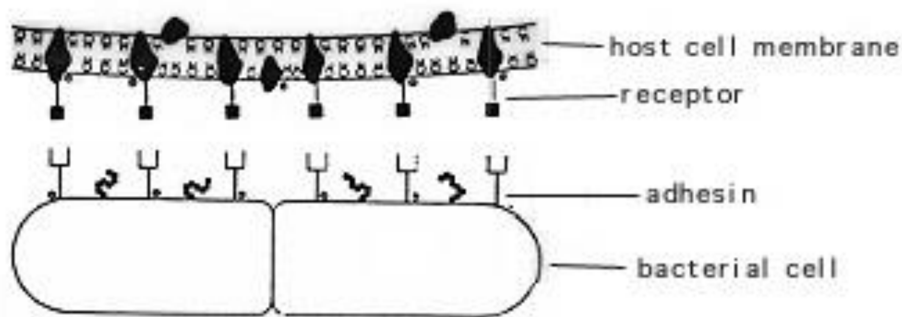


Figure 1.2.2 Specific adherence involves complementary chemical interactions between the host cell or tissue surface and the bacterial surface. In the language of medical microbiologist, a bacterial "adhesin" attaches to a host "receptor" so that the bacterium "docks" itself on the host surface. The adhesins of bacterial cells are chemical components of capsules, cell walls, pili or fimbriae. The host receptors are usually glycoproteins located on the cell membrane or tissue surface. From (Todar 2006).

- Biofilm formation

Some indigenous bacteria are able to construct biofilm on the tissue surface, or colonize a biofilm formed by another bacterial species. Biofilm can be formed by a mixture of species, but generally only one member is responsible for maintaining the biofilm and may predominate (Normark *et al.*, 1992; Todar, 2006).

Commensals and pathogenic bacteria have different adhesion forms and different strategies of colonization or infection that broadly vary (Frommel *et al.*, 2013).

As mentioned above, interactions of animals with environmental microbes have resulted in the coordinate evolution of complex symbioses. Thus, environmental bacteria present a potential influences on animal developmental programs, by both nonspecific influences of bacteria as a critical constituents of the environment, and as specific influences of the bacterial cells that have coevolved with animals (McFall-Ngai, 2002). Gut microbiota and its metabolites have an important role in host physiology. Different environmental factors can profoundly affect the gut bacterial community, thereby changing the gut microbiome activity, which may result in production of bioactive metabolites, which could be health promoting or disease-causing metabolites. Furthermore, many important immune and metabolic disorders, including diabetes, obesity, behavioral disorders and chronic inflammation, are now known to be in part due to the imbalance of interactions between the host and microbiota or metabolites. (Lee & Hase, 2014).

1.3 The revolution of metagenomics

Historically, the study of microbes has predominantly focused on single species in pure laboratory culture. Only recently tools have become available to study microbes in the complex communities where they actually live and thus to begin to understand what they are capable of and how they work. Metagenomics provides a new way of examining the microbial world that not only is transforming microbiology but has the potential to revolutionize understanding of the entire living world. In metagenomics, the power of genomic analysis is applied to entire communities of microbes, bypassing the need to isolate and culture individual bacterial community members. The new approach and its attendant technologies is bringing to light the myriad capabilities of microbial communities that drive the planet's energy and nutrient cycles, maintain the health of its inhabitants, and shape the evolution of life. Metagenomics combines the power of genomics, bioinformatics, and systems biology. (Handelsman *et al.*, 2007)

Metagenomics enables the genomic study of uncultured microorganisms. Faster, cheaper sequencing technologies and the ability to sequence uncultured microbes sampled directly from their habitats are expanding and transforming our view of the microbial world. Distilling meaningful information from the millions of new genomic sequences presents a serious challenge to bioinformaticians. In cultured microbes, the genomic data come from a single clone, making sequence assembly and annotation tractable. In metagenomics, the data come from heterogeneous microbial communities, sometimes containing more than 10,000 species, with the sequence data being noisy and partial. From sampling, to assembly, to gene calling and function prediction, bioinformatics faces new demands in interpreting voluminous, noisy, and often partial sequence data. Although metagenomics is a relative newcomer to science, the past few years have seen an explosion in computational methods applied to metagenomic-based research, (Wooley *et al.*, 2010).

Metagenomic first began to appear in the early 1990s, The first use of the term "metagenome" (of which we aware) was in 1998 by Handelsman and colleagues, who viewed the "collective genomes" of soil microflora. The earliest exercises in sequence-driven metagenomics were undertaken simply for the purpose of assembling complete genomes of uncultivable prokaryotes, using environmental DNA fragments cloned in bacteria artificial chromosomes. the recognition and "omic" characterization of biological entities more inclusive than genomes, organisms, or even species— loosely, communities—seem quite solidly integrated into the practice and developing theory of metagenomics as a discipline, it's very ethos. Indeed, the terms "community genomics," "ecogenomics," or "environmental genomics" are sometimes used as synonyms for "metagenomics," although these former also accommodate whole-genome approaches (Schleper *et al.*, 1998; Doolittle & Zhaxybayeva, 2010).

The metagenomics approach is now possible because of the availability of inexpensive, high-throughput DNA sequencing and the advanced computing capabilities needed to make sense of the millions of random sequences obtained from the extracted metagenomic DNA (Handelsman *et al.*, 2007).

1.3.1 The pre-genomics method of microbial community characterization and the culturability problem

A strong increase in knowledge in the field of microbial physiology and genetics happened during 1960s to mid-1980s wherein some scientists came to believe that cultured microorganisms did not represent the whole microbial world. From then on, several independent studies supported the rise of this uncultured world of microbes (Neelakanta & Sultana, 2013).

Since 1870, cultivation has been the usual approach bacterial identification and microbiological studies. In microbiology isolation and identification of strains constituted the obligate preliminary step of any basic or applied research work. (Uruburu, 2003)).

Most of the bacterial species are still unknown. Consequently, our knowledge about bacterial ecology is poor, bacterial identification is a growing field of interest within microbiology(Busse *et al.*, 1996). Microbiology, from its beginning, faced a lot of difficulties that hamper the correct identification of newly discovered bacteria, and until recently, investigators had no idea how accurately cultivated microorganisms represented the overall microbial diversity. As the cultivation-dependent approach is limited by the fact over 99.8% of the microbes in some environments cannot be cultured. (Å tursa *et al.*, 2009; Shah *et al.*, 2011; Neelakanta & Sultana, 2013). Nevertheless, most definitive microbiological studies have been conducted in laboratories using pure cultures. Such studies have been critical to the development of the microbiological science, and provide the basis for our understanding of the microbial world.

However, the microbial species and interactions that really count in Nature do not occur in pure culture. In fact, most naturally occurring microbes exist in complex communities, and have never before been cultivated or characterized in the laboratory. (DeLong, 2002). So, there has been a limit to the extent of the real biodiversity that was previously accessible in this way, as only a small proportion of environmental microorganisms are isolated in culture in any given media (Leung *et al.*, 2011).

Moreover achieving culture conditions for isolating a single member from a consortium of microbial population would be a very difficult.

The pre-genomics is the use of culture-independent techniques that involve the assay of nucleic acids (DNA or RNA) to investigate microbial communities. A pre-genomic tool targets a small portion of the genome, such as one gene or a specific intergenic DNA region. Pre-genomic techniques can be used with or without full genomic information of the microorganism in question. Pre-genomic tools have proven to be important for assessing diversity in communities and are often essential for the identification of functionally important members of uncharacterized communities; in this case, an important member can then be selected for future genome-sequencing efforts. The most widely used targets for pre-genomic tools were ribosomal RNA (rRNA) and the

corresponding ribosomal DNA (rDNA) genes. Both targets are widely useful, because all independently living organisms possess rRNA, the sequence of which is highly conserved (Rittmann *et al.*, 2008).

1.3.2 The metagenomics approach and 16S rRNA metagenomics

Metagenomics is the study of microbial communities sampled directly from their natural environment, without prior culturing. Metagenomic studies can be grouped into four categories based on different screening methods:

(a) Shotgun analysis using mass genome sequencing.

(b) Genomic activity-driven studies designed to search for specific microbial functions.

(c) Genomic sequence studies using phylogenetic or functional gene expression analysis.

(d) Next generation sequencing technologies for determining whole gene content in environmental samples.

(Riesenfeld *et al.*, 2004; Edwards *et al.*, 2006; Shendure & Ji, 2008; Harismendy *et al.*, 2009; Neelakanta & Sultana, 2013) (Figure 1.3.2).

More related to technical issues, the approaches for metagenomics can be classified into two categories, targeted and untargeted metagenomics. For untargeted metagenomics is intended the sequencing of the whole environmental DNA (eDNA) extracted from a given environment, while the targeted metagenomics relies on the sequencing of a defined gene, or set of genes, after PCR amplification from the eDNA.

In particular, targeted metagenomics is often related to the 16S small subunit ribosomal RNA (rRNA) gene sequencing profiling..

NGS technologies—including 454 and Illumina sequencers—use 16S rRNA amplification primers targeting hypervariable regions, although it is still arguable which regions are best for species profiling: 16S rRNA genes contain nine hypervariable regions (V1–V9) (Figure 1.3.1) that demonstrate considerable and differential sequence diversity among different bacteria (Shah *et al.*, 2011).

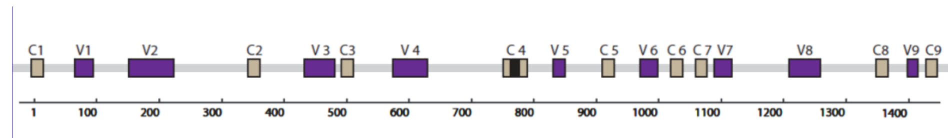
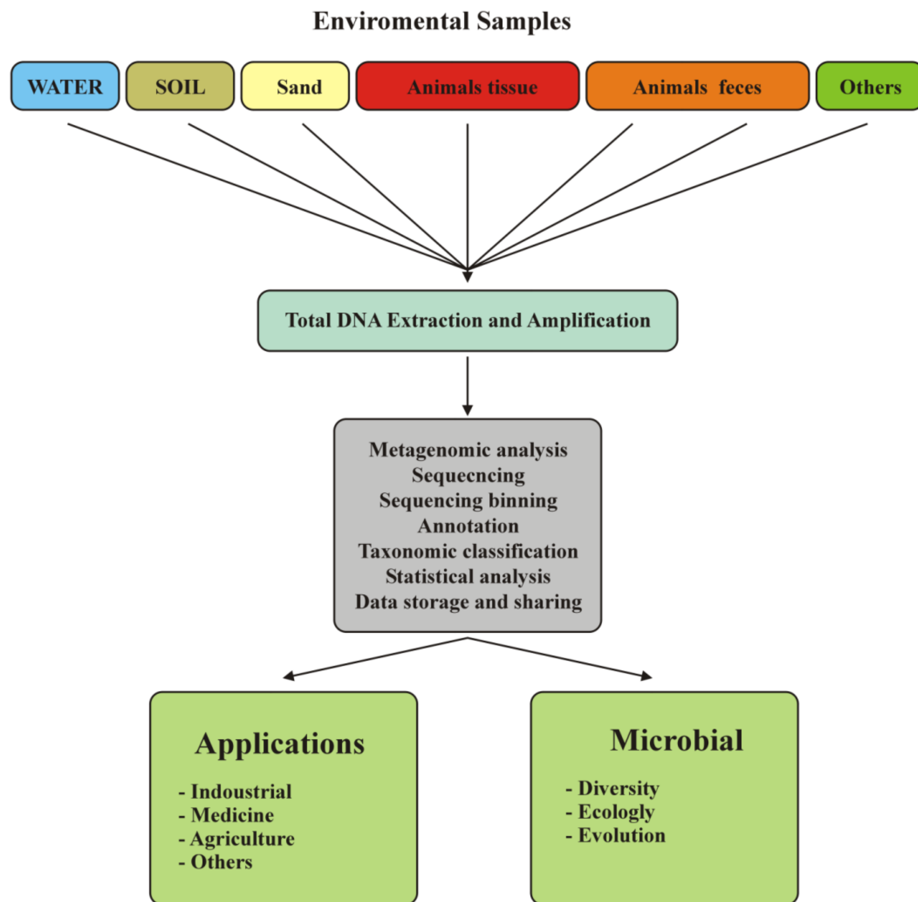


Figure 1.3.1 Schematic representation of the 16S rRNA gene. Location of variable (purple) and conserved (brown) regions in a canonical bacterial 16S rRNA gene. The black region is that invariable in all bacteria. From http://www.illumina.com/content/dam/illumina-marketing/documents/products/research_reviews/metagenomics_research_review.pdf



[i]Figure 1.3.2. Overview of metagenomic analysis.
Schematic representation of a typical metagenomic analysis is shown. Samples from various sources such as from Water, soil, Sand, Animals tissues, Feces, and other environmental samples are processed for total DNA extraction to amplify microbial sequences. The extracted DNA is then processed for metagenomic analysis that is comprised of the following steps: sequencing; sequence binning; annotation of sequences; taxonomic classification of microbial species; statistical analysis of the metagenomic data; and data storage in central metagenome databases. Some of the potential coding sequences that include but are not limited to enzymes, antibiotics, and proteases are cloned into heterologous expression vectors. The expressed proteins are later used in variety of applications. In addition, the information obtained from typical metagenomic analysis would provide substantial insights in the field of microbial diversity, ecology, and evolution. From (Neelakanta & Sultana, 2013).

CHAPTER II

AN OVERVIEW OF THE MICROBIOTA OF SUPRALITTORAL SEDIMENTS

Chapter 2. An overview of the microbiota of supralittoral sediments

2.1 Dynamics of bacterial communities in supralittoral sediments from sandy beaches in Sardinia (Western Mediterranean, Italy)

2.1.1 Abstract

Sandy beaches have an important ecological role as transition zones between land and sea, but to date have poorly been considered for their microbial communities. In particular, it is not clear the influence of environmental variables (i.e. exposure to dominant winds, physico-chemical parameters of sediments and seasonality) on bacterial community diversity. Here, we report results from an analysis of bacterial communities of sandy beaches of Sardinia, carried out with a DNA-based cultivation-independent technique, Terminal-Restriction Fragment Length Polymorphism (T-RFLP).

Results indicate that bacterial community diversity is influenced by electrical conductivity and by total nitrogen and organic matter content of sediments. Furthermore, a seasonal fluctuation was observed in biodiversity indices (Richness and Evenness indices) with an increase of diversity in late spring compared to late summer-autumn. This fluctuation mainly relies on members of the bacterial classes *Firmicutes*, *Alphaproteobacteria* and *Deltaproteobacteria*. A differential occurrence of bacterial genes related to ammonia oxidation, encoding ammonia monooxygenase (*amoA*), and sulfur reduction, encoding the dissimilatory sulfite reductase alpha subunit (*dsrA*), was also noticed.

We conclude that bacterial community of supralittoral sandy sediments are influenced by either macronutrient contribution and seasonality.

2.1.2 Introduction

Sandy beaches constitute two-thirds of the world's ice-free coastlines and are important ecological transition zones between land and sea. In sandy beaches an ecological network is present, mainly related to meio- and macrofauna (McLachlan *et al.*, 1993; Schlacher *et al.*, 2008). Although bacteria inhabiting sandy beaches may account for up to 87% of annual production in these environments (Koop & Griffiths, 1982), the microbial ecology of sandy sediments has still stirred a relatively limited attention. Most of the studies have being focused on presence of bacterial pathogens or on the ecology of submerged sediments and the impact of pollution, such as oil spills (Newton *et al.*, 2013b; Engel & Gupta, 2014; Halliday *et al.*, 2014; Whitman *et al.*, 2014; Xiong *et al.*, 2014; Bacci *et al.*, 2015c). Recently (Bacci *et al.*, 2015c), we showed that bacterial communities from supralittoral sediments harbor quite complex bacterial communities, mainly including *Alphaproteobacteria*, *Gammaproteobacteria*, *Flavobacteria* and *Actinobacteria*, but including also taxa, as *Nitrospira*, which could play role in biogeochemical cycles. Anyway, we should consider that sandy beaches are dynamic systems, constantly subjected to environmental stressors, such as tides, wave action, temperature and conductivity fluctuations, erosion by currents, human activities, etc. (McLachlan & Brown, 2006). Since bacterial communities inhabiting sandy beaches could be strongly influenced by such variables, Sardinia island is one of the areas in the Mediterranean where it is possible to find, in a relatively short geographical range, sandy beaches having contrasting features in terms of human pressure, grain size, exposure and chemical composition (Ioppolo *et al.*, 2013; De Falco *et al.*, 2014). In the present work, by using selected Sardinian sandy beaches as models, we aimed at evaluating in both a cross-sectional and a longitudinal study the impact of substrate, season and exposure of sandy beaches on the bacterial community composition.

2.1.3 Materials and Methods

2.1. 3.1 Sampling

Samples of sand (5 cm below surface) were taken in September 2012, May 2013 and October 2013 in 8 beaches in the island of Sardinia (Western Mediterranean, Italy). Six sampling sites were located in two Marine Protected Areas (MPAs) ("Penisola del Sinis e Mal di Ventre" and "Capo Carbonara"). Samples were also taken in two beaches (Poetto and Giorgino) in the coastal area of Cagliari (Figure 2.1). Except Giorgino, all the beaches are very crowded in the summer season. Samplings took place, before and at the end of recreational season to avoid sand mixture and direct contamination by human trampling. The chosen sites differ in relation to wind exposure, grain size, organic matter and total nitrogen content (Figure 2.1.1, Table 2.1.1).

Figure 2.1.1 Location of sampling sites.

2.1. 3.2 Physico-chemical parameters

The top most sediment (5 cm) was carefully removed with a plastic spoon and transferred to plastic vessels. Composite samples were made from surface sediment at each station. Samples were kept in iceboxes until returned to the laboratory. Grain size distribution was measured by wet sieving as previously described (Bacci et al. 2015).

The Loss-on-Ignition (LOI) method was used to estimate the organic carbon content which was expressed as a percentage of dry weight after heating 5.0 g of sediment to 550 °C for 4 h (Heiri et al. 2001). Total nitrogen (TN) was measured by the Kjeldhal method. Conductivity (dS/m at 25 °C) and pH, were measured on a 5:1 water extract of the sediment. Physico-chemical characteristics of sediments are reported in Table 2.1.1.

Sample code	Site name	Longitude	Latitude	% of gravels passing through 0.425mm	pH	TN g/Kg	Cond. (dS/m) a 25°C	% LOI	N. of cells/g of sand
P	Poetto	9°10'56.08" E	39°13'00.54" N	35.01	8.84	0.053	2.046	0.55	4.04 x 10^7
G1	Giorgino	9°02'21.55" E	39°10'26.31" N	94.47	8.59	0.028	1.130	0.11	1.75x 10^7
V1	Stagno Notteri	9°31' 15.32" E	39°07'05.01" N	92.05	9.17	0.045	0.579	0.61	1.43x 10^7
V2	Marina di Villasimius	9°30'20.81" E	39°06'57.88" N	35.15	8.96	0.031	0.469	0.35	2.12x 10^7
C2	S. Giovanni di Sinis (Mare Morto)	8°26'51.42" E	39°53'07.37" N	77	9.06	0.094	2.078	0.81	7.58x 10^7
C3	S. Giovanni di Sinis	8°26'11.03" E	39°52'55.72" N	31.5	9.21	0.091	0.977	0.79	1.35x 10^7
C4	Maimoni	8°24'02.36"E	39°54'54.76" N	6.5	8.94	0.53	1.515	0.24	3.94x 10^7
C5	Is Arutas	8°24'03.40"E	39°56'59.90"N	0	8.5	0.013	0.443	0.78	5.65x 10^7

Table 2.1.1 Sampling sites and physico-chemical characteristics*
* The pH, total nitrogen content (TN), the electrical conductivity (cond.) and the organic carbon (% LOI) present are reported. Reported measures are related to sampling at October 2013.

2.1.3.3 DNA extraction, T-RFLP profiling and Real-Time PCR

DNA extraction and Real-Time PCR estimation of bacterial load in sediments were performed as previously reported (Bacci *et al.*, 2015c). Terminal-Restriction Fragment Length Polymorphism (T-RFLP) was performed on 16S rRNA genes amplified from extracted DNA with primer pairs 799f and 1495r, as previously reported (Pini *et al.*, 2012). The choice of 799f primer avoid amplification of chloroplast 16S rRNA genes (Mengoni *et al.*, 2009), allowing to better target bacterial community DNA, reducing the amount of amplified DNA from algal origin and *Posidonia* origin. Purified amplification products were digested separately with restriction enzymes *MspI* and *HinfI* and digestions and resolved by capillary electrophoresis and on an ABI3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using LIZ 500 (Applied Biosystems) as size standard. T-RFLP analysis was performed on two technical PCR replicates from each DNA extract, as previously reported (Mengoni *et al.*, 2005). Only peaks present in both duplicate runs were considered for successive analyses.

2.1.3.4 Statistical analyses and processing of T-RFLP data

From T-RFLP chromatogram files a binned peak matrix was obtained after importing into PeakStudio 2.2 software (https://fodorlab.uncc.edu/software/peakstudio). Peaks above 100 fluorescence units and whose size ranged from 35 to 500 nt were considered for profile analysis. MiCA web tool (https://mica.ibest.uidaho.eudu) performed on T-RFs to interpret the taxonomic compositions (Shyu *et al.*, 2007). Statistical analyses were performed on the matrix obtained by linearly combining data from the two restriction enzymes, as previously reported (Mengoni *et al.*, 2009; Pastorelli *et al.*, 2011). Computation of diversity indices, correlations, cluster, univariate and multivariate analyses were performed with the modules present in Past 3 software (Hammer *et al.*, 2001). Differences among communities were also evaluated by the analysis of molecular variance (AMOVA, (Excoffier *et al.*, 1992)), which allow to test the molecular profiles of bacterial communities in a way similar to classical ANOVA, but taking into account the molecular information provided and performing nonparametric test (Mengoni & Bazzicalupo, 2002). AMOVA was run on Arlequin 3.0 software (Excoffier *et al.*, 2007).

2.1. 4 Results

T-RFLP profiling obtained from DNA extracted from the 8 sampling sites generated a total of 178 polymorphic TRFs, spanning from 25 to 499 nucleotides in length.

Taxonomic diversity of bacterial communities was highly variable richness ranging from 3 to 29 T-RFs, evenness from 0.155 to 0.667, Shannon index from 0.694 to 2.466 and Simpson index from 0.372 to 0.876 (Supplementary Table 2.1.S1). Chemical parameters of sediments resulted correlated with community diversity (Table 2.1.2). In particular, significant positive correlations were detected for Simpson index with electrical conductivity and LOI contents, while a significant negative correlation was detected with total nitrogen.

Additionally, Shannon index and Richness (n. of TRFs) were also positively correlated with conductivity. No correlation has been detected for grain-size, pH and bacterial load. A Canonical Correlation Analysis was conducted on T-RFLP profiles and physico-chemical variables (Figure 2.1.2). Results indicated that pH and total nitrogen content were the main variables affecting community structure, as well as that a similar effect of LOI and grain size on community structure.

Site	Sampling time	Bacterial load (n. of cells g-1 of sand)	Simpson	Shannon	Evenness	Richness
C2	Sep 2012	9.99×10^7	0.657	1.398	0.238	17
	May 2013	1.93×10^7	0.772	1.731	0.332	17
	Oct 2013	1.08×10^8	0.493	1.008	0.343	8
C3	Sep 2012	7.86×10^6	0.676	1.748	0.338	17
	May 2013	1.01×10^7	0.800	1.860	0.379	17
	Oct 2013	2.22×10^7	0.785	1.808	0.469	13
C4	Sep 2012	2.48×10^7	0.753	1.890	0.301	22
	May 2013	5.84×10^7	0.420	0.674	0.654	3
	Oct 2013	3.46×10^7	0.728	1.538	0.465	10
C5	Sep 2012	4.83×10^7	0.793	2.049	0.268	29
	May 2013	1.00×10^8	0.746	1.669	0.312	17
	Oct 2013	2.10×10^7	0.606	1.169	0.292	11
G1	Sep 2012	1.09×10^8	0.721	1.935	0.301	23
	May 2013	1.40×10^8	0.806	1.848	0.423	15
	Oct 2013	2.72×10^7	0.724	1.572	0.438	11
P	Sep 2012	5.75×10^7	0.876	2.466	0.654	18
	May 2013	3.13×10^7	0.531	1.007	0.684	4
	Oct 2013	3.20×10^7	0.729	1.570	0.437	11
V1	Sep 2012	3.18×10^7	0.358	0.845	0.155	15
	May 2013	6.75×10^6	0.774	1.765	0.308	19
	Oct 2013	3.89×10^6	0.757	1.656	0.524	10
V2	Sep 2012	2.99×10^6	0.372	0.925	0.158	16
	May 2013	5.27×10^7	0.738	1.674	0.333	16
	Oct 2013	7.52×10^6	0.393	0.694	0.667	3

Table 2.1.S1 Values of alpha diversity of bacterial communities.
* Bacterial loads are estimated by qPCR. Simpson, Shannon H, Evenness and Richness are reported. Sampling times refer to September 2012, May 2013 and October 2013

	pH	Nitrogen content	Conductibility	Organic carbon content	Granulometry
Simpson	0.073	-0.412*	0.402*	0.409*	-0.090
Richness	0.055	0.049	0.385*	0.101	0.155
Evenness	-0.20	0.151	0.029	-0.036	-0.192
Shannon	-0.016	0.266	0.498*	0.254	0.011
Bacterial load	-0.276	-0.057	0.168	-0.299	-0.071

Table 2.1.2 Spearman correlation coefficients between community diversity values and chemical parameters*
*** Spearman r correlation values are reported. Asterisks indicate significant correlations (*<0.05)**

Different grouping of sites were considered in relation to the exposure to dominant winds (see Figure 2.1.1). In particular, C3, C4 and C5 were grouped together (W-group) according to their direct exposure to west, as well as G1, P and V1 (S-E group). C2 and V2 are protected within a harbor, they are then considered in a third group (named “gulf”). Diversity of communities of the different groups was similar for all indices (data not shown). By comparing with AMOVA the community structure of W, S-E and gulf groups no statistically significant groupings according to the exposure were found (P>0.5).

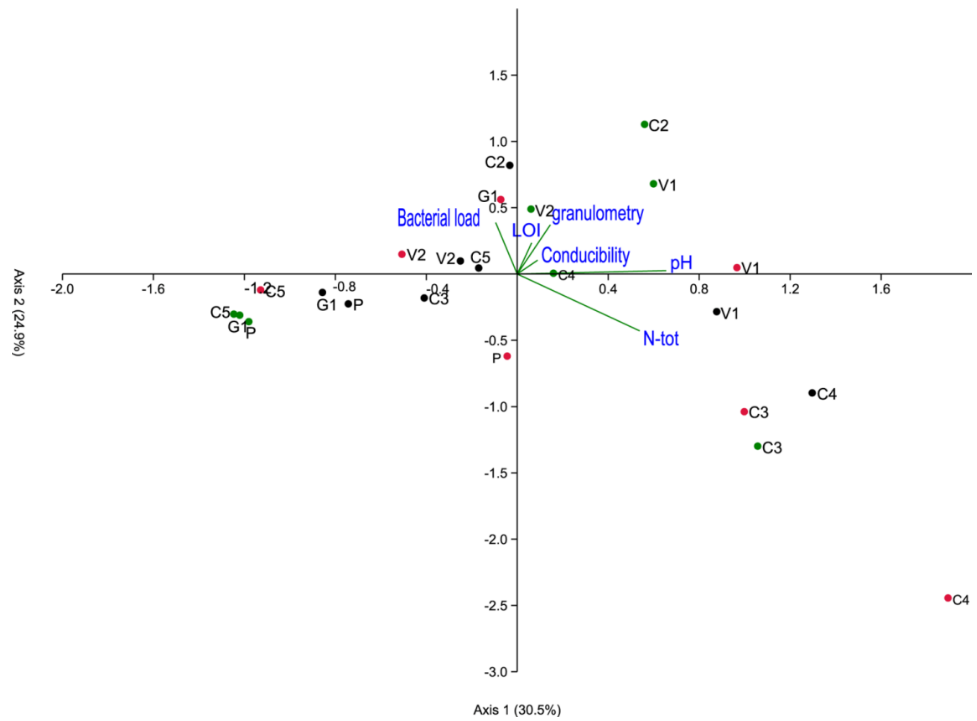


Figure 2.1.2 Canonical Correlation Analysis (CCA) of physico-chemical parameters and bacterial community T-RFLP profiles.
The percentage of variance explained by each axis is reported. Colors indicate the sampling period (red, September 2012; green, May 2013; black, October 2013).

Finally, we evaluated the possible contribution of seasonal variations on the bacterial community diversity. When samples from different seasons were analyzed, samples from September 2012 and October 2013 grouped together (Figure 2.1.3), while samples from May 2013 were scattered along PCA plot.

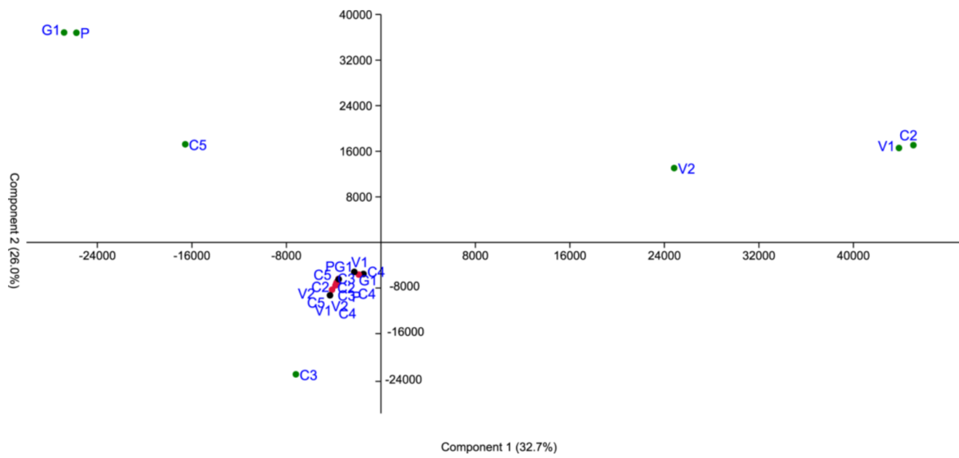


Figure 2.1.3 Principal Component Analysis of T-RFLP profiles.
The percentage of variance explained by each axis is reported. Colors indicate the sampling period (red, September 2012; green, May 2013; black, October 2013).

This result suggested the presence of a seasonal effect on community taxonomic pattern. Indeed a higher beta-diversity of samples taken in spring (May) compared to samples from autumn (September, October) was found (Supplementary Table 2.1.S2). When considering alpha-diversity indices (Evenness, Shannon, Richness and Simpson indices) also, an effect of sampling time was observed. Evenness and Richness indices showed statistically significant differences among sampling point. In particular, both indices showed the highest values in May 2013 (Figure 2.1.4).

Global beta diversities	Sep 2012	may 2013	oct 2013
Cody	142	185.5	120.5

Table 2.1.S2. Mean of Beta diversity indices between sites of each seasons

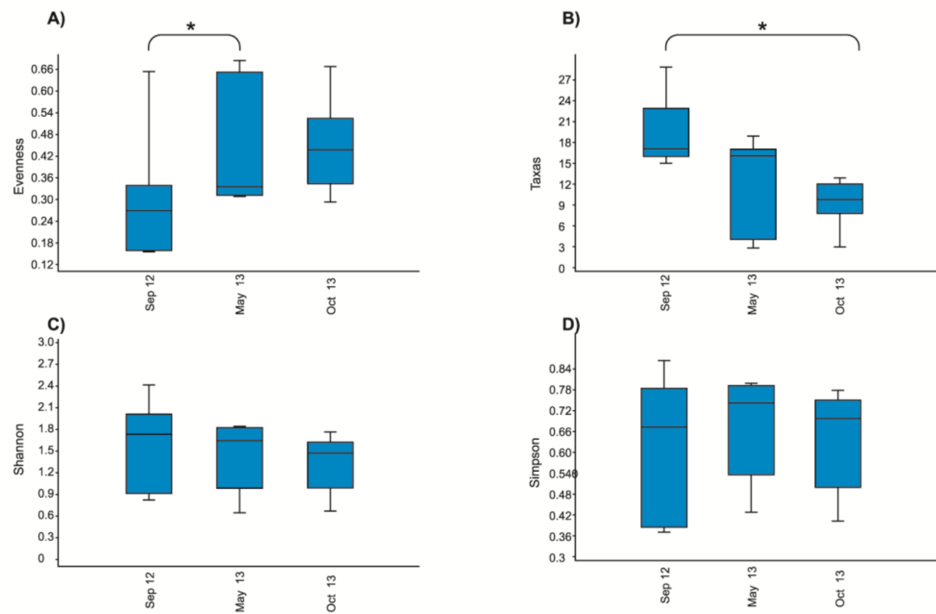


Figure 2.1.4 Box plots reporting the range of alpha diversity values of sandy beaches bacterial communities in September 2012, May 2013, October 2013.
Mean values ± standard deviation are shown. Lines indicate statistically significant pairwise comparison ($P<0.05$) differences Mann-Whitney test. A, Evenness index; B, Richness index; C) Shannon index; D), Simpson index.

To better elucidate which bacterial taxa may potentially contribute to such variation we firstly performed an extensive search with MiCA (Supplementary Table 2.1.S3), then a Principal Component Analysis on phyla composition was run (Figure 2.1.5).

Results showed that the most important contributors in differentiating May 2013 from September and October are *Bacteroidetes*, *Firmicutes*, *Aquificae*. *Protobacteria* accounted for the differentiation September vs. October. A SIMPER analysis was then conducted to estimate the amount of variance due to single TRFs occurrence in differentiating samples with respect to sampling date. Results (Table 2.1.3) indicated that two TRFs attributed *Firmicutes*, *Deltaproteobacteria* and *Aquificae* are the most important in differentiating spring sampling (May) from autumn samplings, while *Alphaproteobacteria* are important in differentiating the two autumn samplings.

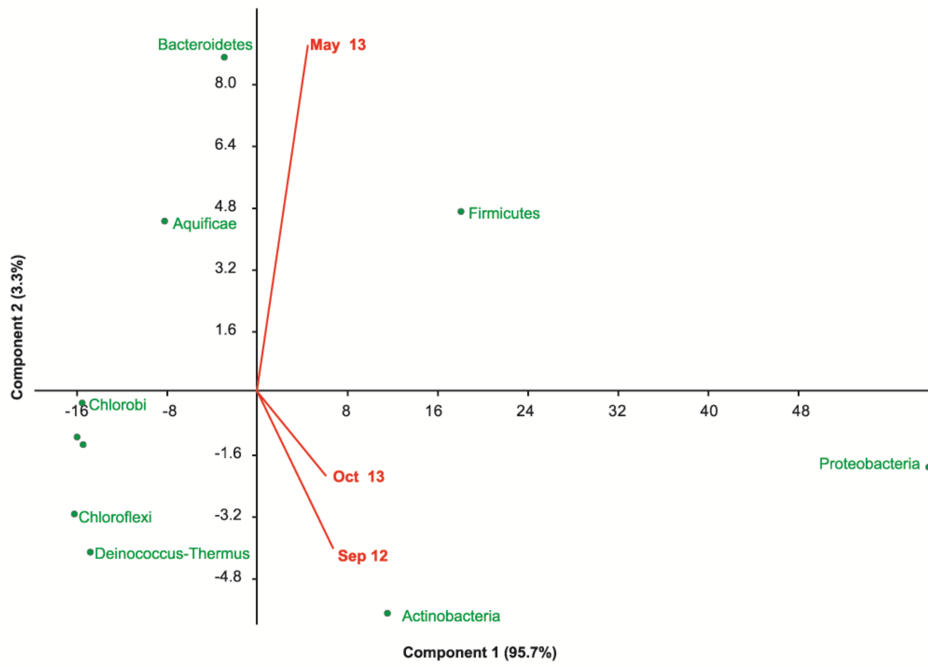


Figure 2.1.5 Principal Component Analysis of phyla composition of the sandy beach in the three different seasons.

Table 2.1.3 Results of SIMPER analysis on taxa occurrence along the three sampling seasons*.

a) May 2013 vs. October 2013

Phylum	Class	Order	Family	Genus	TRF size (in nt, binning 2 nt)	Contribution (%)	Cumulative %
Firmicutes	*Clostridia*	*Thermoanaerobacterales*	*Thermoanaerobacteraceae*	*Thermoanaerobacter*	251-253	9.65	9.65
delta proteobacterium	unclassfied	unclassfied	unclassfied	unclassfied	299-301	8.98	18.63
Aquificae	*Aquificae*	*Aquificales*	*Aquificaceae*	*Hydrogenobacter*	151-153	8.53	27.16
Proteobacteria	*Alphaproteobacteria*	*Rickettsiales*	*Rickettsiaceae*	*Rickettsia*	139-141	8.21	35.37
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	167-169	4.29	39.66
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Streptomycetaceae*	*Streptomyces*	145-147	4.22	43.89
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	149-151	3.86	47.75
Aquificae	*Aquificae*	*Aquificales*	*Hydrogenothermaceae*	*Sulfurihydrogenibium*	159-161	3.48	51.23
unclassfied	unclassfied	unclassfied	unclassfied	unclassfied	165-167	3.22	54.45
Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*	99-101	3.05	57.50
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*Burkholderia*	255-257	2.76	60.26

b) May 2013 vs. September 2012

Phylum	Class	Order	Family	Genus	TRF size (in nt, binning 2 nt)	Contribution (%)	Cumulative %
Firmicutes	*Clostridia*	*Thermoanaerobacterales*	*Thermoanaerobacteraceae*	*Thermoanaerobacter*	251-253	9.08	9.08
delta proteobacterium	unclassified	unclassified	unclassified	unclassified	299-301	8.19	17.27
Aquificae	*Aquificae*	*Aquificales*	*Aquificaceae*	*Hydrogenobacter*	151-153	6.83	24.1
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	489-491	4.87	28.98
unclassfied	unclassified	unclassified	unclassified	unclassified	491-493	4.00	32.97
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	167-169	3.91	36.88
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Streptomycetaceae*	*Streptomyces*	145-147	3.79	40.68
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Burkholderiaceae*	*Burkholderia*	255-257	3.64	44.32
unclassified	unclassified	unclassified	unclassified	unclassified	405-407	3.46	47.79
Proteobacteria	*Alphaproteobacteria*	*Rickettsiales*	*Rickettsiaceae*	*Rickettsia*	139-141	2.92	50.71
Bacteroidetes	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Flavobacterium*	455-457	2.73	53.44

c) October 2013 vs. September 2012

Phylum	Class	Order	Family	Genus	TRF size (in nt, binning 2 nt)	Contribution (%)	Cumulative %
Proteobacteria	*Alphaproteobacteria*	*Rickettsiales*	*Rickettsiaceae*	*Rickettsia*	139-141	10.21	10.21
Firmicutes	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Clostridium*	489-491	7.29	17.5
unclassfied	unclassified	unclassified	unclassified	unclassified	491-493	6.02	23.52
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	405-407	5.56	29.08
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	149-151	5.49	34.57
Aquificae	*Aquificae*	*Aquificales*	*Hydrogenothermaceae*	*Sulfurihydrogenibium*	159-161	4.92	39.5
Bacteroidetes	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Flavobacterium*	455-457	4.47	43.97
Firmicutes	*Bacilli*	*Lactobacillales*	*Lactobacillaceae*	*Lactobacillus*	99-101	4.33	48.3
Proteobacteria	*Gammaproteobacteria*	*Oceanospirillales*	*Halomonadaceae*	*Candidatus Portiera*	49-51	3.83	52.13
Bacteroidetes	*Cytophagia*	*Cytophagales*	*Cyclobacteriaceae*	*Algoriphagus*	481-483	3.47	55.6
Proteobacteria	*Gammaproteobacteria*	*Oceanospirillales*	*Halomonadaceae*	*Halomonas*	355-357	2.48	58.09

The taxonomic attribution of TRFs, the percentage of contribution and the cumulative contribution in terms of variance are reported. Bin size for TRFs is considered at 2 nt. For each TRF the classification from phylum to genus level is reported. 'Unclassified' indicates the lack of match of the TRF with records present in the Ribosomal Database after MiCA search (see materials and methods).

To evaluate the possible role of bacteria involved in the biogeochemical cycles of sulfur and nitrogen over the detected seasonal changes, genes *dsrA* encoding the sulfite reductase, and *amoA* encoding the ammonia monooxygenase gene were amplified on DNA extracted from sandy sediments (Supplemental Table 2.1.S3). Results showed the presence of *amoA* on several, but not all sites. In particular *amoA* was present in all sampling of G1 site. On the contrary *dsrA* was found only in two May 2013 samples.

Sample code	***amoA***			***dsrA***		
	September 2012	**May 2013**	**October 2013**	**September 2012**	**May 2013**	**October 2013**
P2	-	+	-	-	-	
G1	+	+	+	-	+	
V1	+	-	-	-	+	
V2	+	+	-	-	-	
C2	-	-	-	-	-	
C3	+	-	-	-	-	
C4	-	-	-	-	-	
C5	-	-	-	-	-	

Table 2.1.S1 Presence of amoA and drsA genes in sandy beaches bacterial communities

2.1.5 Discussion

Environmental variables, such as temperature, pH, and chemical composition of the sand are known to impact bacterial communities structure and diversity (Lozupone & Knight, 2007; Lauber *et al.*, 2009). Here we found that conductivity, nitrogen and LOI contents are related to bacterial community diversity, suggesting that seawater and organic matter may contribute to bacterial community trophism in the supralittoral sediments and provide a source of new taxa which populate the sandy. Indeed a positive correlation between bacterial diversity and conductivity and LOI was found, while negative correlation was found for nitrogen content. This suggests that seawater (and increased conductivity levels due to seawater evaporation on the humid sand) could be one of the primary source of diversity. A previous 16S rRNA metagenomic survey of sandy sediments in the Mediterranean island of Favignana (Egadi Archipelago, Sicily) indicated an abundance of marine *Alphaproteobacteria* in the supralittoral sediments (Bacci *et al.*, 2015c), again suggesting that marine bacterial taxa seem to strongly contribute to sandy beaches bacterial communities.

Moreover, both community taxonomic composition and diversity (alpha and beta) were related to sampling season. In particular, in spring (May samplings), higher alpha diversity values, as well as a higher heterogeneity among samples were recorded, with respect to late summer/autumn samplings (September and October). Seasonal shifts of bacterial communities have been observed in water bacterial communities, mainly in relation to estuarine and lake environments (Jones *et al.*, 2012; Fortunato *et al.*, 2013). However, to date no indication for supralittoral sediments were reported, although changing environmental conditions undoubtedly influenced community beach composition (Newton *et al.*, 2013a). Here, we indicated that a seasonal variation of bacterial community diversity is indeed present also in supralittoral sediments. This variation is mainly related to members of *Firmicutes*, *Alphaproteobacteria* and *Deltaproteobacteria*, some of which (as Alphaproteobacteria) abundant in sea water worldwide (Morris *et al.*, 2002; Rusch *et al.*, 2007), then reinforcing the hypothesis that sandy beaches bacterial community are closely related to sea water microbiome. These data do not allow attributing functionality to this variation. However, concerning *Deltaproteobacteria*, this class includes the family *Desulfobacteraceae* whose members are active in sulfite reduction. We found indeed a functional signature also in some of the May samples (as presence of the sulfite reductase gene *dsrA*). The detection of *dsrA* gene could be related to some levels of hydrocarbon contamination (Chin *et al.*, 2008), since *Desulfobacteraceae* are known to play pivotal role in alkane degradation in marine environment (Kleindienst *et al.*, 2014).

2.1.6 Acknowledgments

Data reported in this chapter have been obtained in the course of research activities supported by the Project CRP-28345 funded by Autonomous Region of Sardinia (L. R. 7/2007). We thank the crews of MPAs “Penisola del Sinis e Mal di Ventre” and “Capo Carbonara” for technical assistance on sampling .We are also grateful to B.Sc students Marta Ciaramella, Francesco De Certo and Martina Azzini for technical assistance in DNA extraction and PCR amplification.

CHAPTER III

EXPLORING TALITRID AMPHIPODS GUT MICROBIOTA

Chapter 3. Exploring talitrid amphipods gut microbiota

3.1 The gut microbiota of talitrid amphipods provides insight into the ecology of supralittoral sediments detritivors

3.1.1 Abstract

Talitrid amphipods (sandhoppers and beach fleas) are colonizers of the supralittoral zone and obtain most of their food from stranded materials, which include detrital marine angiosperms and macroalgae, as well as occasional death animals. Here, we report the characterization of gut microbiota of *Talitrus saltator*, *Talorchestia ugolinii*, *Sardorchestia pelecaniformis*, *Orchestia montagui*, collected in Sardinia (Italy). Microbiota were analyzed by metabarcoding analysis on amplified 16S rRNA V4 region and by quantification of family 48 glycosyl hydrolases genes, which are involved in cellulose degradation. Obtained results indicated the presence of a complex bacterial flora, including several members of *Verrucomicrobia* in four out of the five species. Moreover, different gut microbiota taxonomic assemblages among the selected talitrid species were found. In particular, *O. montagui* (which lives in close contact with *Posidonia* banquette) gut microbiota was found to be the most different with respect to those of the other talitrids, being more abundant in members of *Firmicutes*, *Planctomycetes* and *Actinobacteria*, and containing the highest level of family 48 glycosyl hydrolases genes. We conclude that talitrid amphipods harbor a complex gut microbiota which may be related to the habitat the different species colonizes.

3.1.2 Introduction

The microbiome, in particular the gut microbiome, is recognized as an "extended genotype" since it encodes a more versatile metabolome than the host (Sommer & Backhed, 2013). This is particularly relevant for animals which uses lignocellulosic compounds, as preferential food source (e.g. ruminants, termites). In the last years, a number of evidences are accumulating on the role of gut microbiota in determining or correlating with host's ecology in animals (see for instance (Bauer *et al.*, 2000; Behar *et al.*, 2008; Basu *et al.*, 2009; Becker *et al.*, 2009; Dittmer *et al.*, 2012).

Talitrid amphipods (sandhoppers and beach fleas) are crustaceans living in the supralittoral zone and key components of the sandy beach food web (Pardi & Ercolini, 1986; Morritt, 1988; Morritt, 1989; Ugolini *et al.*, 1995; Morritt & Spicer, 1998; Calosi *et al.*, 2005; Calosi *et al.*, 2007). Talitrid amphipods obtain most of their food from stranded materials, which include detrital marine angiosperms and macroalgae, as well as occasional death animals (Adin & Riera, 2003). However, among the Mediterranean talitrid taxa, species-specific habitat preferences have been observed. Indeed, some species (as *Talitrus saltator*, *Talorchestia ugolinii*, *Sardorchestia pelecaniformis*,) occur in the damp belt of sandy shores, while others (as *Orchestia montagui*) are found under stranded algae and *Posidonia* banquettes, independently from the type of substrate (Ugolini *et al.*, 1995; Pavesi *et al.*, 2013). Finally, for a third group of species (as *Orchestia stephenseni*) the habitat seems to be more heterogeneous, the species being found in the damp of both sand sea shores and pools and lagoons backshore, as well as less within *Posidonia* banquettes and also stranded detritus (e.g. see (De Matthaeis *et al.*, 2000; Deidun *et al.*, 2009; Lowry & Fanini, 2013; Pavesi *et al.*, 2013) and personal observations). Consequently, it is possible to hypothesize a differential food preference and, consequently ,a different taxonomic and functional pattern of gut microbiota among species. However, despite such intriguing question and the key ecological relevance for carbon cycling on the damp band of sandy beaches have talitrid amphipods (McLachlan *et al.*, 1983), to date there are very limited reports on beach flea and sandhopper -associated microbial flora (Nuti *et al.*, 1971; Martineti *et al.*, 1995; Dittmer *et al.*, 2012; Mengoni *et al.*, 2013), and few studies have been performed in general on marine *Crustacea* gut microbiota (e.g. see (Harris, 1993a; Harris, 1993b; Zimmer *et al.*, 2001)).

Aim of this work was the analysis of the composition and diversity of the gut microbiota from five different talitrid amphipods species (*Talitrus saltator*, *Talorchestia ugolinii*, *Sardorchestia pelecaniformis*, *Orchestia stephenseni*, *Orchestia montagui*) which may be present in syntopy. In particular, the specific aim is to clarify the presence of gut microbiota signatures which may differentiate the five species, in relation to habitat and food preferences, then providing insight into their different ecology.

3.1.3 Materials and Methods

3.1.3.1 Sampling

Adults and sub-adults talitrids amphipods were collected in September 2013 from eight localities in Sardinia (Italy) (Table 3.1.1). For the locality of Giorgino beach, two different sampling points ca. 2 km a parts (named as Giorgino 1 and Giorgino 2) were selected and an additional sampling was also performed on September 2012 for Giorgino 1. In S. Giovanni di Sinis, two species were collected in syntopy (*O. montagui* and *O. stephenseni*). Immediately after collection, guts were excised from animals with sterile forceps and stored in DMSO/EDTA/NaCl preservative solution (20% DMSO, 0.25 M disodium-EDTA, NaCl to saturation, pH 7.5) (Seutin *et al.*, 1991; Dawson *et al.*, 1998). Once arrived at the laboratory, samples were then stored at -80°c prior to DNA extraction.

Code	Locality	Species	Longitude (E)	Latitude (N)
KA11	Centro 1° Sassu (Arborea)	*S. pelecaniformis*	8°32'48.58" E	39°48'7.08" N
KA9	Centro 1° Sassu (Arborea)	*S. pelecaniformis*	8°32'48.58" E	39°48'7.08" N
KA2	Giorgino 1	*T. saltator*	9°03'61.00" E	39°11'11.41" N
KA4	Giorgino 1	*T. saltator*	9°03'61.00" E	39°11'11.41" N
KA5	Giorgino 2	*T. saltator*	9°02'21.55" E	39°10'26.31" N
KA12	Is arenas	*T. ugolinii*	8°28'46.35" E	40° 4'13.00" N
KA13	Is arenas	*T. ugolinii*	8°28'46.35" E	40° 4'13.00" N
KA8	Maimoni (Cabras)	*O. montagui*	8°24'02.36" E	39°54'54.76" N
KA14	Piscadeddus	*O. stephenseni*	9°28'20.67" E	39° 7'56.73" N
KA3	S. Giovanni di Sinis (Cabras)	*O. montagui*	8°26'11.03" E	39°52'55.72" N
KA6	S. Giovanni di Sinis (Cabras)	*O. stephenseni*	8°26'51.42" E	39°53'07.37" N
KA10	Sa Rocca Tunda	*S. pelecaniformis*	8°25'29.71" E	40° 2'38.01" N

Table 3.1.1 Sampled amphipod taxa and sampling locations
*** The locality with geographical coordinates of sampling point and sampled taxon is indicated**

Pools composed by gut of ten animals per each species and locality were prepared. When available, for the same locality, two pools were prepared from animals collected at few distance (ca. 10 m), to take into account for possible population-based variation and substrate (e.g. sand and stranded material) heterogeneity. Twelve different pools were then prepared, accounting for a total of 120 single animals.

3.1.3.2 DNA extraction, metabarcoding analysis and detection of cellulose genes

DNA was extracted from gut tissues by using the QIAamp DNA Investigator Kit (Qiagen), quantified by agarose gel (0.8% TAE w/v) electrophoresis and by spectrophotometric reading using the Infinite® M200 PRO NanoQuant (Tecan). From extracted DNA, the bacterial V4 region of 16S rRNA genes was amplified with V4 specific primers (Klindworth *et al.*, 2013) and sequenced at the IGA Technology Services (http://www.igatechnology.com/), Udine, Italy using an Illumina MiSeq apparatus with pair-end sequencing (Caporaso *et al.*, 2012). Library preparation and demultiplexing have been performed following Illumina's standard pipeline. Sequence reads have been deposited under the BioProject ID: PRJNA260027. Total bacterial titres were estimated by Real-Time PCR using a previously reported SybrGreen protocol (Bacci *et al.*, 2015c). The same Real-Time PCR protocol (with annealing temperature decreased to 52°C) was used for detection and Real-Time quantification of glycosyl hydrolase family 48 (GHF48) genes by using GH48F/GH48R primer pair (Izquierdo *et al.*, 2010). Standard curves for 16S rRNA and GHF48 have been prepared with serial dilutions of genomic DNA of *Streptomyces coelicolor* A3(2), which contain a putative GHF48 gene (SCO5456).

3.1.3.3 Raw data processing

Raw sequence data generated from Illumina sequencing were processed following several steps. First of all, sequences were quality trimmed with StreamingTrim 1.0 (Bacci *et al.*, 2014). Quality trimmed sequences were assembled and subjected to another quality control step with PANDAseq (Masella *et al.*, 2012). Processed sequences were then subjected to the UPARSE pipeline (Edgar, 2013), in order to remove chimeric sequences (both in de novo and in reference mode) and to cluster them into Operational Taxonomic Units (OTUs). An identity threshold of 97% has been used (Konstantinidis & Tiedje, 2007). Representative sequences obtained from the UPARSE clustering were taxonomically classified using the SINA standalone classifier using the "Ref NR 99" as reference database (Pruesse *et al.*, 2012). After taxonomic classification we removed from our dataset all OTUs not assigned at least at Bacteria domain.

3.1.3.4 Biodiversity indexes analysis and statistical analysis

Rarefaction curves have been generated using the OTU assignments. Sample assignments have then be rarefied using a number of random subsamples equal to the number of assignments of the smaller sample Shannon, Richness and Evenness indexes were calculated on the rarefied samples; In order to investigate the presence of species-specific patterns in the metabarcoding data of gut microbiota of the talitrid amphipods OTU abundances were then used to perform a Canonical Correlation Analysis. Real-Time PCR data were statistically analyzed by one-way ANOVA. All statistical analyses were performed with the R software with the following packages: vegan (Dixon, 2003; Oksanen *et al.*, 2013); igraph (Csardi & Nepusz, 2006) and ggplot2 (Ginestet, 2011).

3.1.3.5 Network construction and clustering

In order to generate a OTUs network displaying patterns of correlation between each OTU, we calculated Spearman's correlations between all OTUs in our dataset, regardless of the sample of origin. Each OTU has been considered as a vertex inside the network and an edge between two OTUs has been created only if the Spearman's correlation coefficient between those OTUs was statistically significant ($p < 0.05$) and $r > 0.5$. In this way we generate an edge linking two OTUs only when there is a statistically significant and high degree of correlation between the two OTUs. In order to inspect the OTU distribution in the generated network the Markov Cluster Algorithm (MCL) was used (van Dongen, 2000). This method uses only one parameter in order to choose the number of cluster to generate; this parameter is called "inflation values" and it can range between 1.2 and 5.0 as reported by the author of the algorithm. In order to find the "inflation value" (IF) resulting in an informative number of clusters we used the "intracluster clustering coefficient" (ICCC) method (Lima-Mendez *et al.*, 2008)) Thus, we generated 39 different clusters using all IF values between 1.2 and 5.0 increasing the IF value by 0.1 each time and calculating the ICCC value for each clustering. Finally, the IF value that maximize the ICCC was 1.4 with the generation of 5 clusters. A representation of the generated network was drawn using Gephi (Mathieu *et al.*, 2009) with the "Force Field layout"; nodes were colored based on the cluster attribution.

3.1.4 Results and Discussion

3.1.4.1 Representativeness and diversity of gut microbiota

The metabarcoding analysis of 16S rRNA genes from the twelve gut DNA samples, representing five different talitrid species resulted in a relatively good coverage of bacterial diversity, with almost all samples reaching saturation or near saturation values (Figure 3.1.1). A total number of 7606010 (15212020 paired-end) reads was obtained with 410864 to 846775 reads per sample. After clustering at 97% identity a total of 1004 OTUs were identified in the twelve samples (Supplemental Material Table 3.1.S1).

The analysis of community diversity for each samples (Table 3.1.2) showed the lowest values for all three indices (Richness, Shannon and Evenness) for KA6 sample (*O. montagui*), while the highest values were reached by the other *O. montagui* sample (KA3), for Shannon and Evenness and by *S. pelecaniformis* (KA10) for Richness.. However, no statistically significant differences were found in relation to both talitrid species and locality of sampling (Supplemental Material Table 3.1.S2).

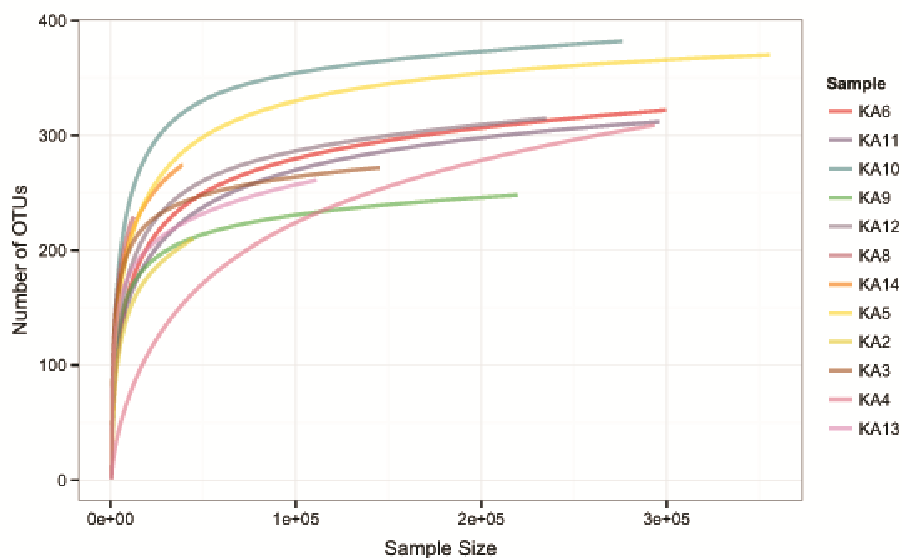


Figure 3.1.1 Rarefaction analysis on sequencing data of the amphipod gut microbiota The number of different Operating Taxonomic Units (OTUs) is reported as a function of the number of subsamples taken. Counts were sampled with a step size of 100 assignments in order to generate smooth curves. The asymptotic trends of curves indicate that a reasonable number of reads has been generated in order to inspect the diversity of each sample.

Code	Richness	Shannon (H)	Evenness	Species
KA8	229	3.84	0.71	*O. montagui*
KA3	206	3.64	0.68	*O. montagui*
KA6	185	2.50	0.48	*O. stephensenii*
KA14	215	3.02	0.56	*O. stephensenii*
KA11	157	1.79	0.35	*S. pelecaniformis*
KA10	256	3.53	0.64	*S. pelecaniformis*
KA9	171	3.09	0.60	*S. pelecaniformis*
KA5	221	3.19	0.59	*T. saltator*
KA2	156	1.72	0.34	*T. saltator*
KA4	76	1.32	0.30	*T. saltator*
KA12	192	2.62	0.50	*T. ugolinii*
KA13	183	3.73	0.72	*T. ugolinii*

Table 3.1.2 Diversity indices of gut microbiota in the twelve metabarcoding samples. Richness, Shannon and Evenness indexes are here reported and ordered based on the different talitrid species.

3.1.4.2 Species-specific signatures of gut microbiota

Numerous evidences indicate that animals co-evolve with their gut microbiota (Ley *et al.*, 2008). Consequently, species-specific patterns in the metabarcoding data of gut microbiota of the talitrids amphipods were inspected. Figure 3.1.2 reports the CCA results, which indicate the presence of clearly separate clusters for the five species under analysis (*O. montagui*, *S. pelecaniformis*, *T. saltator*, *T. ugolinii*, *O. stephensenii*), then supporting the hypothesis that amphipod digestive tracts host species-specific bacterial communities, which may be related to both the phylogeny and to potential dietary differences among the amphipod species, as exemplified in vertebrates (Ley *et al.*, 2008).

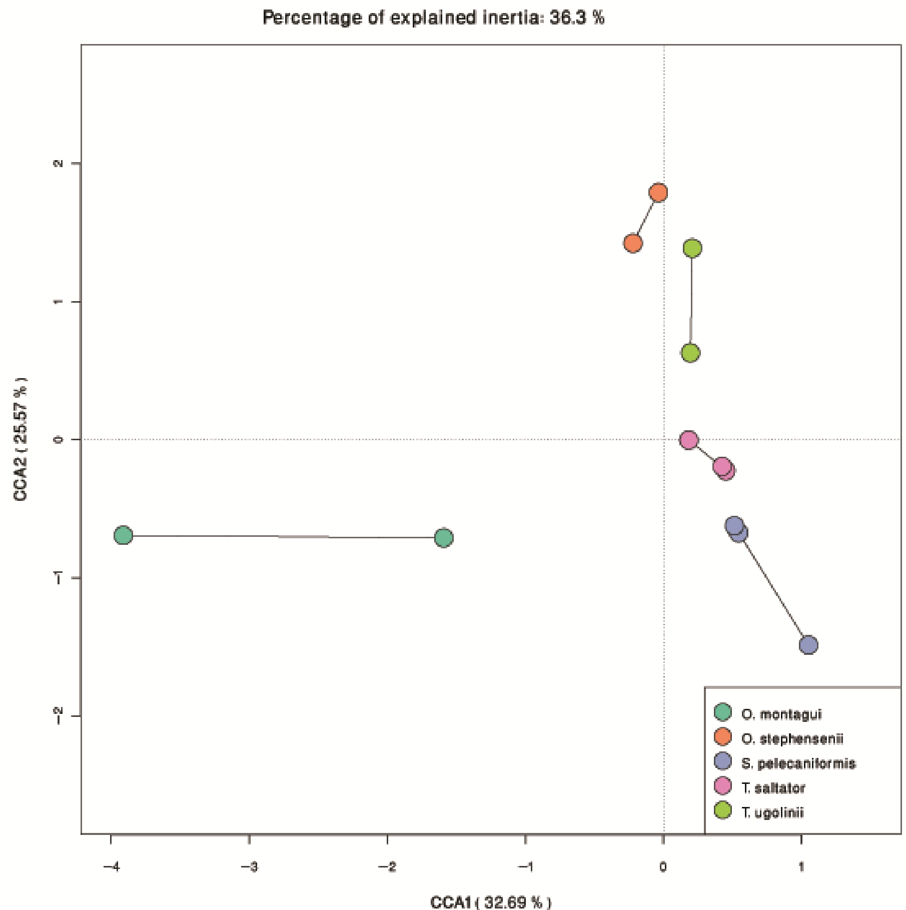


Figure 3.1.2 Species-specific signatures of gut microbial community composition. Canonical Correlation Analysis (CCA) based on OTU assignments to the bacterial taxonomy. Tabulated values of OTUs found in the analyzed samples have been log-transformed and subjected to the species constraints in order to find a possible correlation between the OTU distributions and the talitrid species. The percentage of inertia explained with this analysis has been reported on the top part of the plot, whereas the percentage of constrained inertia explained by the two components (CCA1 and CCA2) has been reported on the x and y-axes.

As recently proposed for soil bacterial communities (Barberan *et al.*, 2012), network analysis of significant taxon co-occurrence patterns could be useful to decipher the structure of complex microbial communities. Several works have shown recently that network analysis of co-occurrence may allow defining community patterns in several environments (for examples (Berry & Widder, 2014; Boutin *et al.*, 2014; Williams *et al.*, 2014; Zhang *et al.*, 2014) see). Consequently, to further inspect the species-specific signatures of our amphipod gut microbiota a network analysis was conducted on Pearson's correlations among OTUs. This analysis, coupled with a k-means clustering, highlighted the presence of five taxonomically differentiated groups (clusters) of co-occurring OTUs (Figure 3.1.3), in particular concerning *Proteobacteria*, *Actinobacteria* and *Firmicutes*. Such clusters showed different representation in the five amphipod taxa (Supplemental Material Figure 3.1.S1). In particular, cluster 4 was practically absent in *S. pelecaniformis*, while the other clusters showed both a high variability among species, as well as among samples within the same species.

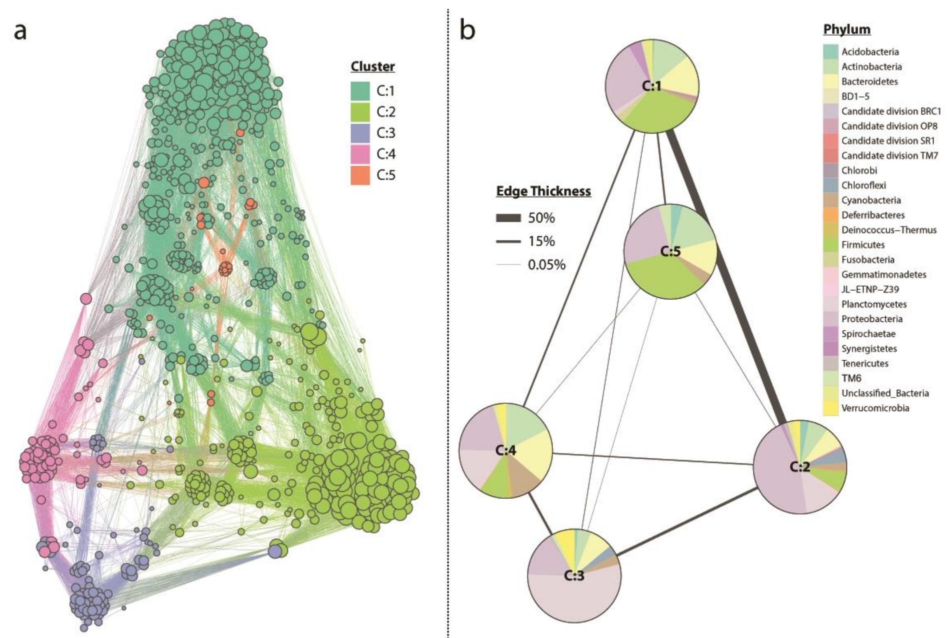


Figure 3.1.3 Network-based signatures of species-specific microbiota composition. Correlation network of OTU assignments. Each connection stands for a high degree of correlation between the two OTUs connected (Spearman's correlation r> 0.6 and p-value < 0.05). The size of each node in the network is proportional to its degree (number of connection of the node). The color of each node corresponds to a cluster obtained with the MCL algorithm with an "inflation value" equal to 1.4 (see Materials and Methods section). Clusters have been defined after a k-means clustering.

3.1.4.3 Taxonomic differences in gut microbiota among talitrid species

To evaluate which bacterial taxa mostly contribute to the interspecific differences of gut microbiota, OTUs were then assigned to bacterial phylogeny (Supplemental Material Table 3.1.S3). Figure 3.1.4 shows the overall representation of taxonomic composition of gut microbiota, which highlights similar patterns, among all samples, with differences both within the same species, and among species. In particular, it could be worth of mentioning that *Verrucomicrobia* were present in four out of five species (absent from all *S. pelecaniformis* samples), as well as the group *Deinococcus/Thermus* absent in both *Orchestia* species. *Verrucomicrobia* are particularly intriguing since this phylum, closely related to *Planctomycetes* and *Chlamydiae*, is considered to be particularly frequent in nonhost-assocaited environments, as soil and waters (Buckley & Schmidt, 2001; Freitas *et al.*, 2012).

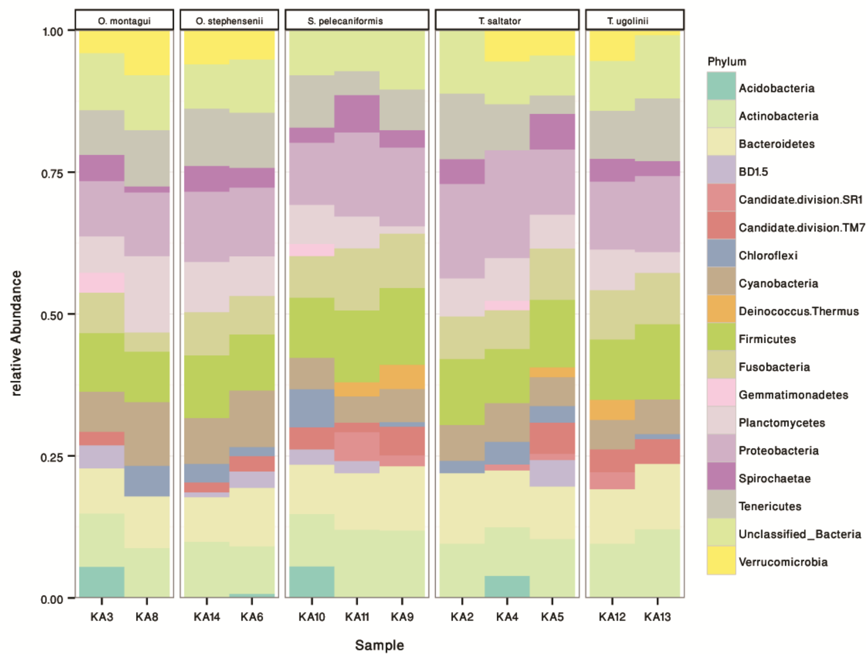


Figure 3. 1.4 Taxonomic composition of gut microbiota.
Relative abundances barplot showing the relative abundance of bacterial phyla in each gut sample.

Verrucomicrobia have been suspected to contribute to energy generation from fermentable substrates in the human gut (Arumugam *et al.*, 2011) and *Verrucomicrobia* have been found as particularly abundant after antibiotic treatment (Dubourg *et al.*, 2013). We cannot consequently exclude that *Verrucomicrobia* (present in all but *S. pelecaniformis* samples) may have a role in some hypothetical differential nutrient assimilation in those talitrid species and in differential resilience toward environmental disturbance, which is frequent in sandy beaches (Moffett *et al.*, 1998; Ugolini *et al.*, 2005; Ugolini *et al.*, 2008; Defeo *et al.*, 2009; Ungherese *et al.*, 2010; Ugolini & Ungherese, 2012).

To more in deep clarify the relative contribution of each phylum in species-specific gut microbiota signatures, a similarity percentage (simper) analysis was conducted (Table 3.1.3).

In general, the phyla mostly contributing to differences between talitrid species are the most abundant, as *Proteobacteria* (5.5%-9.1%), *Firmicutes* (5.2%-9.0%), *Bacteriodetes* (5.0%-6.3%), and *Actinobacteria* (4.9%-8.6%). Interestingly, *O. montagui*, which inhabits within the Posidonia banquettes and macroalgae mat, shows the highest percentage of variance for *Planctomycetes* in all pairwise comparisons, as well as among the highest percentage of variance for *Firmicutes* also. *Planctomycetes* have been found to densely populate the alkaline part of the hindgut of soil-feeding termites (*Cubitermes* spp.) (Köhler *et al.*, 2008) and to strongly vary with diet in humans (Cayrou *et al.*, 2013). Moreover, *Planctomycetes* constitute a large part of bacterial biofilms found on macroalgae (Lage & Bondoso, 2014). The relative importance of *Firmicutes* and *Planctomycetes* in differentiating *O. montagui* gut microbiota from those of the other talitrid species may be due to the habitat of this species, possibly linked to the potential higher cellulose content of its diet. Of course confirmatory experiments under controlled conditions are needed to better evaluate the relative contribution of diet with respect to species in determining

the specific of *O. montagui* gut microbiota, especially in comparison with *O. stephenseni*, which has been found in syntopy in the S. Giovanni di Sinis site (KA3 and KA6, Table 3.1.1).

Table 3.1.3 Species-specificity of bacterial phyla. Results of simper analysis.
The percentage of variance attributed to each phylum in pairwise
Comparisons of talitrid species is reported. In bold pairwise comparison with O. montagui.
Sp, S. pelecaniformis; Ts, T.saltator; Tu, T. ugolinii; Os, O. stephenseni.

	Acidobacteria	Actinobacteria	Bacteroidetes	BD1.5	Candidate.division.SR1	TM7	Chloroflexi	Cyanobacteria	Deinococcus.Thermus	Firmicutes	Fusobacteria	Planctomycetes	Proteobacteria	Spirochaetae	Tenericutes	Unclassified_Bacteria	Verrucomicrobia	Gemmatimonadetes
O.s Vs S.p	0.65%	5.94%	5.54%	0.11%	0.11%	0.11%	0.66%	0.64%	0.11%	5.86%	0.36%	2.41%	7.81%	0.20%	0.34%	0.33%	0.53%	NA
O.s Vs T.u	NA	5.58%	4.99%	0.13%	NA	0.13%	0.13%	0.89%	0.12%	5.23%	0.54%	2.70%	6.82%	0.16%	0.40%	0.40%	0.69%	NA
O.s Vs O.m	0.36%	6.50%	5.75%	0.17%	NA	NA	NA	1.16%	NA	8.68%	0.70%	6.10%	7.03%	0.21%	0.31%	0.51%	0.70%	NA
O.s Vs T.s	NA	7.73%	5.69%	0.15%	NA	0.15%	0.30%	0.59%	NA	7.02%	0.74%	2.80%	9.09%	0.26%	0.43%	0.42%	0.65%	NA
S.p Vs T.u	0.67%	4.88%	5.07%	0.10%	0.10%	NA	0.70%	0.77%	0.09%	5.45%	0.32%	1.75%	6.20%	0.29%	0.17%	0.35%	0.23%	NA
S.p Vs O.m	0.57%	7.23%	6.34%	0.16%	0.16%	0.15%	0.31%	1.06%	0.15%	9.02%	1.00%	5.14%	6.94%	0.35%	0.35%	0.43%	0.34%	0.12%
S.p Vs T.s	0.27%	8.24%	5.94%	0.15%	0.15%	0.14%	0.86%	0.56%	0.14%	8.23%	0.80%	1.25%	8.07%	0.45%	0.29%	0.44%	0.07%	NA
T.u Vs O.m	0.29%	7.88%	5.78%	0.17%	NA	0.17%	NA	1.14%	NA	8.89%	0.95%	5.66%	5.73%	0.10%	0.31%	0.51%	0.40%	NA
T.u Vs T.s	NA	8.58%	5.88%	NA	NA	0.15%	0.15%	0.76%	0.15%	8.23%	0.84%	1.61%	7.21%	0.17%	0.34%	0.50%	0.25%	NA
O.m Vs T.s	0.65%	7.16%	5.98%	0.18%	NA	0.18%	0.52%	0.86%	NA	9.03%	0.87%	6.23%	7.63%	0.31%	0.41%	0.51%	0.59%	0.14%

3.1.4.4 Cellulolytic bacteria may contribute to *O. montagui* gut microbiota difference

From the analysis of taxonomic pairwise differences in gut microbiota (Table 3.3), it emerged that among the most important bacterial phyla, those of *Firmicutes* and *Planctomycetes* were particularly related to possible dietary/habitat differences between *O. montagui* and the other talitrids. Both *Firmicutes* and *Planctomycetes* includes cellulose-degrading strains (Schwarz, 2001; Kulichevskaya *et al.*, 2007). Additionally, a large proportion of *Actinobacteria* (Figure 3.4), which are well known to include many cellulolytic strains (Marshall *et al.*, 1985), has been found in all talitrid species. These evidences raised the question if the proportion of cellulosolytic bacteria with respect to the total bacterial load of the gut, may indeed be different between amphipod talitrids. However, from the molecular point of view, it is difficult to have a global overview of all genes encoding cellulases in a sample. Cellulase systems are in fact complex assemblages of multifunctional glycosyl hydrolases (Schwarz, 2001). Many families of glycosyl hydrolases have been found (Lynd *et al.*, 2002), hampering the possibility to develop universal primers for PCR detection of all known cellulases. However, family 48 glycosyl hydrolases are well represented in many model cellulolytic clostridia and actinobacteria (Lynd *et al.*, 2002; Beloqui *et al.*, 2010).Primer pairs have been developed for 48 glycosyl hydrolases genes identification and quantification in environmental samples (Izquierdo *et al.*, 2010; Pereyra *et al.*, 2010), in particular for clostridia and actinobacteria (Izquierdo *et al.*, 2010). Since in our study a considerable fraction of recovered taxa fall within both *Firmicutes* and *Actinobacteria* (approx. 25% of reads) we decided to investigate the presence of family 48 glycosyl hydrolases genes in amphipod gut microbiota. Results of the qPCR analysis are reported in Figure 3.5. Interestingly, *O. montagui* gut samples contained a higher ratio of GHF48 genes/16S rRNA genes (considered as estimators of the total number of bacterial cells) with respect to the other talitrid species, suggesting that indeed the different microbiota present in *O. montagui* gut may be partly related to a higher prevalence of feeding on cellulose-rich substrates by this species.

In the Appendix a further preliminary investigation is reported on the phylogenetic diversity of GHF48 genes, which showed that GHF48 genes from different talitrid species have different phylogenetic affiliations, again emphasizing a species-specificity of the cellulolytic gut microbiome in such amphipod taxa.

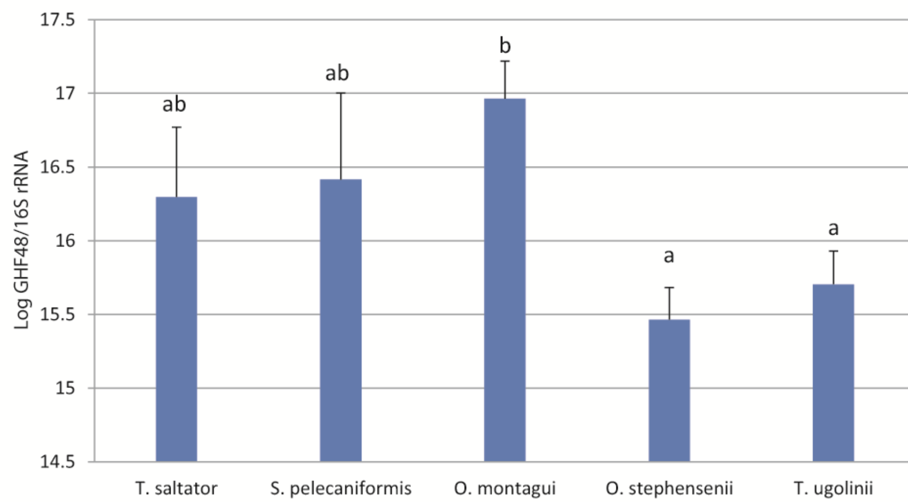


Figure 3.5 Abundance of cellulose-degrading genes in amphipod gut microbiota. Barchart reporting the mean proportion of glycosyl hydrolase 48 genes with respect to 16S rRNA genes in gut microbiota of the different amphipod species. Error bars, standard deviations from three repeated measures on each gut sample (see Table 1). Different letters indicate statistically significant differences ($P<0.05$) after one-way ANOVA.

3.1.5 Conclusions

Talitrid amphipods inhabiting supralittoral environment obtain most of their food from stranded materials, which include debris of various origin, as death animal organisms, macroalgae and plants (as land plants and *P. oceanica* in the Mediterranean Sea) (Adin & Riera, 2003; Colombini *et al.*, 2011). In particular, due to the presence of low digestible components including lignocellulosic compounds in macroalgae and plants, we can hypothesize that a cellulosolytic bacterial flora in the digestive tract of talitrids could contribute in cellulose degradation. Indeed, previous authors (Nuti *et al.*, 1971; Martineti *et al.*, 1995; Olabarria *et al.*, 2009) reported the presence of cellulose-degrading bacterial strain in the gut of talitrid amphipods, supporting the hypothesis of the involvement of gut microbiota in carbon source assimilation by such species. Here, we indicate that among the sampled species, which colonizes different microhabitats, *O. montagui* (which is found within *Posidonia* and macroalgae banquettes) harbors a different gut microbiota with respect to the other species. The *O. montagui* gut microbiota includes more taxa known to be involved in cellulose degradation and an analysis of family 48 glycosyl hydrolases (one of the cellulase genes) indicated that indeed *O. montagui* gut harbor a higher number of cellulose-degrading cells than the other talitrids. We conclude that the different ecological behavior of *O. montagui* (a colonizer of *Posidonia* banquettes) could be related also to a different taxonomic and functional composition of its gut microbiota. However we cannot, a priori exclude that a contribution of host encoded glycosyl hydrolases to food digestion could be present in *O. montagui* and in the other talitrid amphipods, as demonstrated for the marine isopod *Limnoria quadripunctata* (King *et al.*, 2010). Moreover, since *O. montagui* shows a low population structure, probably due to its high capacity of dispersion (De Matthaeis *et al.*, 2000), it remains to explain the quite relevant differences between the two pools of specimens of *O. montagui* gut microbiota investigated here, with respect to those of the other species, as *O. stephenseni*. Consequently, further investigation were performed (see next section 3.2) to elucidate the relative influence of diet on gut microbial communities.

3.1.6 Acknowledgments

Data reported in this chapter have been obtained in the course of research activities supported by the Project CRP-28345 funded by Autonomous Region of Sardinia (L. R. 7/2007). We are grateful to the directions of the Protected Marine Areas “Penisola del Sinis e Mal di Ventre” and “Capo Carbonara” for authorization to samplings and logistic support and to the BSc. student in Biotechnology Marco Confalone for technical assistance during DNA extraction. We are also indebted with Dr. D. Bellan–Santini (Centre d’Océanologie de Marseille-DIMAR) for her help in the identification of some amphipod specimens.

3.2. Are gut microbiota of talitrid amphipods affected by diet?

3.2.1 Investigating the resilience of littoral amphipod gut microbiome on *Talitrus saltator*

3.2.1.1 Abstract

The gut of *Talitrus saltator* host species-specific bacterial communities as we showed in our previous study. We then hypothesize that the different ecological behavior of talitrid species may reflect to some extent the different taxonomic and functional composition of their gut microbiota.

Here we aimed to investigate the hypothesis that diet may influence *T. saltator* gut microbial community composition and diversity. Animals were fed with artificial food for two months and their gut microbiota composition was analyzed by 16S metagenomic analysis of gut DNA. Obtained result showed that microbiota diversity changed over time and dominant taxa were found within members of phylum *Protobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes*. In particular a clear dynamic change represented by an increase in members of phyla *Actinobacteria* and *Bacteroidetes* along the time was found. We conclude that the diversity of gut microbial can rapidly change with shift of diet, and support the hypothesis that food is one of the most important factors to determine gut microbiota composition and diversity.

3.2.1.2 Introduction

Animals gut microbiome is composed by intricate multi-species communities capable of carrying out diverse and complex metabolic processes (Greenblum, 2014), which allow the host to use peculiar food source, adapt to the environment and protect the host from pathogens. The intimate relationships between host and their associate microbial flora is so intimate that a new term (the 'hologenome') has been proposed as a conceptual framework to investigate the role of multicellular eukaryotic host with their associated microbiomes (Rosenberg *et al.*, 2007; Rosenberg & Zilber-Rosenberg, 2013).

Recent studies have shown that the gut microbial communities of several animals are influenced by the nutritional habits of their hosts. Such microbial communities metabolize part of the ingested food and provide the host with important nutrients and may increase the dietary range of the host (e.g. cellulose digestion) (Meziti *et al.*, 2012). Both short- and long-term dietary change can influence the microbial profiles (Conlon & Bird, 2014)**.** Indeed, similar gut communities are found among phylogenetically related animals and among animals with similar diets (Sullam *et al.*, 2012). In a study of the composition of gut microbiota of a beetle (*Dastracus helophoroides*) the quantities of intestinal bacterial communities were different in the adults fed different diets (Wang *et al.*, 2014). In the termite gut, the observed patterns in the host-specific distribution of gut bacterial taxa are mainly explained by diet-related differences in the availability of microhabitats and functional niches, than by the different host taxonomy (Mikaelyan *et al.*, 2015).

Nutritional shifts and the ability to adapt to new food sources, are particularly relevant for detritivorous animals, which derive their food sources from occasional material, often of heterogeneous composition. The supralittoral belt of sandy shores is a habitat were occasional material is deposited by waves. This material is composed by algae, plant parts, death fishes etc. Talitrid amphipods are among the most important detritivorous living in the dump zone of the of supralittoral. Several species of talitrid amphipods are known and some of them are specialized, or more often retrieved, associated with different types of stranded material. In the previous section of this thesis we reported that different talitrid species may host different gut bacterial communities. However, it is unclear if such differences arose because of some species-specific physiological features of host or/and in relation to possibly different foraging behavior. In particular, we showed that *Talitrus saltator*, living in areas with a relatively low abundance of plant material (mainly *Posidonia oceanica* in the Mediterranean Sea), has high differences with respect to *Orchestia montagui*, which can be more often found associated with large Posidonia mats. Consequently, we wanted to investigate the resilience of gut bacterial communities of talitrid amphipods, to shed some light on the effect the different

foraging behavior in nature may have on species-specific gut microbiota patterns.

Here, to investigate the ability of gut microbiome to adapt to dietary modifications, we chose supralittoral detritivours *T. saltator* as model.

3.2.1.3 Materials and Methods

3.2.1.3.1 Sampling and Feeding Experiment

Talitrus saltator individuals, together with sand were collected from Fiume Morto Vecchio beach along the Tuscan coast and transferred to the laboratory. Immediately after collections gut_samples from 3 animals were excised with sterile forceps, and stored in RNALater(Ambion).

Animals were then maintained within their sand and fed with artificial food (commercial fish food). Gut samples from three animals were then taken first in their natural habitat in zero time then after 24 hours, 7days, 23days, 51days of artificial food feeding.

3.2.1.3.2 DNA extraction, metabarcoding analysis

Bacterial DNA was extracted using DNA was extracted from gut tissues using the QIAamp DNA Investigator Kit (QIAGEN S.r.l., Italy), visualized by ethidium bromide stained agarose gel (0.8% TAE w/v) electrophoresis and quantified spectrophotometrically using the Infinite® M200 PRO NanoQuant (Tecan, Milan, Italy), respectively.

Fragments of bacterial 16S rRNA (V3 region) were amplified with specific primers

(Forward Primer = 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG;

Reverse Primer = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) (Klindworth *et al.*, 2012), (http://web.uri.edu/gsc/files/16s-metagenomic-library-prep-guide-15044223-b.pdf).

PCR products were sequenced at the IGA Technology Services (http://www.igatechnology.com/), Udine, Italy using the Illumina MiSeq technology with pair-end sequencing (Caporaso *et al.*, 2012). Obtained paired end reads were 300 bp in length. Library preparation and demultiplexing have been performed following Illumina’s standard pipeline.

3.2.1.3.3 Detection of cellulase genes

for detection and Real-Time quantification of glycosyl hydrolase family 48 (GHF48) genes a previously reported SybrGreen Real-Time PCR protocol used (Bacci *et al.*, 2015c), with annealing temperature decreased to 52°C , using GH48F/GH48R primer pair (Izquierdo *et al.*, 2010). Standard curves for 16S rRNA and GHF48 have been prepared with serial dilutions of genomic DNA of *Streptomyces coelicolor* A3 (2), which contains a putative GHF48 gene (SCO5456).

3.2.1.3.4 Raw data processing

Raw sequences, generated as described above, were processed through automated O2tab Pipeline for "Operational Taxonomic Units" (OUT) clustering of microbiome data (https://github.com/GiBacci/o2tab), performing 5 main process

i) Assembling mate pairs process, ii) Pooling process, iii) Dereplication, iv) OTU clustering process, with a 96% of sequence identity threshold, v) Read mapping process, vi) OTU tabling process.

From OTU (cluster) produced above, a single representative sequence was selected and used for taxonomical analysis.

Collected 16S rRNA sequences were taxonomically classified using the Ribosomal Database Project (rdp) classifier (rdp.cme.msu.edu/classifier/classifier.jsp). 41 OTUs at Bacteria domain where identified with 80% Confidence threshold, and assigned to the OTU table to genus taxonomic level of all samples.

3.2.1.3.5 .Biodiversity indices analysis and statistical analysis

Rarefaction curves were calculated using PAST (PAlaeontological STatistics) ver. 3, (Hammer *et al.*, 2001), by plotting the number of observed OTUs against the number of sequence reads. Tabulated values at genus level (Table 3.2.S2), were used to produce a rarefaction curve for each sample.

Statistical analyses were performed on OTU`s. Alpha diversity analyses include computation of Richness, Shannon, Richness and Evenness, indices, to assess and compare the variation of gut sample microbial diversity along the five different sampling time points (0, 24h, 7-23-51 days).

Similarity percentage (simper) analysis and non metric multidimensional scaling were used to determine the contribution of individual taxa on gut microbiome shifts and the pattern of microbiome similarity, respectively. All analyses were performed with the modules present in Past 3 software (Hammer *et al.*, 2001).

3.2.1.4 .Results and Discussion

A total 2.213.104 reads of 16S rRNA reads passed quality filtering for *T. saltator* sequences (95% of total reads) (Table 3.2.1).

Sample code	Total Reads	Reads Passing Quality Filtering	% Reads Passing Quality Filtering
time 0	178.872	169.878	95.0 %
time 0	123.265	118.803	96.4 %
time 0	99.307	94.297	95.0 %
time 1-24hr	214.169	201.325	94.0 %
time 1-24hr	236.624	223.765	94.6 %
time 1-24hr	150.099	142.932	95.2 %
Time 2-7days	110.078	100.92	91.7 %
Time 2-7days	147.538	138.89	94.1 %
Time 2-7days	118.82	112.891	95.0 %
Time 3-23days	122.054	116.885	95.8 %
Time 3-23days	202.276	193.045	95.4 %
Time 3-23days	112.385	106.19	94.5 %
Time 4-51days	124.567	118.811	95.4 %
Time 4-51days	140.412	135.364	96.4 %
Time 4-51days	250.475	239.108	95.5 %
	2330.941	2213.104	95%

Table 3.2.1 Sequencing Statistics.

After the clustering step OTUs (performed at 96% threshold of identity), a total of 41 OTU were identified and then assigned to the bacterial taxonomy bacterial taxa present were classified at an 80% confidence threshold into six phyla (Table 3.2.S1a,b,c,d,e). Rarefaction curves obtained from genus level assignments reach or nearly reach a plateau for all samples,(Figure 3.2.1), indicating a satisfactory survey of the bacterial diversity, which allowed to estimate biodiversity indices (Table 3.2.2).

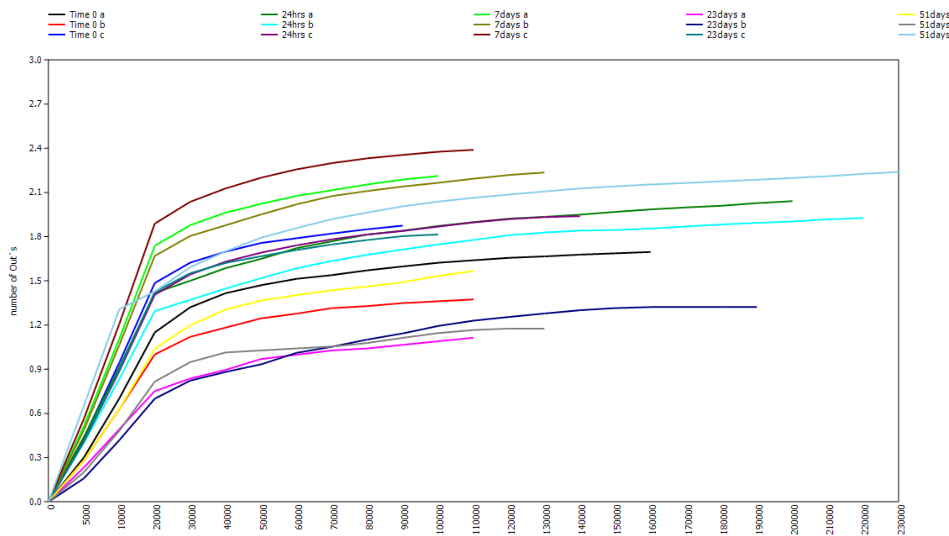


Figure 3.2.1 Rarefaction curve of sequencing reads.

Richness ranged from 29 to 40 OTU`s, evenness from 0.05694 to 0.2956, Shannon index from 0.7177 to 2.4 and Simpson index from 0.2688 to 0.8704 (Figure 3.2.2).

	Time0			24hr			7days			23days			51days		
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
Richness	29	36	30	35	32	36	40	39	34	30	29	33	38	32	38
Simpson	0.6401	0.2688	0.8587	0.8704	0.8441	0.8496	0.836	0.7098	0.8218	0.6055	0.6402	0.6768	0.6457	0.7313	0.7267
Shannon	1.484	0.7177	2.182	2.4	2.152	2.207	2.254	1.878	2.22	1.081	1.211	1.563	1.344	1.438	1.693
Evenness	0.1521	0.05694	0.2956	0.315	0.2687	0.2525	0.238	0.1677	0.2709	0.09823	0.1158	0.1446	0.1009	0.1316	0.143

Table 3.2.2 Values of alpha diversity indices gut samples bacterial communities of the 5 time points.

	Sum Sq	Df	Mean Sq	F value	P-value
Richness					
Between groups	102.267	4	25.5667	2.82	0.08366
Within groups	90.6667	10	9.06667		
Total	192.933	14			
Shannon					
Between groups:	2.25081	4	0.562703	4.069	0.03271
Within groups:	1.38289	10	0.138289		
Total:	3.6337	14			
Simpson					
Between groups:	0.139613	4	0.0349032	1.79	0.2074
Within groups:	0.194998	10	0.0194998		
Total:	0.334611	14			
Evenness					
Between groups:	0.0556893	4	0.0139223	3.609	0.04538
Within groups:	0.0385755	10	0.00385755		
Total:	0.0942648	14			

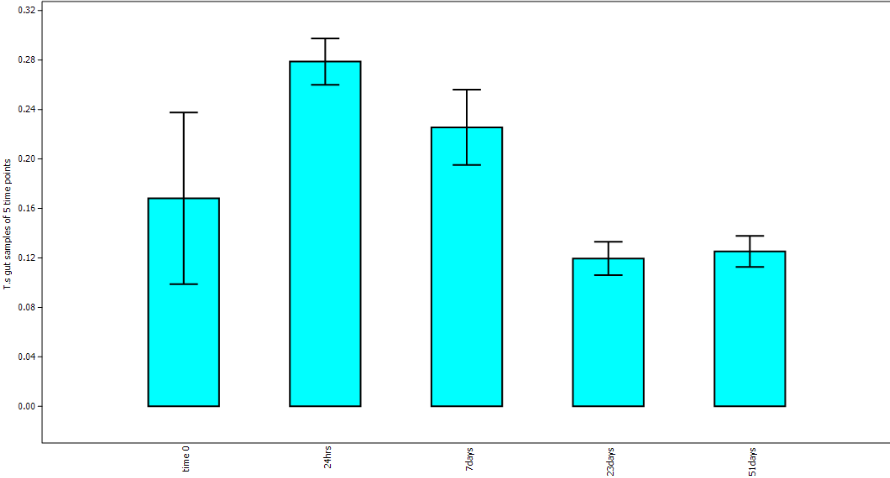
Table 3.2.3 One-way ANOVA on diversity indices*
Test for equal means Sum of squares (Sum Sq), the Mean square (Mean Sq), the F value and P-value for F are reported for Richness, Shannon, Simpson and Evenness.

In particular, a clear dynamic pattern with an initial increase of diversity values at 24hr-7 days was observed. After 7 days, values decreased at 23 days with a slight final increment recovery (Figure 3.2.2). Shannon and evenness indices showed significant differences along the five time points (Table 3.2.3).

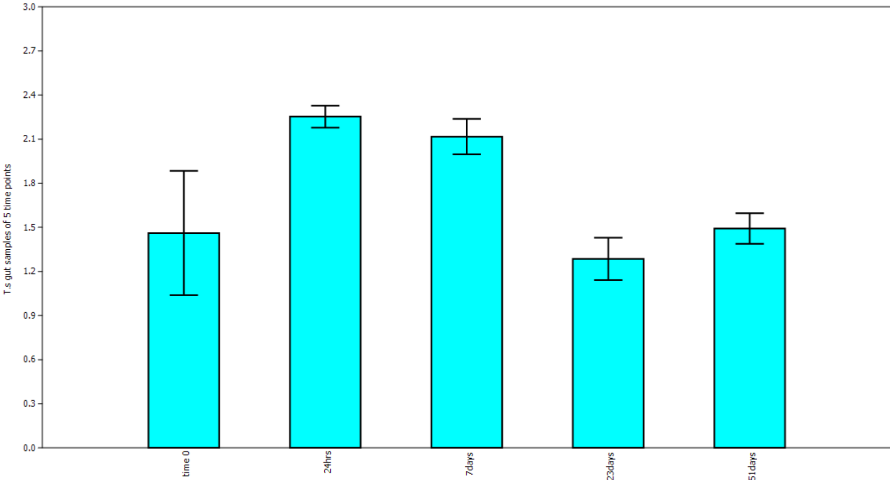
These data suggest that the input of nutrients (and bacteria) coming with the artificial food initially allowed more bacterial taxa to proliferate in the *T. saltator* gut. Then, after an initial reassessment of the microbiome structure the diversity decreased to initial values, suggesting an adaptation, in terms of diversity indices, of the gut microbiome to the artificial food condition. Indeed, this change (24h-7 days) was also observed when comparing microbiome structure. Nonmetric multidimensional scaling indicated that samples at 24h and 7 days were more similar each other, with respect to samples taken in nature (time 0) and those at 23 and 51 days (Figure 3.2.S1).

Figure 3.2.2 Charts of each alpha diversity indices of gut samples bacterial communities of the 5 time points.

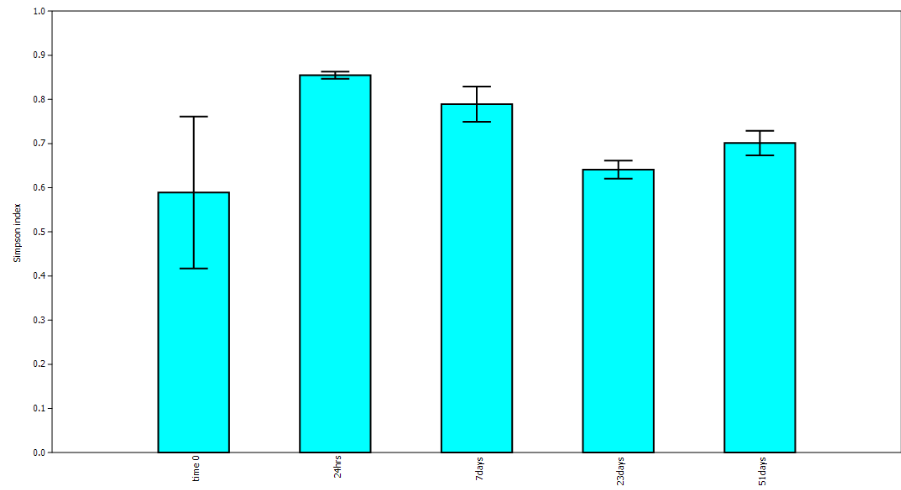
a. Evenness index



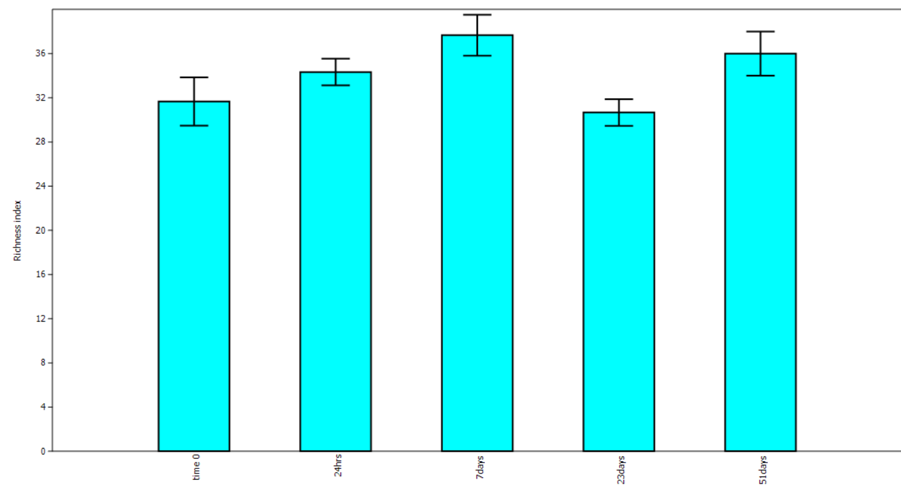
b. Shannon index



c. Simpson index



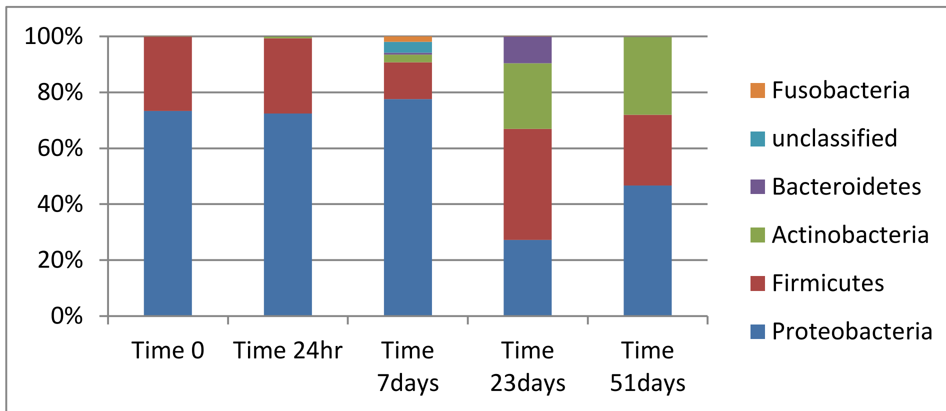
d- Richness Index



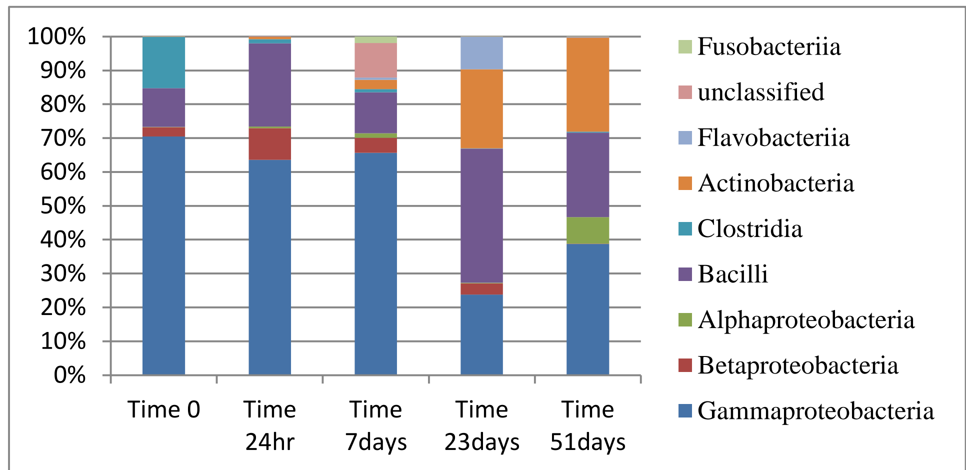
Phylogenetic analysis results obtained indicate that most of the samples of different time points were dominated by phylum *Proteobacteria* with a high percentage of members of class *Gammaproteobacteria*, in particular due to members of order *Enterobacteriales* (Figure3.2.3).

Figure 3.2.3 Taxonomic composition of gut microbiota of the five Time point .

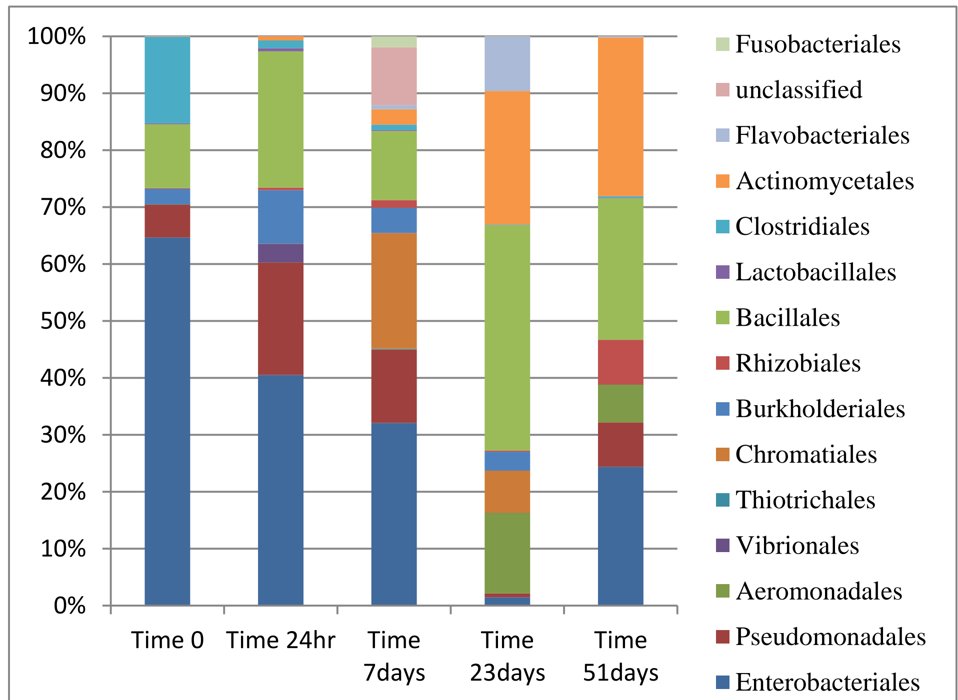
a. Histogram showing the relative abundance of bacterial phyla in the five time point guts samples.



b. Histogram showing the relative abundance of bacterial classes in the five time point guts samples.



c. Histogram showing the relative abundance of bacterial orders in the five time point guts samples.



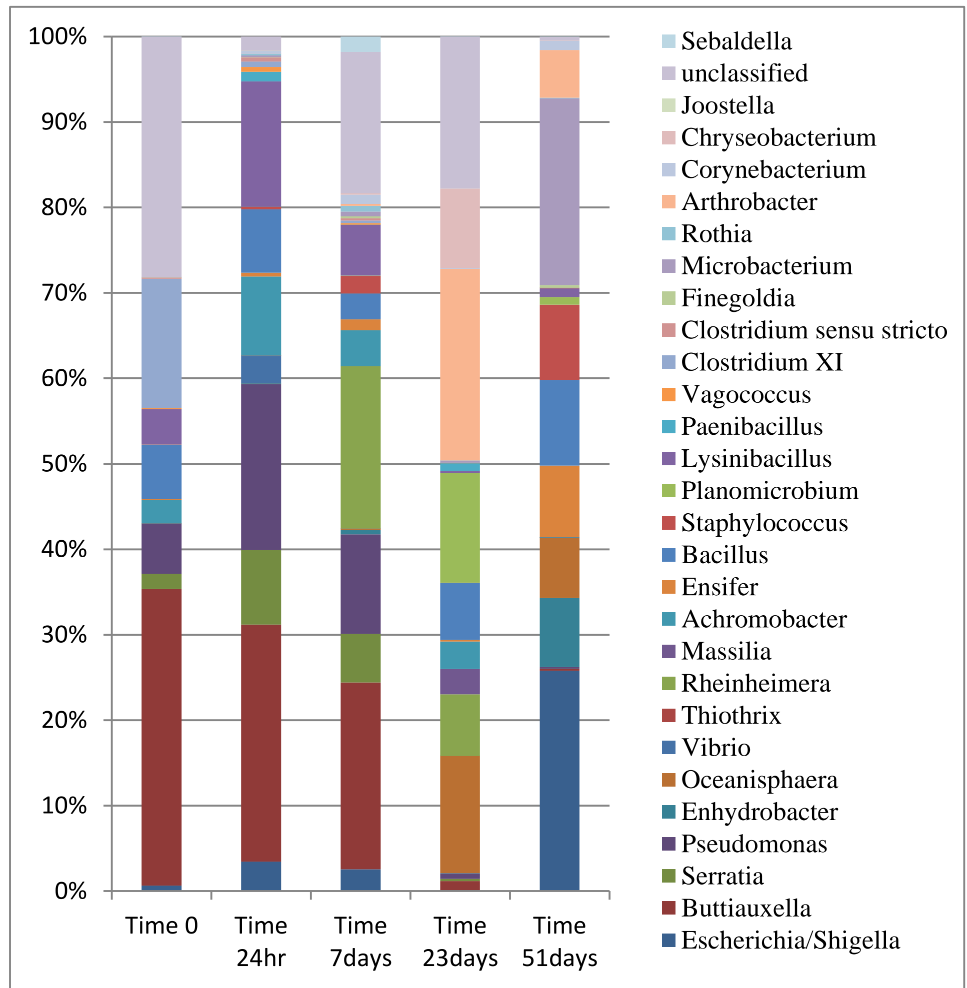
d. Histogram showing the relative abundance of bacterial families in the five time point guts samples.

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%

Time 0
Time 24hr
Time 7days
Time 23days
Time 51days

Leptotrichiaceae
unclassified
Flavobacteriaceae
Corynebacteriaceae
Micrococcaceae
Microbacteriaceae
Clostridiales_Incertae Sedis XI
Lachnospiraceae
Clostridiaceae 1
Peptostreptococcaceae
Enterococcaceae
Paenibacillaceae 1
Planococcaceae
Staphylococcaceae
Bacillaceae 1
Rhizobiaceae
Alcaligenaceae
Chromatiaceae
Thiotrichaceae
Vibrionaceae

e. Histogram showing the relative abundance of bacterial genus in the five time point guts samples.



Indeed similarity percentage (simper) analysis (Table 3.2.4) showed that the genera mostly contributing to the differences between the t 0 and all other time points were two mainly: *Buttiauxella* and *Pantoea* (order *Enterobacteriales*).

The second dominant phylum was that of *Firmicutes*, represented mostly by members of the class *Bacilli*. This class showed a high increment in representation along the time points. Opposite pattern was detected for the class *Clostridia* whose members were more represented at t 0 with respect to the other sampling times. Finally, a large increase of member from phylum *Actinobacteria* has been observed, from t 0 to t 51 days (Table 3.2.S3).

The reported taxonomic differences indicated that, although after 23 days, the biodiversity indices were similar to those at time 0, the taxonomic composition of the microbiome was strongly different, suggesting a sort of “reset” of the microbiota structure after the initial increase of taxa richness at 24h- 7 days.

Table 3.2.4 Results of SIMPER analysis on taxa occurrence along the five time points sampling. The taxonomic attribution of OTUs, the percentage of contribution and the cumulative contribution are reported.

A) Time 0 VS 24hrs

	Phylum	Class	Order	Family	Genus	Contrib. %	Cumulative %
OTU_2	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Buttiauxella	22.05	22.05
OTU_5	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Pantoea	16.75	38.8
OTU_16	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus	10.95	49.75
OTU_6	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	10.37	60.12
OTU_8	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Clostridium XI	9.216	69.34
OTU_9	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia	6.885	76.22
OTU_7	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	6.793	83.02
OTU_28	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	3.795	86.81
OTU_26	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	2.951	89.76
OTU_3	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia/Shigella	2.766	92.53
OTU_12	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus	1.656	94.18

B) Time 0 VS 7days

Taxon	Phylum	Class	Order	Family	Genus	Contrib. %	Cumulative %
OTU_2	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Buttiauxella	24.39	24.39
OTU_5	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Pantoea	20.11	44.5
OTU_13	Proteobacteria	Gammaproteobacteria	Thiotrichales	Thiotrichaceae	Thiothrix	12.1	56.6
OTU_8	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Clostridium XI	11.57	68.17
OTU_6	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	3.857	72.03
OTU_21	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	Orientia	3.768	75.79
OTU_9	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia	2.999	78.79
OTU_12	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus	2.961	81.75
OTU_16	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus	2.567	84.32
OTU_1	unclassified	unclassified	unclassified	unclassified	unclassified	2.038	86.36
OTU_7	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	1.913	88.27

C) Time 0 VS 23days

Taxon	Phylum	Class	Order	Family	Genus	Contrib. %	Cumulative %
OTU_2	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Buttiauxella	17.45	17.45
OTU_5	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Pantoea	12.58	30.03
OTU_4	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Arthrobacter	10.82	40.86
OTU_11	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Oceanisphaera	8.782	49.64
OTU_10	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus	8.366	58.01
OTU_15	Firmicutes	Bacilli	Bacillales	Planococcaceae	Planomicrobium	8.177	66.19
OTU_8	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Clostridium XI	7.24	73.43
OTU_25	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	5.982	79.41
OTU_19	Proteobacteria	Gammaproteobacteria	Chromatiales	Chromatiaceae	Rheinheimera	4.611	84.02
OTU_12	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus	2.986	87
OTU_38	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus	2.506	89.51

D) Time 0 VS 51days

Taxon	Phylum	Class	Order	Family	Genus	Contrib. %	Cumulative %
OTU_2	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Buttiauxella	16.76	16.76
OTU_5	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Pantoea	11.79	28.55
OTU_3	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia/Shigella	9.573	38.12
OTU_17	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	7.778	45.9
OTU_11	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Oceanisphaera	7.474	53.37
OTU_20	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus	7.199	60.57
OTU_8	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Clostridium XI	6.768	67.34
OTU_4	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Arthrobacter	5.896	73.24
OTU_22	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	5.175	78.41
OTU_23	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Enhydrobacter	3.641	82.05
OTU_18	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Ensifer	3.08	85.13

E) 24hrs VS 7days

Taxon	Phylum	Class	Order	Family	Genus	Contrib. %	Cumulative %
OTU_2	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Buttiauxella	16.85	16.85
OTU_16	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus	12.86	29.71
OTU_13	Proteobacteria	Gammaproteobacteria	Thiotrichales	Thiotrichaceae	Thiothrix	12.31	42.03
OTU_6	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	10.52	52.55
OTU_7	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	7.87	60.42
OTU_9	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia	6.103	66.52
OTU_12	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus	4.965	71.48
OTU_28	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	3.893	75.38
OTU_21	Proteobacteria	Alphaproteobacteria	Rickettsiales	Rickettsiaceae	Orientia	3.838	79.22
OTU_26	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	Vibrio	3.651	82.87
OTU_3	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia/Shigella	2.18	85.05

F) 24hrs VS 23days

Taxon	Phylum	Class	Order	Family	Genus	Contrib. %	Cumulative %
OTU_2	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Buttiauxella	15.9	15.9
OTU_4	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Arthrobacter	9.252	25.15
OTU_16	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus	8.568	33.72
OTU_6	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	8.185	41.91
OTU_10	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus	7.457	49.36
OTU_11	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Oceanisphaera	7.178	56.54
OTU_15	Firmicutes	Bacilli	Bacillales	Planococcaceae	Planomicrobium	6.687	63.23
OTU_7	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	5.366	68.6
OTU_9	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia	5.026	73.62
OTU_25	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	4.888	78.51
OTU_19	Proteobacteria	Gammaproteobacteria	Chromatiales	Chromatiaceae	Rheinheimera	3.771	82.28

F) 24hrs VS 51days

Taxon	Phylum	Class	Order	Family	Genus	Contrib. %	Cumulative %
OTU_2	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Buttiauxella	15.61	15.61
OTU_3	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia/Shigella	9.133	24.74
OTU_6	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	7.991	32.73
OTU_16	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus	7.436	40.17
OTU_17	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	6.731	46.9
OTU_11	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Oceanisphaera	6.187	53.09
OTU_20	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus	6.081	59.17
OTU_7	Proteobacteria	Betaproteobacteria	Burkholderiales	Alcaligenaceae	Achromobacter	5.232	64.4
OTU_9	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serratia	4.9	69.3
OTU_4	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Arthrobacter	4.876	74.18
OTU_22	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	4.409	78.59

G) 7days VS 23days

Taxon	Phylum	Class	Order	Family	Genus	Contrib. %	Cumulative %
OTU_4	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Arthrobacter	11.77	11.77
OTU_11	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Oceanisphaera	9.82	21.59
OTU_2	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Buttiauxella	9.817	31.41
OTU_15	Firmicutes	Bacilli	Bacillales	Planococcaceae	Planomicrobium	9.151	40.56
OTU_10	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus	9.024	49.59
OTU_13	Proteobacteria	Gammaproteobacteria	Thiotrichales	Thiotrichaceae	Thiothrix	8.277	57.86
OTU_25	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	6.715	64.58
OTU_19	Proteobacteria	Gammaproteobacteria	Chromatiales	Chromatiaceae	Rheinheimera	5.159	69.74
OTU_6	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	3.798	73.54
OTU_38	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus	2.74	76.28
OTU_16	Firmicutes	Bacilli	Bacillales	Planococcaceae	Lysinibacillus	2.738	79.01

H)7days VS 51days

Taxon	Phylum	Class	Order	Family	Genus	Contrib. %	Cumulative %
OTU_4	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Arthrobacter	12.61	12.61
OTU_3	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia/Shigella	10.17	22.78
OTU_11	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Oceanisphaera	10.17	32.95
OTU_10	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus	8.314	41.26
OTU_15	Firmicutes	Bacilli	Bacillales	Planococcaceae	Planomicrobium	8.154	49.42
OTU_17	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	8.132	57.55
OTU_20	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus	7.704	65.25
OTU_25	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacterium	5.847	71.1
OTU_22	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	5.483	76.58
OTU_19	Proteobacteria	Gammaproteobacteria	Chromatiales	Chromatiaceae	Rheinheimera	4.509	81.09
OTU_23	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Enhydrobacter	3.859	84.95

I) 23days VS 51days

Taxon	Phylum	Class	Order	Family	Genus	Contrib. %	Cumulative %
OTU_3	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia/Shigella	11.07	11.07
OTU_2	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Buttiauxella	9.91	20.97
OTU_17	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Microbacterium	8.72	29.69
OTU_11	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	Oceanisphaera	8.579	38.27
OTU_20	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Bacillus	8.133	46.41
OTU_13	Proteobacteria	Gammaproteobacteria	Thiotrichales	Thiotrichaceae	Thiothrix	7.897	54.3
OTU_4	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Arthrobacter	6.766	61.07
OTU_22	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus	5.858	66.93
OTU_23	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Enhydrobacter	4.088	71.02
OTU_6	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas	3.852	74.87
OTU_18	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Ensifer	3.659	78.53

In particular, *Enterobacteriales, Firmicutes* and *Actinobacteria* were the most important. The two genera detected in *Enterobacteriales* (*Buttiauxella* and *Pantoea*) are known enteric bacteria of vertebrates. And (Kim *et al.*, 2007;

Peterfreund *et al.*, 2012). Concerning *Firmicutes* and *Actinobacteria* these have been reported (see Section 3.1) as those mostly contributing to amphipod gut species differences also (Abdelrhman *et al.,* 2015), and have been claimed as possibly linked to lignocellulotyic material degradation. An analysis of GH48 genes was then performed.

Results of the qPCR analysis are reported in Figure 3.2.4 showed and increment of GHF48 genes/16S rRNA genes ratio along the first three time points, significantly among samples of the 7days time points that contain a higher ratio of GHF48 genes/16S rRNA genes with respect to the other time points samples, interestingly the same pattern reported for Shannon index among the five time point.

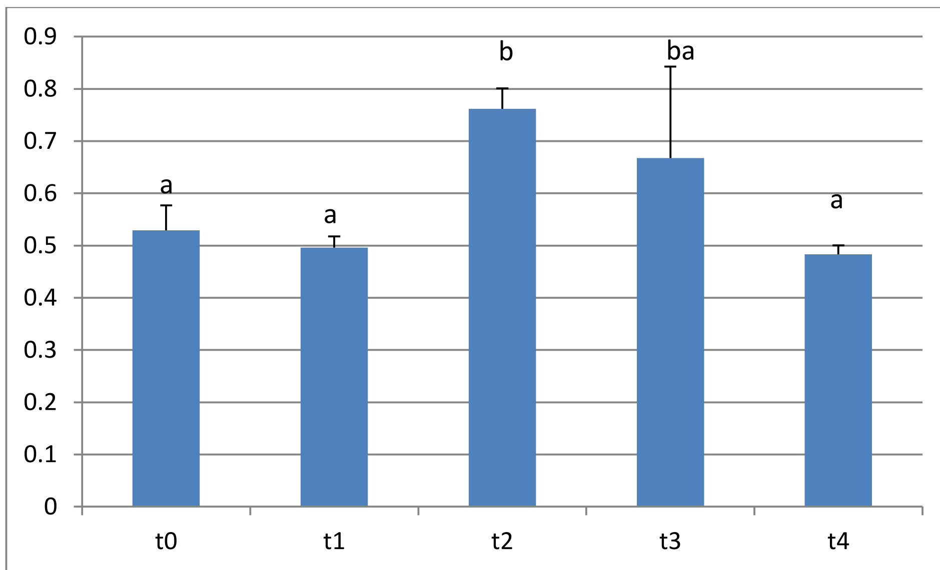


Figure 3.2.4 Abundance of cellulose-degrading genes in the five time points of *T. saltator* gut samples.
Barchart reporting the mean proportion of glycosyl hydrolase 48 family genes with respect to 16S rRNA genes in gut microbiota of time points. *T. saltator* gut samples. Error bars, standard deviations from three repeated measures on each gut sample . Different letters indicate statistically significant differences (P<0.05) after one-way ANOVA Different letters indicate statistically significant contrast (P<0.05, one-way ANOVA, Tukey pairwise).

	Sum of sqrs	*df*	Mean square	*F*	*P (same)*
Between groups	60.013	4	15.003	5.033	* 0.01747
Within groups	29.8077	10	2.98077		
Total	89.820	14			

Table 3.2.5 One-way Anova, Test for equal means (P<0.05)

3.2.1.5 Conclusions

In this study we showed that a dual response of *T. saltator* gut microbiota is present in relation to diet alteration. A fast response is observed after the first 24 hours with a significance increase in the diversity during the first week of artificial food feeding. Then, while the diversity tends to decrease at 23 days and 51 days of artificial diet feeding, the taxonomic composition of the community remained altered. This dual response to the diet change, could be explained by an initial proliferation of new or minor taxa thanks to the new food source (and maybe to food associated bacteria) (Wang *et al.*, 2014), followed by the adaptation of the new taxa to the gut environment, which selects those taxa better exploiting the new diet components, in relation to their functional role (Rettner, et al. 2013). Similar short-term responses have been observed in other systems were diet alteration modify the microbial community structure and overwhelms inter-individual differences in microbial gene expression (Lawrence et al, 2014). Further investigation should be done in future studies to reveals the functional relationship of gut microbiome changes in response to the diet shift. The same experiment of artificial feeding has also been performed with *Orchestia montagui*, which showed (Section 3.1), remarkable differences in GHF48 gene contents (see Appendices, Section 6.4). *O. montagui* animals were fed with artificial food and paper for 2 months and sampling at time interval was done. Subsequent DNA extraction, 16S rRNA metagenomics sequencing and quantification of GHF48 genes were performed. Preliminary results (Section 6.4) indicated a clear pattern of differences in GHF48 gene abundance shift between *O. montagui* and *T. saltator*. This results fits with behavioral observations which indicated a marked preference of *O. montagui* for paper instead that of artificial food mix (as on the contrary was observed for *T. saltator*), suggesting that *O. montagui* diet strongly relies on cellulolytic activities. However, at the time of printing of this thesis sequencing data are still under processing, so no conclusions can be provided.

3.2.1.6 Acknowledgments

Special thanks to the Libyan embassy in Rome (Italy) for the financial support. thanks to dottoressa Ciofini Alice for her help during the experiment and intestines sampling.

CHAPTER IV

THE GUT MICROBIOTA OF MARINE VERTEBRATES

Chapter 4. The gut microbiota of marine vertebrates

4.1 Preliminary Investigation on the Gut Microbiome of the Sea Turtle *Caretta caretta* (Linnaeus 1758)

4.1.1 Abstract:

Gut microbiome contribute to diverse host processes, performs numerous important biochemical functions for the host, nutrition , health, and behavior, gut microbiota differs according to the host phylogeny, in this study we provided a first insights on the gut microbial community compositions of the sea turtle *Caretta caretta*, which face a true risk of extinction. Four samples of feces and six of cloacal contents and intestine sections were analyzed through metagenomic sequencing of amplified 16SrRNA V3 region. Obtained results indicated the presence of a complex bacterial flora mainly dominated by three bacterial phylum phyla across all samples: *Firmicutes*, *Proteobacteria*, and *Bacteroidetes*. However different taxonomic representation were found among faeces and intestine samples which can be related to differential association of bacteria to gut.

4.1.2 Introduction:

Sea turtles range widely over the Earth. They occur in oceanic and neritic habitats from the tropics to subarctic waters and venture onto terrestrial habitats to nest or bask in tropical and temperate latitudes. Although their population drastically reduced since interactions between humans and sea turtles began (Bjorndal *et al.*, 2003)

Population declines have more recently been driven by factors in addition to direct harvest, such as incidental capture in commercial fisheries, habitat degradation, introduction of feral predators on nesting beaches, and marine pollution (Eckert, 1995; Lutcavage & Lutz, 1997; Witherington, 1997).

Caretta caretta (Loggerhead turtle), among other species of sea turtles, plays important roles in maintaining marine ecosystem. These roles range from maintaining productive coral reef ecosystems to transporting essential nutrients from the oceans to beaches and coastal dunes (http://oceana.org/sites/default/files/reports/Why_Healthy_Oceans_Need_Sea_Turtles.pdf).

Loggerheads occupy three different ecosystems during their lives: beaches (terrestrial zone), water (oceanic zone), nearshore coastal areas ("neritic" zone) (http://www.nmfs.noaa.gov/pr/species/turtles/loggerhead.htm).

In the last years the microbial communities (microbiota) associated with the digestive tract of animals have stirred an intense research interest (Zhu, Baoli *et al.*, 2010). The presence of a close functional interrelationship between the host and the microbiome associated has been highlighted, and the new term of hologenome has been proposed (Zilber-Rosenberg & Rosenberg, 2008), meaning the set of functions (genes) of 'host and microorganisms associated with it. Sea turtle populations around the world have dwindled in recent centuries and in many places, continue to decline. For some populations there is risk not only of ecological extinction, but of physical extinction as well. In spite of considerable importance for the study of vertebrates and the protection of marine biodiversity, there are no studies on microbial communities associated with the digestive tract of sea turtles.

The aim of this work has been the characterization, for the first time to the best of our knowledge, of the gut microbiome of the sea turtle *C. caretta*. Both feces and intestine samples were taken from accidentally caught animals to have the wider overview of gut microbiome taxonomic composition.

4.1.3 Materials and Methods:

4.1.3.1 Sampling

Samples of sea turtles *Caretta caretta* L. were collected along the year 2014, from different locations along the Tyrrhenian sea coast in Tuscany and Liguria regions (Italy) (Figure 4.1), through the recovery centers associated with network of the Tuscan Observatory for Cetacean and Sea Turtles (OTCT). Recovery centres host sea turtles accidentally caught in the North-East Tyrrhenian Sea. In the recovery centers turtles are checked for their health status and if needed subjected to veterinary care. When health conditions are good, animals are then:

A total of ten individuals were analyzed (Table 4.1.1). The samples consisted of faeces of four individuals and cloacal contents and intestine sections of six individuals which were death in the recovery centers. All samples were immediately stored at -20°C prior of the extraction of DNA .

Figure 4.1.1 Study area and sampling sites. Located in Tyrrhenian Sea coast in Tuscany and Liguria regions (Italy), were *C. caretta* samples been collected.

Sample code	Sample type	Total Reads	Reads Passing Quality Filtering	% Reads Passing Quality Filtering
T1	Feces	544.605	507.072	93.1 %
T3	Feces	386.371	357.231	92.5 %
T4	Intestine	267.169	249.587	93.4 %
T5	Intestine	100.635	91.047	90.5 %
T6	Intestine	40.231	37.117	92.3 %
T7	Intestine	220.358	207.119	94.0 %
T9	Intestine	116.819	108.915	93.2 %
T10	Intestine	129.634	111.712	86.2 %
T11	Feces	115.155	109.961	95.5 %
T12	Feces	108.224	102.629	94.8 %

Table 4.1.1 Sequencing Statistics.

4.1.3.2 DNA extraction, metabarcoding analysis

DNA was extracted from feces, cloacal contents and gut tissues using the FastDNA™ SPIN Kit for soil (MP Biomedicals, Italy), visualized by ethidium bromide stained agarose gel (0.8% TAE w/v) electrophoresis and quantified spectrophotometrically using the Infinite® M200 PRO NanoQuant (Tecan, Milan, Italy), respectively.

The bacterial V3-V4 region of 16S rRNA genes was amplified with specific primers (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3') (Klindworth *et al.*, 2013) and 16S metagenomic library was prepared according to Illumina MiSeq guidelines. Libraries were sequenced at the IGA Technology Services (http://www.igatechnology.com/), Udine, Italy using the Illumina MiSeq technology with pair-end sequencing (Caporaso *et al.*, 2012). Obtained paired end reads were 300 ±2bp in length. Library preparation and demultiplexing have been performed following Illumina's standard pipeline.

4.1.4.3 Raw data processing

Raw sequences, generated as described above, were processed through automated O2tab Pipeline for "Operational Taxonomic Units" (OUT) clustering of microbiome data (https://github.com/GiBacci/o2tab), performing five main processes

i)Assembling mate pairs process, ii) Pooling process, iii)Dereplication, iv)OTU clustering process, with a 96% of sequence identity threshold, v) Read mapping process, vi) OTU tabling process.

From OTU (cluster) produced above, a single representative sequence was selected and used for taxonomical analysis.

Collected 16S rRNA sequences were taxonomically classified using the Ribosomal Database Project (rdp) classifier (rdp.cme.msu.edu/classifier/classifier.jsp) (Bacci *et al.*, 2015a; Bacci *et al.*, 2015b). 67 OTUs at Bacteria domain where identified with 80% Confidence threshold.

4.1.3.4 Biodiversity indices analysis and statistical analysis

Rarefaction analysis was carried out using PAST (PAlaeontological STatistics) ver. 3,(Ana Durbán, et al, 2010), by plotting the number of observed OTUs against the number of reads. At genus level (Table 4.1.S2). Tabulated values were used to produce a rarefaction curve for each sample.

Statistical analyses were performed on OTU`s, Alpha diversity analyses include computation of Richness, Shannon, Richness and Evenness, indices, to assess and compare microbial diversity of faeces samples vs intestine samples (4faeces samples, 6 intestine samples.).

Specific differences in community composition and the similarity among bacterial communities were determined using similarity percentage (simper) analysis and principle component analysis (PCA). All computations were performing with the modules present in Past 3 software (Hammer *et al.*, 2001)

4.1.4 Results

A total 1.882.390 reads of all samples of *C. caretta* passed quality filtering sequences (92.8% of total reads) (Table 4.1.S1). After the clustering step a total of 67 OTUs that shared ≥96% identity in their 16S rRNA gene sequences were obtained and then assigned to the bacterial taxonomy bacterial taxa present were classified at an 80% confidence threshold into nine phyla, (Table 4.1.S2a,b). Rarefaction curves obtained reached or nearly reached a plateau all samples indicating a satisfactory level of diversity sampling (Figure 4.1.2).

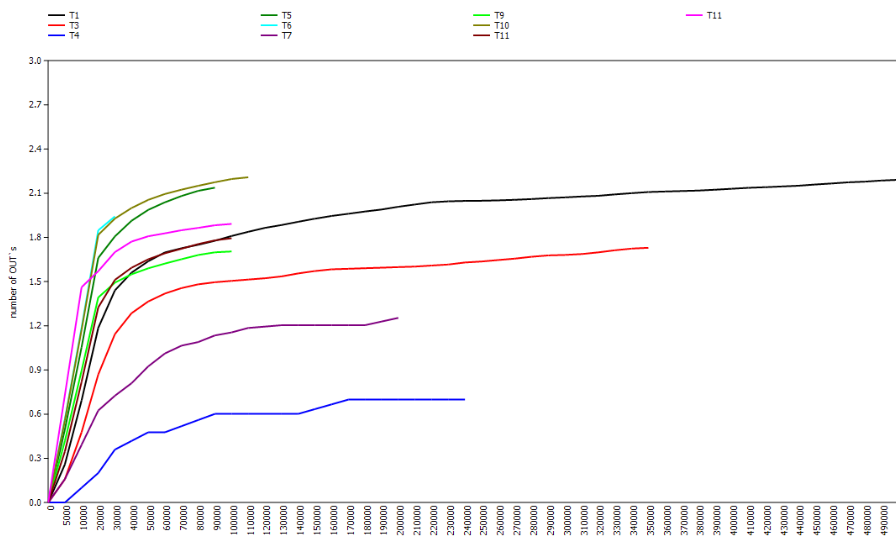


Figure 4.1.2 Rarefaction analysis on sequencing data of *Caretta caretta* gut microbiota. The asymptotic trends of curves indicate that a reasonable number of reads has been generated in order to inspect the diversity of each sample.

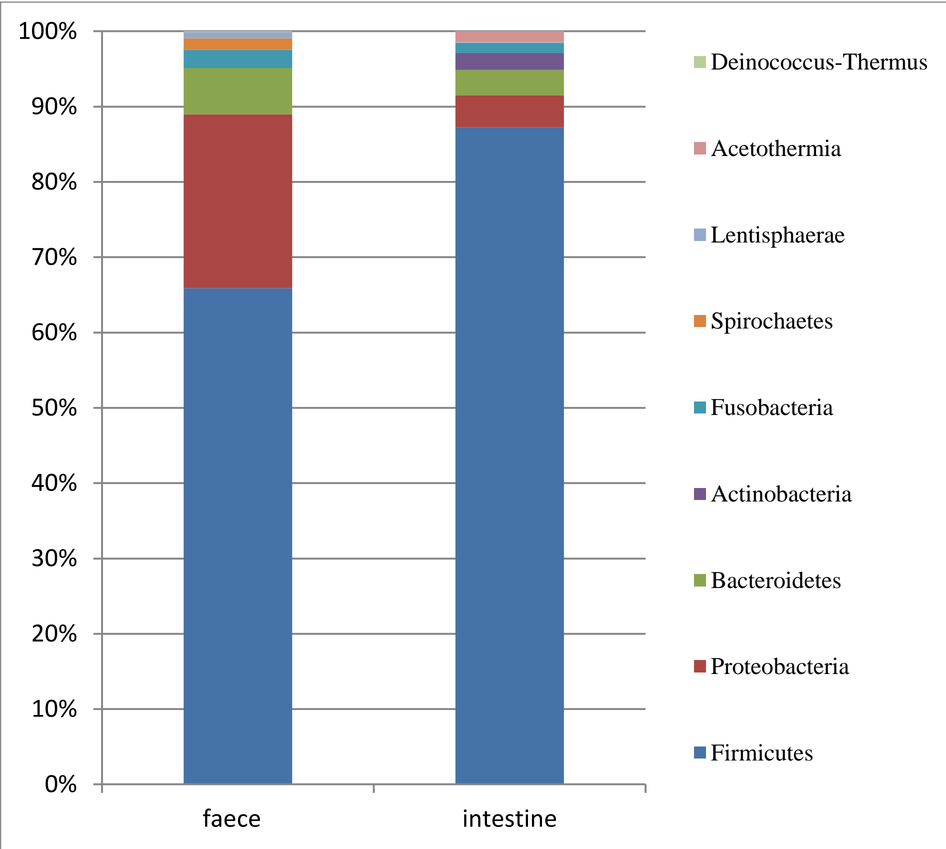
Taxonomic diversity of bacterial communities was highly variable richness ranging from 28 to 69 OUT`s, evenness from 0.0612 to 0.4217, Shannon index from 1.041 to 3.042 and Simpson index from 0.2493 to 0.9115 (Table 4.1.2). Slightly higher value of Simpson and Shannon indices for feces samples with respect to intestine were observed.

	Faeces				Intestine					
	Faeces T1	**Faeces T3**	**Faeces T11**	**Faeces T12**	**Intest ineT4**	**Intestin eT7**	**Intestin eT5**	**Intestin eT9**	**Intestin eT6**	**Intestin eT10**
Richness	61	58	39	39	48	45	47	46	40	28
Simpson	0.918	0.8999	0.8964	0.9115	0.5854	0.4742	0.8643	0.875	0.5702	0.2493
Shannon	3.042	2.603	2.599	2.8	1.078	1.041	2.552	2.339	1.433	0.6568
Evenness	0.3434	0.2328	0.3449	0.4217	0.0612	0.06293	0.2729	0.2255	0.1048	0.06888

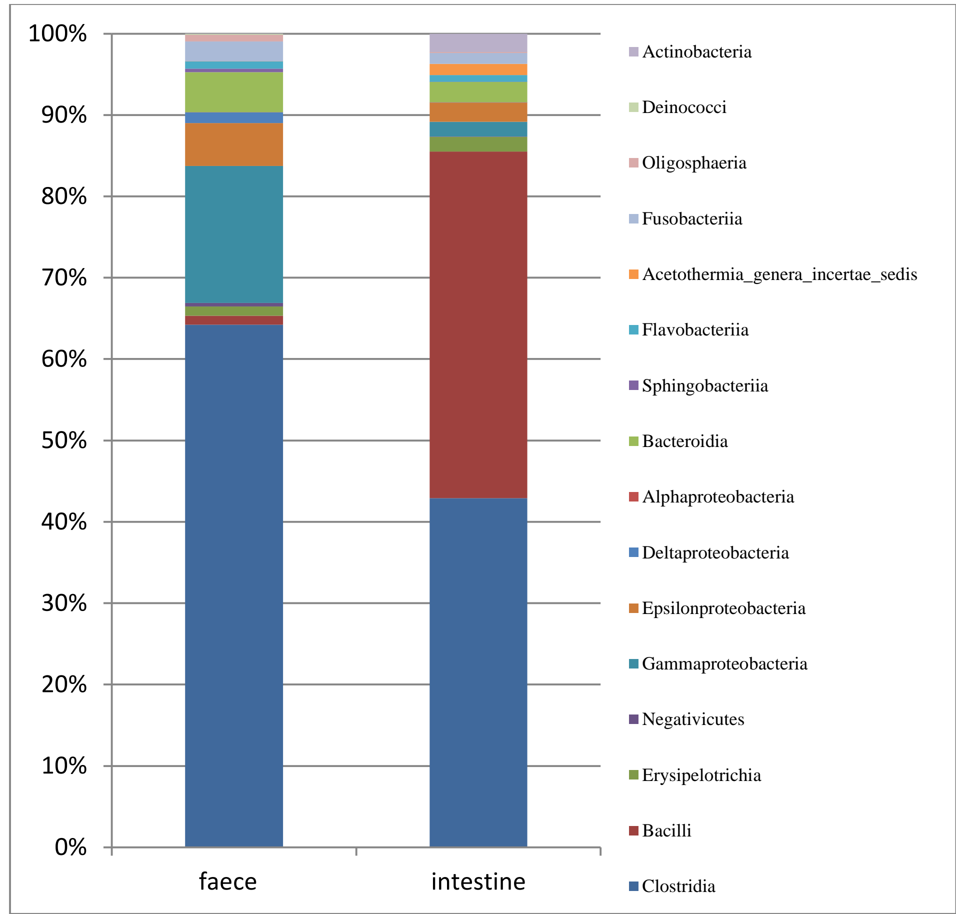
Table 4.1.2 Values of alpha diversity indices, gut and faeces of *Caretta caretta* samples bacterial communities. Richness, Simpson, Shannon and Evenness indexes are here reported and ordered based on the sample type.

The taxonomic composition indicated that the faeces samples were dominated by members of phyla *Firmicutes* (66%), *Proteobacteria* (23%), *Bacteroidetes* (6.2%). Within the phylum *Firmicutes* the class *Clostridia* was the most abundant (63.20%). The intestine samples were still dominated by phyla of *Firmicutes* (87%), *Proteobacteria* (4.2%) and *Bacteroidetes* (3.4%). *Firmicutes* were represented by member of the classes *Clostridia* (43%) and *Bacilli* (42.5%). This latter was entirely represented (100%) by order *Lactobacillale*s (Table S3). The most represented bacterial genus in the intestine samples was *Vagococcus* with 42.3% while for faeces were Clostridium XI 21.3%, and Clostridium sensu strict 14.6% (Figure 4.1.3).

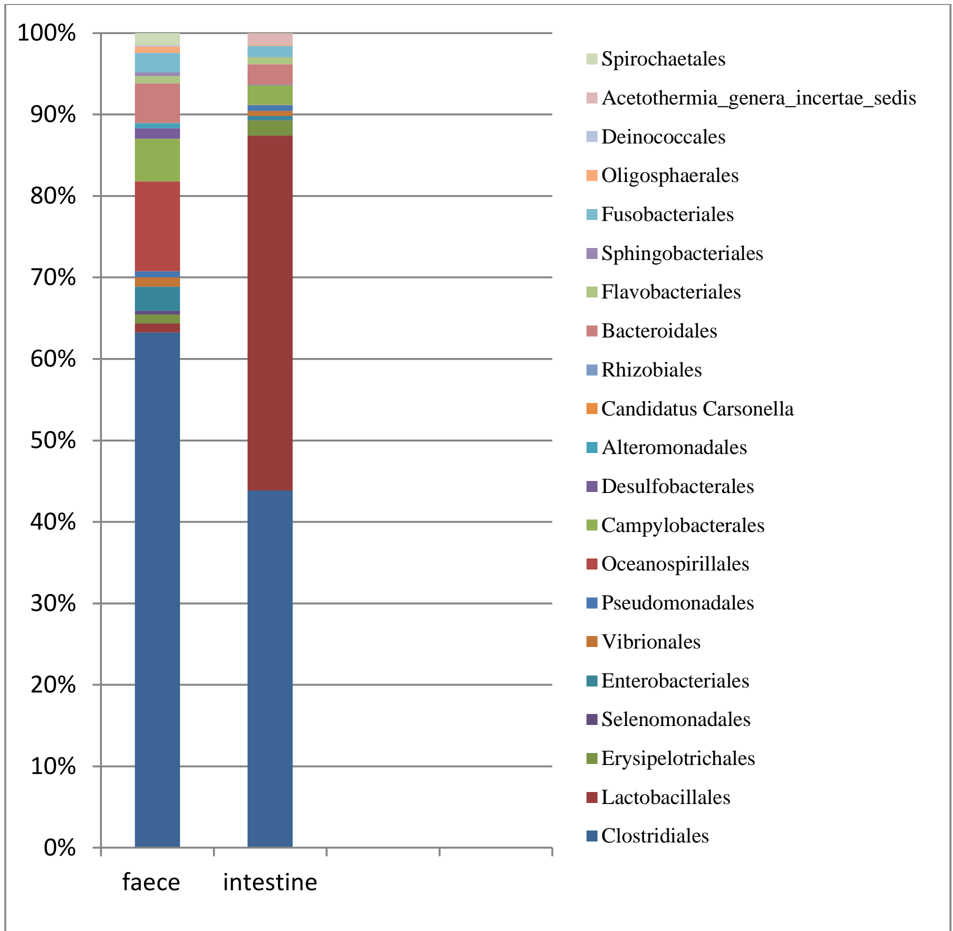
Figure 4.1.3 Taxonomic composition of *C. caretta* gut and stool microbiota. The relative abundance of bacterial phyla is reported.



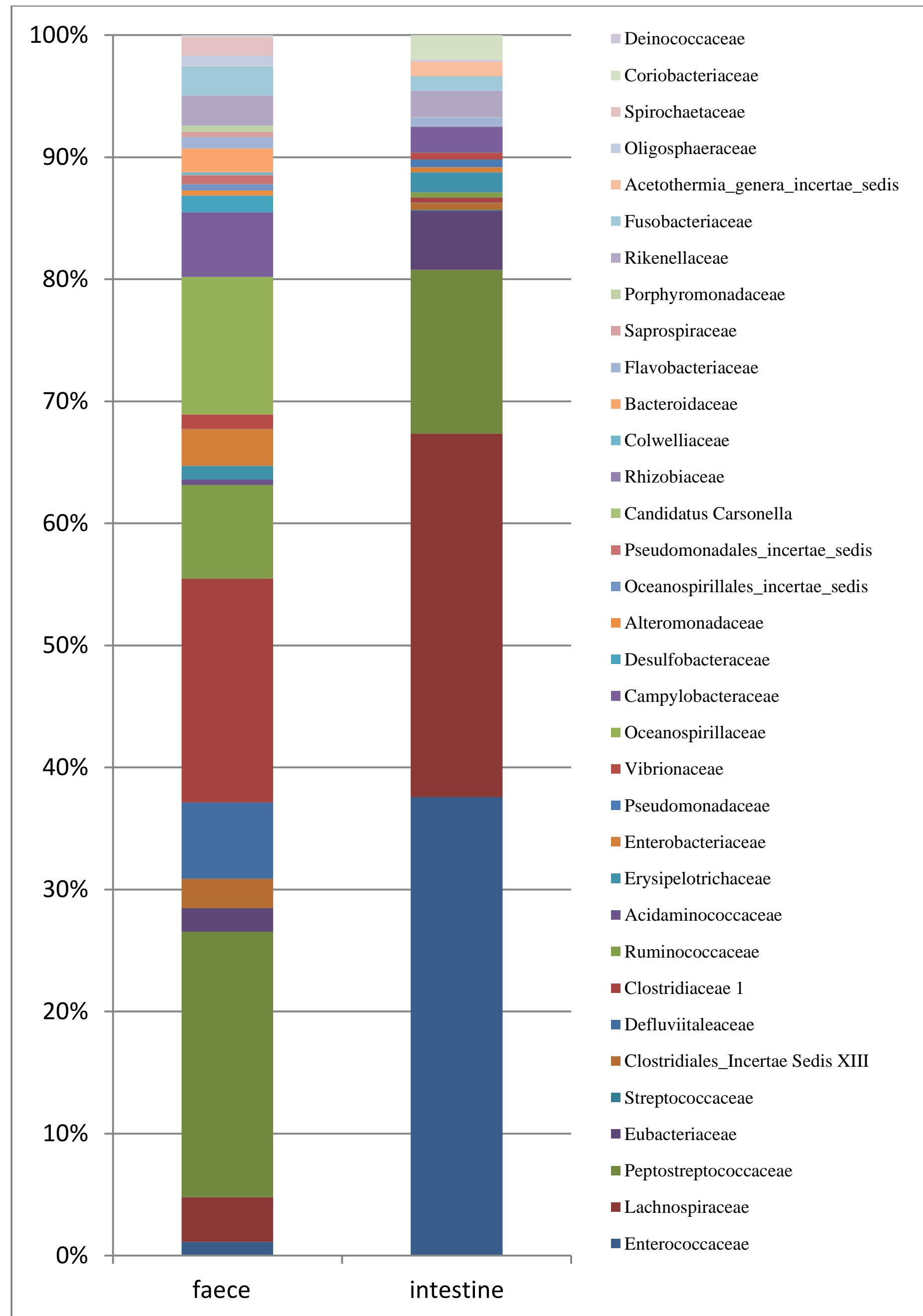
b. Histogram showing the relative abundance of bacterial classes in *C.caretta* gut and faeces .



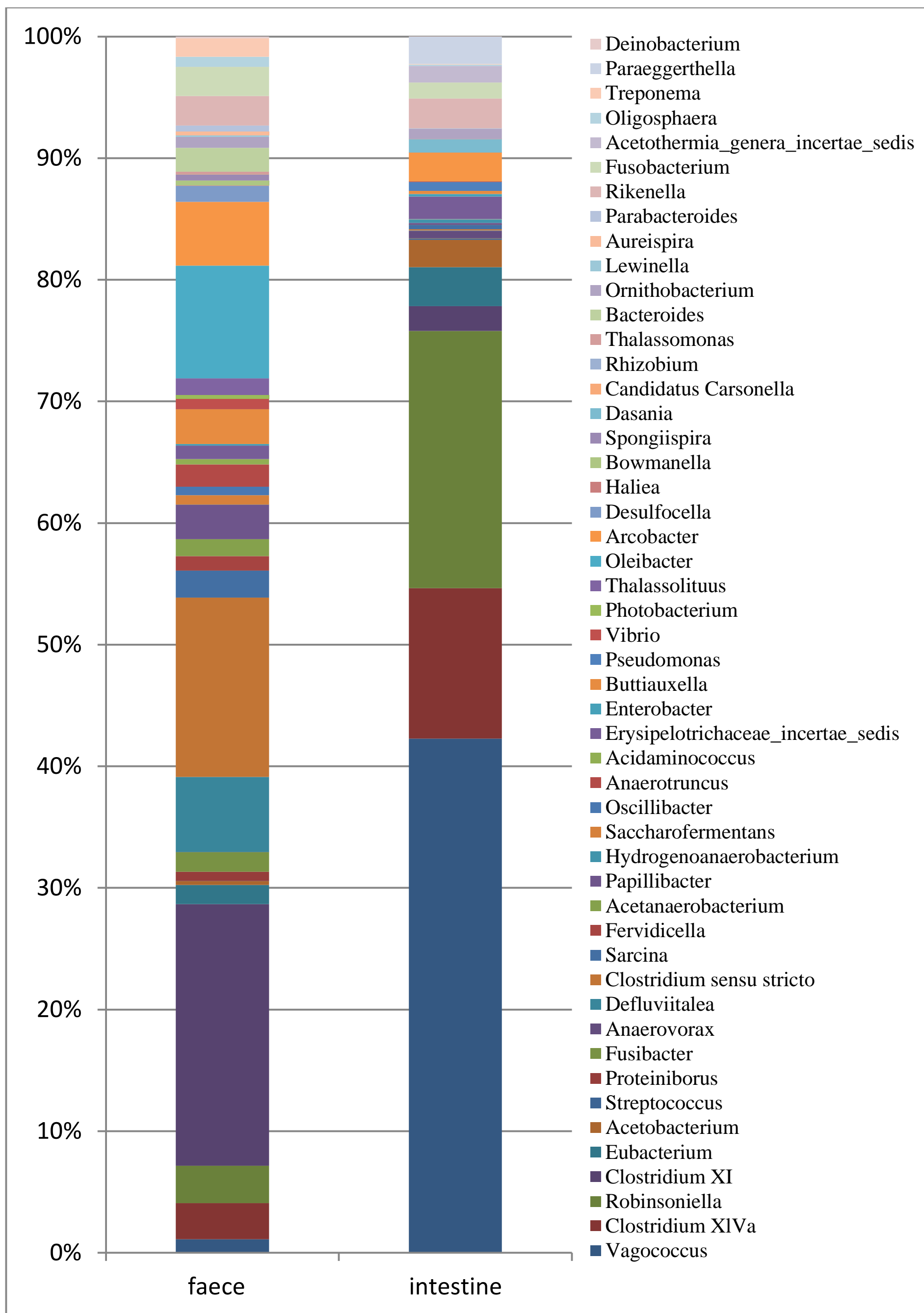
c. Histogram showing the relative abundance of bacterial orders in *C. caretta* gut and faeces .



d. Histogram showing the relative abundance of bacterial families in *C. caretta* gut and faeces.



e. Histogram showing the relative abundance of bacterial genera in *C. caretta* gut and faeces .



The SIMPER test analysis of taxonomic differences in gut microbiota between faeces and intestine, showed that the taxa mostly contributing to differences were belonging to genera *Vagococcus* (Class *Bacilli*) with contribution percentage 11.92%, *Robinsoniella* (Class *Clostridia*) with contribution percentage 6.29% (in intestine samples), Clostridium XI (Class Clostridia) with contribution percentage 7.37 % (in faeces samples) (Table 4.1.3).

Taxon	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Av. dissim**	**Contrib. %**
OTU_1	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Vagococcus	12.25	14.17
OTU_2	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Robinsoniella	6.288	7.273
OTU_3	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Clostridium XI	5.545	6.414
OTU_5	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Clostridium sensu stricto	4.63	5.356
OTU_4	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Clostridium XI	4.55	5.263
OTU_6	Firmicutes	Clostridia	Clostridiales	Defluviitaleaceae	Defluviitalea	3.124	3.614
OTU_14	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Rikenella	2.949	3.411
OTU_10	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Clostridium XlVa	2.937	3.398
OTU_32	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Buttiauxella	2.894	3.347
OTU_28	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	2.767	3.201
OTU_26	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Treponema	2.693	3.115

Table 4.1.3 Results of SIMPER analysis on taxa occurrence in faeces and intestine samples. The taxonomic attribution of OTUs, the percentage of contribution and the cumulative contribution are reported.

4.1.4 Discussion and Conclusions

In recent years, there has been a sharp increase is seen in the number of publications addressing the intestinal microbiota. Such studies have provided various lines of evidence supporting a close link between the intestinal microbiota and human health, (Gerritsen *et al.*, 2011).

This first investigation on the gut microbiota of *C. caretta* showed a pattern of taxa which include well know members colonizing vertebrate guts. In particular the abundance found for *Firmicutes* and *Bacteroidetes* is also present in human gut (Ley *et al.*, 2008; Ana Durbán & Julio Ponce 2011). Interestingly, intestine and faeces samples different in composition. For instance intestine samples were dominated by members of *Vagococcus*, while faeces samples by *Clostridium XI* and *Clostridium* sensu strictu. Such differences could reflect the different adhesion to host gut epithelium by those bacterial taxa, which may then reflect differences in their possible functional/physiological interaction with the host. . Several studies have reported a significant difference in dominant microbial community composition between colonic biopsies and faecal samples in humans (Gerritsen *et al.*, 2011). However, we should remember that sampled animals were hospitalized and subjected to intensive/sub intensive care treatments (including artificial feeding and antibiotic treatments). We cannot exclude that such treatments may have determined the observed differences, considering that intestine samples came from animals dead after intensive care treatments, while faeces in most of the cases came from animals with sub-intensive or recovery therapies. In fact *Vagococcus* strains have been isolated from lesions in mammals and diseased fishes (Schmidtke *et al.,* 1994; Teixeira *et al.,* 1997).

4.1.6 Acknowledgments

This work was performed under the project MICROMAR, GoGreen Mare 2014 CAL, Regione Toscana. We acknowledge the contribution of B.Sc. student in Natural Science Valentina Crobe for her assistance in samples collecting and DNA extraction.

4.2 Cetaceans gut microbiota

4.2.1Abstract

Microbial communities associated with the digestive tract of animals, especially mammals, are the subject of great interest internationally, both by microbial biologists and ecologists, here we investigated the microbial diversity in different cetaceans species, eight samples been collected from Tyrrhenian sea coast in Tuscany and Liguria regions, (4 faeces samples of living individuals + and 4 intestinal tissue from dead individuals), Terminal-Restriction Fragment Length Polymorphism (T-RFLP) was performed on 16S rRNA genes amplified from extracted DNA, diversity indexes were calculated with the array of T-RFs obtained. Results showed that richness and Shannon indices values were higher in faeces sample of *Physeter macrocephalus*, while the highest value of evenness has been found in intestine sample of *Tursiops truncates.*

4.2.2 Introduction

Since many years microbial communities associated with the digestive tract of animal are stirring the attention of several investigators. This is due to both interests in the study of biodiversity but also to the fact that the gut microbiota of animals (of mammals in particular) has high impact on the healthy status of animals (Zhu, B. *et al.*, 2010). The investigation carried out so far (using both culture techniques and metagenomic analyses) (Bae, 2011; De Filippo *et al.*, 2011) have highlighted tight relationships between the composition of the microbial community and the general health status and digestive ability of the animal (see (Armougom *et al.*, 2009; Barbut & Joly, 2011; De Filippo *et al.*, 2011; Dimitrov, 2011; Kinross *et al.*, 2011)).

One of the most fascinating aspects of such studies has been the discovery that the animal gut is an extraordinary reservoir of microbial biodiversity. Its study has strongly increased our knowledge on biology, ecology and biodiversity of microorganisms and of their host animals.

Concerning cetaceans there were no studies on gut microbial communities and in general few are the studies related to microbial communities associated with such animals, as for example wounding (Apprill *et al.*, 2011) or blooming of bacteria on dead animals (Naganuma *et al.*, 1996; Palacios *et al.*, 2009; Goffredi & Orphan, 2010).

In particular, there were no reports on bacteriological exams carried out with modern metagenomic techniques, which can allow to better understand the biology of such animals and better evaluate the causes of their stranding. Modern metagenomic techniques (Mendizabal & Morales, 2010), together the many data available on mammalian gut microbiota (Barbut & Joly, 2011), could allow to fill such lack of knowledge and could have profound impact on the issues of the stress due to human pressure and environmental pollution toward the populations of cetaceans and marine vertebrates.

The aim of this work order to shed a first light on the diversity of the microbial community associated with the digestive system of these large marine vertebrates (whales).

4.2.3 Methods

4.2.3.1 Sampling

From Santuario Pelagos an area of about 87.500 km2 between Toulon (French Riviera), Capo Falcone (West Sardinia), Capo Ferro (Eastern Sardinia) and Fosso Chiarone (Tuscany) (Figure 4.2.1). a total of eight cetaceans of different species have been collected along the year 2014 from different locations (Table 4.2.1). Samples were constituted by faeces of four living animals or gut content and tissue portions of three animals died in the recovery centers.

All samples were immediately stored at -20°C prior of the extraction of DNA .

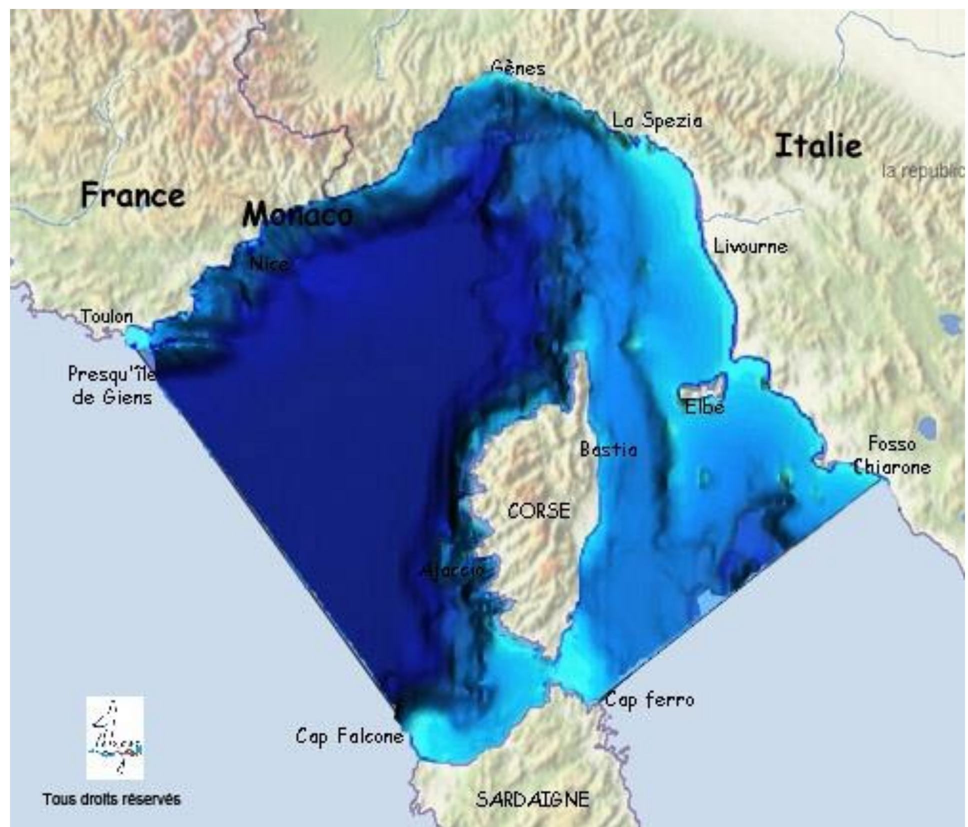


Figura 4.2.1 Sampling area, Il santuario Pelagos.

Original sample code	Origin from OTC Centres	Species	Internal ID	Type of sample	DNA extraction method	PCR reaction
14087711	IZSLT(Pisa)	*Physeter macrocephalus*	CL	Faeces	CHELEX 20%	+
RT87Sc	IZSLT(Pisa)	*Stenella coeruleoalba*	SL	Faeces	CHELEX 20%	+
13012549 RT61Sc	IZSLT(Pisa)	*Stenella coeruleoalba*	P1	intestine	CHELEX 10%	-
14061356	IZSLT(Pisa)	Undetermined (maybe *Tursiops truncatus*)	P2	intestine	CHELEX 10%	+
RT90Pm	IZSLT(Pisa)	*Physeter macrocephalus*	P3	Faeces	FastDNA soil kit	+
14046418 RT82Tt	IZSLT(Pisa)	*Tursiops truncatus*	P4	intestine	FastDNA soil kit	+
13017526	IZSLT(Pisa)	*Tursiops truncatus*	P5	intestine	FastDNA soil kit	+
13017520 RT67Bp	ARPAT (Livorno)	*Balaenoptera physalus*	P6	Faeces	FastDNA soil kit	+

Table 4.2.1 Cetaceans samples and number of DNA extraction performed under the project MICROMAR.

4.2.3.2 DNA extraction, T-RFLP profiling.

DNA was extracted from feces, and gut tissues using the FastDNA™ SPIN Kit for soil (MP Biomedicals, Italy), visualized by ethidium bromide stained agarose gel (0.8% TAE w/v) electrophoresis and quantified spectrophotometrically using the Infinite® M200 PRO NanoQuant (Tecan, Milan, Italy), respectively.

Terminal-Restriction Fragment Length Polymorphism (T-RFLP) was performed on 16S rRNA genes amplified from extracted DNA with primer pairs 799f and 1495r, as previously reported (Pini *et al.*, 2012). The choice of 799f primer avoid amplification of chloroplast 16S rRNA genes (Mengoni *et al.*, 2009). Purified amplification products were digested separately with restriction enzymes *MspI* and *HinfI* and digestions and resolved by capillary electrophoresis and on an ABI3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using LIZ 500 (Applied Biosystems) as size standard. T-RFLP analysis was performed on two technical PCR replicates from each DNA extract, as previously reported (Mengoni *et al.*, 2005). Only peaks present in both duplicate runs were considered for successive analyses.

4.2.3.3 Statistical analyses and processing of T-RFLP data

From T-RFLP chromatogram files a binned peak matrix was obtained after importing into PeakStudio 2.2 software (https://fodorlab.uncc.edu/software/peakstudio).. Statistical analyses were performed on the matrix obtained by linearly combining data from the two restriction enzymes, as previously reported (Mengoni *et al.*, 2009; Pastorelli *et al.*, 2011). Computation of diversity indices, cluster, and multivariate analyses were performed with the modules present in Past 3 software (Hammer *et al.*, 2001).

4.2.4 Results

T-RFLP profiling obtained from DNA extracted from the 8 sampling sites generated a total of 20 polymorphic TRFs, spanning from 27 to 141 nucleotides in length.

T-RFLP has been performed aimed to identify differences in the composition of the microbial community among samples. In Figure 4.2.2 an example of obtained T-RFLP is reported.

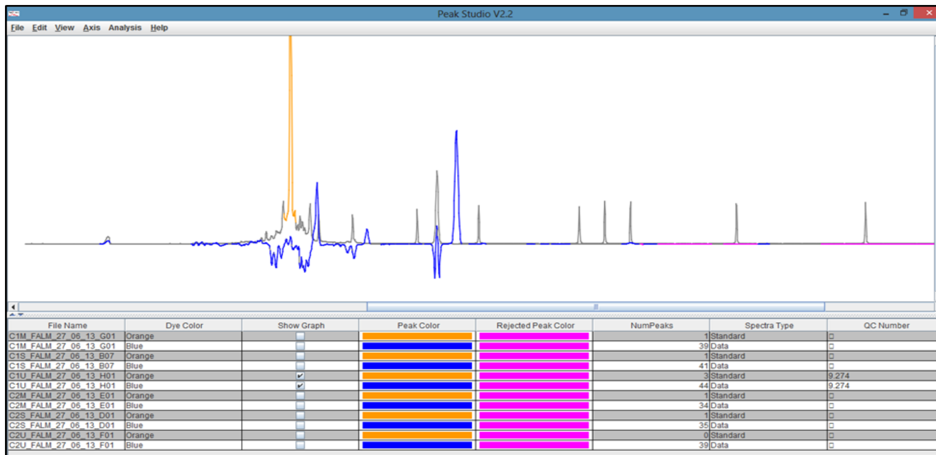


Figure 4.2.2 One of the T-RFLP profiles obtained

4.2.4.1 Diversity of bacterial communities associated with the intestinal contents of Cetaceans

The T-RFLP profiles were compared to identify any differences between the samples in relation to cetaceans. With the array of T-RFs obtained were calculated diversity indexes (Table 4.2.2). The index values of Richness and Shannon indices were found to be higher in feces sample of *Physeter macrocephalus* RT90Pm. And the greater value of evenness is were found in intestine sample P5 of *Tursiops truncatus* RT68Tt.

	RT67Bp P6	*CL*	*RT61Sc P1*	*RT87Sc SL*	*RT84Tt P2*	*RT82Tt P4*	*P5*
Richness	8	11	6	6	9	6	6
Shannon H	1.206	1.539	0.74	1.194	1.479	0.7173	1.227
Evenness	0.4176	0.4236	0.3493	0.5499	0.4877	0.3415	0.5686

Table 4.2.2: Biodiversity indices (Richness, Shannon, and Evenness)

then a cluster analysis using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) was performed (Figure 4.2.3), which showed the presence of a group of mixed samples that includes the *Balaenoptera* (RT67Bp), the *Physeter macrocephalus* (RT90Pm) a *Stenella* (RT87Sc) and a tursiops (RT84Tt), but also the presence of a distinct group of only dolphins, in particular, two tursiops (RT 82Tt) and (RT68Tt) and a *Stenella* (RT61Sc).

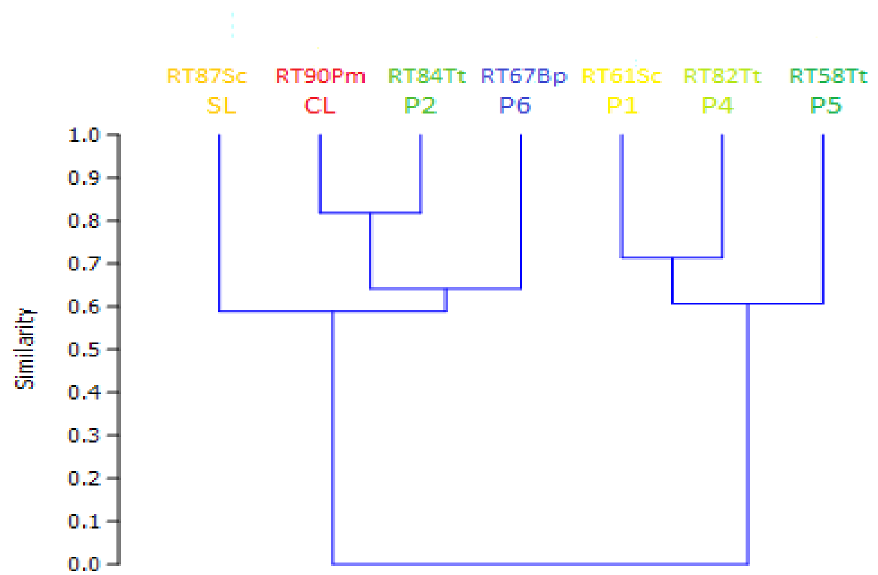


Figure 4.2.3 UPGMA of T-RFLP profiles

To confirm the T-RFLP profile obtained from the analysis it was subsequently carried out a PCA analysis (Figure 4.2.4). This analysis confirmed the previous pattern and has also highlighted that the differences between the samples are attributed to three main T-RF (Figure 4.2.5):

- 77-79 nt; Firmicutes
- 105-107 nt; Actinobacteria
- 139-141 nt; Proteobacteria

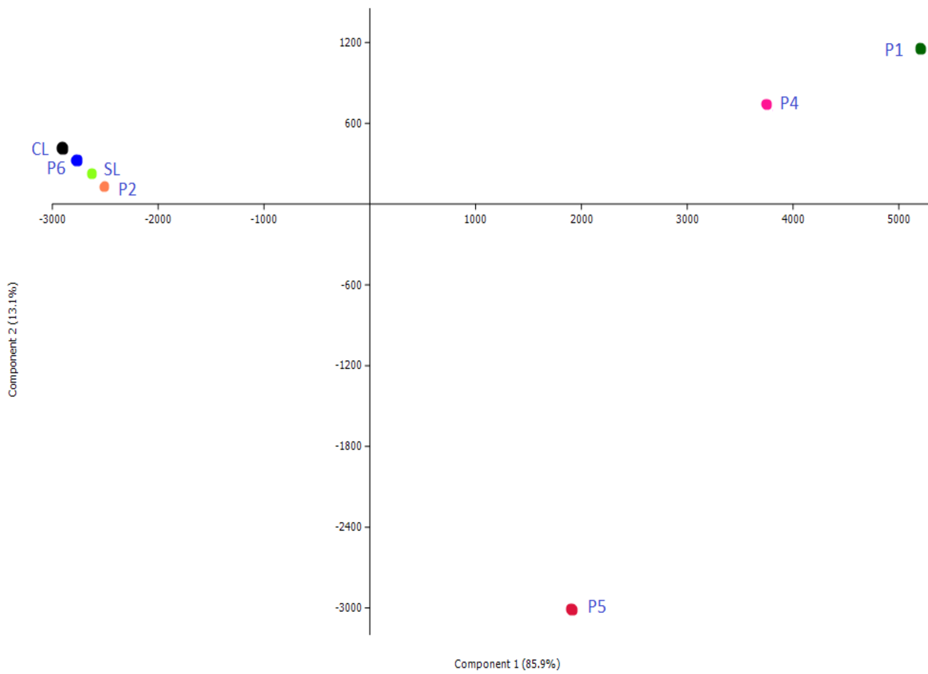


Figure 4.2.4 PCA of T-RFLP profiles

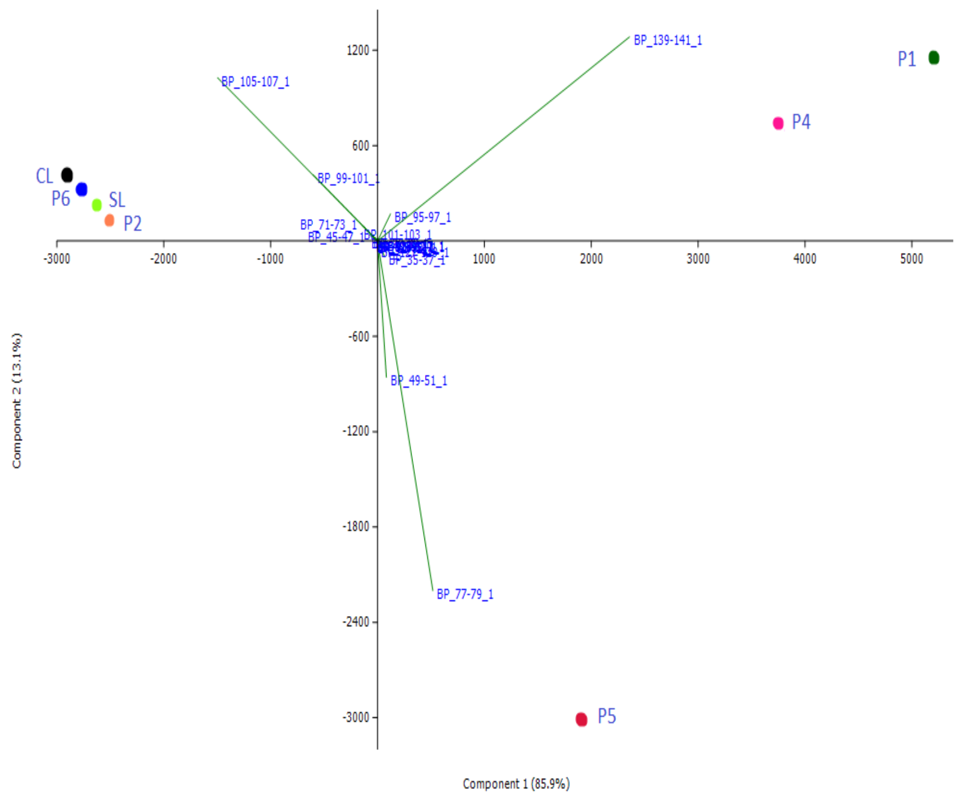


Figure 4.2.5: PCA of T-RFLP profiles and size of T-RF (Terminal-Restriction Fragments)

4.2.5 Conclusions

The results of this investigation has allowed having a first indication on the gut microbiome of cetaceans. In particular, in several samples the presence of *Clostridium* as been detected. This bacterial genus is a common commensal of mammalian gut, but includes also some pathogenic species (Ballal *et al.*; Barnes & Powrie), the T-RFLP analysis showed the presence of some possible taxa (T-RF) which differentiate the samples into two distinct groups: on one hand the group of mixed samples comprising a *balenottera*, the *capodoglio* and two dolphins, the other a group of only dolphins.

It should in fact given the current low level of retention samples (the animals were dead at the time of collection and some already in a state of partial decomposition), which has certainly altered the intestinal microflora.

4.2.6 Acknowledgments

This work was performed under the project MICROMAR, GoGreen Mare 2014 CAL, Regione Toscana. We acknowledge the contribution of B.Sc. student in Natural Science Valentina Crobe for her technical assistance in sampling and DNA extraction.

CHAPTER V

CONCLUSIONS

Chapter 5. Overall conclusions

On the overall, the results obtained in this PhD thesis have allowed to shed additional light on the pervasive presence of bacteria in natural environments. In particular we have for the first time reported evidences of interaction of environmental variables on sandy beaches bacterial communities (Chapter II), and of microbiota-host relationships in talitrid amphipods (Chapter III). Additionally, we have then shifted the attention to two large vertebrates, key species in the protection of marine habitats (cetaceans and sea turtles), showing, again for the first time, the microbiota present in the gut of such animals. The amount of data produced here is then highly relevant for studies on host-microbe interaction and possibly protection of key species of the coastal and marine habitats.

CHAPTER VI

APPENDEX

Chapter 6. Appendix

6.1 Comparison of two molecular methods for microbiota analysis, T-RFLP and 16S rRNA metagenomics

Terminal restriction fragment length polymorphism (T-RFLP) is a method that has been frequently used to survey the microbial diversity of environmental samples and to monitor changes in microbial communities, However, it is difficult to obtain the information of nucleotide sequences because the T-RFs are fragmented and lack a priming site of 3'-end for efficient cloning and sequence analysis. (Lee *et al.*, 2008). There is no consensus on how to treat T-RFLP data to achieve the highest possible accuracy and producibility (Fredriksson *et al.*, 2014)

During the last years Next Generation Sequencing (NGS) developed rapidly enabling the production of massive amount of sequence data that can be used for metagenomic study analyzing microbial communities by ammplicon sequencing (Simon & Daniel, 2011; Knief, 2014). Despite of the price decreasing for the Next generation sequencing (NGS) approaches T-RFLP still far cheaper.

In Chapter III we have investigated the resilience of the littoral amphipod *Talitrus saltator* gut microbiome. Data reported in Chapter III are based on 16S rRNA gene metagenomics. Here we analyzed the very same DNA with T-RFLP to test the T-RFLP reliability in comparing between different microbial communities diversity comparing with the NGS using Illumina MiSeq technology.

6.1.1 DNA extraction, T-RFLP profiling

DNA extraction and Real-Time PCR estimation of bacterial load in T.s intestine were performed as previously reported (Bacci *et al.*, 2015c). Terminal-Restriction Fragment Length Polymorphism (T-RFLP) was performed on 16S rRNA genes amplified from extracted DNA with primer pairs 799f and 1495r, as previously reported (Pini *et al.*, 2012). The choice of 799f primer avoid amplification of chloroplast 16S rRNA genes (Mengoni *et al.*, 2009), allowing to better target bacterial community DNA, reducing the amount of amplified DNA from algal origin and *Posidonia* origin. Purified amplification products were digested separately with restriction enzymes *MspI* and *HinfI* and digestions and resolved by capillary electrophoresis and on an ABI3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using LIZ 500 (Applied Biosystems) as size standard. T-RFLP analysis was performed on two technical PCR replicates from each DNA extract, as previously reported (Mengoni *et al.*, 2005). Only peaks present in both duplicate runs were considered for successive analyses.

6.1.3 Biodiversity indices analysis and statistical analysis

From T-RFLP chromatogram files a binned peak matrix was obtained after importing into PeakStudio 2.2 software (https://fodorlab.uncc.edu/software/peakstudio). Peaks above 100 fluorescence units and whose size ranged from 35 to 500 nt were considered for profile analysis. Statistical analyses were performed on the matrix obtained by linearly combining data from the two restriction enzymes, as previously reported (Mengoni *et al.*, 2009; Pastorelli *et al.*, 2011). Computation of diversity indices.

The same statistical analyses were performed on OTU`s obtained from the automated O2tab Pipeline .

Alpha diversity analyses include computation of Richness, Shannon, Richness and Evenness, indices, to assess and compare the variation of gut sample microbial diversity along the five different sampling time points (0, 24h, 7-23-51 days), (Figure 6.1.1).

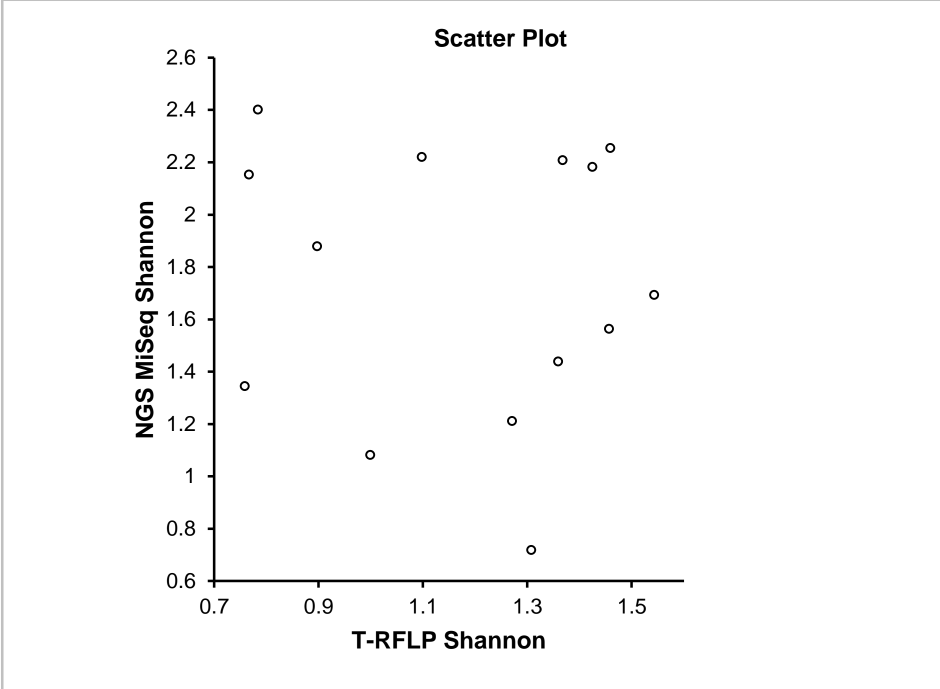
6.2.4 Comparison between Biodiversity indices obtained from T-RFLP and NGS metabarcoding analysis .

Alpha Diversity indices (Shannon, Simpson, Richness and Evenness) calculated for T-RFLP and NGS MiSeq data to compare the two methods, we calculate the correlation between the two methods alpha indices values, results reported in Figure 6.1.1

Figure 6.1.1 The correlation between T-RFLP and NGS MiSeq obtained Alpha Diversity indices.

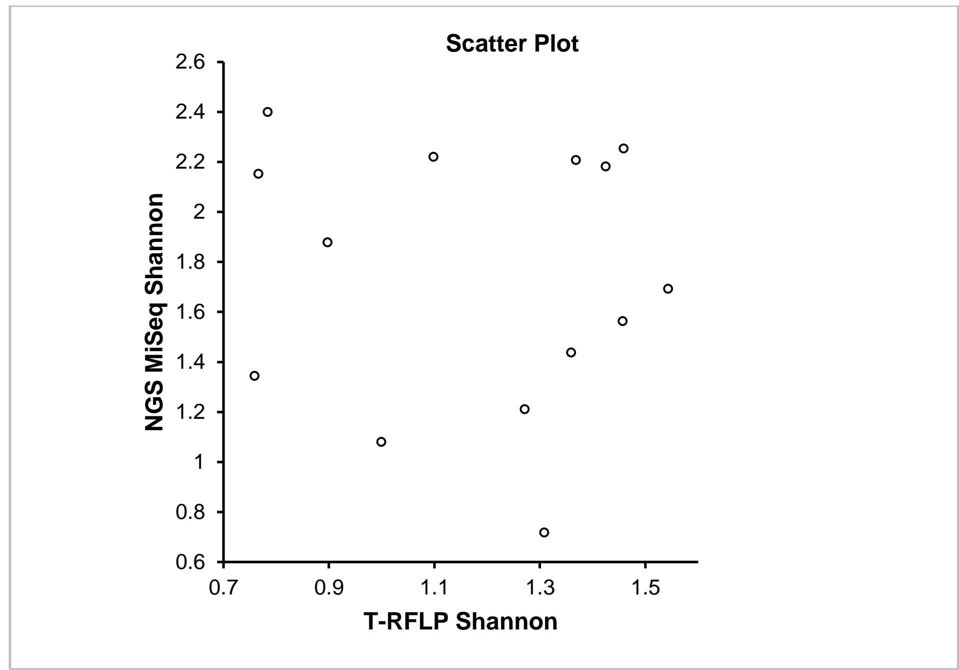
a) Shannon index

n	14		
r statistic	**-0.09**		
95% CI	-0.60	to 0.46	(normal approximation)
t statistic	-0.33		
DF	12		
2-tailed p	0.7480	(t approximation)	



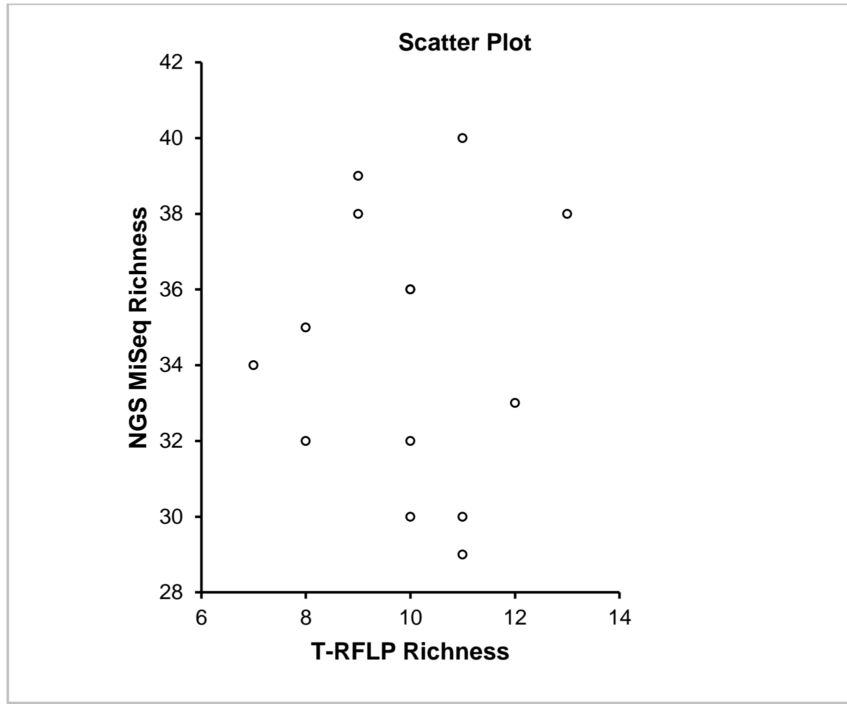
b) Simpson Index

n	14		
r statistic	**-0.10**		
95% CI	-0.60	to 0.45	(normal approximation)
t statistic	-0.35		
DF	12		
2-tailed p	0.7296	(t approximation)	



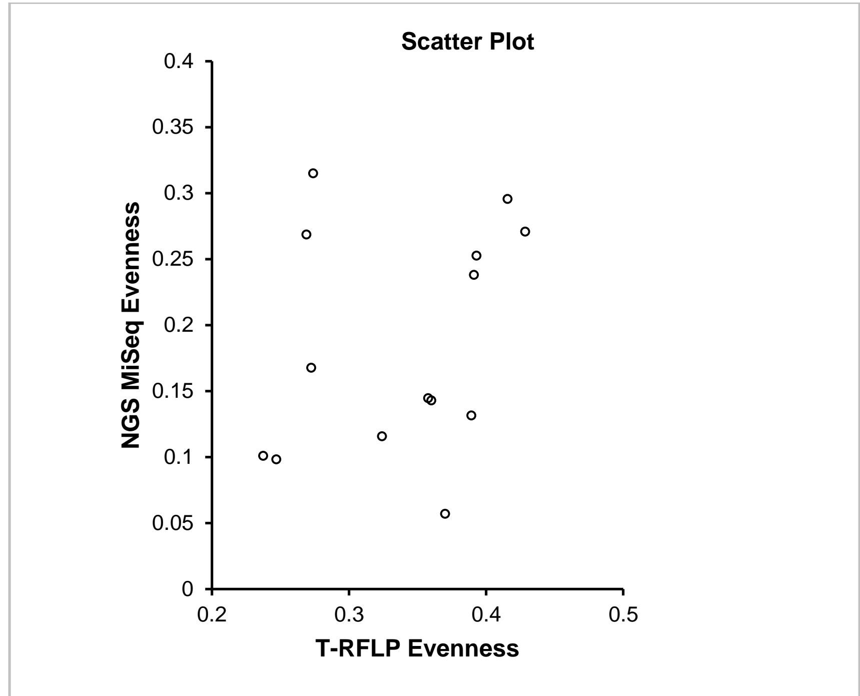
c) Richness Index

n	14		
r statistic	0.01		
95% CI	-0.53	to 0.53	(normal approximation)
t statistic	0.02		
DF	12		
2-tailed p	0.9847	(t approximation)	



d) Evenness index

n	14		
r statistic	0.26		
95% CI	-0.32	to 0.69	(normal approximation)
t statistic	0.92		
DF	12		
2-tailed p	0.3746	(t approximation)	



6.1.5 Result and discussion

The result of comparison alpha diversity (Shannon, Simpson, Evenness and Richness) of the five time points that came through the two methods (T-RFLP and NGS, illumina MiSeq been way difference and un correlated Figure 6.1.1, showed the scattered values results different from each methods , the correlation between T-RFLP and NGS MiSeq obtained Alpha Diversity indices were weak ranges from , -0.09for Shannon index, -0.10 for Simpson index, 0.01 For Richness index, 0.26 for evenness index.

Fredriksson et al 2014 discussed the impact of critical steps in T-RFLP data treatment. The alignment of the T-RFs to compare the samples, and the normalization of T-RFLP profile, were indicated as the most critical, since they produce large differences in the outcome. This of course may affect diversity value estimation and produce biases which can ultimately may result in a lack of correlation with a potentially more robust methods, as those based on NGS of 16S rRNA gene amplicons. However, a careful standardization of the T-RFLP metholodology throughout the samples allows to compare samples among them without biases due to heterogeneity of sample treatment. In this regard the standardization of digested DNA during T-RFLP procedure has been shown to be highly effective (Mengoni *et al.*, 2007). Consequently, the low correlation between T-RFLP and MiSeq community diversity data, observed in our dataset, may be due to the different power of detection of rare taxa. Indeed it is know that t-RFLP can detect only taxa more abundant than 1% of the community (Dunbar *et al.*, 2000) and the discrimination power of T-RFLP is lower than that of the sequencing of 16S rRNA genes, since only few nucleotides are scanned by the 2-3 restriction enzymes used. However, T-RFLP has been proved to be reliable in comparison to 16S rRNA gene massive sequencing when the dynamics and pattern of variation of bacterial communities are investigated (Pilloni *et al.*, 2012).

6.2 Preliminary Investigation of bacterial diversity in supralittoral sediments from different Libyan beaches

6.2.1 Introduction

In sandy beaches an ecological network is present, mainly related to meio- and macrofauna (McLachlan *et al.*, 1993; Schlacher *et al.*, 2008). It has been recognized that bacteria inhabiting sandy beaches may account for up to 87% of annual production in these environments (Koop & Griffiths, 1982). Sandy beaches encompass 75% of the world's unfrozen shorelines, they provide important ecosystem services, including seawater filtration and purification. Dissolved and particulate organic materials are mineralized as seawater passes through the sands; thus, beaches also play an important role in nutrient cycling. Microorganisms present in the lacunars environment between sand grains provide these ecosystem services. However, few studies have characterized the microbial community in beach sands (Boehm *et al.*, 2014). In fact, the microbial ecology of sandy sediments has stirred still a relatively limited attention. Most of the studies have being focused on presence of bacterial pathogens or on the ecology of submerged sediments and the impact of pollution, such as oil spills (Newton *et al.*, 2013b; Engel & Gupta, 2014; Halliday *et al.*, 2014; Whitman *et al.*, 2014; Xiong *et al.*, 2014; Bacci *et al.*, 2015c). As reported in Chapter II, an investigation on Sardinian sandy beaches highlighted the contribution of environmental variables in sandy beaches bacterial community dynamics. However, since the sampled geographical area was relatively limited, it is still to be confirmed the potential presence of a large-scale biogeographical structuring of the community. To clarify this issue a preliminary investigation was carried out by characterizing the bacteria diversity at some South Mediterranean sites, located along the coasts of Libya. Terminal restriction fragment length polymorphism (T-RFLP) analysis was used for bacterial community characterization.

6.2.2 Methods

6.2.2.1 Sampling site description, sampling procedure and physico-chemical characteristics

Samples of sand (5 cm below surface) were taken in December 2013, in 6 sites along 200 km west of Benghazi (Libya), located variable area. Samplings took place in late December far from recreational season to avoid sand mixture and direct contamination by human trampling (Figure 6.2.1) (Table 6.2.1).

DATE	PLACE	SAMPLE CODE	LONGITUDE	LATITUDE	SAMPLE CODE
22/12/2013	Al-Zwitina weat	Z W	20°07'06.23" E	30°57'46.46" E N	ZW
22/12/2013	Al-Zwitina east	Z E	20°07'18.50" E	30°58'08.04" N	ZE
23/12/2013	Tarria w	T W	19°56'33.75" E	31°52'18.31" N	TW
23/12/2013	Tarria e	T E	19°56' 44.12" E	31°53'02.68" N	TE
23/12/2013	Bofakhra	B	19°56'52.35" E	31°56'54.56" N	B
23/12/2013	Garyounis	G	20°02'07.02' E	32°03'44.55" N	G

Table 6.2.1 Location of sampling sites of Libyan sandy beaches.

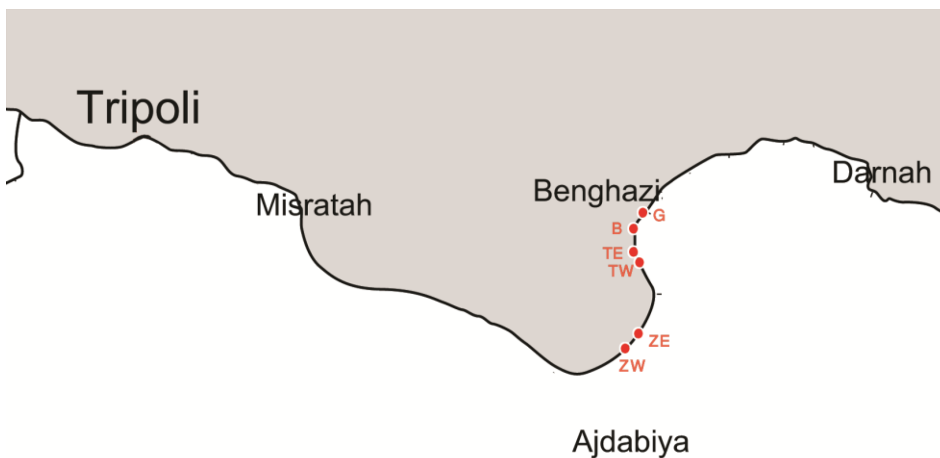


Figure 6.2.1 Sampling sites of Libyan beaches

6.2.2.2 DNA extraction, T-RFLP profiling and Real-Time PCR

DNA extraction and Real-Time PCR estimation of bacterial load in sediments were performed as previously reported (Bacci *et al.*, 2015c) and Chapter II. Terminal-Restriction Fragment Length Polymorphism (T-RFLP) was performed on 16S rRNA genes amplified from extracted DNA with primer pairs 799f and 1495r, as previously reported (Pini *et al.*, 2012). Purified amplification products were digested separately with restriction enzymes *MspI* and *HinfI* and digestions and resolved by capillary electrophoresis and on an ABI3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) using LIZ 500 (Applied Biosystems) as size standard. T-RFLP analysis was performed on two technical PCR replicates from each DNA extract, as previously reported (Mengoni *et al.*, 2005). Only peaks present in both duplicate runs were considered for successive analyses.

6.2.2.3 Statistical analyses and processing of T-RFLP data

This part was performed as previously reported (Bacci *et al.*, 2015c) and Chapter 2. Briefly, from T-RFLP chromatogram files a binned peak matrix was obtained after importing into PeakStudio 2.2 software (https://fodorlab.uncc.edu/software/peakstudio). Peaks above 100 fluorescence units and whose size ranged from 35 to 500 nt were considered for profile analysis. MiCA web tool (https://mica.ibest.uidaho.eudu) performed on T-RFs to interpret the taxonomic compositions (Shyu *et al.*, 2007). Statistical analyses were performed on the matrix obtained by linearly combining data from the two restriction enzymes, as previously reported (Mengoni *et al.*, 2009; Pastorelli *et al.*, 2011). Computation of diversity indices, cluster, and multivariate analyses were performed with the modules present in Past 3 software (Hammer *et al.*, 2001).

6.2.3 Results

T-RFLP profiling obtained from DNA extracted from the 8 sampling sites generated a total of 33 polymorphic TRFs, spanning from 27 to 153 nucleotides in length.

Taxonomic diversity of bacterial communities was highly variable richness ranging from 6 to 11 T-RFs, Simpson index from 0.2698to 0.6339, Shannon index from 0.6165 to 1.462 and evenness from 0.3087 to 0.4735 (Table 6.2.2).

	Bofakhra	Garyounis	tarria east	tarria west	zwitina east	zwitina west
Richness	6	11	12	8	10	11
Simpson	0.2698	0.5981	0.6268	0.6332	0.6339	0.635
Shannon	0.6165	1.362	1.462	1.332	1.347	1.395
Evenness	0.3087	0.3549	0.3597	0.4735	0.3846	0.3667

Table 6.2.2 Alpha diversity indices of Libya sandy beaches sampling sites

All sites were considered almost the same in relation to the exposure to dominant winds (see Figure 1). Diversity of communities of the different groups were similar for all indices, except of the Bofakhara site which show lower diversity indices.

To invastigate the major bacterial taxa present in these sites we firstly performed an extensive search with MiCA (Supplementary Table 6.2 S2), then a Principal Component Analysis on sites T-RFs was run (Figure 6.2.2). Results showed that Bofakhara site was the most far from others sites, and that the most important phyla that constitute the microbial communities where *Protobacteria*, *Actinobaceria*, *Bacteroidetes*, *Firmicutes* , (Figure 6.2.3) mostly as the result of microbial communities of the previous study on Sardinian sandy beaches above in Chapter II and (Bacci *et al.*, 2015c).

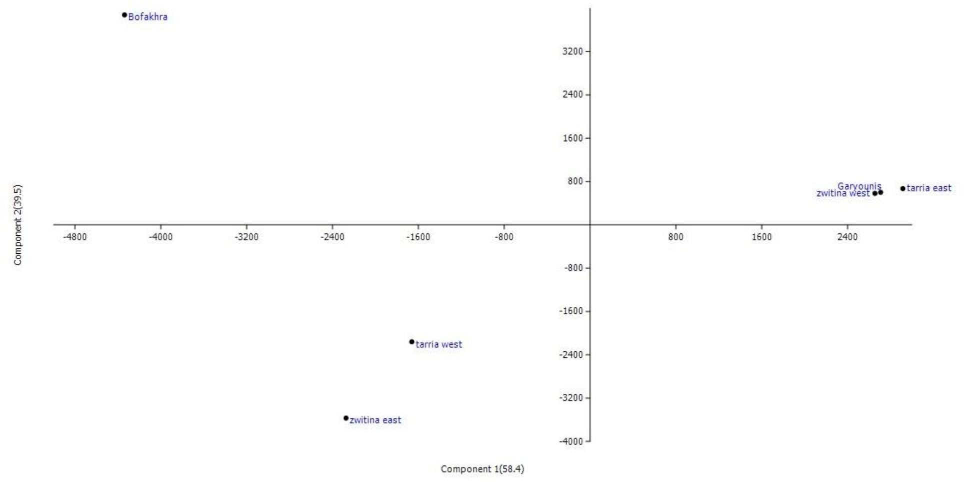


Figure 6.2.2 Principal Component Analysis of T-RFLP profiles. The percentage of variance explained by each axis is reported.

Figure 6.2.3 Taxonomic composition of the sex sampling sites of Libyan beaches

Also To evaluate the possible role of bacteria involved in the biogeochemical cycles of sulfur and nitrogen over the detected seasonal changes, genes *dsrA* encoding the sulfite reductase, and *amoA* encoding the ammonia monooxygenase gene were amplified on DNA extracted from sandy sediments (Supplemental Table 6.2.4) to compare it with the Sardinian beaches. Results showed the presence of *dsrA* in all microbial communities of Libyan sites while in Sardinian study *dsrA* present in two site during summer season, conversely *amoA* which result on several, but not all sites on Sardinian beaches. Present only in one site among the 6 sites on Libyan sandy beaches.

Sample code	*amoA*	*dsrA*
Z W	-	+
Z E	-	+
T W	-	+
T E	-	+
B	-	+
G	+	+

Table 6.2.3 Presence of *amoA* and *drsA* genes in Libyan sandy beaches bacterial communities.

6.2.4 Discussion

It is known that environmental variables, including temperature, pH, chemical composition etc. impact bacterial communities structure and diversity (Lozupone & Knight, 2007; Lauber *et al.*, 2009). Here we found that west Bengasi Libyan sandy beaches shows a proximately the same level microbial diversity although the result where low comparing to the Sardinian sandy beaches (chapter II) except of evenness index which reported high in all Libyan sites.

A previous 16S rRNA metagenomic survey of sandy sediments in the Mediterranean island of Favignana (Egadi Archipelago, Sicily), and our T-RFLP profiling study indicated an abundance of marine *Alphaproteobacteria Gamaproteobacteria* in the supralittoral sediments (Bacci *et al.*, 2015c),(chapter II) also suggested that marine taxa seem strongly contribute to sandy beaches bacterial communities, here we found almost the same dominant taxa , *Protobacteria*, *Actinobaceria*, *Bacteroidetes*, *Firmicutes.*

Moreover, both community taxonomic composition and diversity (alpha) were almost in the same range between all the sampling site except of Bofakhara site for which higher alpha diversity values were recorded in spring, as well as a higher heterogeneity among samples, with respect to late summer/autumn samplings (September and October), confirming the previous observation obtained on Sardinian sandy beaches (Chapter II). Here, we reported that geographical location, even at large range, seems to have no influence on bacterial community diversity of supralittoral sandy sediments.

Interestingly, we found a functional signature in all sites (as presence of the sulfite reductase gene *dsrA*). The detection of *dsrA* gene could be related to some levels of hydrocarbon contamination (Chin *et al.*, 2008). Here we found also a considerable presence of *Deltaproteobacteria* class, which includes the family *Desulfobacteraceae* whose members are active in sulfite reduction (they contain *dsrA* gene). *Desulfobacteraceae* are known to play pivotal role in alkane degradation in marine environment (Kleindienst *et al.*, 2014) and this may reinforce the hypothesis suggested in Chapter II that sandy beaches bacterial communities are “seeded” by marine microorganisms.

6.2.5 Acknowledgments

We are very grateful to Dr. Ibrahim Eldawas, President of the Biology Department in Ajdabiya Art and Science College for his help in sampling for this study.

6.3 Investigating the resilience of littoral amphipod gut microbiome on *Orchestia montagui*

6.3.1 Abstract

In the study reported in Chapter III, we indicated that among the sampled amphipods species, *Orchestia. montagui* (which is found within *Posidonia* and macroalgae banquettes) harbors a different gut microbiota with respect to the other species. In particular, this species seems to harbor more taxa known to be involved in cellulose degradation and as the result of the analysis of family 48 glycosyl hydrolases (GHF48, one of the cellulase genes) *O. montagui* gut microbiota is also enriched of cellulose-degrading cells than the other talitrids. We then hypothesized that the different ecological behavior of *O. montagui* (a colonizer of *Posidonia* banquettes) could be related also to a different taxonomic and functional composition of its gut microbiota. In Section 3.2 we investigate the effect of diet on *Talitrus saltator* gut microbiota, showing its resislience to diet variation. Here we report preliminary results, related to the abundance of GHF48 genes in *O. montagui* fed artificially for two months. Results obtained so far indicated that *O. montagui* gut microbiota strongly differ in functional response related to cellulose degradation with respect to *T. saltator*. In fact a higher proportion over the total bacterial cells of GHF48 genes is present after 7 days of feeding. The differences between the two species increases up to the end of sampling (51 days).

6.3.2 Material and methods

6.3.2 .1 Sampling and Feeding Experiment

O. montagui individuals, were collected from Elba Islands beaches and transferred to the laboratory. Immediately after collections gut_samples from 3 animals were excised with sterile forceps, and stored in RNALater (Ambion).

Animals were then maintained within their sand and fed with artificial food (papers and artificial fish food). Gut samples from three animals were then taken first in their natural habitat in zero time then after 24 hours, 7days, 23days, 51days of artificial feeding.

6.3.2.2 Detection of cellulase genes

For detection and Real-Time quantification of glycosyl hydrolase family 48 (GHF48) genes a previously reported SybrGreen Real-Time PCR protocol used (Bacci *et al.*, 2015c), with annealing temperature decreased to 52°C , using GH48F/GH48R primer pair (Izquierdo *et al.*, 2010). Standard curves for 16S rRNA and GHF48 have been prepared with serial dilutions of genomic DNA of *Streptomyces coelicolor* A3 (2), which contains a putative GHF48 gene (SCO5456).

6.3.3 Results and Conclusions

An overall comparison also of the gene GH48 level between the two species (Figure 6.3.1), showed that *O. montagui* GH48 level was significantly higher than *T.saltator* as previously indicated (Section 3.2).

Concerning (Figure 6.3.2) a rapid increment of GHF48 genes/16S rRNA genes ratio along the 7days, 23days, 51 days time points was found.

In a comparison of the trends between the two species, the result came significantly different (Tukey contrast after one way ANOVA) between the last three time points between the two species (Figure 6.3.3)

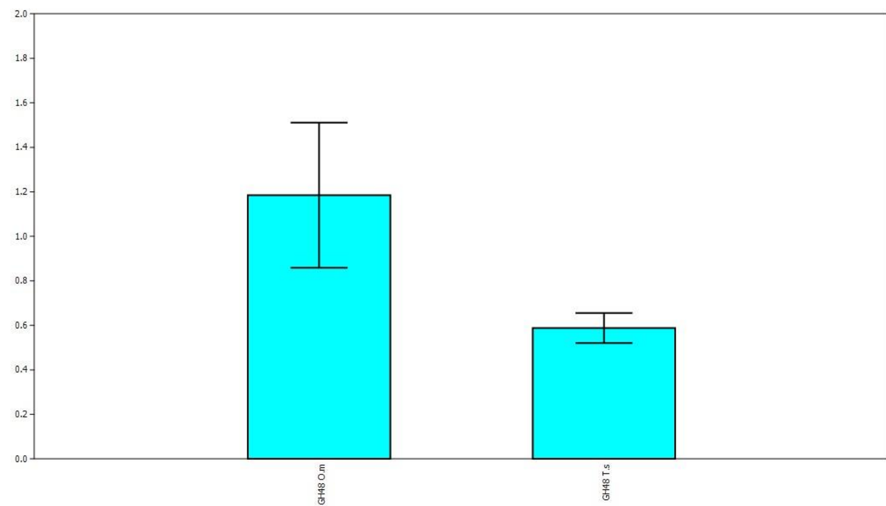


Figure 6.3.1 Comparison of the relative abundance of cellulose-degrading genes between the five time points of *O. montagui* gut and *T. saltator* samples. Barchart reporting the log of the mean proportion of glycosyl hydrolase 48 genes with respect to 16S rRNA genes in gut microbiota of time.

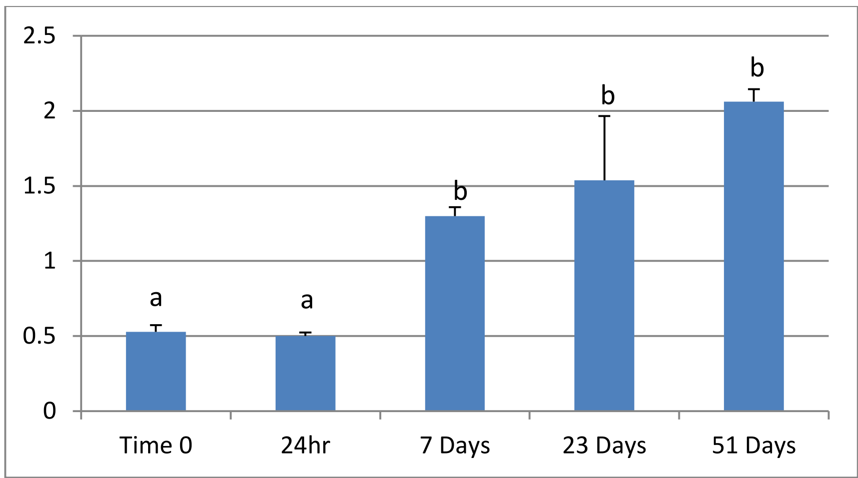


Figure 6.3.2 Relative abundance of cellulose-degrading genes in the five time points of *O. montagui* gut samples. Barchart reporting the log of the mean proportion of glycosyl hydrolase 48 genes with respect to 16S rRNA genes in gut microbiota of time points *O. montagui* gut samples. Error bars, standard deviations from three repeated measures on each gut sample. Different letters indicate statistically significant contrast (P<0.05, one-way ANOVA, Tukey pairwise).

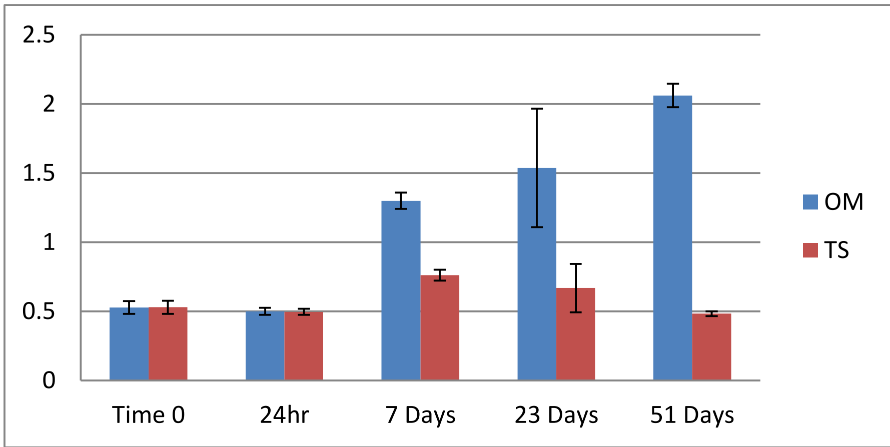


Figure 6.3.3 Abundance of cellulose-degrading genes O.m and T.s gut samples.
Error bars, standard deviations from three repeated measures on each gut samplea . Different letters indicate statistically significant contrast (P<0.05, one-way ANOVA, Tukey pairwise).

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