Standardizing the microbiota of fish used in research

Short title: Standardizing fish microbiota

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Abstract

Until now, little attention has been paid to the effects of fish microbiotas on the reproducibility and comparability of fish studies. Extrinsic and intrinsic factors, such as water quality, environmental microbial populations, diet, host genetic profile, gender, age and stress status, affect fish microbiotas and create significant inter- and intra-species variations. Fish microbiotas play critical roles in many key aspects of host physiology, such as protection against pathogens, digestion and development of the digestive tract and the local immune system. Thus, greater effort should be invested in standardizing the microbiological profiles of research fish. In this context, issues requiring consideration include the establishment of isogenic and isobiotic fish lines, the standardization of rearing conditions and the development of appropriate tests to adequately describe microbial populations. There are many challenges involved in each of these issues, and the research community must decide which aspects should be standardized for each species and each type of research. For all studies in which the microbiota is expected to exert an influence, thorough reporting is of paramount importance. Every step towards standardization increases study quality and simultaneously contributes to reducing the number of fish used in research, which is a legal and ethical obligation.

Keywords

fish, microbiota, standardization

In 2010, Kilkenny et al.[1] proposed the ARRIVE (Animals in Research: Reporting *In Vivo* Experiments) guidelines, which include 20 checklist points describing the minimum, yet essential, information that all publications utilizing animals must include. One of these points requires a detailed description of the characteristics of the research animals prior to the study, including their microbiological status. Monitoring and recording the microbiological status of all research animals is also an obligation according to Directive 2010/63/EU because microbiological surveillance programs must be implemented for all research animals. However, until now, the vast majority of studies involving fish have not included any descriptions of microbiological status, and testing for the absence of certain important fish pathogens has rarely been reported.

The aim of the present review is first to highlight why the normal microbiota of healthy fish is an important experimental variable that affects experimental validity and reproducibility, and second, to discuss the issues and challenges related to standardization of the normal microbiota of research fish.

The fish microbiota

Early studies employed culture-based methods to identify and even quantify the groups of microorganisms comprising fish microbiotas. However, due to the low culturability (often <2%) of many bacteria living in the water, on the skin and in the fish

intestine, various complementary molecular techniques have also been used to provide a more comprehensive picture of the fish microbiota.[2,3,4] Based on the use of such techniques, many obligatory anaerobes that are difficult to culture represent a substantial portion of the fish gut microbiota in some fish species.[5]

Immediately after fish larvae hatch, bacteria present on the egg chorion and in the water begin to colonize different areas of the body, and this colonization continues as the fish start to feed and grow.[6-8]

Microbes are normally found on the skin, gills and in the fish intestine, but their presence has also been reported in other organs such as the liver and ovaries.[9,10] However, because these other organs are considered sterile, the presence of any microbes generally indicates a breach in immune defense mechanisms and the presence of subclinical infections.

Microbiotas of the fish skin and gills

According to many studies, there are quantitative and qualitative differences between the microbiotas of the fish skin and gills and that of the water in the host environment.[6] There are also differences between the adherent bacterial and fungal communities of the gills and skin.[11]

Due to the nutrient-rich environment of the skin and gill mucus, microorganism density on the fish skin and gills is significantly higher than that in the surrounding

74 water, as determined by several studies employing culture-based methods to analyze
75 fish reared either in tanks or in ponds.[12,13] Based on previous studies, Austin[9] reported
76 bacterial populations on fish skin ranging from 10^2 to 10^4 bacteria/cm and 10^6 bacteria/g
77 on the gills. Higher loads were associated with heavily contaminated aquatic
78 environments. However, due to the methods used (primarily culture-based methods and
79 scanning electron microscopy), these studies may have underestimated the investigated
80 bacterial populations.

81 The vast majority of identified bacteria are gram-negative, aerobic and members of
82 the phyla Proteobacteria, Firmicutes, Cyanobacteria, Actinobacteria, and
83 Bacteriodetes.[8,9] The most common genera are the following: *Aeromonas* spp., *Vibrio*
84 spp., *Cytophaga* spp., *Flexibacter* spp., *Escherichia coli*, *Enterobacter* spp.,
85 *Pseudomonas* spp., and *Photobacterium* spp. Many of these bacteria are opportunistic
86 pathogens that are ubiquitous in the aquatic environment. They hold the potential to
87 cause health problems under certain conditions, e.g., when the host immune system is
88 compromised or when the water temperature is favorable.

89

90 *Factors affecting the fish skin and gill microbiotas*

91

92 Various external and host-related factors affect the density and composition of the fish
93 skin and gill microbiotas (Figure 1).

Although there is a clear host species specificity, various factors, such as the environment, the season and various mucus components, affect the fish skin and gill microbiotas.[14-16] Furthermore, host genotype and gender appear to exert strong influences, resulting in significant intra-species variations, although the presence of an autochthonous core population has been demonstrated in certain species such as the brook charr (*Salvelinus fontinalis*) and pangasius (*Pangasius hypophthalmus)*.[16,17]

Different diets (e.g., pellets or natural diets) or starvation influence the fish skin and gill microbiotas through alterations in the composition of the skin and gill mucus.[12] Similarly, various stressful conditions, such as a high density population, hypoxia, or a 5-h transportation period, also influence the fish skin and gill microbiotas through alterations in mucus composition.[18,19] Different fish species are able to differentially tolerate stress, and thus, the effects of various stressors on their skin and gill microbiotas may differ.

In mammals, the stimulation of one mucosal surface may result in an immune response at other mucosal surfaces. In fish, little is known about these common mucosal immune responses, and further research is required to elucidate such interactions and, in particular, to determine how they influence the microbiota.

Effects of fish skin and gill microbiotas on the host

In terrestrial mammals, the normal skin microbiota plays an important defensive role by antagonizing many potential pathogens. A similar role has been demonstrated in fish (Figure 1).[20,21] Beneficial bacteria act through competitive exclusion for nutrients and/or synthesizing antimicrobial compounds. The presence of such beneficial bacteria plays an important role in the initial stages of an infection and even assists in the recovery of affected fish.[20,22]

According to Hansen and Olafsen,[6] some bacteria in the skin microbiota of fish may also assist in fish locomotion by secreting drag-reducing slime, thus enhancing the effects of skin mucus. This role has yet to be confirmed.

The fish gut microbiota

In fish, the gut microbial population has been extensively studied compared to the skin and gill microbiotas, and its effects on digestion, metabolism and various diseases have been confirmed.[8,23,24]

Microbes colonizing the fish gastrointestinal tract are either autochthonous or transient (or allochthonous), depending on their ability to survive the low pH of the stomach (depending on the fish species) and competition with other microbes.[4,8,23] There are differences in the composition of the microbiota between different parts of the gastrointestinal tract, and these differences are associated with the feeding habits of the

host species.[23,25] The number of microbes tends to increase from the stomach toward the distal portion of the intestine.[9,26]

The groups of microbes colonizing the intestinal mucosa (primarily the autochthonous microbiota) are different from those found in the intestinal contents (primarily allochthonous microbiota) and in the water.[27,28] These differences are likely attributable to specific properties of the microenvironment of the intestinal mucus, which provides certain resources for microbes to live and propagate.[29,30]

The major microbial groups are aerobic and facultative anaerobic bacteria, although many obligate anaerobes (e.g., *Cetobacterium somerae*) as well as various yeasts are also present.[7,9,23,28,29,31,32] The predominant bacterial phyla are Proteobacteria, Bacteroidetes and Firmicutes. Viruses, including many bacteriophages, also live in the fish gut.[31]

The cultivable bacterial populations in the intestinal content and mucus range between 10^6 to 10^9 colony forming units (CFU)/g, with the mucus population generally exhibiting lower diversity,[9,23,34,35] although the opposite has also been reported.[27] There are variations in the numbers of microbes colonizing the enterocytes; some enterocytes are colonized by virtually no bacteria at all.[36]

Similar to the skin microbiota, the fish gut microbiota also comprises many pathogenic, primarily opportunistic, species such as *Edwardsiella tarda*, *E. ictaluri*, *Aeromonas hydrophila* and *Vibrio alginolyticus*.[32,37]

155 *Factors affecting the fish gut microbiota*

156

157 Generally, the same factors that affect the fish skin and gill microbiotas also affect the
158 fish gut microbiota (Figure 1). In many cases, the exact underlying mechanism is not
159 fully understood.

160 The fish species strongly determines the composition of the gut microbiota.[38] There
161 are also differences in the predominant bacterial groups present in freshwater and
162 marine fish species. For example, *Aeromonas* spp. and *Pseudomonas* spp. are the most
163 common genera in many freshwater fish species, whereas *Vibrio* spp. appears to be the
164 most common genus in many marine fish species.[7,23]

165 The effects of the host genetic background on the composition of the microbiota are
166 not well-studied in fish. In humans and mice, certain host genes are able to alter gut
167 immunological profiles and consequently influence the composition of the gut
168 microbiota, including the predominant phyla Bacteroidetes and Firmicutes.[39] Smith et
169 al.[40] observed that populations of threespine stickleback (*Gasterosteus aculeatus*) with
170 greater genetic heterozygosity tended to exhibit lower inter-individual microbial
171 variation. This tendency may be associated with increased immunogenetic diversity
172 among individuals in these populations, which reduces microbial diversity. This
173 conclusion, if confirmed, may have serious implications for the selection of fish genetic
174 profiles for use in experiments.

175 Depending on the utilized approach, there have been different reports of the effects
176 of gender on the fish gut microbiota. Employing primarily culture-based methods,
177 Cantas et al.[41] did not observe significant differences in the gut microbiota between
178 male and female zebrafish (*Danio rerio*). However, Bolnick et al.[42] observed significant
179 differences in the gut microbiota between males and females in natural populations of
180 stickleback (*Gasterosteus aculeatus*) and Eurasian perch (*Perca fluviatilis*) using 16S
181 rRNA gene amplification. Additionally, different diets provoked sex-dependent changes
182 in the gut microbiota.

183 As fish progress through different developmental stages, their gut microbiota also
184 changes, often due to changes in the diet.[37,43,44] Moreover, the gut microbiota changes
185 between juveniles and sexually mature fish, potentially due to increasing levels of
186 hormones.[41]

187 According to many studies, environmental factors, such as water quality, available
188 nutrients, and potentially pollution, significantly influence the fish gut microbiota, both
189 in wild and farmed fish.[25,45,46] Roeselers et al.[32] observed a constant, core gut microbiota
190 in zebrafish maintained under diverse conditions in different laboratory facilities; these
191 results are similar to those obtained for fish recently collected from their natural
192 habitats.

193 Even the farming system affects the fish gut microbiota. Using molecular biology
194 methods, Giatsis et al.[47] examined the effects of recirculation and active suspension
195 tanks on the development of the gut microbiota in Nile tilapia (*Oreochromis niloticus)*

196 larvae after the first feeding. Although there were no differences in larval growth, feed
197 conversion and survival between the two systems, significant differences in the gut
198 microbial populations were observed 7 days after the first feeding. Differences in the
199 water microbial populations were also observed, but it was not clear whether these
200 differences were associated with the differences in the gut microbiota of the fish.

201 Diet appears to be the most significant factor directly affecting the gut microbiota.
202 Different dietary ingredients, different types of feeds (e.g., live feeds or pelleted) and
203 different feed additives (e.g., vitamins or probiotics) exert dramatic effects on the
204 microbial community of the fish gastrointestinal tract.[4] These factors favor the growth
205 of certain groups of microbes, which in turn may affect colonization by potential
206 pathogens.

207 Significant changes in the gut microbiota occur within a few days or weeks
208 following a change in diet, depending on the diet and potentially the age of the
209 fish.[27,48,49] Starvation also induces changes in fish gut microbial populations within
210 days.[50] In the latter situation, bacterial groups that utilize more diverse energy sources,
211 such as Bacteroidetes, tend to increase. In different fish species, different diets appear to
212 differentially influence the autochthonous and allochthonous microbiotas,[51-53] a
213 phenomenon that should be examined in every fish species.

214 Stress may influence the fish gut microbiota, primarily due to resulting alterations in
215 the intestinal mucus. In particular, after an acute stress such as netting, there is increased
216 sloughing off of the mucus, resulting in excessive removal of the autochthonous

bacteria, many of which play a significant protective role against potential pathogens.[54] These changes, combined with structural changes (e.g., increased transepithelial permeability) that occur in the intestine during stress, increase the risks of colonization and invasion by potential pathogens. [54]

In mice, circadian rhythms, particularly when combined with a high-fat and high-sugar diet, affect the gut microbiota.[54] This phenomenon has not yet been studied in fish, but such effects cannot be excluded and may have important implications because varying photoperiods are used in different facilities and in different experiments.

Effects of the fish gut microbiota on the host

In fish, the significance of the gut microbiota for host digestion depends on the host trophic level. Herbivorous fish rely on the microbial digestion of certain plant materials, particularly cellulose, whereas carnivorous fish appear to be less dependent on gut microbial metabolism.[56,57]

The gut microbiota plays a protective role against many potential pathogens, primarily by inhibiting pathogen colonization and/or by producing antimicrobial substances.[31,58] Many lactic acid bacteria, such as *Carnobacterium divergens* and *Lactobacillus delbrueckii* ssp. *lactis*, which are members of the indigenous gut microbiota of many fish, are known to have roles against pathogens such as *Aeromonas*

237 *salmonicida* and *Vibrio anguillarum*.[59] Their populations, and thus their actions, may be
238 affected by factors such as nutrition, stress and salinity.[58]

239 Many fish intestinal bacteria synthesize important substances that are used by the
240 host. For instance, *Cetobacterium somerae*, a member of the autochthonous gut
241 microbiota of many fish species including carp and tilapia, produces vitamin B_{12}.[60]
242 These fish species consequently have either low or no requirements for dietary
243 supplementation of this vitamin.[61]

244 Studies employing germ-free zebrafish have demonstrated the positive effects of the
245 gut microbiota on the renewal and differentiation of the intestinal epithelium as well as
246 the expression of fish genes involved in the immune and oxidative stress responses, thus
247 increasing stress tolerance.[62,63] In addition, studies investigating various probiotics have
248 revealed the influence of the gut microbiota on the number of goblet cells, the height of
249 the intestinal villi, the densities of T-cells and acidophilic granulocytes in the intestinal
250 mucosa, serum lysozyme and complement levels, and bactericidal activity.[64-67]

251 In mice, the gut microbiota also influences intestinal motility, which likely occurs
252 through stimulation of the enteric nervous system.[68,69] Furthermore, communication
253 between the gut microbiota and the host brain has also been demonstrated in
254 mammals.[69] The microbiota affects host behavior through vagal afferents, whereas the
255 host affects the content and function of the microbiota through neurotransmitters that
256 bind to specific receptors on microbes. In fish, this research is still in its infancy, but

257 recent studies have already suggested the influence of the gut microbiota on behavior
258 and stress responses.[70]

259 According to Mouchet et al.,[71] functional diversity in the gut microbiota (assessed in
260 terms of the carbon sources used) among individuals of the same population is not
261 related to the genetic diversity of the gut microbiota but is instead affected by the fish
262 species and diet. Thus, although various factors may affect the composition of the gut
263 microbiota in individual fish, an entire fish population living in a specific aquatic
264 environment sustains a certain degradation capacity, which stabilizes, to some extent,
265 this specific environment.

266

267 **Standardization of fish microbiotas: issues and challenges**

268

269 Four key issues are important when considering the standardization of research fish
270 microbiotas (Figure 2): a) the establishment of fish lines with a uniform genetic profile,
271 b) the establishment of isobiotic fish lines, c) the establishment of standardized rearing
272 conditions according to the preferences of each species, and d) appropriate monitoring
273 and adequate reporting of the microbiological status of research fish.

274

275 *Establishment of a uniform genetic profile*

276

In humans, monozygotic twins exhibit significant similarities in terms of their microbial populations.[72] Host genetics affect the microbiota through inherited factors such as different immune system components and mucus composition.[39] These types of interactions are also present in fish. For example, a study by Boutin et al.[17] revealed three quantitative trait loci (QTL) in brook charr associated with *Lysobacter*, *Rheinheimera* and *Methylobacterium* counts on the skin. These bacteria may influence the numbers of certain opportunistic pathogens found on fish skin.

The extensive use of isogenic and isobiotic rodent strains for research has resulted in a rapid increase in our knowledge of many areas of human and animal physiology. The use of such strains provides increased power, facilitates the characterization of more accurate dose-response relationships and results in fewer false-negative results compared to the use of outbred animals.[73] Regarding the gut microbiota, variations between inbred mice are significantly lower than those between outbred mice.[74]

In fish, current experience indicates different isogenic lines exhibit significantly different characteristics and behaviors.[75] Thus, the selection of an appropriate line for study is of great importance and should be taken into account in any experimental design. According to Bongers et al.,[76] if inbred fish are used in studies, the best approach is to utilize a number of inbred fish strains to extrapolate the experimental results to a larger outbred population. Further research is required to examine the interactions between defined microbiotas and host physiology in different fish lines, as well as the stability of the microbiota over time.

298 The production of isogenic lines involves many technical issues, and for some fish
299 species of low commercial value, this may not be practical. However, their use will
300 ultimately promote reproducibility and contribute to a reduction in the number of fish
301 used in experiments, as emphasized by Grimholt et al.[74]
302
303 *Establishment of isobiotic fish lines*
304
305 Ideally, fish used in any type of study should have a fully characterized or defined
306 microbiota. Such animals are designated 'gnotobiotic', and the term also includes germ-
307 free (or axenic) animals. These animals are generally derived from germ-free animals,
308 which are later colonized with a pre-defined microbiota. Animals that are colonized
309 with microbiotas collected from conventionally raised donors are also referred to as
310 conventionalized animals.[77] Once produced, the isobiotic animals transfer their
311 microbiotas to their offspring, as demonstrated by Becker et al. in rats.[78] The biggest
312 advantage of using gnotobiotic animals is the increased control over many variables that
313 affect the development of the microbiota and, in particular, autochthonous bacteria.
314 However, the process has some disadvantages that are primarily related to the
315 complexity of various procedures and the maintenance of gnotobiotic status.[79]
316 Gnotobiotic fish, such as zebrafish, have already been produced and utilized in
317 several studies investigating the gut microbiota.[77,79] The timing required for
318 colonization is important and should be established for each fish species. Artificial

colonization should occur when natural colonization would occur so that the development of the gastrointestinal tract is not disturbed. For example, Pham et al.[77] determined that the optimal time for zebrafish colonization is 3 days post-fertilization because this is the time when conventionally reared fish hatch from their chorions and are colonized by their microbiota. However, thus far, no protocols to standardize or manipulate the fish skin microbiota have been developed; theoretically, the same approach is applicable.

The maintenance of defined microbiotas is an important issue and is strongly related to rearing conditions and fish diets. In addition, the microbiota may change over time due to mutations and/or the exchange of genetic information between microbes. Thus, recolonization through feed or water may be required, likely in combination with antibiotic treatment.[80,81] All of these issues must be examined in different fish species.

Treatment with various antimicrobial agents, such as formalin, is frequently proposed as a standard to reduce the risk of introducing pathogens or even to control the fish microbiota upon the arrival of new animals in a research facility. However, such approaches cause alterations in many fish tissues, induce stress and even increase mortality post-treatment, as demonstrated in challenge studies.[82] Thus, these methods should only be used when necessary and when their influence on both the welfare of the fish and the validity of the results has been assessed.

Standardized rearing conditions

340

341 Research facilities that maintain fish possess controlled environments involving either
342 flow-through or re-circulating systems for the water supply. The majority of these
343 facilities rear their own fish stocks, but they also often must use fish obtained from
344 external sources, such as commercial farms or commercial breeders. In the latter case,
345 the fish remain in quarantine for a certain period of time, during which they may be
346 treated for common pathogens. Ultimately, due to the different practices of different
347 facilities, varying water quality parameters (although these are generally maintained
348 within a preferable range for each species) and different diets, the microbiological status
349 of research fish varies or is unknown.

350 The issue of environmental standardization between different research animal
351 facilities is still controversial. Van der Staay et al.[83] discussed the use of standardized
352 versus heterogeneous environmental conditions in animal experimentation and
353 concluded that the latter fails to detect subtle differences, and thus, the former is
354 preferred, particularly for principle studies. However, the generalizability of results
355 must be confirmed in subsequent 'extended replication' studies, in which various
356 known factors are examined. Using behavior measurements in a multi-laboratory study,
357 Richter et al.[84] observed an increased rate of 'false-positive' results when employing
358 standardized replication. Thus, environmental standardization should be replaced by
359 systematic and controlled environmental heterogenization. However, the conclusions of
360 Van der Staay et al. and Richter et al. differ because they emphasize the significance of

a careful experimental design and the consideration and examination of all contributing factors before any solid conclusions are drawn. Nonetheless, certain rearing variables, such as a common diet for each fish species and the use of re-circulated and treated water, may significantly minimize intra-species variations in the normal microbiota of fish.

Monitoring and reporting fish microbiota

The use of specific-pathogen-free (SPF) animals and the maintenance of an SPF environment are the most important aspects of any fish health monitoring program implemented in a research facility. Additional factors, such as the selection of appropriate groups of target microbes, the test methods employed, the number of representative animals selected for testing and the cost, are also critical for the success of such a program.[85] Johansen et al.[86] provided an overview of the general principles of a health monitoring program for fish research facilities. However, there are additional considerations when monitoring and reporting the normal microbiota in fish to enhance the reproducibility of experiments, and necessary adjustments should also be made based on the fish species.

The importance of standardizing, monitoring and reporting the microbiota of research animals has been previously addressed by Eberl.[87] This author collected opinions from many specialists in this area to answer relevant questions. All specialists

recognizing the role of the microbiota in the host physiology agreed on the importance of reporting the microbiota in all studies, particularly when there is strong evidence of its influence. Two of the initial questions addressed by Eberl were a) which microbes should be monitored, particularly in terms of the level of phylogenetic detail, and b) how often should monitoring occur. In fish, the answers to both questions depend on the fish species (e.g., the trophic level), how isolated and constant the environment of the facility is and the type of study. For instance, if the facility uses re-circulation and water treatment (e.g., UV radiation or ozonation) and a standardized feed containing known microbial content, one assumes that the skin, gill and gut microbiotas will remain relatively constant if the genetic profile of the fish and overall management are also standardized. In particular, nutritional studies should always include a description of the gut microbiota for all treatments (including both aerobic and anaerobic bacteria as well as fungi) at the beginning and at the end of the experimental period, at minimum. Although a detailed description of the fish microbiota may not be practical in terms of cost, the list of target microbes should at least include all of the major groups of microbes that play important roles in digestion, depending on the fish species and the nature of the experiment. Similarly, experimental infections should include groups of microbes with known protective and/or immunostimulatory properties.

When long-term experiments are conducted, the effects of different developmental stages and fish ages on the microbiota should also be examined, and thus appropriate sampling points should be included. According to Giatsis et al.,[47] there are no

403 significant differences in the gut microbiotas of individual fish living in the same tank
404 (particularly if the fish are of the same genetic background), nor are there differences
405 between fish living in replicate tanks and fish maintained under the same conditions.
406 Although these observations should be confirmed under different conditions and a
407 standardized sampling protocol should be developed, only a relatively small sample size
408 appears to be required to determine the microbial status of a homogenous group of fish.

409 Another important issue is the methods employed to examine and standardize the
410 microbiota of research animals. Every test has limitations, and thus, a combination of
411 tests should be used to give a more accurate picture of the microbial populations
412 present.[4,86,87] Recent advances in the use of culturomics to study the human gut
413 microbiota indicate better results are obtained with a combination of culture-based and
414 culture-independent methods, particularly in the case of low-abundance microorganisms
415 that certain molecular methods fail to detect.[88,89]

416 The cost of adequately monitoring the microbiota of research fish may still be high
417 for some facilities, particularly if regular sampling is required. However, this cost is
418 affected by the level of standardization of the microbiota and may be balanced by the
419 reduced numbers of animals required for experiments and the increased reproducibility.

420

421 **Conclusions**

422

Recently, there has been increased focus on the validity and reproducibility of published studies, particularly those involving animals. Apart from scientific and legal reasons, there is an ethical obligation to ensure that a minimum number of animals are used in various experiments to obtain reliable results.

One of the most fundamental factors affecting reproducibility, and consequently the validity of any experiment, is the standardization of experimental conditions. In fish experiments, the fish microbiota is rarely included when describing the status of the animals used, although the ability of the fish microbiota to significantly affect the host, resulting in significant inter- and, more importantly, intra-species variations, is well known. As knowledge of the roles of the skin, gill and gut microbiotas increases, the significance of standardization becomes more apparent.

This review highlights the most important issues and challenges associated with the standardization of normal fish microbiotas and their importance in fish experimentation. Fish constitute a highly diverse group of animals, and each species exhibits different tolerances and responses to various factors. The studies used as examples in this review included only certain species, and thus, further investigation is required before the research community decides which factors affecting the microbiota of each species are important for standardization. Nevertheless, the fish microbiota is an important experimental variable and should be monitored and reported in all studies in which it is likely to have an influence.

Declaration of conflicting interests

The author declares that there are no competing interests.

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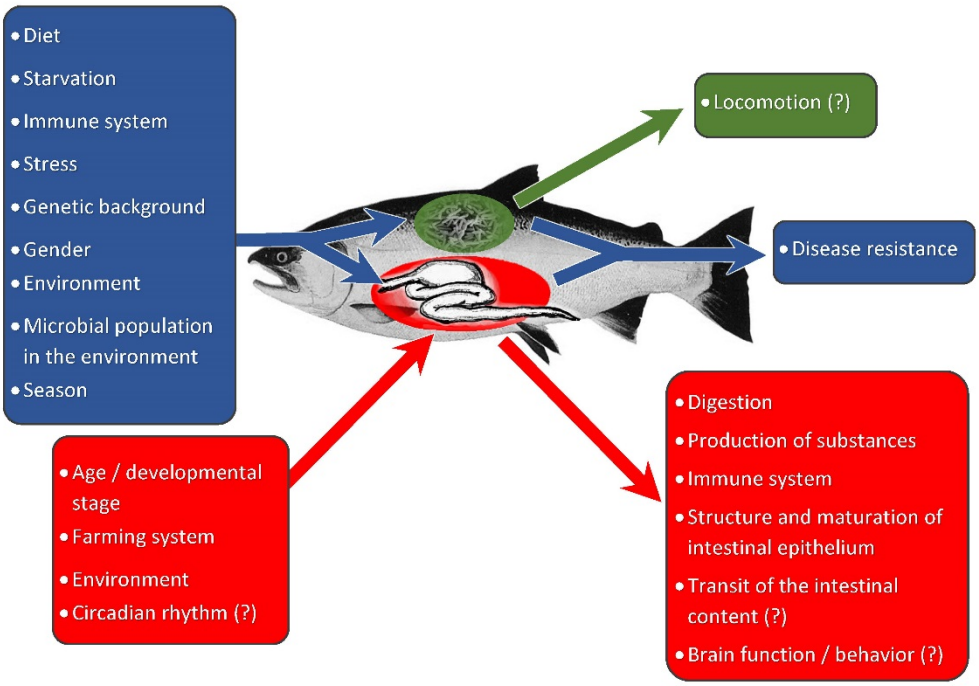

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720 **Figure 1.** Fish skin and gut microbiotas: influencing factors and effects. The blue boxes
721 correspond to both the skin and gut microbiotas, red boxes correspond only to the gut
722 microbiota, and the green box corresponds to the skin microbiota.

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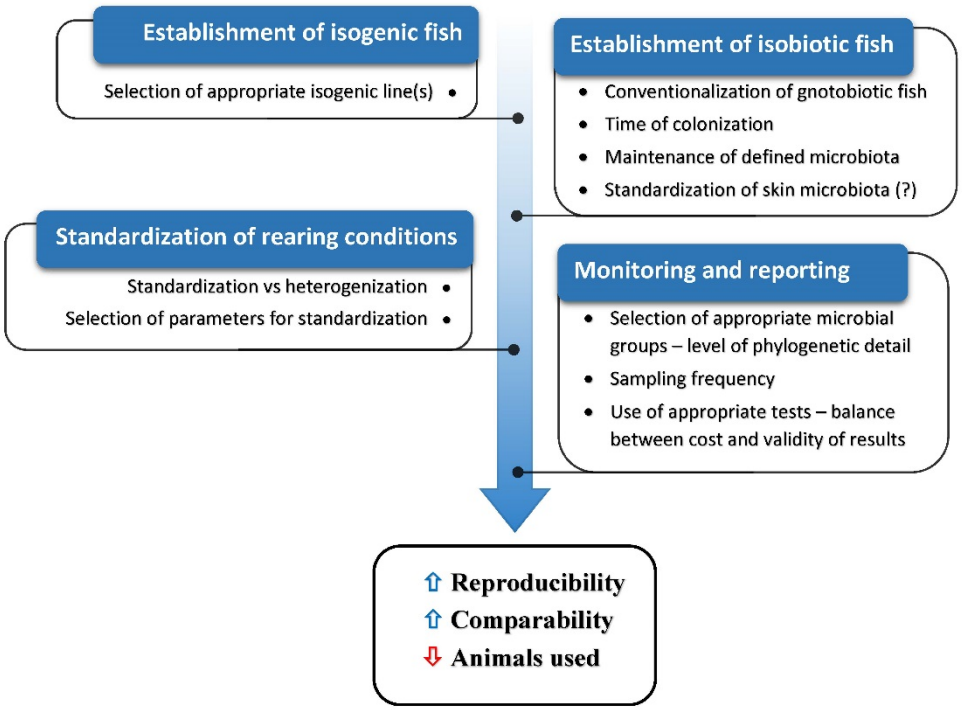

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728 **Figure 2.** Standardization of the fish microbiota: issues and challenges.

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