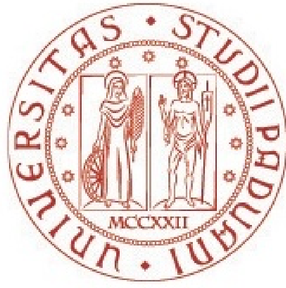

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How is animal microbiome transmitted? An integrative approach to investigate the ecological factors driving microbiome transfer using guppies

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INTRODUCTION

1. MICROBIOTA

1.1. Microbiota body of knowledge

Generally when we talk about bacteria, we refer to them with a negative connotation. This is because we have been used to thinking of them associated with human diseases. In reality, the microbial world is much broader than imagined and consists of a number of very different species and organisms, many of which are beneficial (Scott et al., 2020; Timmis et al., 2019).

The term "microbiota" was coined by Lederberg & McCray to indicate: “the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space […]”. From this basic definition, an increasingly precise understanding of its composition and role has developed (Lederberg & McCray, 2001).

The estimation of microorganisms in human body has changed many times. In the 1970s, Thomas Luckey advanced the hypothesis that the number of microbes in a gram of faeces is approximately 100 billion. Furthermore, he determined that the mean weight of faeces in an average adult is 1000 grams. Consequently, Luckey concluded that the gut must contain an average of 100 trillion microbes (LUCKEY, 1970).

In 1977, Dwayne Savage estimated a ratio of 10:1 between microbes and human cells (Savage, 1977). The most recent review, conducted by Professor Milo, revealed that the ratio of microbes to human cells is 1,3:1 (Sender et al., 2016a).

The methodological and technological evolution, from traditional culture to shotgun metagenomics, led to the introduction of the concept of *microbiome*: the set of microbial genomes present in an organism. Recent studies have shown that the number of genes in the microbiome far exceeds the number of genes in the host. In particular, it has been shown that the human gut microbiome contains at least one hundred times more genes than our genome (Gill et al., 2006).

This finding indicate a delicate equilibrium between our own cells and those of the microorganisms we constantly interact with, which is crucial for maintaining optimal health (Sender et al., 2016b).

The relationship between host and microbiota can be described as *symbiosis* that was defined in 1879 by de Bary as “the living together of unlike organisms” (Bary, 1879). Symbiotic associations are frequently characterised as facultative 'beneficial' metabolic interactions. The classification of microbial symbiotic associations is conventionally undertaken according to three primary interaction types: mutualistic, parasitic and commensal. This classification is based on the perceived net effects on the fitness of the interacting organisms (Steinert et al., 2000).

- **Mutualism**: when both interacting species benefit from the interaction. One example of this phenomenon is the light-producing *Vibrio fischeri* in the Hawaiian Bobtail Squid *(Euprymna scolopes*), which has been shown to aid in prey luring and predator avoidance (Werren & O’Neill, 1997; Curtis & Sperandio, 2011).
- **Commensalism**: when one benefit from the interaction but the other the other exhibits no discernible cost or benefit. *Escherichia coli* has the capacity to exist as commensals within the human gastrointestinal tract, deriving benefit from the host environment without causing detectable harm or benefit to the host when the host possesses a functional immune system and healthy microbiota (Werren & O’Neill, 1997; Drew et al., 2021).
- **Parasitism**: when one of the partner benefits at the expenses of the other, causing harm, damage, or even death to the host. For example, pathogenic bacteria such as *Legionella pneumophila* (the causative agent of Legionnaires' disease) and *Staphylococcus aureus* (which is associated with toxic shock syndrome) illustrate parasitic interactions with clarity (Werren & O’Neill, 1997; Steinert et al., 2000).

Symbiosis can be classified as obligate or facultative. In the obligate case, both partners are completely dependent on each other for survival. In the facultative case, both partners can live independently (Sørensen et al., 2019).

Following the classification of microbial associations according to their ecological outcomes, consideration should be given to the way these symbiotic partners establish themselves within the host environment. It is important to note that, in the gastrointestinal tract, autochthonous microorganisms are defined as those that have

established themselves and adapted to the conditions present. In contrast, allochthonous microorganisms are defined as those that are visitors to the gut and originate from the surrounding environment. Autochthonous organisms have the capacity to produce a wide range of enzymes, which facilitate the efficient utilisation of available nutrients. These bacteria possess the capacity to adapt to ecological changes and defend against bacteriophages, as evidenced by the genetic tools available to them. They are able to permanently occupy niche spaces and represent the long-lasting functional component in the gastrointestinal tract, thereby contributing to the nutritional and protective functions of the host. Conversely, allochthonous organisms exhibit a higher species richness than the mucosa, yet their permanence is constrained due to their inability to consistently manifest the molecular mechanisms necessary for stable adhesion. Their primary contribution consists of increasing bacterial diversity and functioning as a reservoir of potential new functions, many of which remain to be characterised in detail (Nayak, 2010).

1.2. Hologenome concept

The term "holobiont" is used to describe a host and its microbial community, including viruses and cellular microorganisms (Morris, 2018). This term is derived from the Greek word *holos*, which means whole. All natural animals and plants are considered holobionts (Theis et al., 2016).

The "hologenome" is defined as the sum of all the genetic information of the host and its microbiota. The genomes of a holobiont's host and microorganisms are collectively defined as its hologenome (Zilber-Rosenberg & Rosenberg, 2008).

Microbial genomes can be considered stable or variable components of the hologenome, with transmission occurring vertically or horizontally. The traits they encode are context-dependent and, as previously reported for symbioses, may cause harm, benefit, or no consequences for the holobiont.

The *Theory of hologenome evolution* was recently developed, and considers the holobiont as a unit of selection in evolution. It is based on four key principles: all animals and plants establish symbiosis with microbial communities; a significant part of the microbial genome is transmitted, together with that of the host, to subsequent

generations via vertical pathways (vegetative reproduction, contact at birth, lactation, coprophagia); the host-microbiota interaction modulates the fitness of the holobiont, expanding its adaptive potential; the variability of the hologenome arises from changes in the genomes of the host or microbiota, with the microbiome able to respond rapidly to environmental stresses. These points suggest that the genetic richness of different microbial symbionts may play an important role in both the adaptation and evolution of higher organisms. During times of rapid environmental change, the diverse microbial symbiont community can help the holobiont survive, multiply, and gain the time it takes for the host genome to evolve (Rosenberg & Zilber-Rosenberg, 2019).

This adaptation is achieved through three main mechanisms: the relative amplification or reduction of specific symbionts, an increase in the number of a particular microbe is equivalent to the amplification of an entire set of genes, thus constituting a powerful mechanism for the adaptation and evolution of holobionts, the acquisition of new environmental microbes, which can enrich the holobiont with new metabolic traits; and horizontal gene transfer, which allows the exchange of genes between microorganisms and even between microbes and the host. (Rosenberg & Zilber-Rosenberg, 2016)

The theory of the hologenome is part of the framework of the "superorganism" proposed by Wilson and Sober (Wilson & Sober, 1989).

It is noteworthy that the hologenome theory encompasses elements of both Darwinism and Lamarckism. While individual organisms evolve through the selection of random variants (Darwinism), holobionts can evolve through adaptive processes, such as the amplification or acquisition of microorganisms that respond to environmental changes and that can be transmitted to the next generation (Lamarckism). The significance of this phenomenon for long-term evolutionary processes remains a subject of debate. However, in the short term, the inheritance of acquired traits by holobionts can facilitate their survival, multiplication, and the acquisition of time necessary for the host genome to evolve (Y. Singh et al., 2013).

2. MICROBIOME ECOLOGY

Microbiome ecology is the science that studies how microorganisms interact with each other, with the environment and with hosts. These interactions are decisive for human and animal health.

The study of the process by which micro-organisms colonise a host organism is principally conducted on the gut. The theory of gut microbial colonization is founded on studies and examples in zebrafish. The scientific literature suggests that the process of gut colonization in fish larvae is shaped by stochastic events, such as microbial contamination from the surrounding environment. It is further suggested that this process evolves, becoming increasingly determined by non-neutral processes as the host matures into adulthood. So it is hypothesised that a transition from a stochastic to a deterministic process occurs during the colonisation of the gastrointestinal tract (Talwar et al., 2018).

In the context of assembling microbial communities, it is insufficient to focus exclusively on the order of colonisation, that is to say, the chronological sequence in which microorganisms arrive in a habitat; it is imperative to consider priority effects, that is, the manner in which the initial colonisers modify resources and environmental conditions, thereby influencing the settlement and persistence of subsequent arrivals. Priority effects occur when early colonizers alter resource availability or habitat conditions in ways that influence later arrivals: by pre-empting niches—consuming limited resources or occupying key spaces—and by modifying the environment—depleting nutrients or producing metabolites that selectively promote or inhibit subsequent taxa (Debray et al., 2022).

The microbiome ecology also focuses on the shape and composition of the microbiota. These factors can be categorised into two distinct groups: host-specific factors, which are associated with the intrinsic characteristics of the animal in question; and non-specific factors, which are of an environmental or behavioural nature (Cholewińska et al., 2021).

2.1. Host-specific factors

These factors are directly related to the host itself. We can identify as important factors: anatomy, genotype, vertical transmission and the immune system.

ANATOMY: The gastrointestinal anatomy of the host is a primary factor in this regard. The organ and epithelial peculiarities of the digestive trat (length, surface area, transit times) create different ecological niches along the intestinal tract, thereby selecting microbial communities with heterogeneity in terms of density and composition (Maritan et al., 2024).

1. Organ and epithelial length and surface area contribute to the variation in gut microbiome across animals. A larger surface areas or longer segments would potentially offer more niches for microbial colonization and interaction (Maritan et al., 2024).
2. Short transit times in the upper GI regions are a key factor contributing to the low numbers of microbial cells per gram of gut content in these areas. This is coupled with acidic conditions and high concentrations of host secretions. Conversely, high transit time in the ileum and rectum, along with increased pH and accumulation of food content, favour microbial proliferation. This suggests that a slower passage of digesta allows more time for microbial growth and colonization (Venkatesh & Ramadass, 2023).

In addition to these factors, physicochemical factors such as pH and redox potential, must also be considered.

3. In the upper gastrointestinal regions, acidic conditions (luminal pH < 3) have been observed to reduce the concentration of microbial cells per gram. The presence of high concentrations of host secretions (e.g., antimicrobial effectors, bile acids, ancreatic fluids) and short transit times have been shown to contribute to the low bacteria concentration. Conversely, elevated pH levels, prolonged transit time in the ileum and rectum, and the accumulation of foodstuffs have been demonstrated to promote microbial proliferation (Barron et al., 2023).

   In fish, a general progressive increase in bacterial population size and variation in community composition is observed from the stomach to the hindgut. However,

unlike mammals or insects where diversity is highest in the hindgut, fish often show highest overall diversity in the midgut. This may be linked to differences in dominant microbial taxa; fish microbiomes are primarily composed of Proteobacteria and Firmicutes, while mammals are dominated by Bacteroidetes and Firmicutes.

4. It has been demonstrated that dietary microbes, such as Lactobacillus spp., are capable of inducing the production of reactive oxygen species (ROS) by the NADPH oxidase DUOX, thereby helping to regulate their density within the gastrointestinal tract. The importance of DUOX in gut immunity appears to be conserved across various animal models, including *C. elegans*, zebrafish, mice, and humans (Maritan et al., 2024).

As demonstrated by (Stephens et al., 2016), the age, stage of development and sex of the subject are all important factors in the selection of microbiota. Nevertheless, the contributions of factors such as diet, age and host genetic background have obscured the presence of sex differences in a variety of model systems (Maritan et al., 2024).

GENOTYPE: It is imperative to consider the genetic variables intrinsic to the host when analysing the composition of the intestinal microbiota in fish. QTL and GWAS studies have demonstrated that genetic variation in the host genotype accounts for between 1.6% and 9% of microbiotic variability across different taxa (Benson et al., 2010).

The genotype functions as a selective filter, shaping the structure of the intestinal microbial community from the earliest stages of life. In fish, bacterial diversity increases progressively during development and stabilises within the first 50 days of life, thereby establishing a "core microbiota" that is resistant to environmental changes (Egerton et al., 2018).

As demonstrated by transplantation experiments between zebrafish and rodents, concrete examples can be provided. It has been established that the transferred microbial communities maintain their original phylogenetic lines, but their relative abundance adapts to the typical microbiota of the recipient host. This confirms a highly specific genotype–microbiota interaction (Rawls et al., 2006).

Finally, we have the concept of phylosymbiosis, which describes how relationships between microbial communities can reflect the phylogeny of the host. This concept emerges from stable and intimate associations over the course of evolution. Such co-speciation patterns have been extensively documented in a variety of model species. In contrast, the evidence of phylosymbiosis in fish is, as yet, fragmentary and sometimes conflicting. This finding indicates that, while a genetic filter maintains microbial composition according to the evolutionary history of the host, environmental factors and horizontal transmission mechanisms also influence fish systems, thereby attenuating the phylogenetic signal (Maritan et al., 2024).

VERTICAL TRANSMISSION: Vertical transmission can occur by a variety of mechanisms, by transovular transmission, by asexual reproduction, or by milk in mammalian species or direct contact with parents. The manner in which a mutualist is transferred from one generation to the next is contingent upon the significance of the services it provides to the host. In the context of hosts exhibiting a pronounced reliance on their symbionts, vertical transmission plays a pivotal role in ensuring the maintenance of microorganisms that fulfil critical nutritional functions or other functions indispensable for the hosts' survival. This process also facilitates the persistence of an optimal niche for the symbiont (Fisher et al., 2017; Maritan et al., 2024).

IMMUNE SYSTEM: Immune system is responsible for the regulation of microbial colonisation, both in its innate and adaptive components. The immune system exerts a selective pressure that functions as an ecological filter, thereby favouring the most functional microorganisms for the digestion and defence of the host. Research has also demonstrated the influence of the immune system on the spatial distribution of bacteria along the mucosa of the gastrointestinal system (Maritan et al., 2024).

2.2. Non-host-specific factors

These factors are defined as not directly related to the host itself. The primary determinants of this phenomenon are environmental conditions, diet and trophic level, and horizontal or social transmission mechanisms.

ENVIRONMENTAL CONDITIONS: The microbial communities in the surrounding water strongly influence those present in the fish's gut. Fish acquire the first bacteria from the water they drink for osmoregulation after hatching, and the microbiome diversifies further with feeding. The salinity of the water largely determines the gut microbial composition. Changes in pH can also significantly alter the endogenous microbiome, although strong resilience to the restoration of pH levels can be observed (Talwar et al., 2018). Another important aspect to consider is captive breeding, with unnatural breeding densities and increased stress levels, can cause alterations in the microbiota in the gastrointestinal tract of fish (Egerton et al., 2018). Seasonal variations and temperature changes are determining parameters for the intestinal microbial composition of fish. For example, increased water temperatures (up to 21° C) have been associated with a disappearance of lactic acid bacteria (LAB) and Acinetobacter spp. and an increase in Vibrio spp. in salmon, which could have a negative impact on host health (Hovda et al., 2012).

DIET AND TROPHIC LEVEL: The introduction of foodstuffs serves not only as a substrate to produce energy, but also as a vector for microbial inoculum. Dietary regimes have been shown to have a significant impact on the composition of gut microbial communities. This influence is often evident in hosts that consume comparable diets, resulting in the establishment of functionally similar microbiota, irrespective of their phylogenetic relatedness (e.g., carnivores vs. herbivores) (Dapa & and Xavier, 2024). It has been demonstrated that diet exerts a significant influence on the composition of the gut microbiota, as well as on the relative microbial abundance. It has been demonstrated that as the fish's diet transitions from a carnivorous to an omnivorous to an herbivorous diet, an increase in microbial diversity within the gastrointestinal tract is observed. This phenomenon is indicative of a correlation with trophic level (Liu et al., 2016). The trophic level, source, amount, and structure of dietary protein have been demonstrated to exert an influence on both gut health and microbial composition. A correlation has been demonstrated between reduced levels of protein in the Atlantic salmon diet and a more divergent structure of the gut microbial community (Egerton et al., 2018). Research has indicated that the source of nutrients exerts an influence on the relative abundance of Lactobacillales, Bacillales and Pseudomonadales. These bacteria have been observed to

increase in correlation with the ingestion of plant-derived proteins. In contrast, the ingestion of animal-derived proteins has been observed to promote the proliferation of Bacteroidales, Clostridiales, Vibrionales, Fusobacteriales and Alteromonadales within the gastrointestinal tract (Talwar et al., 2018).

SOCIAL INTERACTIONS: encompassing direct contact, co-housing, parental care and coprophagy, have been demonstrated to facilitate the horizontal transmission of microbial strains, thereby contributing to the homogenisation of the microbiome within family or social groups (Maritan et al., 2024).

Individuals belonging to the same social group or sharing the same environment tend to have more similar microbial communities than isolated individuals. However, identifying the contribution of social interactions to changes in the microbiota is difficult because this could be masked by factors such as exposure to common environmental sources and similar dietary habits (Archie & Tung, 2015).

A few studies that exclude indirect pathways such as coprophagia, i. e. (Kort et al., 2014; Tung et al., 2015), provide evidence of direct transmission. Such research highlights a two-level model: on the one hand, the 'group effect', i.e. the overall influence of social co-residence on microbial structure; and on the other hand, differentiated individual interactions within the group, which can modulate the composition of the microbiome more finely (Archie & Tung, 2015).

From a functional point of view, the transfer of microorganisms between socially interconnected individuals forms part of the cost–benefit balance of group living. While it can promote the acquisition of protective or metabolically advantageous bacteria, it can also promote the spread of pathogens or antimicrobial resistance factors (Archie & Theis, 2011).

3. STRUCTURE ANATOMY AND MICROBIOME OF TELEOST FISH

Fish, like all animals, demonstrate a diverse range of interactions with microorganisms inhabiting both their bodies and their surrounding environment.

These interactions may range from mutualistic symbioses to pathogenic infections. In fish, as in humans and other mammals, mutualistic gut microbiota have been shown to contribute significantly to nutritional provisioning, metabolic homeostasis, and immune defence (Egerton et al., 2018).

3.1. Gut

The digestive system of fish shows variability in function of the diet , but in general all fish alimentary canal starts with buccal and pharyngeal cavities. The gut can be divided in:

- **Foregut**: is positioned behind gills and is compose by oesophagus, stomach and pylorus, but is estimate that 20% of fish does not have stomach.
- **Midgut:** is the longest part of intestine and here there is absorption of nutrient, midgut end with an increase of diameter indicating the beginning of the hindgut.
- **Hind gut**: is formed by the distal part of intestine and by the anus.

The length of the gut displays considerable interspecies variability, with dietary factors playing a primary role in determining its length. In general, the gut length of herbivorous fish is three times their body length, while the length ratio of omnivorous fish is one to three and that of carnivorous fish is approximately one (Egerton et al., 2018; Wilson & Castro, 2010).

The composition of gut bacterial communities in fish is significantly different from that of other vertebrates, including reptiles, birds and mammals. While in humans and other mammals, *Firmicutes* and *Bacteroidetes* predominate, in fish the main microorganisms that comprise the microbiota are: *Proteobacteria, Fusobacteria* and *Actinobacteria* (Wu et al., 2012).

Within the intestinal tract of fish, facultative anaerobes and aerobes are present in greater numbers than obligate anaerobes. It has been demonstrated that bacteria

belonging to genera such as *Aeromonas, Vibrio* and *Pseudomonas* are present in both freshwater and marine fish.

Specifically, in freshwater fish, the most prevalent phyla are Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes. The following species and genera are commonly encountered: *Escherichia, Klebsiella, Proteus, Serrata, Alcaligenes, Eikenella, Bacillus, Listeria, Propionibacterium, Bacteroides, Citrobacter freundii, Hafnia alvei, Cytophaga/Flexibacter, Staphylococcus, Mycoplasma, Streptococcus, Lactococcus, Peptostreptococcus, Deefgea, Cetobacterium* and *Moraxella*.

A study of the gut microbiota of seawater fishes revealed the presence of microorganisms belonging to the following phyla: *Proteobacteria*, which included *Vibrio, Photobacterium* and *Shewanella* (B. K. Singh et al., 2025).

The composition and functions of the gut microbiota in fish vary according to diet, thus clearly distinguishing between herbivorous/omnivorous and carnivorous species. In herbivorous fish, bacteria capable of degrading complex carbohydrates and vegetable fibres prevail, such as *Clostridium, Citrobacter, Lactobacillus*, and *Epulopiscium*, capable of producing digestive enzymes (e.g. cellulase) and short-chain fatty acids (SCFAs); *Cetobacterium somerae*, for example, also contributes to the synthesis of vitamin B12. Bacteria such as P*seudomonas, Burkholderia, Photobacterium* and *Vibrio* are prevalent in carnivorous species, where they specialise in the digestion of proteins, fats and chitin, which are often derived from crustaceans (Talwar et al., 2018; B. K. Singh et al., 2025).

3.2. Gills

In teleost fishes, the gill apparatus is characterised by a complex morpho-functional organisation. There are four pairs of holobranchie symmetrically distributed on either side of the skull, each of which is composed of a gill arch and two gill petals arranged in a comb pattern. The gill arch, which possesses a semicircular section, is the primary skeletal support structure and provides support to the gill filaments. These filaments extend parallel from the convex surface of the arch, terminating at their free ends. On each side of the filaments there are gill lamellae, regularly distributed bag-shaped structures, which allow the controlled passage of water through the interlamellar spaces, thus optimizing gas exchange. The concave side of the arch is equipped with gill

rakes, which are conical serrated devices that facilitate the filtration of particulates. The gill filament, characterised by its thin and elongated structure, exhibits an expanded blind end and is separated from the adjacent filament by a distance ranging from 0.10 to 0.13 millimetres. The external protection of the entire system is ensured by a mobile operculum that guarantees the protection of the gill cavity and contributes to ventilation.

The gills are primarily composed of floor cells (90%), with a smaller proportion consisting of chloride cells and mucous cells (10%). The filaments, arch, and gill rakes' surfaces are characterised by the presence of pores (1.03–2.15 µm) and intercellular fossae, the latter of which are more pronounced in the rakes. The gill lamellae are covered with microvilli and mucus, while the rakes and arch have floor-like cells with fingerprint structures.

The gills are pivotal in the process of gas exchange, facilitating the uptake of oxygen and the elimination of carbon dioxide. They contribute to osmoregulation thanks to the activity of chloride and floor cells, which regulate the water and ion balance in environments with different salinity. Furthermore, they represent a significant site for the excretion of ammoniacal nitrogen and waste products, a process that is facilitated by symbiotic bacteria. The gill epithelium acts as a physical barrier against environmental stress, while the opercular cover offers mechanical protection. Gills also participate in mucosal immunity, thanks to localised immune cells and molecules. These organisms play a crucial role in the regulation of acid-base balance and demonstrate remarkable morphological plasticity in response to environmental changes, such as fluctuations in oxygen, temperature, and salinity (Chen et al., 2023).

The microbial community associated with the gills of teleost fishes is characterised by a peculiar composition, mainly dominated by Gammaproteobacteria but significantly enriched by Betaproteobacteria, Flavobacteria and Saprospirae. The most prevalent taxa include several species of Shewanella, which are distinguished by their high metabolic adaptability, and members of the family Endozoicimonaceae, which are associated with the mucosal surfaces of corals and here indicate a distinctive gill niche. Other taxa of interest include *Cetobacterium*, *Clostridium*, the intracellular *Neorickettsia* and the methylotroph *Methylobacterium*, which are also present, suggesting a wide range of metabolic and defensive interactions (Pratte et al., 2018; Mes et al., 2023).

From a functional point of view, this microbiota plays a crucial role in mucosal immune protection, acting as an antifungal and antibacterial barrier, and participates in the nitrogen cycle through the nitrification and denitrification of ammonia excreted by the host, contributing to the detoxification and excretion of nitrogen products. Concurrently, the presence of ammonia-oxidising bacteria and intracellular symbionts underscores the integration of the microbiome into respiratory metabolism and osmotic regulation of gills (Pratte et al., 2018; Chen et al., 2023).

4. GUPPY AND GUPPY MICROBIOME

4.1. Relevance of teleost fish and guppies into the scientific research

Teleostean fish are considered important models in scientific research for a number of reasons that allow them to overcome many of the limitations of traditional studies on mammals and to provide unique insights into host-microbe interactions and social behaviour.

Unlike inbred mice, teleostsoffer a natural genetic variability that allows you to investigate the influence of hereditary differences on the composition and dynamics of the intestinal microbiome. This variability, combined with the high numbers of progeny and the rapid life cycles typical of many fish species, increases the statistical power of the experiments and makes it easier to sample both internal and environmental microbial communities (Lescak & Milligan-Myhre, 2017).

From a physiological and immunological point of view, teleostsshare key structures and genes with mammals: the digestive tract, including liver, gallbladder, pancreas and intestines, develops according to similar processes, while the signalling pathways of the immune system are highly conservative (Wallace et al., 2005).

The complex and functionally active intestinal microbiome of fish plays a crucial role in the innate immune response. Unlike many rodent studies, which rely on faecal samples to infer the microbial structure, fish allow the direct analysis of the communities distributed along the various intestinal segments, thanks to the embryonic and larval transparency of the zebra fish. This peculiarity allowed, for the first time, in vivo observations of bacterial colonisation processes and microbial succession dynamics in the developing host (Lescak & Milligan-Myhre, 2017).

On the evolutionary and ecological side, the extraordinary diversity of teleosts allow to understand how genetic, environmental and behavioural factors co-evolve in the modulation of host-microbe symbiosis. Species such as the spinel and the poeciliids have been shown how genotype, diet and parasites influence the composition of the microbiome, while cleaner fish offer a natural model for studying direct microbial transfer during mutualistic interactions. This evidence complements the sophisticated social networks and the behaviours of learning, deception and cooperation observed in many fish species, which reflect neural components homologous to those of higher

vertebrates. The correlation between microbiome and behaviour is interesting for translational studies: investigations on zebra fish have shown how co-housing and the integration of probiotics can modulate social behaviour and gene expression, suggesting new ways for the study of psychiatric disorders based on dysbiosis (Soares et al., 2019).

Guppy is a well-established model organism in ecology, evolutionary biology, and animal behaviour. They are useful for investigating the links between the microbiome and social behaviour. The Trinidad guppy system, in particular, has been used to study the complex role of gene flow on adaptation and fitness in a natural environment, naturally isolated populations provide natural study systems (replicates) that have been intensively used to evaluate the impact of different environmental factors, predation pressure and parasitism on the morphology, genotype and host behaviour (Reznick & Ricklefs, 2009). From a practical point of view, guppies are extremely easy to maintain and reproduce in captivity: their viviparous, rapid and prolific reproduction, combined with tolerance to a wide range of qualitative water conditions, allows them to obtain numerous descendants in a short time. This not only facilitates studies with adequate statistical power, but has also favoured the development of selected lines characterised by different colours, ornaments and hybridisations, further enriching the possibilities of exploring genetic bases of complex phenotypic traits (Soares et al., 2019).
Guppies were the first fish whose microbiome was evaluated in relation to host adaptation to the environment. A comparison of wild and laboratory-bred individuals reveal the existence of differences in intestinal bacterial composition, with significant variations not only between different geographical populations, but also over time in those same waterways. These results pave the way for research on the role of the microbiome in environmental adaptation and suggest that microbial communities can mediate immune and physiological responses to external factors (Kim et al., 2021).
Guppies display sophisticated behaviour and complex social networks. Studies of their social behaviour have revealed how different life strategies influence the tendency to cooperate, form stable groups and even transmit “cultural” behaviour within populations. Analyses of social networks have shown that individuals with well-defined social positions tend to maintain these roles over time and that parasite infestation can alter group cohesion. As these social dynamics are likely to be correlated with differences

in the microbiome, guppies represent an ideal bridge between ethology, ecology and microbiology (Soares et al., 2019).

4.2. Guppy

The Guppy, *Poecilia reticulata*, is a small fresh-water fish belong to the Poecilidae family. They are teleost fish native from Tobago and Trinidad, but in the last century they were used all over the word for the aquarium trade and as a mosquito control agent, and their introduced range now extends to at least 70 countries on six continents.

They manifest physical dimorphism in relation to their sexual characteristics (Figure 1: A - Male *P. reticulata*, a brightly marked male; B - Female *P. reticulata*, a female during parturition). The males are comparatively smaller in size, measuring between 15-20 mm, and possess a distinct array of bright polymorphic colouration (black, white, orange, yellow, green and iridescent spots, lines and speckles). In contrast, the females are larger in size, measuring between 20-25 mm, and display a uniform silver-grey body colouration (Deacon, 2023). Males have gonopodium, a sexual organ derived from a modified anal fin (Kobayashi & Iwamatsu, 2002).

There is significant discrepancy between the characteristics exhibited by the domestic aquarium strain of Guppy, and those exhibited by the wild form. This discrepancy can be attributed to the fact that the domestic aquarium strain has undergone a process of selective breeding over an extended period. Domesticated females may express colour and patterns normally only present in males, especially in the caudal region.

Figure 1: A - Male P. reticulata, a brightly marked male; B - Female P. reticulata, a female during parturition

The diet of Guppy is omnivorous, comprising approximately 50% algae, invertebrate larvae and benthic detritus (Deacon, 2023).

P. reticulata can be found in a variety of environments across its natural range, ranging from muddy, slowly flowing water bodies at low elevations to clear mountain streams, most of which without significant aquatic vegetation. Few guppies are found in the deeper sections of streams. Although they can withstand a broad range of salinities and temperatures (18–28°C), including up to 150% average saltwater, they are typically found in freshwater streams close to the coast. Guppies are frequently the sole species in highly contaminated water bodies in non-native regions (Chung, 2001).

An important characteristic of this species is that they are viviparous fishes with a internal fertilization. Male introduce the sperm into the female's reproductive tract using gonopodium and bundles of sperm travel down a channel in the gonopodium to inseminate the female. The sperm bundles enter the female via a gonoduct situated in front of the anal fin. The ovary of a female guppy consists of a single large ovary containing a folded surface with a sperm storage micropocket (SSP). This SSP facilitates the storage of sperm from one or more males for a period of up to eight months prior to its utilisation in the fertilisation of eggs. Following parturition, the offspring are immediately capable of feeding independently, with no requirement for continued parental care. They have a short generation time (3-4 months), so population can grow very fast and all of this is important for scientific research because they represent a valid alternative at the murine model (Kobayashi & Iwamatsu, 2002).

Guppies are susceptible to a wide range of pathogens and parasites. In the context of high-density conditions, prevalent within the ornamental fish industry, such infections have the potential to result in significant morbidity and mortality. Mycobacterium and Nocardia bacterial infections have been observed to affect guppies, manifesting as chronic nodular features. Other diseases include yellow grub disease caused by metacercariae of *Clinostomum complanatum* (Deacon, 2023; Reznick, 1997).

4.3. Guppy microbiome

The guppy microbiota is a complex microbial community influenced by a variety of environmental and host-related factors. Guppy intestinal microbial communities are

generally dominated by Proteobacteria, Actinomycetes, Firmicutes and Fusobacterium (S. Evans et al., 2020).

The intestinal microbiota is essential in the presence of contaminated water because it directly reflects the quality of the environment in which they live and influences their health and adaptability. In heavily polluted sites, the abundance of suspected pathogens is significantly higher in both the surface waters and in the intestinal microbiota of the guppys. This implies that water contamination can lead to an increase in harmful bacteria in the intestines of fish, posing risks to their health and, potentially, to the safety of consumers (Jia et al., 2021).

In environments where food resources are of lower quality or limited Nitrogen-fixing bacteria play a crucial role. These bacteria, particularly the order Rhizobiales, are able to convert atmospheric nitrogen (N2) into nutrients absorbable by the host body. Empirical studies show that guppy populations inhabiting low-predation environments, where diets are dominate by detritus and diatoms rather than nitrogen-rich invertebrates, exhibit a higher relative abundance of Rhizobiales compared to high-predation populations (S. E. Evans et al., 2022).

Recent studies have revealed that the diversity of the skin's bacterial microbiome predicts lower levels of activity in guppy females, but not in males. This sex-specific relationship between the skin microbiome and host behaviour could indicate sex-specific physiological interactions with the skin microbiome. In addition the skin microbiome is probably important in modulating social behaviour: the skin is vital to the production of chemical signals, many of which are produced and modulated by microbes. These signals can indicate sex, age and infection status, being essential for the recognition of species, the recognition of kinship and the choice of partner (Kramp et al., 2022).

5. ABSTRACT

Animals and microorganisms are linked by associations that influence health, ecology and evolution. The environment and social interactions are a determining factor in the composition and alteration of the microbiota. In this thesis I addressed this topic using the guppy (*Poecilia reticulata*) as a model organism and applying an integrative approach combining microscopy, molecular quantification, sequencing and behavioural analysis. Optimisation of FISH visualisation revealed dense microbial localisation along the intestinal mucosa, while highlighting methodological limitations in gill tissue. Experimental conditions were established in which female individuals with simplified microbiota were co-housed with conventional males. qPCR showed that the overall bacterial load did not vary significantly between treatments, but sequencing of the 16S rRNA gene showed that exposure to sterile water reshaped community composition and reduced richness, favouring the dominance of specific taxa such as *Mycobacterium*. In addition, video tracking software was used to quantify the number of contacts between individuals. Taken together, these results suggest that microbiome ecology is a multifactorial process shaped by both environmental disturbances and host behaviour. They also confirm that *P. reticulata* is a valuable model for studying the ecological factors that drive microbiome alterations.

MATERIALS AND METHODS

1. MICROSCOPY VISUALIZATION

To gain a comprehensive understanding of the spatial organisation and interactions between microorganisms, it is important to observe them directly in their natural habitat. For this purpose, an effective technique is Fluorescent in Situ Hybridisation (FISH), which facilitates the identification of the microbial taxa present and the analysis of their social behaviour.

1.1. FISH protocol

As outlined in paragraph 2.1, samples of *Poecilia reticulata* were sectioned under sterile hood.

The fish (either a section or the entire specimen) were then fixed in 4% paraformaldehyde (PFA) in 1× PBS at room temperature for 4 hours. After fixation, samples were stored in a 1:1 solution of PBS and ethanol at −20 °C until further processing for dehydration.

Dehydration was performed through a graded ethanol series in 1× PBS (50%, 60%, 70%, 80%, 96%, and 100%) at room temperature, with progressively increasing incubation times (20–40 minutes), tripled for the 96% and 100% ethanol steps.

Samples were then immersed in Roti®Histol (Carl ROTH, DE) two passages of 40 minutes each, followed by overnight incubation, and subsequently in a 1:1 mixture of Roti®Histol and paraffin for 1 hour at 60 °C. Final paraffin embedding was achieved by three successive incubations of 1 hour each at 60 °C, followed by overnight incubation at the same temperature. At the end of the paraffin embedding process, samples were stored at 4 °C until further examination.

Sections of 4 µm thickness were obtained from the paraffin blocks using a microtome and mounted onto Poly-Prep Slides (Sigma-Aldrich, US), slides were pre-treated with poly-L-lysine to promote adhesion.

Prior to the dewaxing procedure, the slides were subjected to a baking cycle at a temperature of 60°C for a duration of one hour, with the slides maintained in a vertical orientation and the box left open.

Subsequently, the dewaxing process was performed by three washes in Roti®Histol, followed by a wash in 96% ethanol. Glass slides were dried in oven (30 min at 37°C), and the areas containing the samples were delimited with both a pap-pen and a diamond-tipped pen.

The hybridization process was carried out in a humidified chamber containing *KimWipes* soaked in 35% formamide (1-2 mL for KimWipe).

The slides were then placed facing upward and treated with hybridization mix (reported in Table 1) and incubated at 46°C for 4 hours, in the dark. The Eubacteria FISH probe - Cy3 (Sigma-Aldrich, US) and the Eubacteria FISH probe - ATTO488 (Sigma-Aldrich, US) were utilised for the purpose of visualising all bacteria in the sample.

Post hybridization the slides were quickly rinsed in pre-warmed washing buffer (48 °C) (reported in Table 2) and subsequently incubated in fresh pre-warmed washing buffer at 48 °C for 15 minutes.

After washing, the slides were incubated in 1X PBS for 20 minutes at room temperature, followed by a brief rinse in Milli-Q water (1 minute at room temperature).

Sections were stained with Hoechst 33342 (Thermo Fisher Scientific, US) for 10 minutes to visualize nuclei, followed by brief washes in ultra-pure water and 96% ethanol. Subsequently, the slides were dried in an oven at 37 °C for 30 minutes.

Before covering the slide, 40–50 µL of ProLong™ Gold Antifade mounting medium (Thermo Fisher Scientific, US) were added per section. The slides were then covered with coverslips, left at room temperature in the dark, sealed, and stored in the fridge until subsequent visualisation under subsequently a fluorescence microscope.

The images were obtained using LEICA Thunder DMi8 (Leica Microsystems, DE) with 10x, 20x and 40x objectives, and using confocal LEICA Stellaris 8 (Leica Microsystems, DE) with 20x, 40x and 63x objectives.

Hybridization mix	
16S rRNA probe/s (100 µM)	4.5 µL (for probe)
Hybridization buffer 35% formamide	up to 500 µL

Table 1: Composition of Hybridization Mix for FISH

Washing Buffer	
5M NaCl	700 µL
1M Tris-HCl pH 8.0	1 mL
0.5M EDTA pH 8.	0.5 mL
MQW	up to 50 mL
20% SDS	25 µL

Table 2: Composition of Washing Buffer for FISH

1.2. Haematoxylin and eosin staining

The tissue section was obtained in the same way as the FISH protocol. The section was stained on Microscope Slide Singol Frosted (Corning, USA) The staining process was carried out by the Pathology Facility of Department of Comparative Biomedicine and Food Science (BCA) at the University of Padua using a Leica Autostainer XL (Leica Biosystems, DE) following the protocol below:

STEP	REAGENT	TIME
1	XYLOL	2 minutes
2	XYLOL	3 minutes
3	ALCOHOL 100%	2 minutes
4	ALCOHOL 95%	2 minutes
5	ALCOHOL 70%	1 minutes
6	WATER	15 second
7	DISTILLED WATER	20 second
8	HAEMATOXYLIN DI MAYER	2 minutes and 30 second
9	WATER	7 minutes
10	ALCOHOL 95%	15 second
11	EOSINE-FLOXINA	50 second
12	ALCOHOL 95%	30 second
13	ALCOHOL 95%	30 second
14	ALCOHOL 100%	1 minutes

15	ALCOHOL 100%	1 minutes
16	XYLOL	2 minutes
17	XYLOL	2 minutes

Table 3: Protocol of Haematoxylin and Eosin staining

The tissue section was visualised under a Keyence Digital Microscope (Keyence, US) after staining with haematoxylin and eosin.

2. EXPERIMENTAL DESIGN

The experimental design of the thesis was performed at the Department of Biology (University of Padova). Individuals with a simplified microbiota were obtained by keeping the fish in sterile water for 24 hours (with seven female fish in each tank). They will be referred as MD (microbiota-depleted). The conventional individuals will be annotated as CV.

The experimental conditions were set up in four different "social conditions" as follows:

A → one MD female in contact with one CV male for 24 hours,

B → two MD females in contact with one CV male for 24 hours,

C → one MD female and one CV male in the same tank but separated by a grid preventing physical contact,

D → one MD female kept in sterile water in isolation for 24 hours.

For each condition, individuals were sampled at three time points. First, a conventional (CV) sample was obtained from individuals before cohabitation. Second, a microbiota-depleted (MD) female sample was obtained at the start of cohabitation. Finally, after 24 hours of cohabitation, we obtained the post-treatment individuals (e.g. A, B, C, D).

Five biological replicates were prepared for each condition. Figure 2 shows a graphical set up of the experimental design.

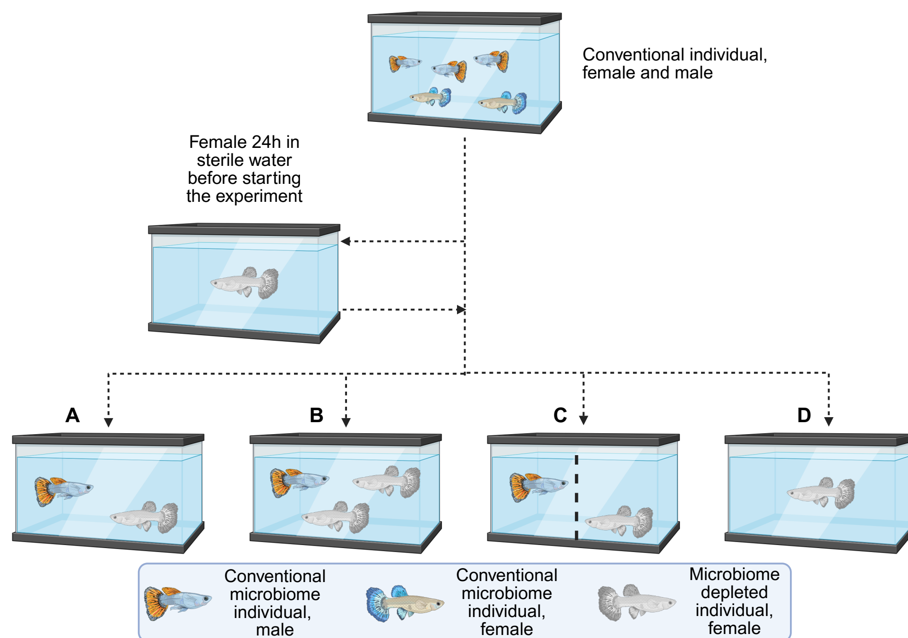


Figure 2: Experimental design. Conventional individuals (CV) and those with simplified microbiota (microbiota-depleted, MD) were used to set up four social conditions: (A) one MD female with one CV male; (B) two MD females with one CV male; (C) one MD female and one CV male separated by a grid; (D) one MD female in isolation.

3. ANALYSIS OF MICROBIOTA COMPOSITION

To investigate the composition of the microbiota and evaluate the influence of host-host contact, total RNA and DNA were first extracted from the samples. Subsequently, the RNA was reverse transcribed into cDNA. Then, quantitative polymerase chain reaction (qPCR) and 16S ribosomal RNA gene sequencing were performed: the first to quantify relative microbial abundance, and the second to identify metabolically active bacterial taxa and determine which taxa were transferred between hosts.

3.1. DNA/RNA extraction

Molecular analysis of the microbiota composition began with the extraction of microbial DNA and RNA from different guppy tissues isolated during the experiment. The fish were first anaesthetised and then euthanised in cold water (0°C). The fish was mounted on a rigid support under a biological hood. The gills were meticulously harvested using sterile tweezers. The initial step involved lifting the operculum, followed by the extraction of the gills. The intestine was carefully collected by the fish using a sterile scalpel, and a swab was performed on the skin.

The extraction was performed using the *Quick-DNA/RNA™ Microprep Plus Kit* (Zymo Research). After sectioning, tissues were stored in 200 µl of DNA/RNA Shield.

Each sample was homogenized with a sterile pestle on ice. Briefly, the extraction process started with protein digestion, carried out by adding 10 µl of Proteinase K and 20 µl of PK Digestion Buffer, after which the mixture at 30 °C for 45 minutes.

Next, the solution was centrifuged, the supernatant transferred to a new eppendorf and 200µl of DNA/RNA lysis buffer (1:1 ratio) was added. The solution was then transferred to a Zymo-Spin™ TMIC-XM column, and after a 3-minute centrifugation step, DNA was bound to the column and RNA was recovered in the eluate.

After, 400 µL of 100% ethanol was added to this eluate, the mixture was put onto a Zymo-Spin™ MIC column and centrifuged in order to separate the RNA. After centrifugation, RNA was bound to the column matrix. Then, 400 µL of DNA/RNA Wash Buffer was added and the column was centrifuged again.

The membrane of Zymo-Spin™ MIC column was then directly coated with 40 µL of DNase I Reaction Mix (prepared by mixing 5.0 µL of DNase I with 35.0 µL of 1X DNase Digestion Buffer) and incubated for 15 minutes at 30 °C to remove any residual genomic DNA.
After incubation, 400 µL of DNA/RNA Prep Buffer were added to both the Zymo-Spin™ TMIC-XM and MIC columns, followed by centrifugation to proceed with the final washing steps.
To both columns, 1200 µL of DNA/RNA Wash Buffer were added in two sequential steps, each followed by a centrifugation step. Subsequently, the columns were transferred into official collection tubes, and 15 µL of DNase/RNase-Free Water were applied directly onto the column membrane. The columns were incubated for 5 minutes at +4 °C to facilitate nucleic acid elution. Finally, a centrifugation step was performed to collect the eluted DNA and RNA.
Subsequently, nucleic acids were quantified with NanoDrop (Thermo Fischer Scientific, US).
NanoDrop is a microvolume analytical instrument used to quantify nucleic acid (DNA or RNA) concentrations using only 2 µL of sample. The NanoDrop operates without the need of traditional cuvettes or capillaries by combining fiber optic technology and the natural surface tension of liquids to hold small volumes between two optical pedestals. This system allows for rapid and accurate spectrophotometric analysis.
Nucleic acids maximum absorbance is at 260 nm, proteins absorb at 280 nm, and organic contaminants absorb at 230 nm. The nucleic acid concentration is calculated with the Beer-Lambert law, that allow to obtain the concentration by absorbance value. In addition, the absorbance ratios (260/280 and 260/230) are used to verify the purity of DNA and RNA preparations (Desjardins & Conklin, 2010).

3.2. REVERSE TRANSCRIPTION

The reverse transcription protocol was carried out using the *SuperScript® IV First-Strand cDNA Synthesis System* (Thermo Fisher Scientifc, US).
Initially, Master Mix 1 and Master Mix 2 were prepared according to the composition (reported in the Table 4 and Table 5). Extracted RNA was diluted to a final concentration of 90,9 ng/µl.

A volume of 2 µl of Master Mix 1 was aliquoted into each well, followed immediately by the addition of 11 µl of RNA. The strip containing the reaction mix was then transferred to a thermocycler and incubated at 65°C for 5 minutes.
Next, 7 µl of Master Mix 2 was added to each well (at +4°C), and the strip was placed back in the thermocycler for the reverse transcription reaction protocol (i.e., 23°C 10', 50°C 10', 80°C 10'.

MIX 1	**1X**
Random hexamers	1 µM
dNTPs 10mM	1 µM

Table 4: Composition of MIX 1 used for retrotranscription

MIX 2	**1X**
5x SS Buffer	4µM
DTT 100 mM	1µl
RNAse out	1µl
SS IV (enzyme)	1µl

Table 5: Composition of MIX 2 retrotranscription

3.3. REAL-TIME PCR ANALYSIS

In order to ensure effective primer annealing and amplification, it is required that a minimum of 1 ng of DNA is present in the reaction.
For the amplification of the target gene (16S rRNA) a 1:20 dilution was prepared and for the amplification of the host reference gene (18S rRNA) a 1:100 dilution was obtained.
It is important to note that these dilutions were performed in accordance with the established protocol, as bacterial DNA is generally found in lower concentrations compared to host DNA in animal tissue samples.
For each sample, a PCR MIX (reported in Table 6 was prepared using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, US) containing a Master Mix that includes all the components required for the PCR reaction, such as Mg^{2+}, oligonucleotides, DNA polymerase, and the fluorescent dye SYBR Green for real-time quantification. At the end forward and reverse primers were added to the PCR mix.

PCR MIX	**1X**
Ultra sterile water	2 µl
Master MIX	5 µl

Primer FWD 10 µM	0,25 µl
Primer RVS 10 µM	0,25 µl

Table 6: Composition of PCR MIX for real time PCR

For each well 7,5 µl of PCR MIX and 2,5 µl of cDNA dilution were used be careful to put on opposite side of the well the two solutions to avoid premature initiation amplification reaction.

The reaction plate was then transferred to the thermocycler and incubated with the following protocol:

1. UDG 1 cycle at 50 °C for 2 minutes
2. DENATURATION 1 cycle at 95 °C for 2 minutes
3. AMPLIFICATION 45 cycles at 95 °C for 10 seconds followed by 60 °C for 40 seconds. In this step was also record the fluorescence.
4. MELTING CURVE 1 cycle with a ramp of temperature till 95 °C

To obtain a relative estimation of the abundance of bacteria present in the sample relative quantification was performed.

The amplification signals obtained with universal primers for the bacterial 16S rRNA gene were compared to those obtained with primers targeting the eukaryotic 18S gene. The relative quantification protocol involves the use of a primer pair for the target gene (16S rRNA) and a primer pair for the reference gene (18S rRNA).

3.4. 16S RIBOSOMAL RNA GENE SEQUENCING

The sequencing of 16S ribosomal RNA gene (V3 and V4) was performed by BMKGENE (Germany, DE) on cDNA samples.

The raw readings (paired-end) were uploaded from a dedicated directory and subjected to quality control using the *plotQualityProfile()* and *fastqPairedFilter()* functions of the `dada2` package, applying parameters of trimming, truncation length (250 bp), and filters for expected errors (maxEE = 2) and minimum quality (truncQ = 2).

The filtered sequences were dereplicated (*derepFastq*) and subsequently processed with error models for the inference of Amplicon Sequence Variants (ASV), using the DADA

algorithm. The forward and reverse readings have been merged (*mergePairs*) and the chimaera sequences removed (*removeBimeraDenovo*). A sequence matrix (ASV table) was built and then annotated taxonomically through the SILVA v138.2 database.
Sample names have been corrected by mapping from external files, and sample metadata have been imported from Excel files and merged into the main dataset. Finally, a phyloseq class object was built containing the ASV matrix, the assigned taxonomy and the metadata associated with the samples. The resulting object has been saved in .rds format for further statistical analysis.

3.5. STATISTICAL ANALYSES

Statistical and visual analyses were conducted in the R environment using specialised packages for the management and visualisation of microbiomics data (`phyloseq`, `vegan`, `ggplot2`, `glmmTMB`, `microbiomeutilities`, `microViz`, `brms`) (R Core Team, 2024).
The phyloseq object containing the ASV matrix, taxonomic assignments and sample metadata has been filtered to select only intestinal samples. A taxonomic cleaning was carried out, removing mitochondria and chloroplasts, and only taxa with at least two occurrences were maintained.
Alpha diversity was calculated using four metrics: Shannon, Observed, Chao1 and Simpson. Statistical comparisons between treatment were performed using a Student t-test.
For the analysis of relative abundances, the data were agglomerated at the gender level (*tax_glom*) and the 20 most abundant genera were selected.
Beta diversity was evaluated by sorting analysis (PCoA) based on Bray-Curtis distance. The structure of the community was visualised according to treatment and sex. The statistical significance of the treatment effect, gender and their interaction was evaluated by permutational multivariate analysis of variance (PERMANOVA, adonis2). In all cases, the replicas were inserted as a random variable to consider intragroup variability. The percentage of variance explained for each main axis has been calculated and saved.

A normalised heatmap (Z-score) of the most representative genera was built, selected based on prevalence (≥10%) and average abundance (>0.05%). For each gender, the values have been normalised line by line with Z-score, so as to express how many standard deviations each abundance value deviates from the gender average, and subsequently displayed via hierarchical clustering (row and column) with the following parameters: the Euclidean distance and the complete linkage method. The dendrograms demonstrate the similarity of the microbiomics profiles in the vicinity.

3.6. ETHOVISION

To quantify the number and type of contact between fish it was used EthoVision XT (Noldus, NL) tracking software. Treatment A tanks (five replicated) were subject to monitoring over the course of a single day, during which three videos were recorded. The duration of each video was 30 minutes, with one video recorded at 9:00 am, one at 6:00 pm, and one at 9:00 am of the following day.

RESULTS

1. MICROSCOPY

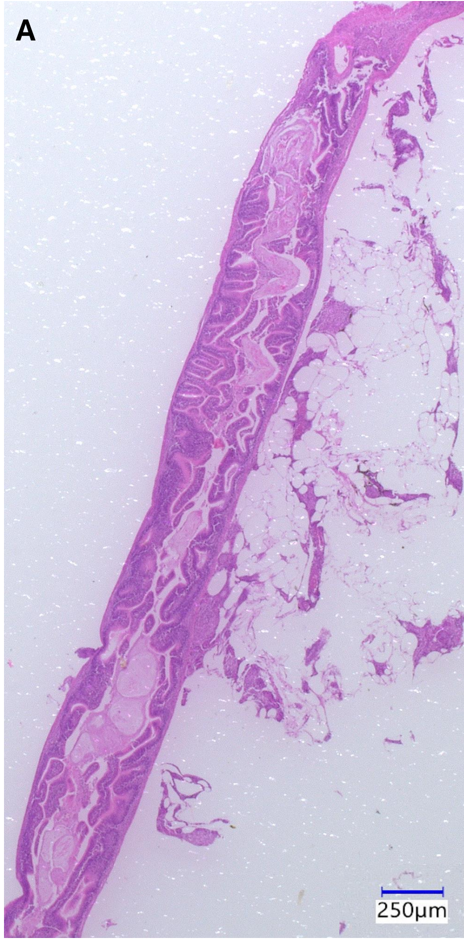
It is crucial to visualize microorganisms in their natural habitat to observe their spatial organization and interactions. The utilisation of FISH visualisation in conjunction with haematoxylin and eosin histology facilitates the visualisation of host tissue structure.

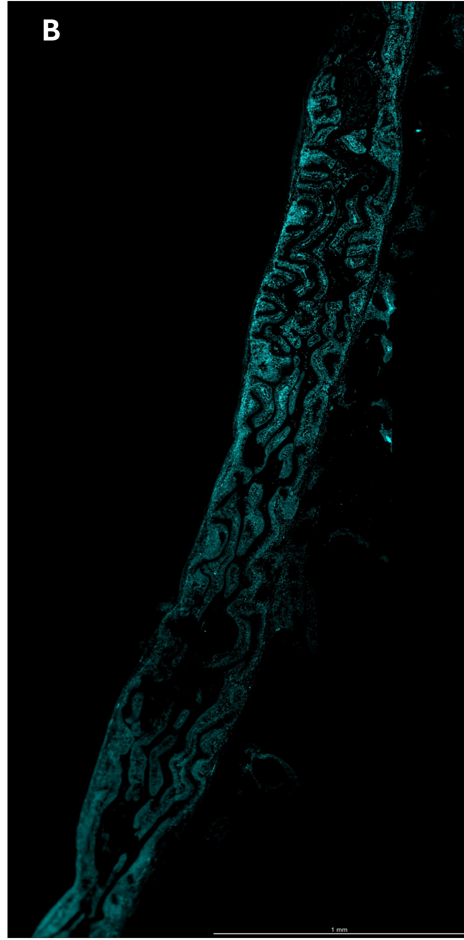
In this work, I performed the optimization of a FISH protocol on the anterior intestine of *Poecilia reticulata* in conventional subjects (CV), with the aim of observing the spatial distribution of bacteria in the gut. As illustrated in Figure 3A, the application of haematoxylin-eosin staining serves to emphasise the structural characteristics of the villous epithelium, in conjunction with the underlying connective layer. In the individual FISH visualisations (see Figures 3B-D), the nuclei are represented by blue, tissue autofluorescence by red, and the bacterial probe signal by yellow. A marked concentration of yellow fluorescence is observed in the intestinal lumen, with aggregates of bacteria in close proximity to the epithelial cells of the villi. The overlay of all channels (Figure 3E) confirms the colocalization of the yellow signal along the epithelial margin.

As illustrated in Figure 4, a focus view of the villous fringe reveals the same colour patterns: in panels A-D, the blue nuclei dispersed throughout the mucosa, the red autofluorescence in the background tissue, and the yellow signal from the bacteria are distinctly discernible. The overlay (panel E) accentuates bacterial clusters that are predominantly located along the apex of the villi.

Figures 5 and 6 illustrate the gill arch sample from the same organism. Nuclei and autofluorescence are evident in the individual channels (Figure 5 A–C). Figure 5D (overlay) does not show a distinct yellow signal; the slight yellow fluorescence detected is compatible with tissue autofluorescence rather than with a specific bacterial probe.

Focusing on the gill lamellae (see Figure 6 A-C), it is evident that the blue and red patterns are analogous, though no substantial accumulations of yellow are discernible. In the overlay (6D), the yellow signal maintains a diffuse and uniform disposition, thereby substantiating the hypothesis of a non-microbial origin.


A
250µm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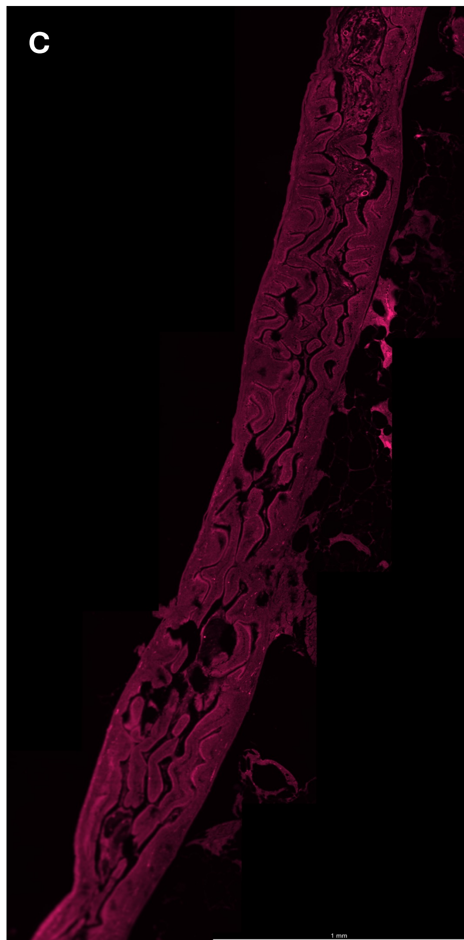
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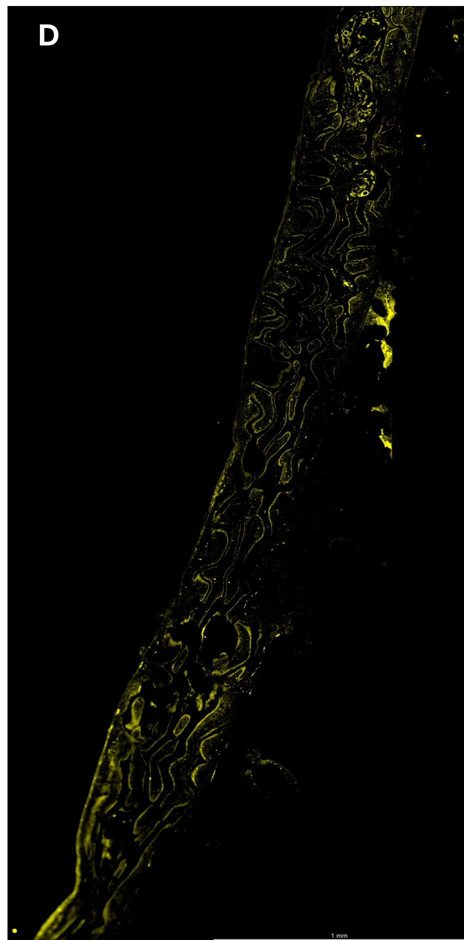
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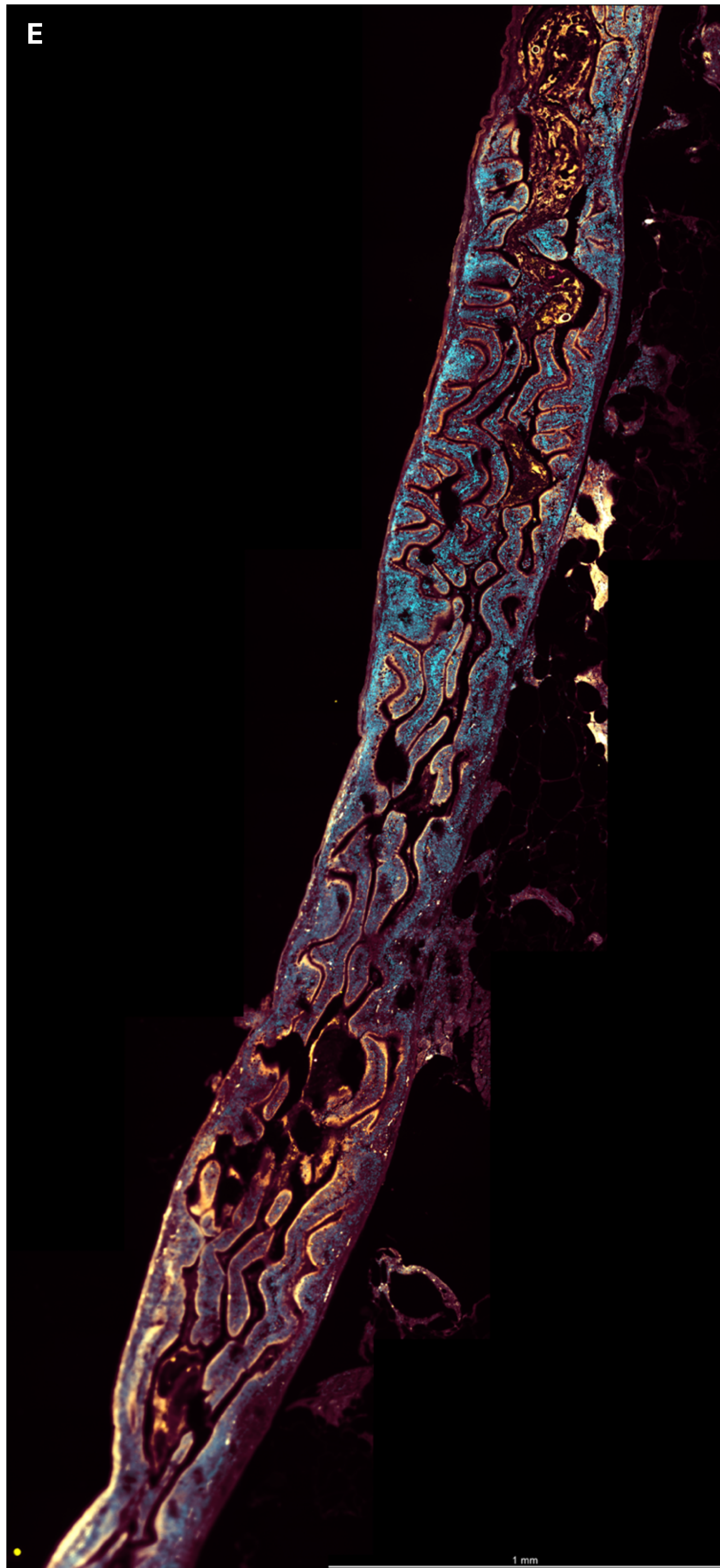

Figure 3: Overview of the anterior intestine of Poecilia reticulata. (A) Histological section stained with haematoxylin-eosin. Scale bar = 250 µm. (B) Blue channel signal (Hoechst) used for nuclear DNA visualisation. (C) Red channel signal, corresponding to tissue autofluorescence. (D) Yellow channel signal, representing bacteria labelled with a universal eubacterial probe conjugated with the fluorophore Atto488. (E) Combined image with all channels overlaid, showing simultaneously the tissue structure, the nucleus and the bacterial distribution. Scale bar=1mm

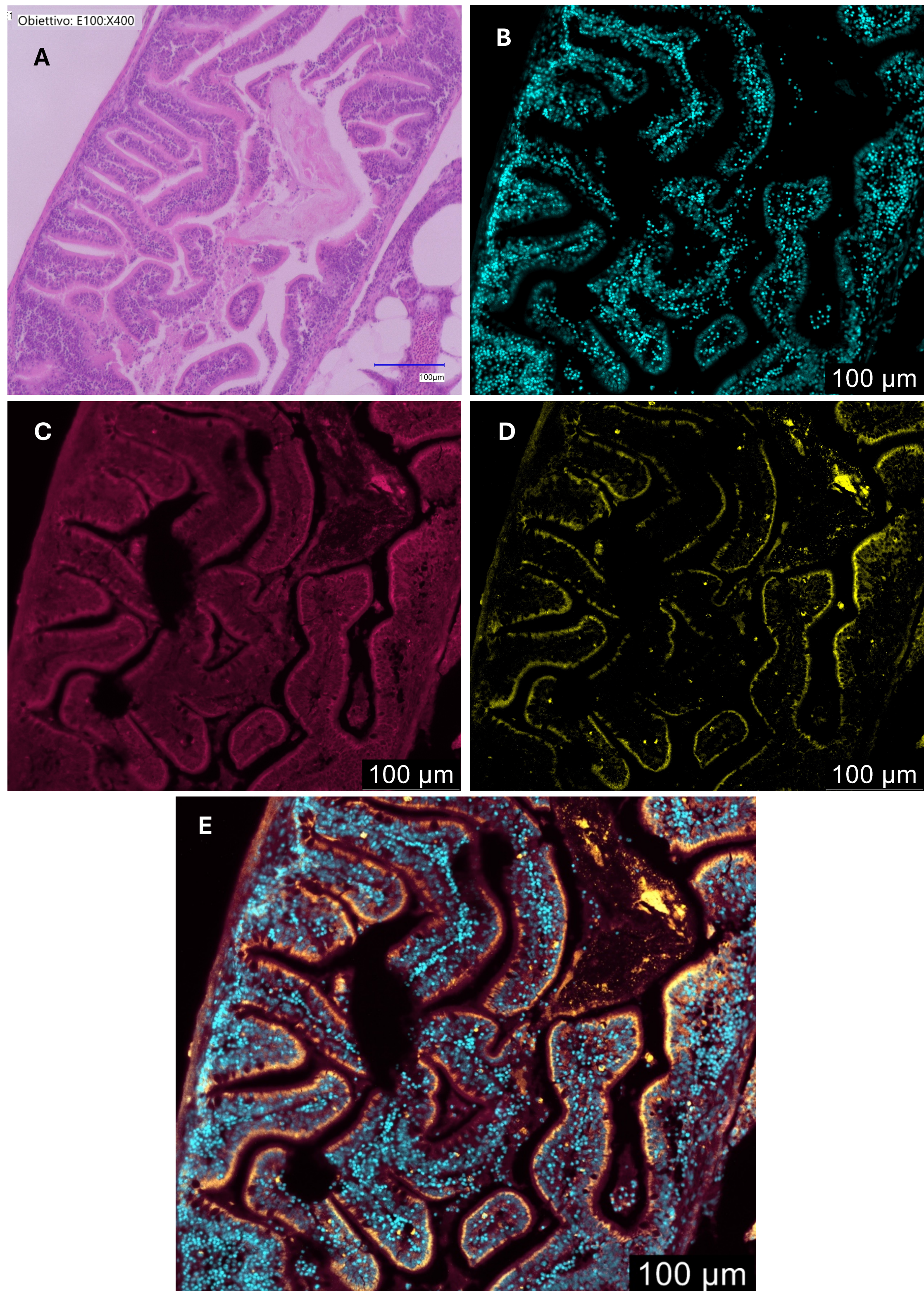

Figure 4: Focus view of the anterior intestine of Poecilia reticulata. (A) Histological section stained with haematoxylin-eosin. (B) Blue channel signal (Hoechst) for nuclear DNA staining. (C) Tissue autofluorescence (red). (D) Yellow channel signal, corresponding to bacteria labelled with the universal eubacterial probe conjugated with Atto488. (E) Composite image with all channels overlaid, showing the simultaneous distribution of tissue structures, nuclei and bacteria.
Scale bar = 100 μm.

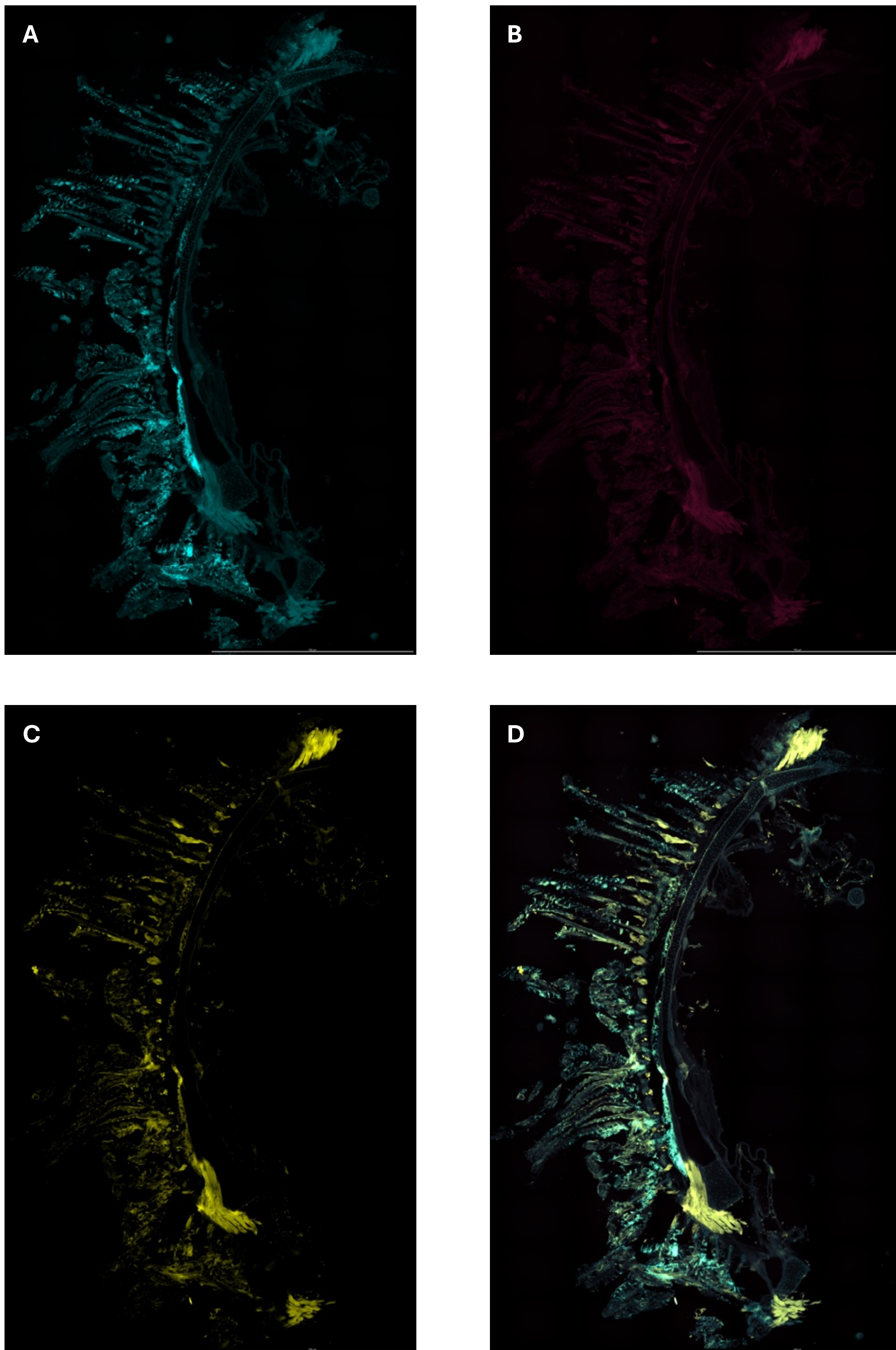

Figure 5: Overview of the gill arch of Poecilia reticulata. (A) Blue channel signal (Hoechst) for nuclear DNA staining. (B) Tissue autofluorescence (red). (C) Yellow channel signal, corresponding to bacteria labelled with the universal eubacterial probe conjugated with Atto488. (D) Combined image with all channels overlaid, showing tissue structures, nuclei and bacterial distribution simultaneously. Scale bar = 1 mm.

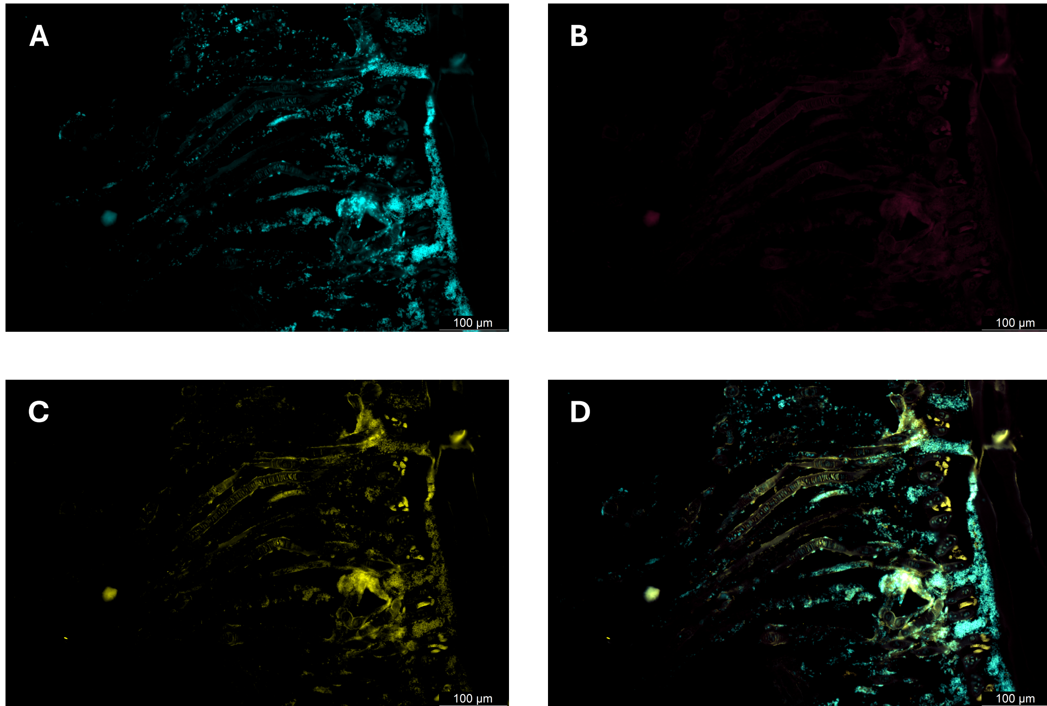

Figure 6: Focus view of the gill arch of Poecilia reticulata. (A) Blue channel signal (Hoechst) for nuclear DNA staining. (B) Tissue autofluorescence (red). (C) Yellow channel signal, corresponding to bacteria labelled with the universal eubacterial probe conjugated with Atto488. (D) Composite image with all channels overlaid, showing tissue structure, nuclei and bacterial distribution. Scale bar = 100 µm.

2. REAL TIME PCR

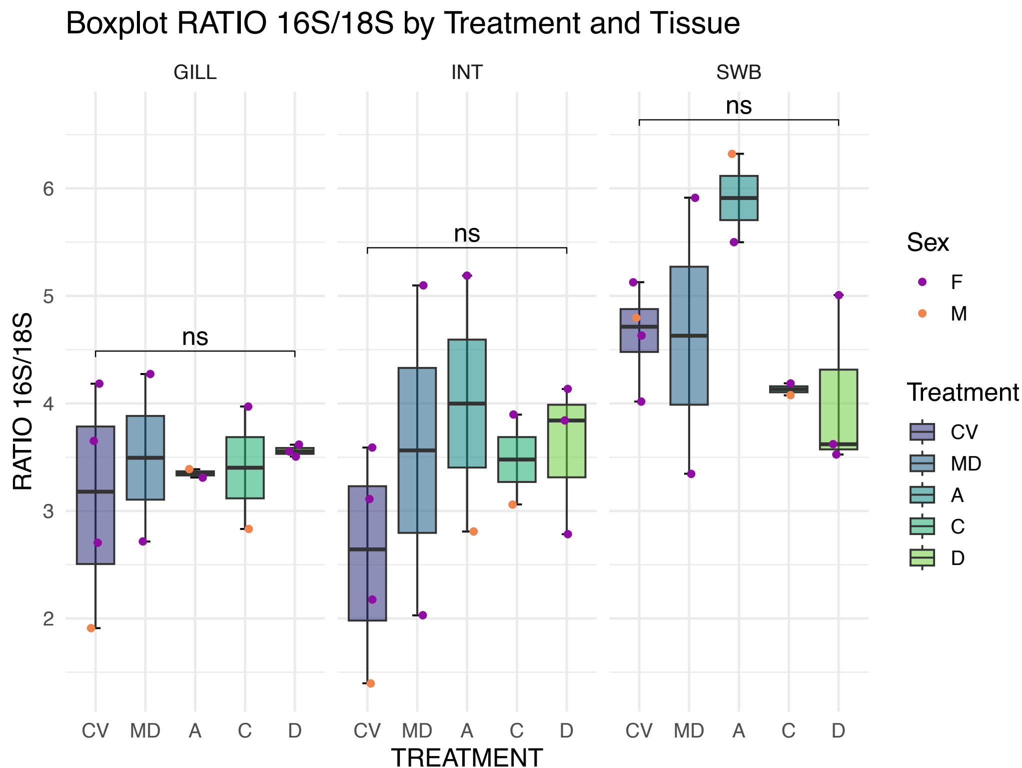

Figure 7: Box plot of 16S/18S ratio values in different tissues (gills, intestine and SWB) divided by treatment (CV, MD, A, C, D). The y-axis shows the normalised 16S/18S ratio values. In the box plots, the central line represents the median, the edges of the box correspond to the first and third quartiles, while the vertical bars indicate the data range. The statistical comparison between treatments was performed using Student's t-test; the significance indications are shown above the corresponding boxes.

To evaluate the effect of different treatments on the microbiota in different tissues, following RNA extraction I performed real-time qPCR. The relative abundance of the microbiota was analysed using the eukaryotic 18S gene as a normaliser and the bacterial 16S gene as a target.

Figure 7 shows the 16S/18S ratios in the three districts tissues analysed (gills, intestine and skin swabs) divided by treatment (CV, MD, A, C, D). The ratio values for the skin exhibit a higher propensity for microbiome abundance in comparison to the gills and intestine. In particular A sample in swab exhibit the higher ratio values (mean ratio A_SWB = 5.9). Gill and gut sample were observed to be very similar to each other (average ratio of all treatments in the gills 3.38; average ratio of all treatments in the intestine 3.44)

Moreover, while gill samples appear more uniform across treatments, greater variation was exhibited by the gut and skin microbiome (SD gills = 0.17, SD gut = 0.53; SD skin

swab = 0.74). Where possible (n>3), a Student t-test was conducted to compare the microbiome relative abundance between the different experimental conditions. Male and female individuals were not separated in order to apply the T student test. No significant differences were observed between treatments.

3. 16S rRNA SEQUENCING

Microbiome sequencing permitted the determination of the composition of the samples at the genus level, and the influence of treatments on the structure of the microbiome itself.

The 16S rRNA sequencing data was exclusively conducted on the intestine samples of CV, MD and A individuals. Figure 8 presents the relative abundance of the predominant bacterial genera across the three experimental conditions. The analysis of the samples revealed the presence of *Pseudomonas* and *JSC-12 spp*. in the majority of the CV individuals, with a few samples also showing high abundance of *Mycobacterium*, *Aeromonas* and *Flavobacterium*. R1_F_MD and R3_F_MD are primarily distinguished by

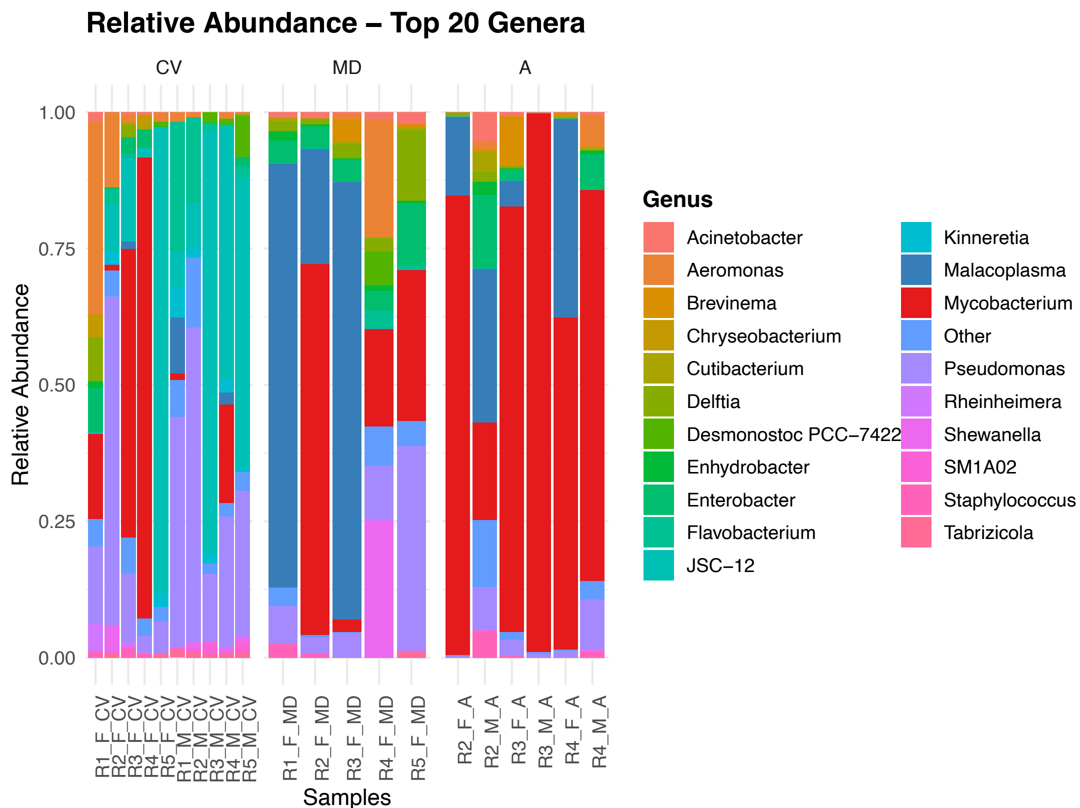

Figure 8: Relative abundance of the microbial community in the different samples, divided according to the treatment received (CV, MD, A). The y-axis shows the relative abundance of the main microbial taxa, while the x-axis shows the individual samples analysed. The colours represent the different microbial genera identified, as shown in the legend on the right.

the presence of *Malacoplasma*, while R2_F_MD exhibits a high prevalence of *Mycobacterium*. The remaining MD individuals demonstrate a more uniform distribution of taxa. The samples from treatment A are characterised by a strong presence of Mycobacterium, which in some cases is almost the only taxon present.

I next tested differences in the alpha diversity (considering four indexes: (Observed OTUs, Chao1, Shannon and Simpson)) between CV males and CV females to understand whether microbiome richness differed between sexes (Fig. 9A). The average values of the different indices are shown in Table 7. The number of taxa present in the two different groups is very similar. In fact, there are no significant differences in the number of taxa present (Student t-test $p>0.2$).

Then I compares CV females, MD females and A females (Figure 9B) to verify whether and how exposure to sterile water and contact with a CV male affected bacterial richness. The average values of the different indices are shown in Table 7. The values for conventional females and microbiota depleted females are comparable, but there is a lower trend in alpha diversity values for A samples. It is important to note that comparisons between: F_CV vs F_MD and F_MD vs F_A are not statistically significant (Student t-test $p \geq 0.07$). Conversely, a comparison of F_CV with F_A reveals significant disparities (Student t-test $p \leq 0.017$).

In both figures, the axes of the diversity indices are on a linear scale and the values are dimensionless, as they represent indices of richness and equity in the composition of bacterial communities.

SAMPLE/INDEX	M_CV	F_CV	F_MD	F_A
Shannon	2.78657349	2.37227991	2.18913696	1.44314059
Observed	37.6	39.4	31.6	13.3333333
Chao1	37.6	39.4	31.6	13.3333333
Simpson	0.89568046	0.82233429	0.7939202	0.69018161

Table 7: α diversity indices of the microbial community in the different experimental groups (M_CV, F_CV, F_MD, F_A). The Shannon index, the number of species observed (Observed), the Chao1 richness estimate and the Simpson index are reported. The values describe the microbial diversity and richness within each group as a function of the treatment received.

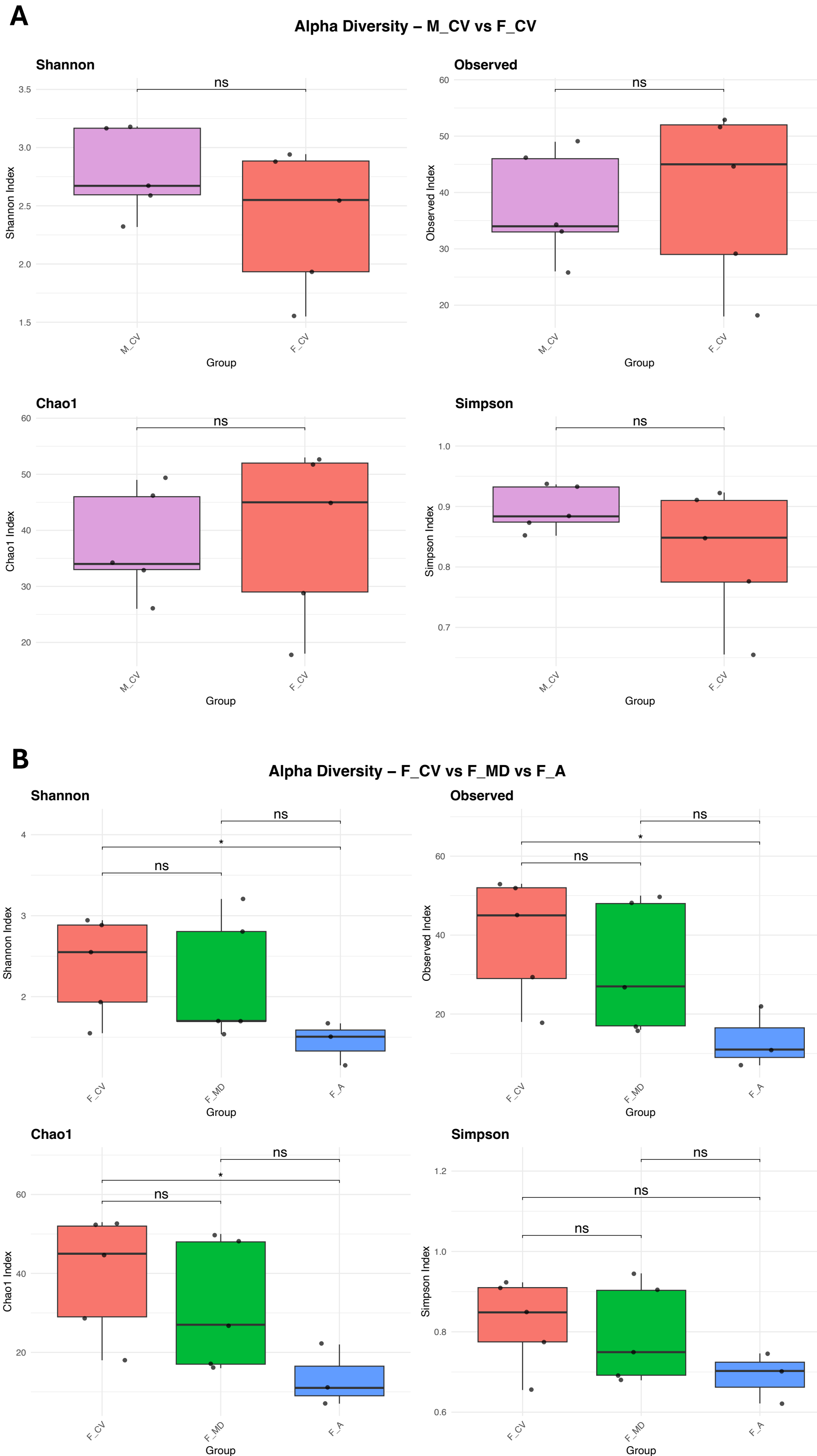

Figure 9: α diversity indices of the microbial community in the different experimental groups.(A) Comparison between M_CV and F_CV for Shannon indices, number of species observed (Observed), Chao1 richness estimate and Simpson index. B) Comparison between F_CV, F_MD and F_A for the same indices.
The box plots show the median (centre line), the first and third quartiles (box limits) and the dispersion bars (whiskers). The statistical comparison was performed using Student's t-test, with the significance indicated above the groups (ns = not significant).

Figure 10 presents a multivariate sorting analysis (PCoA) performed on the Bray–Curtis distances calculated from the intestinal bacterial communities. The first two principal components (PCoA1 and PCoA2) account for 30,01% and 21,67% of the total variance in the data, respectively, for a cumulative total of 51,68%. As shown in Figure 10, the axis-1 clearly separates conventional individuals from those exposed to sterile water. The results of the permutation test (adonis2) indicate that the treatment explains a significant portion of the compositional variance (R^2 = 0,248; F = 2,97; p = 0,001). When considered in isolation, sex is not significantly associated with the composition of the sample (R^2 = 0,067; F = 1,37; p = 0,232). When considering the interaction between treatment and sex, the overall model explains 32,75% of the variance (R^2 = 0,328; F = 1,95; p = 0,009).

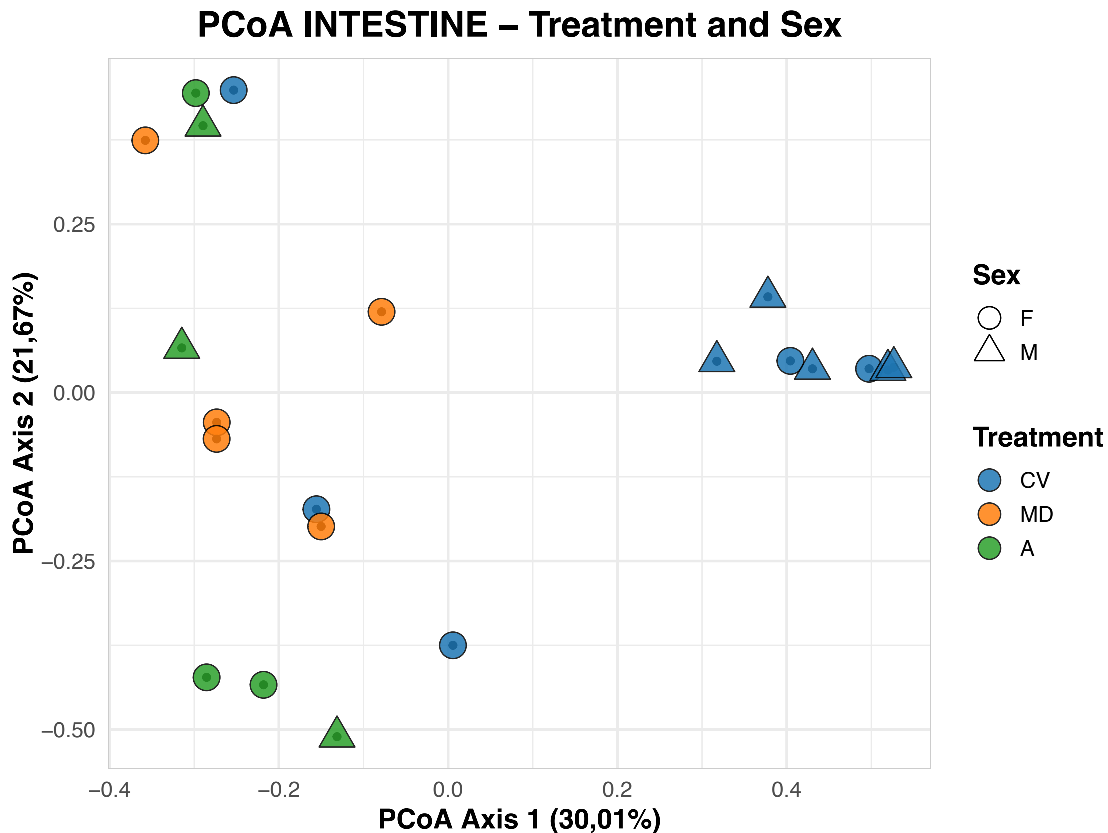

Figure 10: PCoA-based beta-diversity analysis calculated from Bray–Curtis distance. Each circle represents a microbial sample from a female individual, while each triangle represents a male. Blue indicates CV individuals, orange indicates MD individuals, and green indicates A individuals. The distance between points reflects the dissimilarity in microbial composition between samples: closer values indicate more similar microbial communities, while more distant values reflect more different communities.

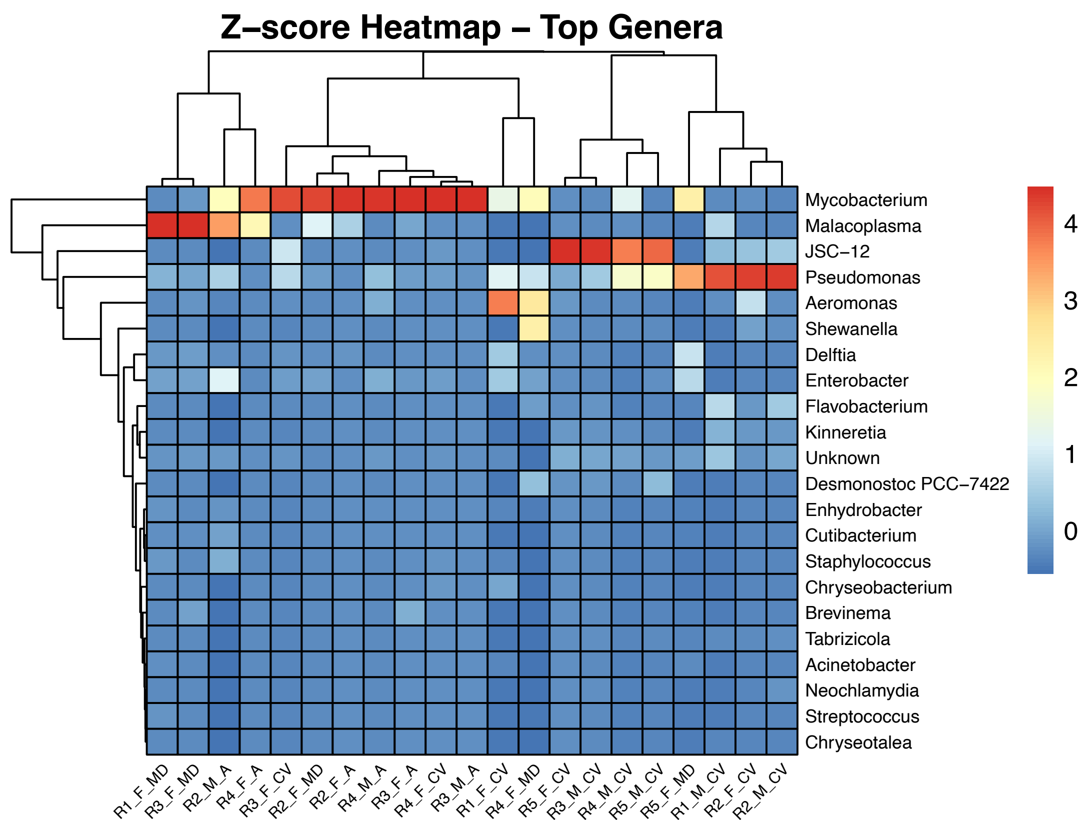

Figure 11: Heatmap of the most prevalent bacterial genera in intestinal samples. Abundance values were normalised using Z-scores, with a colour gradient from blue (below-average values) to red (above-average values). Each column represents a sample, while the rows correspond to the different bacterial genera identified. The colour scale on the right shows the range of variation in normalised values.

Figure 11 presents a Z-score-based heatmap of the relative abundances of a filtered subset of the 60 most abundant genera (prevalence ≥ 10%) measured in the 21 intestinal samples. The colour scale utilised in this study ranges from blue, indicative of a negative Z-score, through white to red, denoting a positive Z-score. The heatmap highlights a variability in distribution among the samples belonging to the three experimental groups. The conventional samples cluster together on the same side of the figure, elevated Z-score values are observed for genera such as *JSC-12 spp.*, *Pseudomonas* and *Aeromonas*, which have been found to occur with greater frequency in this group than in the others. Microbiota-depleted samples show a more diverse microbial profile among themselves. *Malacoplasma* in this condition is more present. In the samples that were subjected to treatment A, an enrichment of Mycobacterium was observed. The highest values of Mycobacterium are found in samples R3_M_A, R3_F_A, R4_M_A and

R2_F_A, where it is consistently above average. High z-scores for Mycobacterium are also observed in two conventional samples (R3_F_CV, R4_F_CV), while in microbiota-depleted samples its presence is more discontinuous, with increased values only in R2_F_MD and R5_F_MD.

4. ETHOVISION

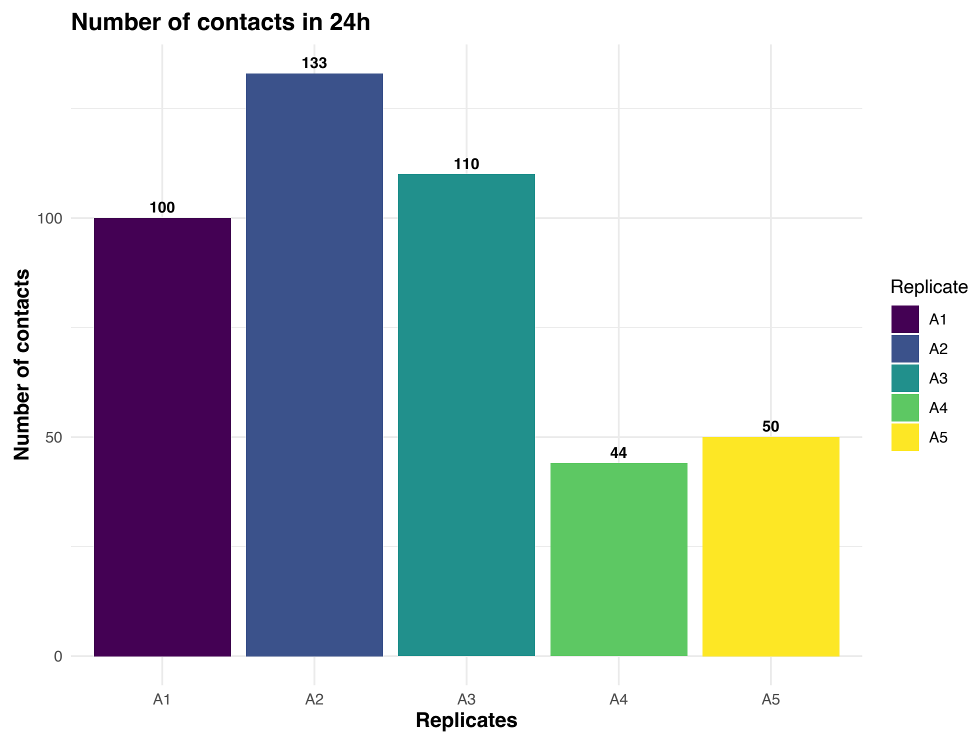

Figure 12: Number of contacts recorded via EthoVision between male and female individuals in experimental condition A. The y-axis shows the average number of interactions observed, while the x-axis indicates the different experimental groups (A1–A5).

The total number of contacts detected between the two individuals (male and female) within experimental condition A is illustrated in the form of a bar graph (Figure 12). The data was acquired by three video sessions of 30 minutes each, recorded at regular intervals over 24 hours. For each pair A, the software has identified the moments of contact between the two subjects, adding the total number of interactions for each session.

The graph illustrates the bars corresponding to the various experimental replicas (e.g. A1, A2, A3, A4, A5), emphasising the individual variations in the total number of contacts (SD ~38,2).

DISCUSSION

This thesis forms part of a broader research project investigating the impact of physical contact on microbiome transmission and composition (see Figure 2). My work focused on specific experimental conditions involving conventional individuals (CV), perturbed individuals (i.e., fish exposed to sterile water; MD), and individuals allowed physical contact (CV male as microbiota donor and MD female as microbiota recipient).

1. HOW IS THE MICROBIOTA ORGANISED IN DIFFERENT TISSUES?

First, I analysed the spatial distribution of bacteria in the guppy intestine. The images obtained through FISH demonstrated a discrepancy in yield between intestinal tissue and gills.

The intestinal tissue displays a strong bacterial signal in the proximity of the villi, which is consistent with the presence of nutrient-rich mucosal niches. This result is concordant with that described by (Yang et al., 2024), which highlights how the microbiota is distributed among the intestinal folds adhering to the mucosa in zebrafish. Experiments on other species (e.g., mice) also demonstrate that microorganisms are mainly found on the mucosa of cells (Duncan et al., 2021).

Conversely, in the gills, there is no prominent yellow signal, but it manifests itself in a diffuse manner. This outcome is somewhat unexpected, given that gills are in constant contact with the environment (a source of microorganisms) as they filter water, and since it has previously been demonstrated that gills of fish are characterised by an abundant microbiota (Mes et al., 2023; Pratte et al., 2018). Literature contains studies on a variety of other marine species (for example, mussels), and FISH has demonstrated that microorganisms are distributed along the gill lamellae. However, as these are symbiotic microorganisms, it seems probable that this has facilitated their visualisation. (Alcaraz et al., 2024; Fujiwara et al., 2010). Consequently, it is necessary to differentiate the FISH protocol from that of the intestine and adapt it accordingly. For instance, the number of washes in the protocol could be reduced, which risk "taking away" bacteria that are not found adhering to the gill tissue. Furthermore, the branchial epithelium is characterised by a thinning of the tissue, accompanied by an abundance of keratins and pigments. These factors contributed to an increase in background noise.

2. ANALYSIS OF BACTERIAL LOAD ACROSS TISSUES

The qPCR results (Fig. 7) do not provide significant evidence to suggest that sterile water or contact between the male donor and female recipient significantly influence the bacterial load. It has been observed that, in general, the bacterial load does not decrease; in some cases (A_SKIN), there is an increase. It is important to note that the sample analysed is very small in number, which reduces the possibility of obtaining greater data variability and performing statistical tests on all the conditions analysed. Despite this, higher mean ratio values are observed on the skin than in the intestine and gills, consistent with the findings of (Wang et al., 2025), who reported that fish skin represents a surface in direct and extensive contact with the surrounding environment.

3. STERILE WATER AFFECTS THE COMPOSITION AND STRUCTURE OF THE MICROBIOTA

The composition of the microbiota was assessed using 16S sequencing data. First of all, consideration must be given to how different experimental conditions influence the richness of taxa present. I evaluated the diversity between conventional males and females to determine whether there could be differences in microbial richness based on sex. As shown in Figure 9A, this condition does not occur, allowing us to exclude biases based on sex. So I compared the alpha diversity of female individuals in the three experimental conditions. The reduction in alpha diversity values between conventional females and microbiota-depleted females (exposed to sterile water for 24 hours) is an indication of the influences of water on the number of taxa present, and this is also confirmed by the significant difference observed in alpha diversity values between conventional females and A females (exposed to sterile water for 48 hours and in contact with conventional males). The reduction in the number of taxa should not be confused with the bacterial load (expressed by qPCR ratio values); in fact, the reduction in the number of taxa could favour the development of specific genera (e.g. *Mycobacterium*).
So I analysed the composition of the microbiota in the different samples. The analysis of the relative abundance of the different taxa shows that the composition of the microbiota of conventional individuals is in accordance with the findings reported by (Evans et al., 2020). The use of sterile water disrupts the composition of the microbiota

by reducing the number of taxa present; in fact, it decreases the relative abundance of many species at the expense of others (e.g., *Mycobacterium*). The variations in the structure of the microbiota of individuals exposed to sterile water are confirmed by Beta diversity (see Fig. 10), which clearly separates conventional individuals from microbiota-depleted individuals and A.

The marked dominance of *Mycobacterium* observed in F_A receivers can be interpreted through two possible mechanisms, not necessarily alternative. Firstly, it can be hypothesised that a relatively efficient transfer of a taxon that is already relatively abundant into a donor occurred, which would have given it an initial competitive advantage in colonization processes. Secondly, the phenomenon of ecological selection is plausible: in the presence of ecological niches that are not yet saturated, *Mycobacterium* had the capacity to colonize the niches thanks his greater adhesion capacity and the capacity to form biofilm (Pereira et al., 2020; Debray et al., 2022; Viljoen et al., 2022).

The heatmap data highlight how, within the same treatment, some individuals develop different microbial profiles, suggesting that factors other than the simple presence of sterile water may modulate the composition of the microbiota. In particular, samples such as R2_F_A and R4_F_A show opposite trends for genera like *Mycobacterium* and *Malacoplasma*, underlining a variability in microbial composition among individuals within the same treatment. The behavioural analysis obtained with EthoVision supports this interpretation, showing differences in the number of contacts between pairs exposed to the same experimental conditions. In this thesis, however, no statistical model was applied to formally test the correlation between the number of interaction and microbial structure. But, the hypothesis that behaviour acts as a modulating factor is consistent with what has been reported in the literature: studies on social models such as primates have shown that the frequency and intensity of physical contact significantly influence microbiota sharing (Archie & Tung, 2015).

CONCLUSION

This thesis is part of a larger project that seeks to answer the question: *How is animal microbiome transmitted?* In my research, I used guppies as a model species, adopting an integrative approach that combined microscopy, molecular quantification, sequencing and behavioural analysis. The results suggest that microbiome transmission is not explained by a single factor, but rather depends on a combination of factors, including environmental disturbances and social interactions.

I showed that our procedure for FISH at the intestinal level was very effective, revealing dense microbial localization along the mucosal villi, whereas its application to gills was not successful, highlighting the need for methodological adaptation in this organ.

Molecular and sequencing data showed that exposure to sterile water alters community composition and taxa richness, favouring the expansion of certain taxa such as *Mycobacterium*. The use of tracking software allows the number of contacts between individuals to be obtained. Behavioural observations have suggested that variability in physical contact between individuals may contribute to differences in microbial profiles within the same treatment. In the future, integrating more sequencing data with more social interaction data (including data such as contact duration) will provide a more reliable indication of the influence of social interactions on the microbiota.

Taken together, these findings confirm that Poecilia reticulata is an excellent model organism for studying the interaction between microbial ecology and animal behaviour. The results highlights the importance of considering both environmental and behavioural factors when studying microbiome dynamics in animals.

To expand on these findings, we plan to characterize the genetic determinants that underpin bacterial transmission strategies, distinguishing between dispersal- and host-specialist taxa. Furthermore, we will combine genomic data with imaging techniques using taxon-specific fluorescent probes to precisely localize bacterial populations within intestinal tissues. This approach will enable us to investigate whether specific bacterial genera exhibit spatial preferences within the host gut.

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