**EXTENDED ABSTRACT**

Journal of the International Society of Microbiota
Issue n°3, Vol. 3
DOI 10.18143/JISM_v3i1_1595

# Zebrafish gut as a house for human intestinal bacteria

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## Abstract

During the last years human intestinal microbiota has been deeply studied and associated with a wide variety of diseases. As a consequence, novel techniques that allow study the whole microbiome in depth have emerged, such as next generation techniques. Otherwise, study the interactions of the microbiome with the host and the environment is still difficult. Animal models offer a closer approximation to the reality. In the present study, zebrafish (*Danio rerio*) intestine has been colonized with five bacterial species, members of the human gut microbiome, including obligate and facultative anaerobes (*E. coli, E. faecalis, B. breve, L. casei* and *E. limosum*). Germ-free zebrafish larvae were infected with each bacterium (monoassociation) and with all bacteria (consortia). The colonization was monitorized by culturing on different media, by q-PCR analysis and metabarcoding. After the infection, all the strains remain inside the zebrafish gut almost during 48 hours post infection (hpi). As a matter of fact, we resolved that different bacteria commonly found in the human intestine, including obligate anaerobes, are able to colonize and compete inside W
suitable model for studying certain species of the human gut microbiota.

## Introduction

The human gut houses a vast microbial community that is vital for maintaining host health (Toh M. *et al*, 2013). Considering the human body as an environment, the human microbiota is the entire collection of microorganisms living on the surface and inside our body (Dargenio *et al.*, 2015). Consequently, we have two genomes, one inherited from our parents and the other acquired, i.e., the microbiome. This

Z *et al.*, 2014).

The intestinal microbiome thrives in a nutrient rich and thermostable environment and provides the host with metabolic nutrition, the facilitation of energy extraction, the competitive exclusion of pathogenic microorganisms and many other beneficial functions (Jones *et al.*, 2016). The gut resident microbes are crucial for normal immune development and homeostasis, as well as regulatory effects on epithelial growth, differentiation and cytoprotection, thus exemplifying a balanced symbiotic relationship between the host and its resident bacterial flora. Compositional perturbations of the microbiota (dysbiosis) have been associated with diseases, including obesity, diabetes, colorectal cancer, and allergies. Hence, maintaining compositional and functional stability within the gut microbiome is essential to host health, as demonstrated by dysbiosis detected at the onset of nonpathogenic chronic diseases (Arnold *et al.*, 2016).

*Received: November 25, 2016; Accepted: March 17, 2017*



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The complexity and the high inter-individual variability of the human gut microbiota are inherent problems in the study of host-microbe interactions. Germ-free animals offer the opportunity to circumvent some problems. For that reason, it would be desirable to have a simple animal model to study the interactions between the gut microbiota and the host (Rawls *et al.*, 2006).

has become a popular new model organism for biomedical research, due to their physiological and genetic homology to mammals, high sensitivity to tions, and ease of experimental (behavioral, genetic and pharmacological) manipulation. In addition, its genome is fully sequenced and available, they have quick reproduction, potential for high-throughput screening, low cost, and at larvae state they are optically transparent (Nguyen *et al.*, 2013, Seth *et al.*, 2013).

In the present study, we have successfully colonized the intestine of 5 days post fertilization (dpf) zebrafish larvae with five bacteria, members of the normal human gut microbiome (*Escherichia coli, enterococcus faecalis, Lactobacillus casei, Bifidobacterium breve* and *Eubacterium limosum*).

## Materials and methods

### *Zebrafish husbandry*

Zebrafish embryos were obtained from wild-type adult zebrafish (*D. rerio*, Hamilton 1822) bred in the AZTI Zebrafish Facility (REGA number ES489010006105; Derio, Spain) following standard conditions. es were maintained at 27°C in 60 l tanks, with aerated freshwater; according to standard protocols (Brand *et al.*, 2002).

Fish were fed with a pellet-formulated diet (Gemma Micro 300; Skretting) and reared on a 12-h light/12-h dark cycle.

Convencionalized zebrafish larvae (monoassociation and consortia) were maintained under gnotobiotic conditions with autoclaved diet (ZF biolabs) three times per day (Pham 2008) and one medium change, for up to 10 (dpf).

Zebrafish embryos were collected directly from the breeding tanks immediately after fertilization and a well stablished protocol for obtain germ-free larvae was followed (Oyarbide et al. 2014).

Sterility was tested after 96 hours post fecundation (hpf), by culturing on general culture media (Brain Heart Infusion, Plate Count Agar and Sabouraud; from oxoid)

targeting 16S ribosomal RNA gene.

### *Ethical issues*

All experimental procedures were approved by the regional animal welfare body.

### *Germ-free larvae colonization*

Germ-free zebrafish laevae were colonized at 5dpf with desired bacteria (*E. coli, E. faecalis, B. breve, L. casei and E. limosum*) separately (monoassociation) and in association (consortia), inoculating each pure culture into the surrounding water at a final density of $10^7$ - $10^8$ CFU/ml.

### *Colonization confirmation*

Colonization success was tested at different time points (24, 48, 72 hpi, 5 7 dpi). 3 pools of five larvae were taken at each time point, euthanized with tricaine methanesulfonate (MS-222, Aldrich), washed and homogenized in pre-reduced Buffered Peptone Water, supplemented with cysteine (0.25 g/l) and K1 vitamin (0.001 ) (Oxoid). Serial dilutions were spotted onto Wilkins Chalgren general media; to MacConkey, KF, MRS (Oxoid) and Bifidobacterium agar (BD), as specific culture media in order to count and confirm each colony. Media were incubated at 37°C in anaerobiosis during 48 hours. All the procedures, manipulation and incubations carried out in this section were performed inside invivo & microaerophilic workstation (Ruskinn technology) maintaining anaerobic conditions. The identification of each suspected *E. limosum* colony was performed by qPCR (LightCycler 480 Instrument II, Roche), because there was no specific medium for it. ELIM-F (5´-GACTTAGGCGCAGAAAAATTCC) and ELIM-R (5´-CAAACCAGCCATACGGCATT). The PCR conditions consisted of initial activation for 10 min at 95°C, followed by 45 cycles (15 s at 95°C and 30 s at 60°C) and cooling at 40°C for 30 s.

Furthermore, total genomic DNA was extracted from 3 pools of five larvae (QIAamp DNA mini kit , Qiagen) at 48 hpi and the microbial composition was characterized by Illumina sequencing of 16S rRNA gene amplicon (Biopolis, Valencia, Spain). The 16S rRNA gene Illumina reads were processed using methods implemented by mothur (1.37.2) (Schloss *et*

*Received: November 25, 2016; Accepted: March 17, 2017*

*al.*, 2009). The final operational taxonomic unit (OTU) table was rarefied to a depth of 3036 sequences per sample and results are shown to 97% sequence similarity. Graphics were drawn in excel.

## Results and discussion

Using this protocol, all the strains included in the study were identified inside the zebrafish gut for 48 hpi, whereas previous studies only found two species in very low concentrations (Toh *et al.*, 2013).

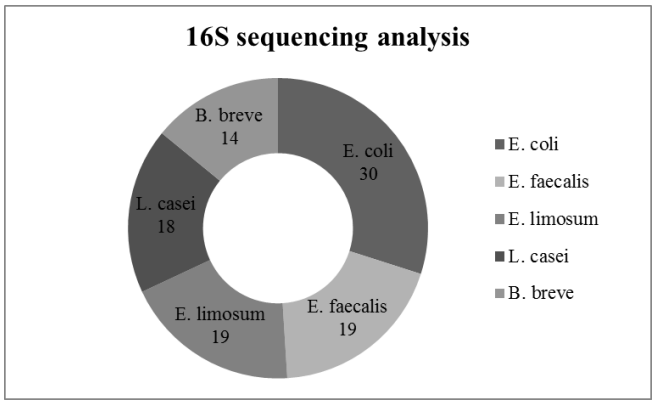


**Figure 1:** *E. coli, E. faecalis, L. casei, B. breve and E. limosum* percentage contribution in consortia at 48 hpi by 16S sequencing.

These strains were selected because (i) they are commensal bacteria in the human intestine (ii) belong to different taxonomic filum and classes and (iii) they are facultative and obligate anaerobes. *E. limosum* was included because Toh *et al.* (2013) introduced this strain in their experiment, and was the only specie they found, together with *Lactobacillus pasacasei*, inside the zebrafish gut 72 hpi.

On the one hand, facultative anaerobes are able to survive up to 10 days, but obligate anaerobes only survive 48 hours. This could be due to the oxygen pressure that makes possible facultative anaerobes growth, limiting obligate anaerobes survival (Toh *et al.*, 2013). At the beginning, obligate anaerobes might be able to survive in low/restricted oxygen conditions, but when facultative anaerobes colonize and grow obligate anaerobes die out because of the interspecific competition for the niche and resources (Rawls 2006).

Furthermore, these kinds of bacteria are not commonly found in the zebrafish gut, so they could find difficulties to stablish themselves and grow.

In monoassociations, in general, higher counts for all species used were monitorized. This could be due to the absence of interspecific competition, as well as the metabolism derived from the bacterial growth.

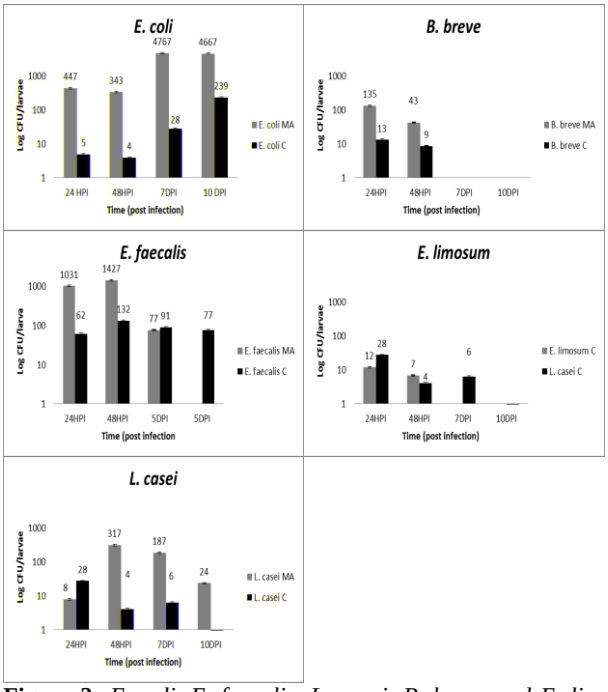


**Figure 2:** *E. coli, E. faecalis, L. casei, B. breve and E. limosum* concentration inside zebrafish intestine (CFU/larvae), for monoassociation experiment (grey) and for consortia (black).

Therefore we conclude that different human microbiota derived bacteria, including some obligate anaerobes, are able to colonize the zebrafish gut.

This data suggests that using this method, working in anaerobiosis, to colonize
could be a suitable model for studying the human gut microbiota, how to model it, the interactions with the host, and related diseases, as a result, diabetes, obesity, IBD...

As the anaerobes survive during 48 hours inside the zebrafish gut, this could be an appropriate model to test whether toxics, phages or antibiotics affects the microbiota.

In the near future we will use this protocol to, elucidate if zebrafish could be used as a model to study whole human intestinal microbiota.

## Acknowledgements

This study was financially supported by LIFE+ program (project LIFE13 ENV/ES/001048,

*Received: November 25, 2016; Accepted: March 17, 2017*

ENVIPHAGE), and NA is the recipient of a PhD fellowship from to the Department of Environment, Territorial Planning, Agriculture and Fisheries of the Basque Country Government. This article is the contribution of n 798 from AZTI-Tecnalia (Food Research Division).
We thank the European Commission for their financial support.

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*Received: November 25, 2016; Accepted: March 17, 2017*