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Par

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Évaluation de l'efficacité d'alternatives alimentaires fonctionnelles pour lutter contre l'infection à *Aeromonas*, principalement *Aeromonas salmonicida* subsp. *salmonicida*: Vers la réduction de l'utilisation d'antibiotiques pour diminuer le développement et la diffusion de bactéries et de gènes résistants aux antibiotiques chez les poissons et l'environnement

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TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1

Bibliographic analysis:

Chapter 2

Article N° 2:

Chapter 3

Article N° 4:

LIST OF TABLES

Chapter 1

Bibliographic analysis:

Article N° 1:

Chapter 2

Article N° 2:

Chapter 3

Article N° 3:

***In vitro* studies (b):**

***In vivo* studies, Article N° 4:**

On-farm studies:

LIST OF ANNEXES

Chapter 3

On-farm studies:

LIST OF ABBREVIATIONS

AMR	antimicrobial resistance
ANSES	L'Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail
ANSM	Agence nationale de sécurité du médicament et des produits de santé
ARB	antibiotic-resistant bacteria
ARG	Antimicrobial Resistance Genes
ASS	*Aeromonas salmonicida* subsp. *salmonicida*
BMH	bouillon Mueller-Hinton
CFU	Colony-Forming Unit
CIPA	Comité Interprofessionnel des Produits de l'Aquaculture
CLSI	Clinical and Laboratory Standards Institute
CMI	Concentration Minimale Inhibitrice
C_T	Cycle Threshold
DNA	Deoxyribonucleic acid
ECOFF	Epidemiological Cut-OFF value
EFSA	European Food Safety Authority
ESBL	Extended Spectrum b-Lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization of the United Nations
HGT	horizontal gene transfer
MDR	MultiDrug-Resistance
MGE	Moblie Genetic Element
PCR	Polymerase Chain Reaction
pMAR	presumptive multi-resistant
NWT	non-wild-type population
RNA	Ribonucleic acid
WHO	World Health Organization
WT	wild-type population

FRENCH SUMMARY-RESUME FRANCAIS

La réduction des usages d'antibiotiques et la prévention des antibiorésistances, notamment en médecine vétérinaire, est un enjeu national et international. En France, en 2012, a été mis en place un premier plan ECOANTIBIO (2012-2017) qui a permis, sur les 4 premières années de ce plan, de diminuer de plus de 20% l'exposition des animaux aux antibiotiques. Ce 1er plan s'est poursuit par le plan ECOANTIBIO2 (2017-2021). Les besoins de recherche identifiés dans ce contexte comportent des questions relatives à la prévention sanitaire, zootechnique et médicale des maladies infectieuses et aux traitements alternatifs aux antibiotiques afin de induire un moindre recours aux antibiotiques en élevage (Ecoantibio 2, 2017; ANSES, 2018). En aquaculture, pour limiter l'utilisation d'antibiotiques chez les poissons d'élevage et leurs effets négatifs potentiels sur la santé publique et l'environnement, une évaluation des « alternatives fonctionnelles » est nécessaire.

En France, la filière piscicole a produit en 2018, 41000 t de poissons dont 27900 t de poissons d'eau douce avec une part majoritaire de truites Arc-en-Ciel (*O. mykiss*) (FranceAgriMer, 2019). Comme dans les autres élevages, les poissons sont exposés à de nombreux agents infectieux, notamment bactériens, nécessitant parfois de les traiter aux antibiotiques. L'élevage piscicole en circuit ouvert tel qu'il existe actuellement notamment en France est en interaction constante avec l'environnement. Dans ces élevages, les antibiotiques sont le plus souvent administrés via un aliment médicamenteux. Une partie des antibiotiques administrés aux poissons est excrétée dans les matières fécales et l'urine et rejetée dans la rivière. Cela peut entraîner une contamination de l'eau de surface et parfois de l'eau destinée à l'usage humain comme source d'eau potable et créer un risque potentiel de développement et de propagation de bactéries et de gènes résistants aux antibiotiques entre les poissons, leur environnement et l'homme (Lamy, 2012; Romero et al., 2012; Cabello et al., 2013). La présence

prolongée d'antibiotiques dans l'eau des étangs, associée à un nombre élevé de bactéries dans les matrices polybactériennes des biofilms, où le milieu aquatique peut également être contaminé par des agents pathogènes d'origine humaine et animale, pourrait entrainer une pression sélective sur l'échange d'informations génétiques entre bactéries terrestres et aquatiques et favoriser la survie de bactéries et de gènes résistants aux antibiotiques (Muziasari et al., 2017; Watts et al., 2017).

Les bactéries du genre *Aeromonas* sont des habitants communs des milieux aquatiques tels que les eaux douces, les eaux d'estuaires, les eaux marines et les sédiments. Ce sont des agents environnementaux pathogènes opportunistes des animaux et des humains. Dans l'environnement, *Aeromonas* peut persister attaché aux biofilms sur des surfaces biotiques ou abiotiques. Les biofilms naturels sont développés et se différencient pour construire une communauté bactérienne qui est souvent multi-espèces. Cette capacité à créer des biofilms mixtes polybactériens représente une niche et des réservoirs d*'Aeromonas* et d'autres bactéries telles qu'*E. coli* et promeut l'échange et la diffusion de gènes d'antibiorésistance (Talagrand-Reboul et al., 2017). Le genre *Aeromonas* est également remarquable par son profil de résistance aux antibiotiques dans les souches environnementales et cliniques (Talagrand-Reboul et al., 2017). Des études ont indiqué la présence d'*Aeromonas* dans la production aquacole avec des niveaux élevés de résistance aux antibiotiques et des déterminants génétiques de résistance (Jacobs and Chenia, 2007; Penders and Stobberingh, 2008). Des souches multirésistantes d'*Aeromonas* porteuses de plusieurs gènes comme *sul1, tetA et floR,* ont été détectées chez différentes espèces de poissons d'élevage (Patil et al., 2016; Duman et al., 2020). Certaines études évaluant la sensibilité aux antimicrobiens des espèces *Aeromonas* isolées de la truite arc-en-ciel d'élevage et de son environnement ont montré un profil de résistance aux quinolones et fluoroquinolones, à la streptomycine, à l'oxytétracycline, au chloramphénicol, au

florfénicol, au sulfaméthoxazole-triméthoprime et aux β-lactamines. Le support génétique de ces résistances acquises est transférable par des transposons / intégrons chromosomiques ou des plasmides porteurs de gènes associés à la résistance aux antibiotiques (Saavedra et al., 2004; Naviner et al., 2011 ; Vega-Sánchez et al., 2014).

Dans les élevages salmonicoles, la furonculose à *Aeromonas salmonicida* sub *salmonicida* est responsable de lourdes pertes économiques par septicémie hémorragique (forme aiguë) ou par dépréciation du poisson en raison du développement de furoncles dans les muscles (forme chronique). Même s'il existe des vaccins, ils restent peu utilisés du fait d'une efficacité controversée, des adhérences intra-abdominales observées après injection par voie intra-péritonéale, et de la difficulté de vacciner individuellement des jeunes animaux du fait de leur petite taille (Smith and Hiney, 2000; Plant and LaPatra, 2011; Rømer Villumsen et al., 2015). Le recours aux antibiotiques est actuellement le moyen le plus utilisé pour maîtriser cette maladie. Cependant, de nombreux plasmides porteurs de gènes de résistance aux antibiotiques (ARG) ont été décrits chez *Aeromonas salmonicida sub salmonicida* et l'aspect ubiquitaire du genre *Aeromonas* pourrait contribuer à la diffusion de ces ARG dans l'environnement (Piotrowska and Popowska, 2014; Vincent et al., 2014). Le développement et l'évaluation de produits « alternatifs » contre cette infection sont de ce fait un enjeu important pour limiter l'utilisation d'antibiotiques en truiticulture et leurs impacts négatifs potentiels sur la santé publique.

Actuellement, des méthodes de biocontrôle pour maîtriser des maladies infectieuses des poissons ont fait l'objet de publications et revues, montrant l'intérêt de ces pratiques alternatives en aquaculture. Les produits destinés aux poissons d'élevage décrits dans la bibliographie comme ayant des propriétés correspondant à une alternative aux antibiotiques sont principalement des prébiotiques (e.g. manno- et fructo- oligosaccharides, parois de

levures), des probiotiques (e.g. *Lactobacillus* sp, *Saccharomyces cerevisiae*, *Bacillus sp*.,…) ou des huiles essentielles (e.g. huiles essentielles de *Cinnamomum kanehira*, *Origanum heracleoticum*…)(Bidhan et al., 2014; Romero et al, 2012). Les effets antimicrobiens de ces « produits alternatifs » peuvent être des effets directs microbicides ou microbiostatiques et/ou des effets indirects via une modulation du système immunitaire inné des individus ou une modification du microbiote intestinal permettant une augmentation de la résistance des individus (Bidhan et al., 2014; Huynh et al., 2017 ;Lazado et al., 2015; Romero et al, 2012).

Cependant, moins d'une quinzaine de publications sont recensées concernant l'utilisation de produits alternatifs (probiotiques principalement) contre la furonculose chez la truite à notre connaissance. L'effet prophylactique de probiotiques a été évalué uniquement dans des conditions d'infection contrôlées. Les probiotiques testés permettent de contrôler la furonculose en diminuant la mortalité des truites infectées via la production de substances inhibitrices (sidérophore et chitinase) et un effet immunomodulateur (augmentation de l'activité du lysozyme, de l'activité du complément, de l'activité phagocytaire et/ou du métabolisme oxydatif des macrophages) (Balcázar et al., 2007 ; Brunt et al., 2007 ; Gao et al., 2017; Kim and Austin, 2006). Une seule publication relate l'effet protecteur d'un additif alimentaire contenant des huiles essentielles d'origan, d'anis et de citron (Menanteau-Ledouble et al., 2015) mais les modes d'action ne sont pas étudiés. Ces études sont ainsi très incomplètes (efficacité évaluée uniquement sur la mortalité, faible nombre de paramètres immunitaires étudiés, étude uniquement en condition d'infection contrôlée). Des bases scientifiques sont nécessaires afin que ces méthodes de biocontrôle soient adoptées par les professionnels (éleveurs et vétérinaires) de la filière piscicole : évaluation de leur efficacité et de leur innocuité en condition d'infection contrôlée mais aussi en condition d'exposition naturelle en situation d'élevage, modes d'action, impact de leur utilisation sur l'évolution de l'antibiorésistance de

bactéries ichtyopathogènes et de bactéries de l'environnement potentiellement pathogènes pour l'Homme.

Dans cette thèse deux objectifs principaux sont envisagés. Le 1er objectif est d'évaluer l'efficacité de produits alternatifs contre la furonculose à *Aeromonas salmonicida* subsp. *salmonicida* chez la truite Arc-en-ciel en conditions d'infection contrôlée et d'étudier si l'administration de l'additif améliore la protection induite par un vaccin contre la furonculose (auto-vaccin). L'efficacité de produits alternatifs contre la furonculose chez la truite Arc-en-ciel en conditions d'infection naturelle dans les élevages piscicoles sera également étudiée. Le 2ème objectif est d'étudier le risque de diffusion de bactéries antibiorésistantes et de gènes de résistance depuis les poissons vers leurs environnements.

Les travaux de recherche dans la thèse sont organisés en 3 chapitres principaux :

(i) Une étude bibliographique investiguant la production aquacole, l'application des antibiotiques en aquaculture et l'étude d'agents pathogènes courants des poissons d'eau douce, notamment les bactéries du genre *Aeromonas.* Ce chapitre inclut une analyse bibliographique sur les additifs fonctionnels pour contrôler des infections à *Aeromonas* chez les poissons d'eau douce sous la forme d'une revue scientifique (Article N° 1) (Hayatgheib et al., 2020a).

(ii) Un suivi mensuel durant 7 mois de l'antibiorésistance étudiée au niveau phénotypique et génétique des souches bactériennes d'*Aeromonas* issues de 2 élevages bretons et des écosystèmes aquatiques (eau, biofilm, poissons) sous la forme d'un article de recherche (Article N° 2).

(iii) Les études *in vitro* et *in vivo*, en condition contrôlée et en élevage de produits alternatifs fonctionnels contre la furonculose à *A. salmonicida* subsp.

salmonicida (ASS). Les études *in vitro* sur des produits alternatifs fonctionnels tels que diverses huiles essentielles commerciales et leurs constituants chimiques afin d'étudier leurs effets antimicrobiens contre ASS ont fait l'objet d'un article de recherche (Hayatgheib et al., 2020b) (Article N° 3), ainsi que l'étude *in vivo* dans des conditions expérimentales contrôlées (Article N° 4).

Afin de sélectionner le produit additif pour ce projet, une recherche bibliographique sous la forme d'une revue scientifique a été réalisée. Cette étude comprend les résultats d'études in vivo sur les principales espèces de poissons d'élevage en eau douce (salmonidés, cyprinidés et cichlidés), mettant l'accent sur l'efficacité des alternatives fonctionnelles contre *Aeromonas spp*. Elle décrit également les progrès récents de la lutte biologique et les traitements alternatifs potentiels en aquaculture. Des produits alternatifs fonctionnels peuvent augmenter la résistance contre *Aeromonas spp*., notamment en augmentant l'immunocompétence des poissons. De nombreux produits alternatifs tels que les probiotiques, les prébiotiques, les plantes, les huiles essentielles, les phages d'algues, les minéraux et les nanoparticules ont été testés, mais la diversité des protocoles expérimentaux rend difficile la comparaison de l'efficacité des produits testés. Cette revue suggère la standardisation des investigations sur les produits alimentaires fonctionnels pour chaque espèce de poisson contre un pathogène spécifique. Elle recommande également des recherches « sur le terrain » sur les alternatives fonctionnelles d'aliments dans des conditions naturelles d'exposition afin d'évaluer la diminution de la consommation d'antibiotiques dans les exploitations piscicoles (Hayatgheib et al., 2020a).

Ensuite, une étude visait à évaluer l'efficacité *in vitro* d'huiles essentielles (EO) et de leurs composés (EOC) seuls ou en association contre ASS, l'agent causal de la furonculose chez les salmonidés. L'activité antimicrobienne de 13 EO et 16 EOC a été étudiée pour quatre souches *A. salmonicida* subsp. *salmonicida* par la méthode de microdilution en bouillon (CLSI,

2006). Le test « checkerboard assay » a été utilisé pour évaluer une synergie potentielle entre les EO et les EOC les plus efficaces contre les souches testées. Les huiles d'écorce de cannelle, d'origan, de clou de girofle et de thym et leurs principaux composés, le cinnamaldéhyde, l'eugénol, le carvacrol et le thymol ont montré de fortes activités contre ASS avec des concentrations minimales inhibitrices et des concentrations minimales bactéricides les plus basses. L'association cinnamaldéhyde et eugénol (V/V : 30%/70%) a montré une activité synergique contre trois souches testées. Les associations de cannelle avec des EO d'origan, de clou de girofle ou de thym ont montré une activité neutre ou additive contre toutes les souches testées. Pour réduire l'utilisation d'antibiotiques en aquaculture, les composés phytochimiques tels que le cinnamaldéhyde et l'eugénol pourraient être testés seuls ou en combinaison dans des études *in vivo* en tant qu'alternatives fonctionnelles (Hayatgheib et al., 2020b).

Dans ce projet, la sélection d'un additif alimentaire commercial pour une étude *in vivo* et à la ferme afin de contrôler la furonculose chez la truite arc-en-ciel a donc été basée sur une étude bibliographique, une étude *in vitro*, ainsi que l'expérience pratique de différents producteurs d'additifs alimentaires, des vétérinaires aquacoles et des pisciculteurs, tout en considérant la réglementation française sur les additifs alimentaires à usage animal. En conséquence, nous avons testé l'efficacité *in vitro* de trois additifs alimentaires commerciaux contenant des composés phytochimiques et prébiotiques provenant de trois entreprises différentes d'aliments et notamment l'AQUABOOST® fabriqué par « Le Gouessant Aquaculture ». Les résultats ont révélé que tous les produits présentaient un effet antibactérien assez comparable contre quatre souches d'ASS. La concentration minimale inhibitrice (CMI) a été détectée à 0,5 µl ml^{-1} pour tous les produits et aucune différence significative n'a été trouvée entre les produits testés ($P \geq 0,05$). Nous avons constaté que cette valeur de CMI est également cohérente avec notre étude *in vitro* précédente sur les différents EO et EOC contre

ASS. Par conséquent, sur la base de tous nos résultats *in vitro* ainsi que de nos communications techniques et scientifiques externes, le produit AQUABOOST® composé d'huiles essentielles et de prébiotiques (PEA) a été choisi pour des études *in vivo* et en ferme.

Par la suite, une étude *in vivo,* dans des conditions contrôlées, a examiné l'effet du PEA sur les performances de croissance, la résistance aux maladies et les paramètres immunologiques chez la truite arc-en-ciel vaccinée et non vaccinée contre ASS. Huit groupes de poissons (+/- PEA, +/- vaccin et +/- ASS) ont été étudiés. Les mortalités ont été enregistrées quotidiennement tandis que des investigations cliniques et bactériologiques ont également été menées. Le poids corporel et les paramètres immunitaires comme l'activité du lysozyme, l'activité du complément hémolytique alternatif (ACH50) et le taux d'anticorps anti-ASS dans le sérum ont été mesurés. Avant l'inoculation de l'ASS, la mortalité était très faible (<3%) et aucune altération de l'état de santé des poissons n'a été détectée dans tous les groupes étudiés ($p > 0,05$), permettant de montrer la bonne innocuité du PEA et du vaccin. Une réponse immunitaire humorale a été induite 4 semaines après l'injection du vaccin mais aucune différence n'a été observée entre les poissons nourris avec ou sans PEA. Cependant, chez certains poissons, la vaccination n'induisait qu'une très faible production d'anticorps anti-ASS dans les deux groupes. Cependant, le nombre de ces poissons était plus faible dans le groupe nourri avec PEA que dans le groupe sans PEA. Après l'inoculation de l'ASS, il n'y avait pas de différences significatives de mortalité (12 à 28%) et de morbidité entre les groupes inoculés. L'inoculation d'ASS a induit une diminution de l'activité du lysozyme mais une augmentation de la production d'ACH50 et d'anticorps anti-ASS à la 3ème semaine après l'inoculation. Le nombre de poissons avec une augmentation importante du taux d'anticorps anti-ASS était plus élevé dans le groupe inoculé nourri sans PEA que nourri avec PEA. Cela suggère qu'il y a significativement moins de poissons infectés dans le groupe PEA que dans le groupe non PEA.

Malgré le faible effet immunostimulant du PEA utilisé, l'effet favorable du PEA sur la protection des poissons contre ASS et l'amélioration de la prise vaccinale ainsi que la performance de croissance des poissons après l'inoculation de l'ASS ont été observés. Le meilleur taux de survie (87,8%) était dans le groupe vacciné nourri avec PEA, alors qu'il n'y avait pas de différences significatives dans tous les groupes infectés. L'effet immunostimulant du PEA utilisé dans cette étude et son rôle sur la résistance à la furonculose sont discutés dans l'article N° 4.

Le produit AQUABOOST® a été également étudié dans des conditions naturelles, en ferme piscicole. Dans deux fermes d'élevage, deux bassins dans chaque pisciculture ont été dédiés pour examiner l'effet du PEA sur les performances de croissance et la résistance aux maladies chez la truite arc-en-ciel. Les truites arc-en-ciel étaient nourries avec le PEA (bassin d'essai) ou l'aliment de base (bassin de contrôle). Pendant l'infection naturelle à ASS dans une des deux fermes, les résultats n'ont pas montré de différences significatives pour le taux de mortalité ou la prise de poids entre les deux bassins études dans la ferme concernée ($P \geq 0,05$). L'autre ferme n'a pas rencontré de cas de furonculose. Fait intéressant, la vaccination ASS a été appliquée avant de commencer l'étude. De plus, cette dernière ferme était plus petite (production annuelle plus faible) et était située dans une zone isolée. De plus, une autre souche de truite arc-en-ciel a été élevée par rapport à la forme antérieure. En ce qui concerne les performances zootechniques, ce produit n'a pas non plus amélioré les performances de croissance de la truite arc-en-ciel au cours de l'étude dans les deux fermes étudiées.

Sachant qu'*Aeromonas* est une bactérie omniprésente dans les environnements aquatiques et bien connue pour ses profils de résistance aux antimicrobiens, une étude a été faite afin de souligner la transmission des gènes de résistance aux antibiotiques (ARG) chez *Aeromonas* dans les écosystèmes aquatiques. Pour cette étude, les deux mêmes fermes

commerciales de truites arc-en-ciel indiquées précédemment ont été considérées. Cette étude a présenté l'occurrence et l'abondance des bactéries *Aeromonas* résistantes aux antibiotiques (ARB) et leurs gènes de résistances (ARG) isolés de l'eau, du biofilm et des poissons dans deux fermes de truites avant et une semaine après un traitement à la fluméquine. Les souches sauvages (WT) plutôt non-sensibles et non sauvages (NWT) plutôt sensibles ont été déterminées pour les quinolones (flumequine, acide oxolinique et enrofloxacine), l'oxytétracycline (OXY), le florfénicol (FFN), le triméthoprime-sulfaméthoxazole (TMP) et la colistine (COL) ; les souches multi-résistantes ont été classées. Quarante-quatre ARG pour les antibiotiques mentionnés, les bêta-lactamines et la multi-résistance ont été quantifiés pour 211 isolats. *BlaSHV-01, mexF* et *tetE* étaient les ARG dominants. Une occurrence et une abondance plus importantes de *tetA2, sul3, floR1, blaSHV-01* et *mexF* ont été observées dans les NWT par rapport à WT. L'apparition des souches multi-résistantes d'*Aeromonas* et des NWT pour les quinolones, l'OXY, le FFN, le TMP et le COL et les ARG dépendait de l'origine d'*Aeromonas*, de l'utilisation d'antibiotiques et de la présence d'activités en amont. Nos résultats ont révélé l'impact d'un traitement à la flumequine sur les *Aeromonas* présentes dans une ferme piscicole à travers une augmentation des souches NWT et des souches multi-résistantes. Le lien entre les poissons et leur environnement a été démontré par la détection d'ARB et d'ARG identiques dans les deux types d'échantillons. Il semblait y avoir un risque élevé de développement et de propagation de gènes de résistance dans les milieux aquatiques.

Cette étude a démontré que les fermes d'aquaculture peuvent être considérées comme un énorme réservoir environnemental de bactéries multi-résistantes et d'ARG. De plus, un traitement antibiotique peut avoir un impact sur l'évolution de bactéries résistantes contenant des gènes de résistance aux antibiotiques. Cependant, certains *Aeromonas* sont des agents pathogènes bactériens bien connus en aquaculture, mais ce sont principalement des bactéries

omniprésentes qui se trouvent facilement dans le milieu aquatique. Ces résultats suggèrent que le risque de transmissions des ARGs doit être évalué en lien avec la santé humaine associé aux fermes piscicoles. Les recherches futures devraient se concentrer sur la quantification des autres éléments génétiques mobiles des isolats d'*Aeromonas* ainsi que sur les ARGs chromosomiques et plasmidiques associés aux antibiotiques qui sont principalement prescrits en aquaculture et également en médecine humaine. Finalement, les pratiques d'aquaculture durable qui investissent dans de nouvelles approches pour réduire la propagation de la résistance aux antibiotiques doivent être stabilisées.

L'ensemble des résultats issus de nos études *in vitro*, *in vivo* et à la ferme sont des informations pour rappeler jusqu'où nous devons encore aller dans le développement d'un aliment fonctionnel alternatif efficace et pratique pour réduire les traitements antibiotiques dus aux maladies en aquaculture, en particulier la furonculose, ainsi que une meilleure compréhension de l'effet du traitement antibiotique sur la transmission généralisée de bactéries et de gènes environnementaux résistants à *Aeromonas* dans les écosystèmes aquatiques liés à l'homme et à diverses espèces animales.

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Introduction

Antimicrobial resistance is a major public health issue concerning both human and veterinary medicine at the global level. A survey of the use of antimicrobials in medicine accounted for almost 50% of the global consumption of antibiotics in human medicine and veterinary medicine represents approximately the other half prescription/consumption (ANSM 2017; Nathwani 2017; Coulter et al. 2017). The link between antibiotic exposure and selection of antibiotic resistance in bacterial species has been demonstrated and characterized for many bacterial resistance associations in human medicine (Monnet et al. 1997). These evolutions towards greater resistance could lead to restrictions in the choice of effective antibiotics in human medicine and lead to therapeutic failures in some cases with a current estimate of 700,000 antimicrobial resistance (AMR) deaths attributed annually in the world and estimated to rise to 10 million deaths per year in 2050 (O'Neill, 2016). In parallel, reducing the use of antibiotics and preventing antibiotic resistance, particularly in veterinary medicine, is a national and international issue. In France, Ecoantibio plan aimed to reduce the exposure of animals to antibiotics by more than 25% in five years which encourages the continuation of these activities with an Ecoantibio 2 plan (2017-2021) (EcoAntibio 2017, 2017).

Aquatic animal species are in constant interaction with potential pathogens that can strongly impair growth performance and result in significant economic losses for livestock. Aquaculture has become an economic and safe source of protein for human consumption around the world (Haguenoer 2010). In intensive aquaculture, under conditions of stress, farmed fish can be affected by various bacterial diseases, which may lead to a decrease of fish resistance and growth performance. Therefore, antibiotic prescriptions may be needed to control infectious diseases and avoid significant economic losses due to the bacterial diseases (Romero et al. 2012). However, the use of antibiotics in fish farming can be related to the

public health hazards including, on one hand the development and spread of antimicrobial resistance bacteria and resistance genes, and on the other hand the presence of antimicrobial residues both in the environment and in farmed fish products (Romero et al. 2012; Caruso 2016). Therefore, control measures in aquaculture sectors are more needed in fish farms to reduce the antibiotic use against fish diseases.

Antibiotics can be effective in treating the bacterial infection if the bacteria are not resistant to the prescribed antibiotic but a notable outcome of the antibiotic use can be the further antibiotic resistance in environmental and clinical strains (Heuer et al. 2009). Administration of antibiotics with inadequate dose and duration of treatment may lead to the development of antibiotic-resistant bacteria because the selective pressure effected on them. The use of large amounts of mixed antibiotic with fish feed in aquaculture resulted to exposure of drug residues and their metabolites coming from urine and faeces and thus discharging into the wastewater. This may lead to contamination of the surface water, groundwater and sometimes water intended for human consumption. For example, the concentrations of antibiotic, such as oxytetracycline and sulfadimethoxine, in water can reach values ranging from ng to µg /l due to the presence of intensive fish farms in nearby river (Thurman et al., 2002). However, the use of antibiotics in aquaculture depends on local regulations. Specifically, Europe, North America, and Japan, regulations on the use of antibiotics are stricter and only a few antibiotics are licensed for use in aquaculture. In Europe, for example, the approach of a non-curative prophylactic use of antibiotics was banned in 2001 by the EU Veterinary Medicinal Products Directive, as amended and codified in Directive 2001/82/EC (Watts et al. 2017). Besides, an increase in resistance to beta-lactam antimicrobials such as penicillins and derivatives, cephalosporines, carbapenems, and monobactams by the presence of bacteria genes that code for the production of the extended-spectrum beta-lactamases (ESBL) is mostly reported in developing countries due to their slack regulations on antibiotic use. This causes the

spread of AMR genes such as ESBL, resulting in antibiotic-resistant infections for humans, fish and other aquatic animal (Haguenoer 2010; Alassan et al. 2017; Watts et al., 2017).

Most bacterial diseases in fish take a systemic form and are the subject of mainly metaphylaxis, preventive and curative approaches. Vaccination has become established as a preventive method against various bacterial pathogens in aquaculture in recent years. However, vaccination has been observed with difficulty of application and also its controversial effectiveness in fish (Plant and LaPatra, 2011). Major disadvantages of vaccination are handling of certain minimum weight fish through a stressful vaccine injection or immersion vaccination that has shorter duration of immunity compared to injection. Even though, oral vaccination as the most convenient way has not been proved very effective, as antigens are often destroyed in the digestive system before they reach the immune sensitive areas of the lower gut (Gudmundsdóttir and Björnsdóttir, 2007).

Biocontrol methods to control diseases through application of some promising novel biocontrol methods, such as probiotics, prebiotics, plants, essential oils, algae phages, minerals and nanoparticles, have been paid attention in aquaculture in recent years. These alternatives practices aimed to avoid the disadvantages of traditional one that potentially affects fish, human and environment (Romero et al., 2012; Bidhan et al. 2014; Lazado et al. 2015; Huynh et al. 2017). The development of the alternative treatments and evaluation of their benefits to limit antimitotic use are also recommended by in French agency for food, environmental and occupational health & safety (ANSES). Aquaculture sectors, especially fish farming in open circuit, is in constant interaction with the environment as it exists currently and particularly in France. In these farms, the contamination of the surrounding environment by these drugs are more likely to occur. In consequence, the impacts of antibiotic use on public health through the development and spread of antibiotic-resistant bacteria and resistance genes and also by the presence of antibiotic residues in aquatic food products have to be considered (Romero et al

2012). Since, the development and evaluation of the efficacy for alternative treatments to substitute or partially replace antibiotics is necessary.

Over the past two decades, many studies have evaluated the efficacy of functional alternatives against fish diseases. The most common alternative products for farmed fish are mainly probiotics (e.g. *Lactobacillus spp*., *Saccharomyces cerevisiae*, *Bacillus spp*., ...), prebiotics (e.g. manno- and fructo-oligosaccharides, yeast walls) or essential oils (e.g. *Cinnamomum kanehira Origanum heracleoticum*...) (Romero et al., 2012; Bidhan et al. 2014). The antimicrobial effects of these "alternative products" may be direct (microbicide or microbiostatic action) and / or indirect via modulation of the innate immune system of individuals or modification of the gut microbiota, allowing an increase in the resistance of individuals (Romero et al., 2012; Bidhan et al. 2014; Lazado et al. 2015; Huynh et al. 2017). Particularly, several studies have investigated the efficacy of functional alternatives against *Aeromonas* spp showing the interest of applying alternative practices in aquaculture. Although the low number of end-use and commercial preparations as an alternative for aquaculture species limit their application in fish farms.

Although to our knowledge, only a dozen *in vivo* studies have evaluated the use of alternative products (mainly probiotics) against *A. salmonicida* subsp. *salmonicida* a causal agent of furunculosis in rainbow (*Oncorhynchus mykiss*). However, the prophylactic effect of probiotics was evaluated only under controlled infection conditions. The probiotics tested showed that they can control furunculosis by reducing the mortality of infected trout via the production of inhibitory substances (siderophore and chitinase) and an immunomodulatory effect (increase of lysozyme activity, complement activity, activity phagocytic and / or oxidative metabolism of macrophages) (Kim and Austin 2006; Balcázar et al. 2007; Brunt et al. 2007; Gao et al. 2017). Only two publications reported the protective effect of a feed additive containing essential oil and/or organic acid (Menanteau-Ledouble et al., 2015; 2017) and two

publications also studied therapeutic phages (Imbeault et al. 2006; Kim et al. 2015) against *A. salmonicida* subsp. *salmonicida* but the modes of action are not studied. Most of these alternatives were effective in a preventive approach against mortality due to their immunostimulation mechanism and/or modifications of the gut microbiota as well as of the intestinal structure to protect gut tissue from bacteria; however, these mechanisms of action have not been thoroughly and comprehensively discussed. Furthermore, the efficacy of alternative products has not been studied in fish farms under natural conditions, where *A. salmonicida* subsp. *salmonicida* could be influenced by environmental bacterial flora, environmental temperature as well as quality of water. Since, scientific bases are necessary for an alternative biocontrol method to be adopted by professionals (breeders and veterinarians) in the fish farming sector to allow less use of antibiotics in aquaculture: their effectiveness and their safety in conditions of controlled infection but also in natural exposure in rearing conditions need to be evaluated. Furthermore, robust clinical trial designs are necessary and must include analysis of the mode of action of an alternative product, impact of its use on the evolution of antimicrobial resistance bacteria in aquaculture and understanding of the widespread transmission of antibiotic-resistant genes in aquatic ecosystems.

Objectives of the thesis

Antibiotics are regularly used in animal husbandry, especially in aquaculture against diseases, increase productivity or prevent contamination of the food chain but almost participate to the development of resistant microbial strains in both animals and humans which conventional antibiotics no longer have any effect. In aquaculture, antibiotics are commonly distributed in the water of the basins, thus entering directly into the aquatic compartment of the environment. This thesis focused on priority and strategic scientific knowledge to reduce the use of antibiotics in fish farming and limit the associated risks by evaluating the potential

benefit of the use of an alternative product in terms of animal health but also to explore the risk of development and diffusion of antibiotic-resistant bacteria from fish to its environment (water, sediment, biofilm).

In this thesis two main objectives are considered. The 1st objective is to evaluate the effectiveness of alternative products against furunculosis caused by *Aeromonas salmonicida* subsp. *salmonicida* in rainbow trout under controlled infection conditions and to study whether administration of the additive improves the protection induced by a vaccine against furunculosis (auto-vaccine). The efficacy of alternative products against furunculosis in rainbow trout under conditions of natural infection in fish farms will also be studied. The second objective is to study the risk of the spread of antibiotic resistant bacteria and resistance genes from fish to their environments.

The first thesis objective is to study the functional alternatives efficacy to reduce the antibiotic use in rainbow fish farm that were infected with commonly known *A. salmonicida* subsp. *salmonicida* a causal agent of furunculosis. In order to achieve this aim, firstly, the alternative substances have to be defined among various products. Therefore, a bibliographic analysis has done on this subject. This work is aimed to explore the possible substances that can be used as partial replacement of antibiotics in curative and/or preventive approaches to reduce the frequency of occurrence of some fish bacterial diseases, which may lead to a lower use of antibiotics. In this reach, the alternative products (probiotics, prebiotics, plants, essential oils, algae phages, minerals, nanoparticles, ...) and their methods of administration (prophylactic, metaphylactic and/or therapeutic, administration dose, duration of administration, ...) have chosen after a bibliographical study, *in vitro* studies and integration of current knowledge concerning "alternatives" usable in aquaculture farms. *In vitro* antibacterial activity of the chosen alternative product has tested against four *Aeromonas salmonicida* subsp. *salmonicida* clinical and environmental strains by using broth micro dilution.

In order to test the chosen functional alternatives *in vivo* studies, this study was divided into 2 phases:

- The first phase consists of a study under controlled experimental conditions in rainbow trout (*O. mykiss*) experimentally infected with *A. salmonicida* subsp. *salmonicida*. This study aimed to answer the three questions “Does the additive have an immunostimulation effect”, “Does the additive have an effect on the resistance against *A. salmonicida* subsp. *salmonicida* furunculosis, “Does the additive have an effect on the protection conferred by a furunculosis auto-vaccine”.
- The second phase of the study has been carried out in a condition of natural exposure to *A. salmonicida* subsp. *salmonicida* and will aim to answer the questions "Does the administration of the alternative product improve the fish health, "Does the administration of the alternative product reduce the frequency of the antibiotics treatments during a rearing cycle "and" Does the use of alternative products reduce the development and spread of antibiotic-resistant bacteria ".

The second objective is to focus on the evolution of antimicrobial resistance bacteria in aquaculture production and understanding the widespread transmission of antibiotic-resistant genes in aquatic environments. Hence, antimicrobial susceptibility profiles and resistance genes in *Aeromonas* isolated from environment (water and biofilm) and rainbow trout of two fish farms in France were considered for 7 months including summer, an optimal season for furunculosis out breaks to compare *Aeromonas* strains in fish detected with furunculosis, isolates from fish, pond water and biofilm before and after antibiotic treatment.

The research works which are presented in this document are organized in the form of under/published articles. Firstly, a general bibliographic studies is documented. Secondly, the problem of resistance to antibiotics by detecting the *Aeromonas* resistant bacteria and resistance

genes on two selected fish farms in France is presented. Afterward, the *in vitro, in vivo* and on-farm studies on the efficacy of a functional alternative product to reduce the antibiotic use to control *Aeromonas* infections mainly furunculosis in aquaculture are presented. Overall, the manuscript is organized in 3 main chapters and ended by a general discussion and conclusion:

1. **The literature review including aquaculture production, the application of antibiotics in aquaculture and the study of common pathogens in freshwater fish, in particular bacteria of the genus *Aeromonas***. This chapter includes a review of the literature on functional additives to control *Aeromonas* infections in freshwater fish in the form of a scientific review (Article N° 1) (Hayatgheib et al., 2020a).

2. **Monthly monitoring of the phenotypic and genetic antibiotic resistance of bacterial strains of *Aeromonas* during 7 months on 2 Breton farms and aquatic ecosystems** (water, biofilm, fish) in the form of a research article (Article N ° 2).

3. ***In vitro* and *in vivo* studies, under controlled conditions and in breeding, of alternative products that are functional against furunculosis caused by *A. salmonicida* subsp. *Salmonicida***. *In vitro* studies of functional alternatives including various commercial essential oils and their chemical constituents to investigate their antimicrobial effects against *A. salmonicida* subsp. *Salmonicida* were the subject of a research article (Article N ° 3) (Hayatgheib et al., 2020b), as well as the *in vivo* study under controlled experimental conditions (Article N ° 4).

BIBLIOGRAPHIC STUDIES

Chapter 1: Bibliographic studies

A- Aquaculture exploitation

a) International aquaculture production

Aquaculture is used for all aquatic plant and animal production activities. It is used in saltwater, seashore and oceanic coasts, freshwater, lakes, ponds, rivers and ponds to produce farming of fish, crustaceans, molluscs, aquatic plants, algae, and other organisms. According to the food and agriculture organization of the United Nations (FAO), aquaculture is probably the fastest growing food production sector in half a century. The share of world fish production utilized for direct human consumption has increased significantly in recent decades up to 88 percent, or more than 151 million tons in 2016 (FAO 2018). The majority of this supply was produced by seven countries with the largest amount of aquaculture products in the following in order, China, India, Indonesia, Vietnam, Bangladesh Egypt and Norway (FAO 2018).

One of the world's greatest challenges is feeding more than 9 billion people by 2050. In 2015, fish was estimated for about 17 percent of animal protein consumed by the global population. World per capita food fish supply reached a new record high of 20.2 kg in 2015 and predicted to increase in the future (FAO 2018). In a context of climate change, economic and financial uncertainty, and growing competition for natural resources, member states of the United Nations adopted the 2030 Agenda. One of its key issue is the contribution and conduct of fisheries and aquaculture towards food security and nutrition through sustainable management and use of natural resources. Since, a global goal to conserve and sustainably use the oceans, seas and marine resources has dedicated to ensure sustainable development in economic, social and environmental policies for today and tomorrow (FAO 2016). Regarding to sustainable programs, world total marine catch was 79.3 million tons in 2016, representing a decline of almost 2 million tons from the 81.2 million tons in 2015 due to the limited sources

of sea fish and catch quotas restrictions. In contrast, the total aquaculture production representing an increase from 76.1 million tons in 2015 to 80 million tons, for an accounted value of 231.6 billion USD in 2016 (FAO 2018).

Word aquaculture production relies increasingly on fish food supplied from inland aquaculture with the majority of finfish production and, marine and costal aquaculture with the majority of molluscs production. In 2016, inland and, marine and costal aquaculture accounted for 51.4 and 28.7 million tons of total production respectively. Based on FAO, inland aquaculture production included 47 516 thousand tons of finfish mainly freshwater farmed fish, 3 033 thousand tons of crustacea mainly shrimps, 286 thousand tons of molluscs mainly oyster and 531 thousand tons of other aquatic animals in 2016. Asia has accounted for about 89 percent of world aquaculture production. The European Union (EU) supply only 3.7% of total production (FAO 2018).

b) International freshwater farmed fish production

Freshwater fish are those that spend some or all of their lives in fresh water, such as rivers and lakes, with a salinity of less than 0.05%. These environments differ from marine conditions in many ways, the most obvious being the difference in levels of salinity. To survive in fresh water, the fish need a range of physiological adaptations. In nature, many species of fish do reproduce in freshwater, but spend most of their adult lives in the sea such as salmon and trout (Olden et al. 2010). Freshwater fish species are usually classified by the water temperature to which they survive. The water temperature affects the amount of oxygen available; for example, cold water contains more oxygen than warm water. Warmwater fish often tolerate a wide temperature range, but usually have a minimum temperature requirement for reproduction (often around 20°C). They prefer a water temperature around 27 °C. Thus, their growth frequently stops or is poor below 10°–15°C. The most important groups of warmwater fish are carp, catfish and tilapia families. Coldwater fish often tolerate water

temperature between 10 and 16 °C. The most important group of coldwater fish is the salmonid family. Common salmonid fish includes rainbow trout (*Oncorhynchus mykiss*), salmon (*Salmo salar),* brook trout (*Salvelinus fontinalis*) and brown trout (*Salmo trutta*) (FAO 2016).

Rainbow trout (*O. mykiss*) is one of the well-known species of salmonid, native to cold-water of the Pacific Ocean of North America. Since 1874, it has been introduced to waters on all continents except Antarctica, for recreational angling and aquaculture purposes. It is characterized by a silver body covered in black spots with a pink horizontal band and size of 20 to 30 cm on average for a weight of 200 to 300 g at the age of 1 to 2 years or even can reach 7 to 10 kg at three years, by considering that coloration and size varies widely based on subspecies, forms and habitat. The optimal of water temperature is like other coldwater fish species about 10–16 °C (Jalabert and Fostier, 2010). Rainbow trout like all trout species shares common characteristics and live only in fresh, oxygenated and good quality waters. Their taste and nutritional qualities have oriented their production in aquaculture (UICN 2011).

Aquaculture production is seen as a key role to contribute to increase food production while helping reduce pressure on fish resources. The expansion of aquaculture production, especially for freshwater fish such as salmonid, tilapias, carps and catfishes is evident in the relative growth rates of per capita consumption in recent years. Since 2000, global average annual growth rates of aquaculture production have been most significant for freshwater fish (3.1%). Carp and tilapia freshwater fish species were the major species produced in world aquaculture by representing 44% of the total production in 2016. Production of higher-value freshwater fish species, such as salmon and trout, is projected to continue to grow by 2030 due to their rich source of protein for human health. The increasing production of salmon and trout has led to a significant growth in annual per capita consumption of freshwater fish up to 7.8 kg in or 38 percent of the total farmed food fish consumption in 2016 (FAO 2018).

Most salmon and farmed trout consumed today comes from aquaculture, supplied by Norway, Chile and a number of smaller producers mainly in Europe and North America.

c) France aquaculture and salmonid production

France is the second EU aquaculture producer after Spain. Shellfish culture sector is well developed on the various coasts in France by producing mainly oyster and mussel. Fish farming sector mainly produces trout and salmon, carp, other freshwater species and sturgeon and, some marine fish such as seabass and seabream. In 2016, shellfish farming was the dominant aquaculture activity in France (191,800 tons), followed by fish farming, with 40,730 tons of sales, with rainbow trout (*Oncorhynchus mykiss*) more than three-quarters of total production. Based on European Commission report in 2016, France is the 4th largest producer of rainbow trout after Norway, Denmark and Italy by representing more than 18% of the EU rainbow trout production for around 180 million people each year. France produced 27, 900 tons of trout and salmon with majority of rainbow trout production in 2016. Large trout (1 to 2.5 kg) is the major part of production which used to produce smoked trout fillets or fillets. Trout portion (260 g) and trout eggs are also produced in France (European Commission 2016; FranceAgriMer 2019; CIPA 2018). France average annual fish consumption per capita reached 34 kg in 2015. Even, fish from catches (marine aquaculture) consist the major consumption (20 kg per capita) but farmed freshwater trout is one of the top 5 fish consumed regularly by the French (FranceAgriMer 2019; CIPA 2018).

Around 500 freshwater fish farming production sites, mainly in open water circuit system, are spread across the whole of France and managed by around 300 commercial companies. New Aquitaine, Hauts de France and Brittany regions account for 70% of national production. Rainbow trout (*O. mykiss*) currently represents 96% of total national salmonid production (other 4% accounted for trout fario, brook trout or brook salmon, Arctic char (CIPA 2018).

B- Antibiotic application in aquaculture

a) Antibiotic administration

In aquaculture, intensive production and increasing incidence of aquatic animal pathogens are driving antimicrobial use (Cabello, 2006; Schar et al., 2020). Based on a recent review conducted by Lulijwa et al., (2020), regarding drug classes, tetracyclines, amphenicols, sulfonamides and quinolones are the most commonly used antimicrobial classes among 15 highest aquaculture-producing countries in which four countries namely, China, India, Indonesia and Vietnam, showed the highest antimicrobial consumption in terms of dose and quantity. They observed that 67 different antibiotic compounds were used in 11 of the 15 countries between 2008 and 2018. Among these countries, 73% used oxytetracycline, sulphadiazine and florfenicol. On average, countries used 15 antibiotics and the top users included Vietnam (39 antibiotic compounds), China (33 antibiotic compounds) and Bangladesh (21 antibiotic compounds). From the Americas, six antibiotics were used by Chile and four by Brazil in which the most used antimicrobial agents are oxytetracycline and florfenicol. Similarly, In Norway, six antibiotics have been cited and mostly oxolinic acid, florfenicol and sulphadiazine were administered (Park et al., 2012; Lulijwa et al., 2020; Schar et al., 2020).

Aquaculture, as all animal production intended for the production of foodstuffs for humans, is subject to strict regulations concerning the safety of foodstuffs resulting from the processing or production of these animals and the chemotherapeutic treatments for the maintenance of the health of these animals. Although data on quantities of antimicrobials used in aquaculture are not available in most countries, available evidence suggests that the amount of antimicrobials used in aquaculture in most developed countries is limited and in some countries the quantity has been decreasing. Conversely, in some less developed countries, large quantities of antimicrobials are used in aquaculture often without professional consultation and supervision or insufficient regulations for the authorization of antimicrobial agents used in

animals (Joint FAO / WHO / OIE Expert Consultation on Antimicrobial Use in Aquaculture and Antimicrobial Resistance Towards a risk analysis of antimicrobial use in aquaculture, 2006).

In some countries, the use of antibiotics in aquaculture is highly controlled by FDA, FAO's Codex Alimentarius Commission (Codex), European Medicines Agency (EMA) and European Commission (EC). For instance, the use of growth promoting antibiotics has been banned since 2006 in Europe under directives 96/22 / EC, 2003/74 / EC and 2008/97 / EC regarding the development of antibiotic resistance associated with the use of these drugs. Likewise, chemical decontamination of food of animal origin is prohibited under Regulation EC No 853/2004, and this prohibition includes antibiotic substances. In fact, any administration of an antibiotic must follow a veterinary prescription as part of a treatment protocol.

Therefore, only certain antibiotics have a marketing authorization allowing their administration to fish. Generally, this authorization achieves though various experimental assays which are long and costive procedures for pharmaceutics companies to obtain the efficacy and safety drugs' proof from higher authorities. Hence, submitting for marketing authorization is depending on the country's legislative system and also marketing demand/aquaculture production of each county. In a similar way, in France, antibiotics with marketing authorization for fish are not numerous. They are only five molecules: florfenicol, flumequine, oxolinic acid, oxytetracycline and the combination sulfadiazine-trimethoprim. For each administration and depending on the molecules, a withdrawal time is fixed by the marketing authorization, expressed in days or degree-days (the water temperature multiplied by the number of days), and must be observed before slaughter fish for food production. This withdrawal time must ensure that the starting does not contain residues of the administered antibiotic above a legal limit called the "maximum residue limit" (MRL), which depends on the

pharmacokinetics of the antibiotic and raw material of animal origin intended to be transformed into food (eg muscle, egg, etc.).

Regarding to the mode of antibiotic administration in aquaculture, there is a major difference between aquatic and terrestrial animals. The two most common routes for the administration of antimicrobials in aquaculture are: (i) water medication like immersion therapy often for small biomass and (ii) medicated feed by the addition of a small amount of the antimicrobial drug to a homogenized and extruded diet, or the sprayed or the top-coat of the drug onto the feed. The advantage of in-feed medication is the reduced wastefulness of antibacterial agent when compared with water medication. It also reduces undesirable exposure of the environment and other fish to the drug. Other methods like gavage, injection or topical application are rarely used in the aquaculture industry due to it is labor intensive and stressful to the fish and sometimes anesthesia is needed (Noga, 2010; Park et al., 2012). Hence, compared with antimicrobial use in terrestrial food animal and aquaculture production, antibiotics administrated mostly in feed or water, can provide a potentially wider direct or indirect environmental exposure pathway for drug distribution through water with important ecosystem health implications. This can contribute in increasing the resistant bacterial population and maintaining selective pressure that causes the development and dissemination of resistance in aquatic environments (Cabello, 2006; Watts et al., 2017).

b) Antibiotic resistance evolution

Antibiotic resistance occurs when bacteria use metabolic or other defenses to survive in presence of antibiotics and continue growing. The release of antibiotic residues to the environment, the overuse and the non-therapeutic use of antibacterial agents have accelerated the generation and evolution of antibiotic resistance resulting to worldwide public health problem (Drancourt, 2016). Notably, the emergence of antibiotic resistance phenomena occurs rapidly, generally around only ten years, after an antimicrobial agent has been placed on the

market (Kennedy and Read, 2017). Antibiotic resistance can be acquired in bacteria by mutations in target regulatory genes, which can be transferred in a vertical way that genetic information, including any genetic mutations transfer from a parent to its offspring. Other than vertical transmission of DNA, horizontal gene transfer (HTG) is the primary mechanism for the spread of antibiotic resistance in bacteria (Varga et al., 2012; Kahl, 2015; Cairns et al., 2018).

Genes responsible for antibiotic resistance or antibiotic resistance determinants by means of mobile elements such as plasmids, integrons and transposons in one species of bacteria can be transferred to another species of bacteria through various mechanisms of HGT such as conjugation, transformation, transduction and gene transfer agents (Figure 1). Conjugation is a process requiring cell to cell contact via cell surface pili or adhesins, through which DNA is transferred from the donor cell to the recipient cell. Transformation is the uptake, integration, and functional expression of naked fragments of extracellular DNA. Through specialized or generalized transduction, bacteriophages/viruses may transfer bacterial DNA from a previously infected donor cell to the recipient cell. In specialized transduction, the bacteriophages pick up only specific portions of the host's DNA. In contrast, with generalized transduction, the bacteriophages can pick up any portion of the host's genome. During generalized transduction, bacterial DNA may be accidentally loaded into the phage head. Gene transfer agents are bacteriophage-like particles that carry random pieces of the producing cell’s genome. Gene transfer agents (GTAs) are DNA-containing virus-like particles that are produced by some bacteria and mediate HGT. Different GTA types have originated independently from viruses in several bacterial. These cells produce GTA particles containing short segments of the DNA present in the cell. GTA particles may be released through cell lysis. After the particles are released from the producer cell, they can attach to related cells and inject their DNA into the cytoplasm. The DNA can then become part of the recipient cells' genome

(Figure 1) (Von Wintersdorff et al., 2016) (Varga et al., 2012; Kahl, 2015; Von Wintersdorff et al., 2016; Cairns et al., 2018).

Moreover, multidrug resistance in bacteria can evolve though co-resistance mechanisms by the selection of multiple resistance genes within a mobile genetic element (MGE) and/or through cross-resistance mechanisms, by the presence of resistance genes with a broad substrate range (Baker-Austin et al., 2006; Pal et al., 2017).

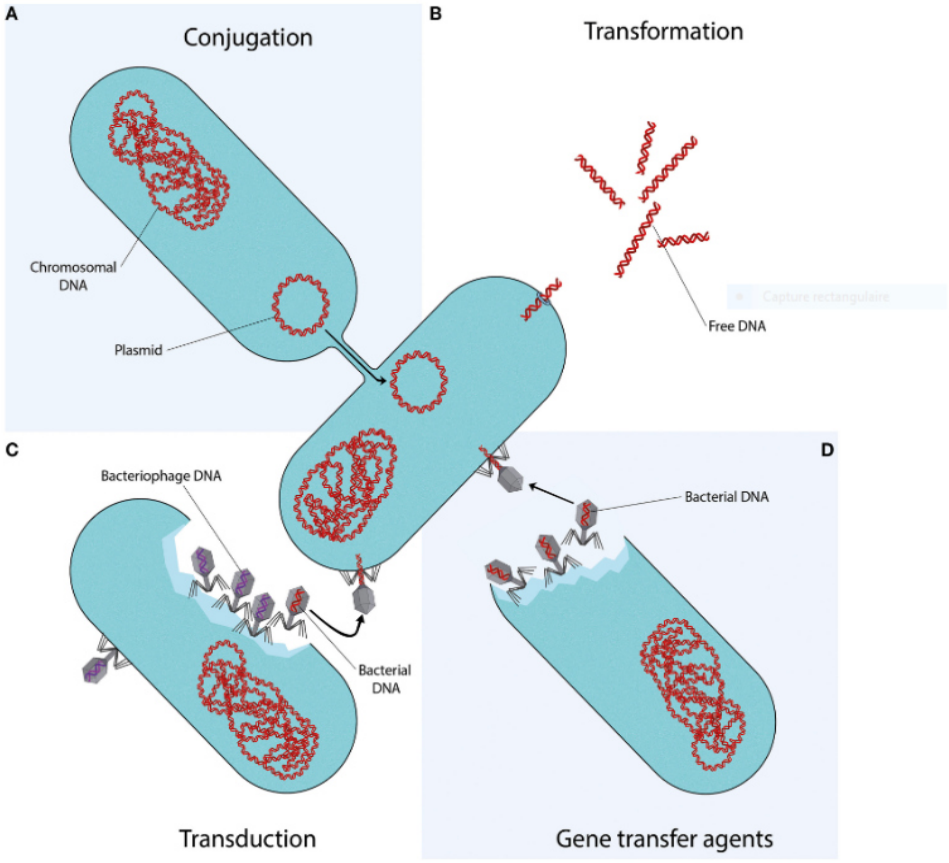

Figure 1. Mechanisms of horizontal gene transfer. Each quadrant represents one different method of gene transfer. (A) Conjugation. (B) Transformation. (C) Transduction. (D) Gene transfer agents (Von Wintersdorff et al., 2016).

c) Mechanisms of action and resistance to antibiotics

Antimicrobial agents can be divided into five groups based on the mechanism of antimicrobial activity. There are agents 1) that inhibit cell wall synthesis (β-Lactams and glycopeptides like vancomycin), 2) depolarize the cell membrane (lipopeptides like colistin), 3) inhibit protein synthesis (bind to 30S ribosomal subunit: aminoglycosides and tetracyclines; bind to 50S ribosomal subunit: chloramphenicol, lincosamides and macrolides 4) inhibit nuclei acid synthesis (quinolones and fluoroquinolones), 5) inhibit metabolic pathways in bacteria (sulfonamides and trimethoprim). Moreover, there are a number of mechanisms that bacterial cells use to defeat the efforts of antibiotics. Some bacteria are naturally resistant due to intrinsic resistance by limiting drug uptake, drug inactivation through producing enzymes, and drug efflux. Moreover, bacteria can acquire resistance mechanisms by drug target modification, drug inactivation and drug efflux (Kapoor et al., 2017; Reygaert, 2018).

Regarding to differences in structure and function of bacteria cell, there is variation in the types of mechanisms used by gram negative bacteria versus gram positive bacteria. Gram positive bacteria less commonly use limitation of the uptake of a drug, because they don't have a lipopolysaccharide outer membrane and don't have the capacity for certain types of drug mechanisms efflux. In Gram-negative bacteria, antibiotics need to penetrate the outer membrane composed of phospholipids and lipopolysaccharides. Changes in porin outer membrane proteins (number or size) can promote antibiotic resistance by decreasing the penetration of antibiotics such as many β -lactams, fluoroquinolones and chloramphenicol and thereby limit their ability to find their targets (Džidić et al., 2008). Bacteria can also produce efflux pumps, which are present in Gram-negative and Gram-positive bacteria; however, this mechanism is a key mechanism of resistance in Gram-negative bacteria. Efflux pumps can be specific or conversely they can pump out of the cell a big variety of molecules such as multidrug efflux pumps. Changes in regulatory genes and overexpression of efflux pumps proteins can

promote antibiotic resistance in bacteria especially for antibiotics such as fluoroquinolones, macrolides and tetracycline which need to get into the cell to be able to exert their action (Kapoor et al., 2017; Reygaert, 2018).

d) Detection of resistance to antibiotic

The most widely used antibiotic susceptibility testing methods following Clinical Laboratory Standard Institute (CLSI) are based on the phenotypic detection of antibiotic resistance by measuring bacterial growth in the presence of the antibiotic being tested. Susceptibility and resistance are usually measured as a function of minimum inhibitory concentration (MIC) in dilution method (broth or agar dilution method) which is the minimal concentration of drug that will inhibit growth of the bacteria. The susceptibility is actually a range of the average MICs for any given drug across the same bacterial species (Mahon et al., 2014; Reygaert, 2018)**.** These conventional methods take typically from 24 h to obtain results. Even more, some species like *Aeromonas* requires a lot of time before obtaining an MIC result. They need to isolate the pathogen on a selective medium that requires 24h to 48h incubation and then test for antibiogram using antibiotic concentrations that require another 24h to 48h incubation before obtaining the result (Murray, 2013).

There are many instrumental techniques that allow an antibiogram to be made quickly. Disk diffusion method (commercially prepared disks of antibiotics) is widely used for determining antimicrobial resistance in private veterinary clinics because of convenience, efficiency and cost. A commercial E-test is also an available test that utilizes a plastic test strip impregnated with a gradually decreasing concentration of a particular antibiotic. This method is a convenient quantitative test of antibiotic resistance of a clinical isolate. However, a separate strip is needed for each antibiotic, and therefore the cost of this method can be high. Other used methods are mechanism-specific tests, chromogenic test or genotypic methods such as multiplex or single quantitative PCR and DNA hybridization (microarray) methods (Khan et

al., 2019). Since resistance traits are genetically encoded, tests for the specific genes that confer antibiotic resistance are sometimes needed. However, although nucleic acid-based detections systems are generally rapid and sensitive but expensive. Notably, the presence of a resistance gene does not necessarily equate to treatment failure, because resistance is also dependent on the mode and level of expression of these genes (Bartkova et al.,2017a; Vasala et al., 2020).

C- Common freshwater fish pathogens: *Aeromonas* bacteria

In aquaculture sectors, threat of aquatic pathogens can impact the ultimate goal of aquaculture sustainability and productivity. Particularly in fish farming, mortality due to bacterial diseases has been considered to be one of the significant causes, contributing to reduced production and profits. Bacteria commonly known to be pathogenic to fish are likely *Aeromonas* spp., *Flavobacterium* spp., *Pseudomonas* spp., *Acinetobacter* spp., *Lactococcus garviae* and *Streptococcus iniae* etc. Among these bacteria, *Aeromonas* spp. are the most common known pathogen that can cause diseases in freshwater fish species, especially in salmonid farms (Austin. and Austin 2012; Pękala-Safińska 2018). *Aeromonas* spp. are commonly found in aquatic ecosystems including water, and therefore fish are constantly exposed to these bacteria. Interaction with these bacteria is especially threatening under conditions of stress, which include unfavorable environmental conditions as well as human interventions in catching, sorting, and transporting of the fish. *Aeromonas* species are also involved in the physiological microflora of the fish intestine (Austin, B & Austin D, 2012).

a) Phenotypic characteristics and pathogenicity of *Aeromonas*

The genus *Aeromonas* belongs to the family *Aeromonadaceae,* to the order *Aeromonadales* and to the class *Gammaproteobacteria* (Martin-Carnahan and Joseph 2005). *Aeromonas* is a ubiquitous bacterium in aquatic environments. These bacteria are gram-negative, bacilli rod-shaped bacteria (0.3–1.0× 1.0–3.5 μm), facultative anaerobic, oxidase-positive, catalase-positive. *Aeromonas* genus are predominantly mobile by a single polar flagellum, while peritrichous or lateral flagella may be formed on solid media in some species. This genus can effectively degrade nitrates to nitrites and ferment glucose (Martínez-Murcia 2016). This genus includes 36 species that are considered opportunistic aquatic environmental pathogens of animals and humans. They are isolated from foods, animals, and various infectious processes in humans (Lamy, 2012a; Watts et al., 2017). *Aeromonas* are widely distributed in

aquatic environments such as freshwater, estuarine waters, marine waters and sediments with some species able to cause diseases in humans and aquatic animals. Eventually, the ubiquitous distribution of these bacteria facilitate its role as a great health problem issue (Talagrand-Reboul et al., 2017).

The taxonomy of the genus *Aeromonas* has always been changed, with 22 new species added since 1992, due to the behavioral variation of its strains (Beaz-Hidalgo et al. 2010; Fernández-Bravo and Figueras 2020). In late 1970s, aeromonads were divided into two major groups based on physiological properties and host range. Motile aeromonads grow at optimum temperature of 35–37◦C and those isolated from human infections were identified to be *A. hydrophila,* but non-motile aeromonads which grow at 22–28◦C and are isolated from fish were called *Aeromonas salmonicida*. Before 1990s, phenotypic identification relied on physiological, morphological, and biochemical characteristics, while thereafter, the identification of *Aeromonas* isolates was made by their DNA-DNA hybridization groups (HGs) and 16S ribosomal DNA relatedness containing virulence factors (Neyts and Huys2000; Igbinosa et al. 2012). Despite the current knowledge, the identification of *Aeromonas* genus is still complex due to the high variable behavior of *Aeromonas* strains and their high molecular similarity (Beaz-Hidalgo et al. 2010; Fernández-Bravo and Figueras 2020). The various biochemical characteristics of some common *Aeromonas* species are shown in table 1 (Igbinosa et al. 2012; Carnahan and Joseph 2005).

Recent advances in biochemistry, molecular biology and virulence factors associated with *Aeromonas* spp. have led to new understanding of this bacterial group. The pathogenic potential of *Aeromonas* has been related to the presence of several virulence factors such as expression of genes (*exoA, alt, act,* etc.) that encode extracellular components and toxins like proteases, lipases, enterotoxins, hemolysins, and Shiga toxins, structural components like flagella, pili, capsule, S layer surface protein, and lipopolysaccharides (flaA, maf-5, flp, etc.),

secretion systems (*T3SS, T6SS*, etc.), quorum sensing and proteins associated with metals. These mechanisms allow *Aeromonas* bacteria to adhere, invade, and destroy the host cells, especially in digestive tract, skin and soft tissue, blood stream, and overcoming the immune host response (Janda and Abbott 2010; Tomás 2012; Beaz-Hidalgo and Figueras 2013; Janda and Abbott, 2010).

Table 1. The biochemical identification of motile *Aeromonas* spp. HG: hybridization group; +: > 75% of strains positive; d: 26–74% of strains positive; −: < 25% of strains are positive; nd: not determined; R: resistant; S: sensitive (Carnahan and Joseph 2005).

Characteristic	***A. hydrophila***	***A. bestarium***	***A. caviae***	***A. sobria***	***A. veronii biovar sobria***	***A. jandaei***	***A. veroni biovar veroni***	***A. schubertii***
Motility	+	+	+	+	+	+	+	+
Hybridization group	HG-1	HG-2	HG-4	HG-7	HG-8	HG-9	HG-10	HG-12
Esculin hydrolysis	+	+	+	−	−	−	+	−
Gas from glucose	+	+	−	+	+	+	+	−
Voges-Proskauer	+	+	−	+ weak	+	+	+	d
Indol	+	+	+	+	+	+	+	−
Pyrazinzmidase	+	−	+	nd	−	−	−	−
L-arabinose	d	+	+	−	−	−	−	−
D-mannitol	+	+	+	+	+	+	+	−
Sucrose	+	+	+	+	+	−	+	−
Lysine decarboxylase	+	−	−	+ weak	+	+	+	+
Ornithine decarboxylase	−	−	−	−	−	−	+	−
Arginine dihydrolase	+	+	+	−	+	+	−	+
Arbutin hydrolysis	+	+	+	nd	−	−	+	−
H2S production	+	+	−	nd	+	+	+	−
Hemolysis	+	+	d	−	+	+	+	+
Ampicillin 10 µg	R	R	R	S	R	R	R	R
Carbenicillin 30 µg	R	R	R	S	R	R	R	R
Cephalothin 30 µg	R	R	R	S	S	S	D	S
Colistin 4 µg/ml	d	d	s	nd	S	R	S	S

Especially, *Aeromonas salmonicida* is best known as a pathogen of salmonid fish, multiple other species can be infected (Wiklund and Dalsgaard 1998). In particular, the development of aquaculture to new species has led to the increased isolation of new strains of *A. salmonicida* and differentiating between 'typical' as *A. salmonicida* subsp. *salmonicida* and 'atypical' isolates such as *A. salmonicida* subsp. *achromogenes, A. salmonicida* subsp. *masoucida*, *A. salmonicida* subsp. *smithia*, and *A. salmonicida* subsp. *pectinolytica*, to simplify the dominance of causal agents in salmonid species. Indeed, different sub-species can express different virulence factors, and these might contribute to the specialization of some groups of *A. salmonicida* (Pavan et al., 2000; Gudmundsdóttir et al., 2003).

Indeed, *A. salmonicida* subsp. *salmonicida* can secrete at least 4 distinct types of acylated homoserine lactones, of which N-butanoyl-L-homoserine lactone (C4-HSL) is the most important virulence factor (Schwenteit et al. 2011). Other mechanism as quorum sensing has been linked to the secretion of protease in the bacterial supernatant (Rasch et al. 2007). Iron deprivation also increased the transcription levels of the enzyme enolase, found to be both secreted and present in the outer-membrane fraction of this bacterium (Vanden Bergh et al. 2013; Menanteau-Ledouble et al. 2014). Enzyme lactoylglutathione lyase of the bacterium a allows the bacterium to reduce the immune response (Vanden Bergh and Frey, 2014). Among the bacterial virulence factors, the type 3 secretion system (T3SS) has received the most recent attention. T3SS is encoded on a large plasmid and describes the multiple effector proteins like AopO and their putative effects. One particularly interesting function of the T3SS is to interfere with immune signaling inside the host and repress the inflammatory response (Menanteau-Ledouble and El-Matbouli, 2016). Another virulence mechanism involves the lipopolysaccharides that considered as the most important contributors to endotoxicity and also they can display hemolytic activity against fish erythrocytes (Anwar and Choi, 2014: Menanteau-Ledouble et al., 2016). The A-layer protein VapA, a type I pilus, three type IV pilus

systems, superoxide dismutase and some extracellular proteins including serine protease (AspA). Glycerophospholipid, cholesterol acyltransferase (GCAT or SatA) and several hemolysins (aerolysins) are the other identified virulence factors for *A. salmonicida* (Vanden Bergh and Frey 2014; Menanteau-Ledouble and El-Matbouli, 2016).

b) *Aeromonas* in environments, human and fish

In the environment, the genus *Aeromonas* is widely distributed across numerous aquatic ecosystems, surface, underground, potable, bottled, residual, seawater, and irrigation waters. Although, they have also been isolated from several clinical samples (Janda and Abbott, 2010). The occurrence of *Aeromonas* in chlorinated drinking water supplies have been reported typically less than 10 CFU/ml in several countries. They can form biofilms and may be protected from disinfection water (Chauret et al. 2001). *Aeromonas* may persist attached to biofilms on biotic or abiotic surfaces. *Aeromonas* polar and lateral flagella can contribute to biofilm formation and adhesion on surfaces (Kirov et al. 2004). Natural biofilms are developed and differentiated to build a packed community that is often multi-species. This ability to create polybacterial mixed biofilms represents a niche and reservoirs of *Aeromonas* and other bacteria such as *E. coli* and promotes the exchange and dissemination of antimicrobial resistance genes (Talagrand-Reboul et al., 2017).

In humans, the most frequently clinically isolated species are *Aeromonas veronii biovar sobria*, *Aeromonas hydrophila*, *Aeromonas caviae, Aeromonas aquariorum*, *Aeromonas jandaei* and *Aeromonas schubertii* in order of decreasing frequency. The different types of infections include wound infections, bacteremia, gastroenteritis, peritonitis, hepatobiliary, respiratory, and ophthalmic infections (Janda and Abbott 2010; Fernández-Bravo and Figueras 2020). These are rare but emergent infections occurring in immunocompromised patients (cancerous or hepatobiliary pathology) or even in immunocompetent patients (Lamy, 2012).

In fish farms, the 2 most frequently encountered species are *Aeromonas hydrophila* and *Aeromonas salmonicida*. Although, other *Aeromonas* bacteria have also been isolated from aquatic animals such as *A. veronii*, *A. piscicola, A. sobria, A. schubertii, A. bestiarum, A. encheleia, A. sobria, A. allosaccharophila, A. dhakensis, A. caviae, A. jandaei, A. media,* and *A. trota* (Fernández-Bravo and Figueras 2020). *Aeromonas hydrophila* is a ubiquitous bacterium found commonly in fresh water ponds and is a normal inhabitant of their gastrointestinal tract. This bacterium has been isolated from numerous species of freshwater fish and occasionally in marine fish, amphibians, reptiles, cattle and humans throughout the world (M. Randy White, 1991). *A. hydrophila* causes disease in fish known as "hemorrhagic septicemia" where the bacteria or bacterial toxins are present within numerous organs of the fish. For example, *A. hydrophila* have been isolated from rainbow trout (*O. mykiss*) in a natural pond with spring water by observing an epidermal erosion at the base of the left ventral fin, as well as petechias and desquamation around the affected area (**Figure**) (Zepeda-Velázquez et al. 2005).

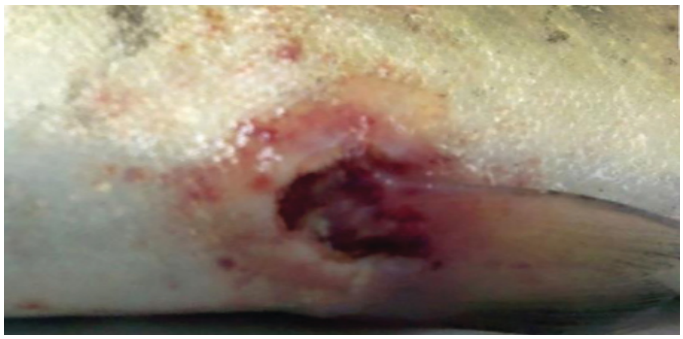

Figure 2. Macroscopic lesions in rainbow trout naturally infected with *A. hydrophila*. Ulcer at the base of the left ventral fin (Zepeda-Velázquez et al. 2005).

In carp (*Cyprinus carpio*), erythrodermatitis (CE) caused by *A. hydrophila* with the main symptom of skin ulcer was observed in a farm with high losses of carp (Sioutas et al.

1991). Outbreak by *A. hydrophila* infection have been reported in Nile Tilapia (*Tilapia nilotica*) especially during the summer season in semi-intensive fish farms (Ibrahem 2008). *A. hydrophila*, is a zoonotic pathogen that infects fish and also humans who are in contact with the aquaculture facility or via foodborne infections (Haguenoer, 2010). *A. salmonicida* have been isolated from wild range of cultivated and wild fish species, non-salmonids as well as salmonids, inhabiting fresh water, brackish water and marine environments. *A. salmonicida* subsp. *salmonicida* is a causative agent of furunculosis which is an opportunistic pathogen of aquatic environments that mainly affects salmonids (Austin, B. & Austin D, 2012).

c) Furunculosis in fish farming

Furunculosis due to *Aeromonas salmonicida* subsp. *salmonicida* can cause severe economic losses by hemorrhagic septicemia in acute form or by fish depreciation because of the development of boils in the muscles in chronic form (Figure 3) (Austin, B. & Austin D, 2012). The salmonid family can be affected by various diseases worsen by stress factors in fish, resulting to worldwide losses in aquaculture. The temperature of the pond particularly in summer season when water temperature increases, the accumulation of fish and the supply of oxygen are factors to be controlled by fish farmers to prevent the development of diseases especially in coldwater freshwater fish like salmonid such as salmon, brook trout and rainbow trout. In fact, an infectious disease called furunculosis mainly affects salmonids, caused by the pathogenic bacterium *Aeromonas salmonicida* subsp. *salmonicida*. It can be distributed widely in water and sediments of ponds and can be transmitted by discharge from the intestinal tract and external lesions on the skin (Morin 2010; Beaz and Jos 2012). Furunculosis was first documented during an outbreak of ulcerative lesions between 1888 and 1889 in a fish farm in Germany that reared brown trout. This outbreak was harmful to the farm economy as the affected fish became unfit for consumption (Dworkin et al. 2006). The most studied subspecies

of this bacterium is *salmonicida*, which is sometimes qualified as 'typical', and is well known to cause furunculosis, (Austin & Austin 2012).

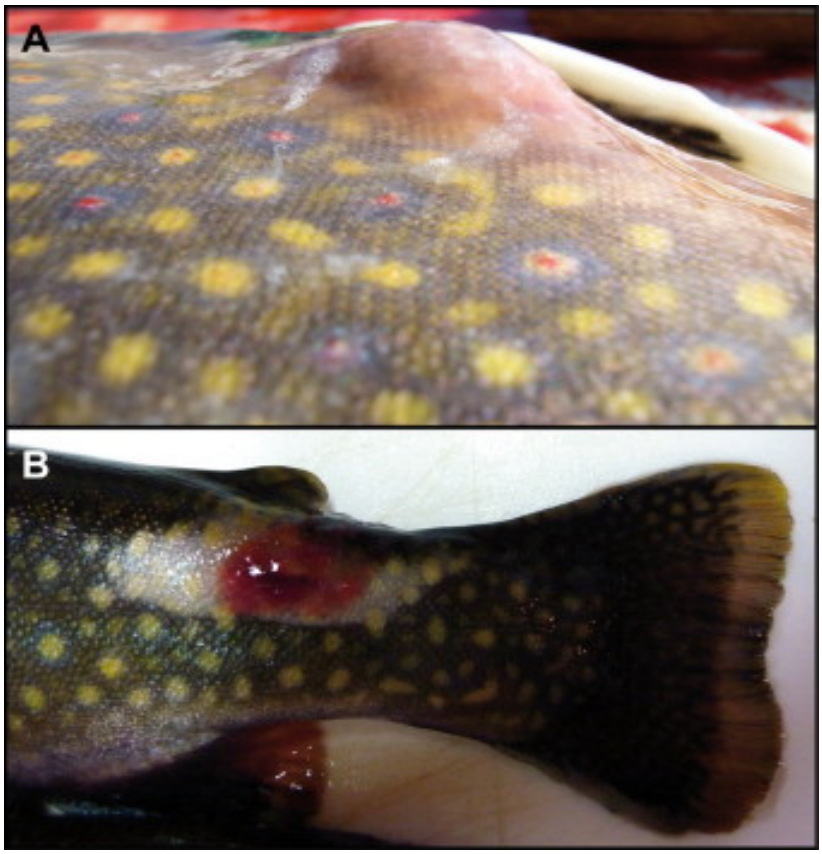

Figure 3. Clinical manifestations *of A. salmonicida* subsp. *salmonicida* infections. Furunculosis in sick brown trout (Salmo trutta). The presence of a subcutaneous boil located in the muscle tissue is characteristic of a chronic infection. (A) Large furuncle on the surface of an infected fish. (B) The swollen skin lesion or furuncle under the skin is filled with pink fluid. containing blood and necrotic tissue (Dallaire-Dufresne et al., 2014).

This infectious disease can be either chronic or acute. Young fish usually presented the acute form with loss of appetite and blackening of the skin, while older fish were more frequently affected by the chronic form and mortality is usually slower. The chronic form can

be converted into acute form because of stressful environmental conditions such as water temperature. Chronic infections affect fish by causing boils that are lesions on their bodies. Acute infections cause many symptoms in fish, such as sepsis, hemorrhage, tissue necrosis and melanosis which lead to the death of fish (Dallaire-Dufresne et al., 2014).

In France, furunculosis (*A. salmonicida* subsp. *salmonicida)* has a central position (12/30) in the list of existing diseases in farmed fish resulting in repeated treatments of diseased fish and economic loss, particularly during warmer summer months during which *Aeromonas* species can grow to large numbers and generally peak in the warmer temperatures. Moreover, vaccination has shown some disappointing results. Even, furunculosis has been fairly well controlled in whole of France but recently evolves with a resurgence and recurring of clinical cases particularly in rainbow trout (*O. mykiss*). A decrease in the effectiveness of antibiotics (antimicrobial resistance), a disappointing result of vaccination, an extension of the production cycle for rearing larger trout, an increase in summer storage, an interfacing with warmer summer due to climate change etc. can be related to the resurgence of furunculosis in rainbow in recent years. This can be also associated to the tending of rainbow culture rather than brown trout and char with much more incidence in the rainbow trout than other salmonid species. Therefore, furunculosis becomes a recurring and worrying issue likely in rainbow trout. Currently, antibiotic treatment seems to be applied to avoid the economic loss due to furunculosis in these farms. Application of antibiotics and then after, antimicrobial resistance is a growing public health concern worldwide. Since, finding an appropriate substitution of antibiotic therapy against fish diseases due to their negative impact is necessarily needed (ANSES, 2015).

d) Control of furunculosis (*Aeromonas salmonicida* subsp. *salmonicida*)

The existing medical methods against *Aeromonas salmonicida* subsp. *salmonicida* are mainly antibiotics and vaccination in fish farming.

d.1. Vaccination

Various publications state the importance of vaccination to limit losses linked to bacterial diseases rather than antibiotic treatments due to their side effects in the development of drug resistance. Vaccination as a preventive method is suggested to farmers to prevent the economic costs caused by high mortalities in breeding stock. Vaccines against bacterial diseases are playing an increasingly important role in aquaculture production. There are many vaccines commercially available for the control of bacterial pathogens. However, improved a safe, efficient, and economical vaccine candidate against furunculosis, *A. salmonicida* subsp. *salmonicida* is still demanded (Plant and LaPatra, 2011; Villumsen et al. 2017).

Since 1990, different strategies of vaccination have been developed to control furunculosis in marine or farmed salmonids such as surface (A-layer)-disorganized, attenuated mutants of *A. salmonicida* live vaccines (Thornton et al., 1991; Vaughan et al. 1993;) or oil-adjuvanted bacterin (dead or inactivated antigen) vaccines (Smith and Hiney 2000). Besides, many disagreements have been reported to evaluate vaccine efficacy and its mode of action against *A. salmonicida*. Even in the case of successful vaccinations, some research considered the important role of non-specific protective mechanisms rather than the specific immune response and *vice versa* whereas some work attributed the vaccine efficacy to the both mechanisms (Michel et al., 1990). For example, Olivier et al. (1985) explained that the immunity to *A. salmonicida* with a modified complete Freund's adjuvant (FCA, mineral oil-in-water emulsion) *A. salmonicida* bacterin vaccine can be associated with the induction of the non-specific or innate immunity and the potential role of macrophage phagocytic and bactericidal activities (Olivier et al. 1985). In the same study, formalin-killed *A. salmonicida*

was injected by intraperitoneal route in the absence or presence of FCA and the best protection was found for the FCA adjuvanted vaccine (Olivier et al. 1985).

Currently, the most used commercial vaccines to control furunculosis containing an adjuvant consisting of oil and antigens from inactivated *Aeromonas salmonicida* subsp. *salmonicida* administered by intraperitoneal injection. Among different vaccine administration routes, intraperitoneal injection of vaccine has provided reasonable levels of protection rather than bathing or immersion which can be explained by limited uptake of antigens by immersion compared to injection (Bøgwald and Dalmo, 2019; Mohd-Aris et al. 2019). Inactivated vaccines compared to live vaccines, are more stable under farm conditions and may be less expensive to produce. In addition, inactivated vaccines are safer than live vaccines due to their non-replicated form in host. However, they generally present a shorter length of protection and weaker immune responses. So far, many worldwide investigations have been conducted on the improvement of inactivated oil-adjuvanted vaccines. The induction of protective immunity through inactivated *A. salmonicida* bacterin can be enhanced by addition of oil-based adjuvants. However, the presence of lesions adhering to the viscera in vaccinated fish due to side effects of its adjuvant made them not applicable by farmers. Certain side effects caused by the oil adjuvant resulting in congestion at the injection site or severe peritonitis. In some cases, these inactivated vaccines have resulted to controversial level of protection and shorter-lived immunity due to the potential immunosuppressive passenger antigens. Therefore, vaccination was not successful and long-lasted and the recurrent outbreak have been reported due to the insufficient level of induced immunity by the used vaccine (Plant and LaPatra, 2011; Villumsen et al. 2017; Ma et al. 2019). Recent investigations on alternatives to mineral oil based vaccine showed that nucleotide or liposome-based vaccine formulations could reduce but not thoroughly eliminate its adverse effects in rainbow trout chair (Plant and LaPatra, 2011; Villumsen et al. 2017).

Today, several monovalent and multivalent vaccines (like vaccines against vibriosis and furunculosis) containing *A. salmonicida* have been commercialized to control furunculosis. The main method of vaccination in salmonids against *A. salmonicida* involves formalin-killed/inactivated bacteria with oil adjuvant administered through immersion, bathing or injection routes and induced some level of humoral immunity. For example, AquaVac® FNMPLUS is a non-mineral oil based injectable vaccine containing two strains of *A. salmonicida*, using the surface S-layer (VapA) protein of *A. salmonicida* (iron-regulated outer membrane protein) for protection against atypical strains of *A. salmonicida,* while Furogen Dip® is a formalin inactivated *A. salmonicida* bacterin administered via injection or bathing (Mohd-Aris et al. 2019). Braden et al., (2019) described that the protective effect of Forte Micro® oil based adjuvant injectable vaccine against *A. salmonicida* containing formalin inactivated cultures of *A. salmonicida*. can be involved in the immunological priming of innate humoral components such as complement, coagulation, and metal homeostasis effectors and adaptive *A. salmonicida* antibodies molecules like serum IgM. These mechanisms, firstly may allow a rapid aggressive response, followed by prompt genes regulation to induce the antibody-mediated complement activity targeting extracellular bacteria, and secondly may induce cytotoxic-mediated intracellular to target infected self-cells. Therefore, survival can be resulted in significant protection from host mortality (Braden et al., 2019).

In the case of the market requests for furunculosis vaccine, most of these commercial vaccines are licensed nationally and then marketed but sometimes with a narrower distribution. For example, actually, no commercially licensed and marketed vaccine is available to control furunculosis in France and the production of AquaVac® FNMPLUS has been ceased based on a French Animal Health Network (RFSA) report; however, it is marketed in Europe each year (Mutoloki et al. 2006; Quentel et al., 2007). Therefore, the development of commercial vaccines

is limited by economic considerations, biological problems and regulatory restrictions in aquaculture which leads fish farm sectors to a wide use of autovaccines (Sudheesh and Cain 2017; Ma et al. 2019). Autogenous vaccines (autovaccines) are prepared from cultures of microorganisms obtained from an individual in a farm and then used to immunize all the individuals in the same farm against further spread and progress of the same microorganisms. Autogenous vaccines provide more flexibility in production regulation, and are created within a collaborative veterinary-client-patient-relationship. These vaccines may suggest a solution to emerging pathogens of interest, when no commercially licensed product is available, or when commercially licensed products have not provided adequate protection. However, the use of autovaccines is considered as a cost-effective alternative to commercial vaccines (Yanong 2011; Sudheesh and Cain 2017; Adams 2019; Ma et al. 2019). Even vaccines exist, they are used hardly because of their short efficacy, the intra-abdominal adhesions observed after intraperitoneal injection and the difficulty of vaccinating young animals individually because of their small size (Plant and LaPatra, 2011; Villumsen et al. 2017).

d.2. Antibiotic treatment

The use of antibiotics is currently the most used way to control this disease. Antibiotics can be effective in treating the infection if the bacteria are not resistant to the prescribed antibiotic. Furunculosis have been treated with several fish approved antibiotics. Florfenicol, sulfamethoxazole associated with trimethoprim and flumequine are the most prescribed antibiotics against furunculosis in aquaculture (Morin 2010; Naviner et al., 2011). Other antibiotics such as oxytetracycline, amoxicillin, erythromycin, oxolinic acid etc. are also prescribed widely in human medicine against *Aeromonas spp*. infections (Lamy 2012). Antibiotics can be administered either orally by the addition of antibiotics in the feed, by injection or by bath. The most used method is by addition into the feed. This method of administration is not always effective since infected fish with furunculosis lose appetite which

does not encourage them to consume the antibiotics present in the feed. Fish treated with antibiotics can be reinfected after treatment, unlikely vaccination immunized fish. However, from an economic point of view, antibiotics are less expensive to use than vaccines (Plant and LaPatra, 2011; Villumsen et al., 2017).

e) *Aeromonas* antibiotic resistance profile

Resistance to antibiotic is a genetic–evolutionary response evolved by the presence of genes, some of which are found in plasmids, integrons or in the genome of the bacteria (Martin-Carnahan and Joseph 2005; Janda and Abbott 2010). The genus *Aeromonas* is also notable for its antibiotic resistance profile in environmental and clinical strains. The natural processes of horizontal gene transfer (HGT) and mutation events have been studied since ancient times. By their nature, aquaculture systems contain high numbers of different bacteria, which exist in combination with the current and past use of antibiotics, probiotics and other treatment regimens. These systems have been designated as "genetic hotspots" for gene transfer. The genetic support of these acquired resistances is transferable by chromosomal transposons / integrons or plasmids carrying genes associated with resistance to current antibiotic treatment in *Aeromonas spp*. infection such as beta-lactams, quinolones, macrolides, tetracycline, sulfonamides and chloramphenicol (Talagrand-Reboul et al. 2017).

Many plasmids carrying antibiotic resistance genes (ARGs) have been described in *A. salmonicida* subsp. *salmonicida* and the ubiquitous appearance of the genus *Aeromonas* could contribute to the diffusion of these ARGs in the environment (Piotrowska and Popowska, 2014; Vincent et al., 201; Piotrowska 2017). Genotyping analyses and the antibiotic resistance profiles of *A. salmonicida* subsp. *salmonicida* demonstrated that the variants of the pAB5S9, pSN254 and pRAS3 plasmids carry several antibiotic resistance genes. Moreover, these variants have been previously reported in other bacteria genera or species, including *Salmonella enterica*, a well-known human pathogen. Moreover, pSN254 plasmids have been found to be transferable

by conjugation from *A. salmonicida* subsp. *salmonicida* to *Escherichia coli*, *A. hydrophila*, and *Edwardsiella tarda* (McIntosh et al. 2008). It suggests a high level of interspecies exchange that may contribute to the spread of antibiotic resistance genes in the environment of *Aeromonas* ubiquitous bacteria (Vincent et al., 2014).

Recently, an increased resistance to beta-lactam antibiotics in the genus *Aeromonas* has been reported globally in aquatic environments and aquatic species due to the presence of beta-lactamases genes (Chen et al. 2012). For example, 39.6% of the *Aeromonas* strains isolated from farmed rainbow trout (*O. mykiss*) showed the presence of one or more resistance genes. The gene bla $_{CphA/IMIS}$ was showed in 29.2% of the isolates, followed by the intI1 (6.2%) and bla$_{SHV}$ (4.2%) genes. The results from the sequencing of class 1 integrons revealed the presence of the gene cassette aadA1 (aminoglycoside transferase) that plays a role in streptomycin/spectinomycin resistance (Vega-Sánchez et al. 2014). Patil et al. (2016) demonstrated, high levels of antibiotic resistance and ARG in a large collection of *Aeromonas* strains isolated from fish and pond water facilities by showing a strong link between sulfadiazine-trimethoprim antibiotic use in fish farms and resistance in both environmental and pathogenic *Aeromonas* strains including *A. salmonicida A. veronii* sulfadiazine-resistant strains in aquaculture ecosystem (Patil et al., 2016).

A. hydrophila is a pathogenic bacterium that caused diseases in animals (fish included) and human. Antimicrobial susceptibility testing revealed that *A. hydrophila* plasmid can show antimicrobial resistance against ampicillin, chloramphenicol, streptomycin, sulfamonomethoxine, and tetracycline (Tipmongkolsilp et al., 2012). Recently, a multi-drug resistance (MDR) plasmid, pR148 was isolated from *A. hydrophila* from a tilapia *(Oreochromis niloticus)* farm. pR148 was a 165,906-bp circular plasmid containing 147 coding regions showing highest similarity to pNDM-1_Dok1, an MDR plasmid isolated from *Escherichia coli*, a human pathogen. pR148 was also very similar to other IncA/C plasmids isolated from

Salmonella enterica or *Escherichia coli* detected in humans, animals (fish included) and food. pR148 encodes a Tn*21* type transposon. This transposon provides resistance against β-lactams (bla_{OXA-10}), aminoglycosides (*aadA1*) and sulfonamides (*sul1*) in a class 1 integron. Downstream of the class 1 integron, there is a Tn1721-like transposon that provides tetracycline resistance through the *tetA/R* genes and chloramphenicols resistance (*catA2*) (Del Castillo et al. 2013). The bla_{OXA-10} and *aadA1* genes showed 100% similarity to those from the *Acinetobacter baumannii* AYE genome, a multidrug-resistant human pathogen that caused a nationwide outbreak in France in 2001 (Vallenet et al. 2008). These similarities of pR148 to a human pathogen-derived plasmid reveal that the plasmids were either transferred between different genera or that they are derived from a common origin. These observations illustrate the dangers of non-appropriate use of antibiotics in humans and in animals and the necessity of understanding how drug resistance determinants are disseminated and transferred (Del Castillo et al., 2013).

f) Biocontrol measures: Functional alternative against *Aeromonas* spp.

Recent developments have been conducted and are still in progress on the efficacy of the functional alternatives in aquaculture by exploiting their potential health benefits for fish. These alternatives may be used "in substitution or partial substitution" of antibiotics in breeding. Regarding to ANSES, report “alternatives to antibiotics” concerns only the control of bacterial diseases and excludes chemicals, growth promoter antibiotics, vaccines/autovaccines and antiparasitic agents (ANSES, 2018). Functional alternatives, as the feed additive products, have shown their promise yields to improve the zootechnical parameters like growth and health performances as well as immune response of the fish. Therefore, one of the sustainable practices recommended to enhance fish health, immunity and growth involves the administration of dietary immunostimulants (Magnadottir, 2006; 2010).

These functional additives can be derived from different natural sources. They are organic and eco-friendly to fish and environment. These functional feed additives included, prebiotics, probiotics, phytochemicals, etc. Most of these additives are added during feed preparation to improve quality of the feed and feeding efficiency of the fish as well as health and immunity performance of fish. These products can not only inhibit pathogens, but also regulate the host immune system. For example, immunomodulation by probiotics/ beneficial microorganism was considered as a community effort of the introduced microorganism to host enterocyte or intestinal absorptive cells. The host can detect whether the organism is pathogenic or not through pathogen pattern recognition receptors. To activate these recognition receptors, the microbial associated molecular patterns (MAMPs) like lipopolysaccharides, peptidoglycan, flagellin, and microbial nucleic acids which are present in both pathogenic and non-pathogenic microorganisms bind to pattern recognition receptors (PRRs) of innate immune system. This trigger intracellular signaling cascade, urging the release of specific cytokines and transmit signals to adjacent cells, or to exert antiviral, pro- or anti-inflammatory exercise effects (Figure 4) (Akhter et al., 2015).

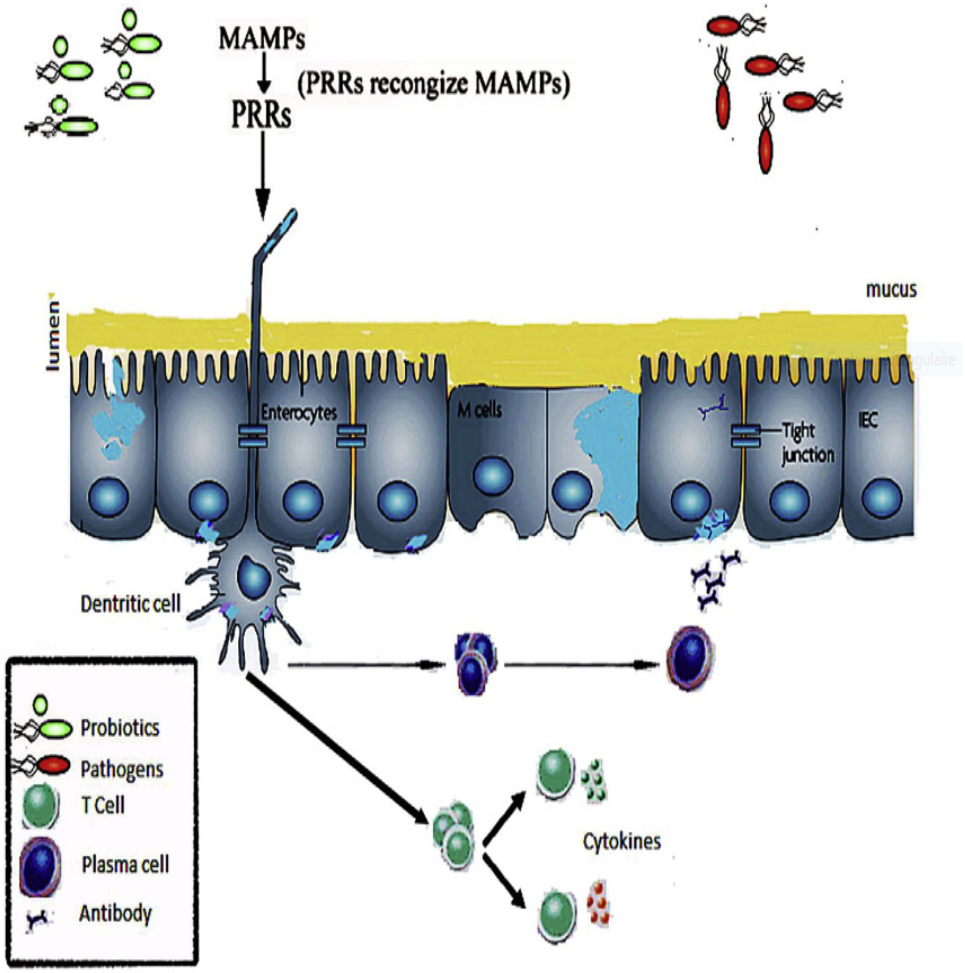

Figure 4. Probiotics showing the activity of host immunomodulation. Note: MAMPs / microbe associated molecular patters, PRRs / pathogen pattern recognition receptors, IEC / Intestinal epithelial cell (Akhter et al., 2015).

These functional feed additives can become an alternative for antibiotics to overcome the infectious diseases including *Aeromonas* spp. Therefore, the reduce of antibiotic resistance phenomenon as a global threat by applying the functional feed alternatives can be considered (Myers 2007; Ringø et al. 2010; Reverter et al. 2017; Bharati et al. 2019).

Previous published studies of the promising alternatives to antibiotics against *Aeromonas* spp. are presented in a bibliography analysis of this thesis project as a published review (Article N° 1). It focuses on protective efficacies and effects of the functional

alternatives such as probiotics, prebiotics, plants, essential oils, algae and phages on the immune system against the most frequent ubiquitous organism, *Aeromonas* spp., when delivered *in vivo* in the three major families of freshwater fish (salmonids, cyprinids and cichlids). The Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) checklist as guidelines was used in this study (PRISMA-P GROUP, 2015). The search was carried on by using the Web of Science Core Collection (until 09/09/19) with the keywords of fish, aquaculture, alternative, antibiotic, prebiotic, probiotic, synbiotic, essential oil, plant, algae, and phage. Studies conducted on saltwater or sea fish species, on crustaceans or seafood, environmental studies, *in vitro* studies and *in vivo* studies without *Aeromonas* spp. infectious challenge have been excluded. Furthermore, we assessed the references from cited articles and added them on the matter of relevance. Finally, 145 studies were selected out of 1434 publications according to functional alternative products in major species of freshwater fish for *Aeromonas* spp. *in vivo* infections (**Figure** 5).

The survey showed that the majority of studied cases of alternatives were carried on probiotics, plants, and prebiotics (54, 53 and 13 publications respectively). The other alternatives studied are synbiotics (mixture of prebiotics and probiotics) essential oils, algae, bacteriophage and other non-classified alternative families, such as mineral and nanoparticles. Alternative products were tested mainly on Nile tilapia (*Oreochromis niloticus*), rainbow trout (*Oncorhynchus mykiss*), rohu (*Labeo rohita*) and common carp (*Cyprinus carpio*). The other fish species studied were hybrid tilapia, red tilapia (*Oreochromis* spp.), catla (*Catla catla*), shabot (*Tor grypus*), shabout (*Barbus grypus*), Yoshitomi tilapia, javanes carp (*Puntius gonionotus*), crucian carp (*Carassius carassius*), allogynogenetic crucian carp (*Carassius auratus gibelio*), mrigal carp (*Cirrhinus cirrhosus* / *Cirrhinus mrigala*), grass carp (*Ctenopharyngodon idellus*), ningu (*Labeo victorianus*), brown trout (*Salmo trutta*) and brook trout (*Salvelinus fontinalis*). For each study, the information was collected by analyzing the

substance and source of the product and its application on fish species, whilst the main results on fish health such as survival rate and growth performance are indicated considering the experimental conditions. The potential mechanisms of action of the product such as the immunostimulant effect, the modification of the gut microbiota, the intensity of the histological lesions etc., were also recorded. In this manuscript, the bibliography research is presented in a literature review (Article 1: A review of functional feeds and the control of *Aeromonas* infections in freshwater fish).

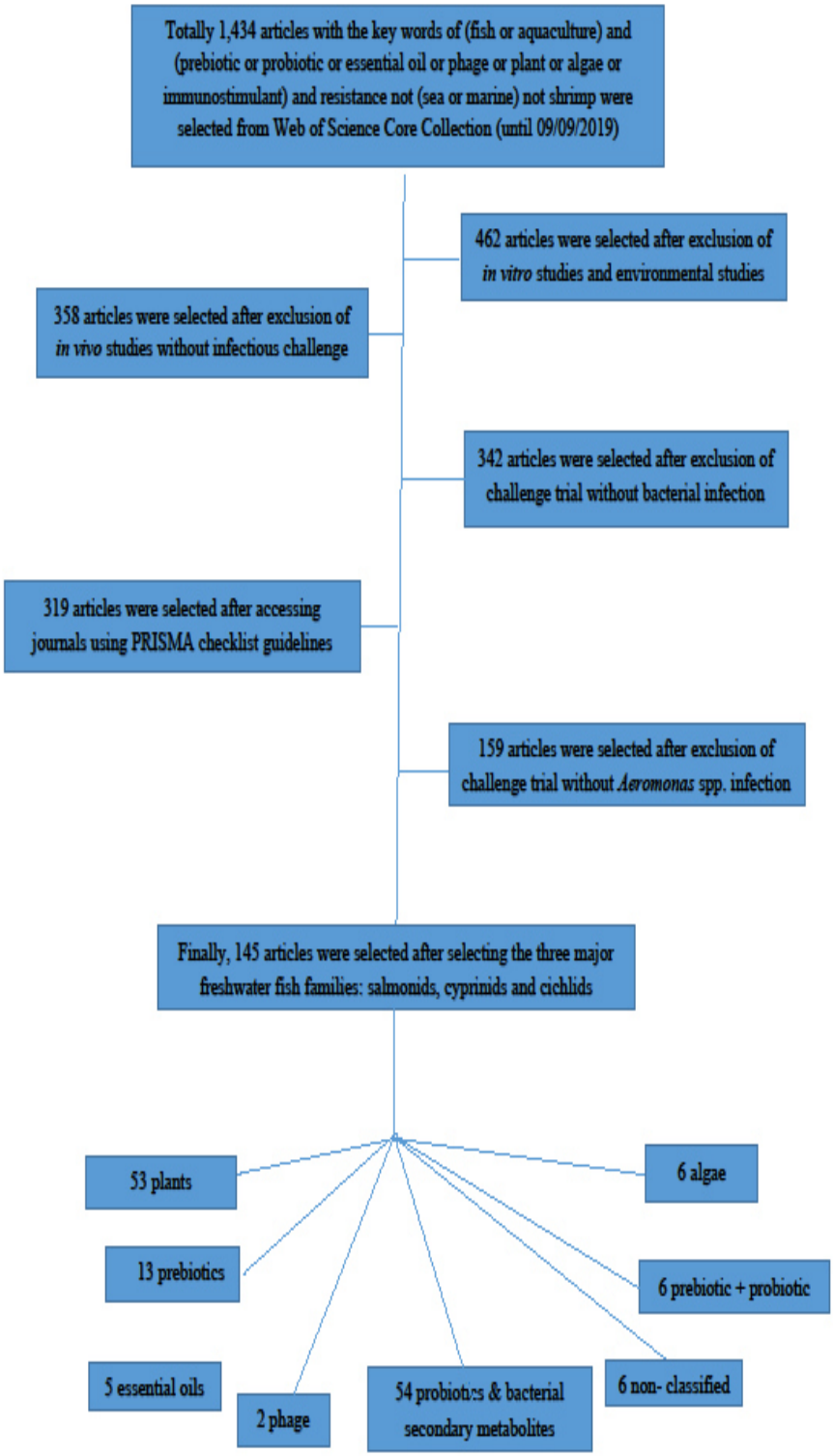

Figure 5. Selection strategy flow chart

g) Article N° 1: A review of functional feeds and the control of *Aeromonas* infections in freshwater fish

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A review of functional feeds and the control of *Aeromonas* infections in freshwater fish

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Abstract
To limit the use of antibiotics in farmed fish and their potential negative impacts on public health and the environment, an evaluation of "functional alternatives" is required. The availability of effective treatments to control fish diseases is one of the most significant challenges facing aquacultures and veterinarians to reduce consequences of antimicrobial resistance. This paper includes results from in vivo studies in major freshwater-farmed fish species (salmonids, cyprinids, and cichlids), focusing on the efficacy of functional alternatives against *Aeromonas* spp. infections. It also outlines the recent biocontrol advances and potential alternative treatments in aquaculture. Functional alternative products can increase the resistance against *Aeromonas* spp. particularly by increasing the immunocompetence of fish. Many diverse alternative products such as probiotics, prebiotics, plants, essential oils, algae phages, minerals, and nanoparticles have been tested, but the diversity of the experimental designs makes it difficult to compare the efficacy of the tested products. It suggests the standardization of investigations on functional feed products for each fish species against a specific pathogen. This review also recommends farm research on functional feed alternatives in natural conditions in order to evaluate the decrease of antibiotic consumption in fish farms.

Keywords *Aeromonas* · Infection · Functional feed alternatives · Antibiotic · Freshwater fish

Introduction

Aquaculture has become an economic and safe source of protein for human consumption around the world. Global food fish production has been increasing at an average annual rate of 6.6% since 1995 (FAO 2017) and reached 80 million tons in 2016 (FAO 2018). The production of Nile tilapia, salmon, and other freshwater species has led to a significant growth

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in annual per capita consumption, approximately from 1.5 kg in 1961 to 7.8 kg in 2015 (FAO 2018).

In intensive aquaculture, farmed fish can be affected by various infectious diseases worsened by stress factors which may lead to a decrease of fish resistance. Antibiotic prescriptions may be needed to avoid impaired growth performance and significant economic losses due to bacterial disease (Romero et al. 2012). In aquaculture, antibiotics were mainly added to feed supplement into water, resulting in the discharge of drug and their metabolites into the wastewater (Romero et al. 2012). Even when the antibiotic concentrations are well below the minimum inhibitory concentration, the prolonged presence of antibiotics in water, combined with high numbers of bacteria in the polybacterial matrices as the pond, sediment, or biofilm, may put selective pressure on bacterial populations and allow the exchange of antimicrobial resistance genes between bacteria (Baquero et al. 2008; Muziasari et al. 2016; Watts et al. 2017). The passage of antimicrobial residue, antimicrobial-resistant bacteria, and resistance genes from aquatic animals and their environment to terrestrial livestock and humans presents the increasing risk of a widespread emergence of drug-resistant pathogens (Rasul and Majumdar 2017; Santos and Ramos 2018).

Common infections in freshwater fish are caused by the genus *Aeromonas*. These bacteria are common inhabitants of aquatic animals (fish and shellfish) and aquatic environments such as freshwater, estuarine waters, marine waters, and sediments (Swann and White 1989). In fish farms, the two most frequently encountered species are *Aeromonas hydrophila* and *Aeromonas salmonicida*. *A. salmonicida* subsp. *salmonicida* mainly affects salmonids and is the causative agent of furunculosis. This disease is responsible for severe economic losses by haemorrhagic septicaemia in the acute form and by fish depreciation due to the development of boils in the muscles in the chronic form (Austin and Austin 2012). *A. hydrophila* is a ubiquitous bacterium which is commonly isolated from freshwater ponds and which is a normal inhabitant of the gastrointestinal tract of aquatic animals. It may also cause a disease in fish known as "haemorrhagic septicaemia" (Randy White 1991). *A. hydrophila* is also a zoonotic pathogen that infects humans via foodborne infections or through aquaculture facilities and is a public health hazard (Okocha et al. 2018). *Aeromonas* are opportunistic environmental pathogens of animals and humans. Genotyping analyses and antibiotic resistance profiles of the two main species *A. salmonicida* subsp. *salmonicida* and *A. hydrophila* demonstrated the presence of multidrug resistance plasmids with a high level of interspecies transfer, including human bacteria (Del Castillo et al. 2013; Vincent et al. 2014). *Aeromonas* may persist being attached to biofilms on biotic or abiotic surfaces, and the presence of these bacteria with *E. coli* in polybacterial mixed biofilms promotes the exchange and dissemination of antimicrobial resistance genes (Talagrand-Reboul et al. 2017). Limiting the emergence of antibioresistant *Aeromonas* and the transfer of their resistance genes by decreasing the antibiotic uses in aquaculture is therefore an issue for fish and public health.

To decrease the use of antibiotics, alternative strategies have been developed to improve fish health and aquaculture systems while reducing the potential for the spread of antimicrobial resistance. These include: (i) vaccination, by considering the difficulty of its application and its controversial effectiveness in fish populations (Gudmundsdóttir and Björnsdóttir 2007; Plant and LaPatra 2011), (ii) immune stimulation by using products derived from plants, bacteria or algae with effects on the microbiome and the immunity of the farmed host, (iii) phage therapy, and (iv) biosecurity approaches such as disinfection of water system (Watts et al. 2017).

In this review, we summarize the promising functional feed alternatives, such as probiotics, prebiotics, plants, essential oils, algae, and phages to reduce antibiotics consumption in

aquaculture. The focus of this paper is mainly on their protective efficacies against the most frequent ubiquitous organism (*Aeromonas* spp.) when delivered in vivo in the three major families of freshwater fish, salmonids, cyprinids, and cichlids.

Methodology analysis to evaluate alternative products against *Aeromonas* spp. infection

The survey showed that the majority of studied cases of alternatives were carried on probiotics, plants, and prebiotics, respectively. The other alternatives studied are synbiotic (mixture of prebiotics and probiotics) essential oils, algae, bacteriophage, and other non-classified alternative families, like as mineral and nanoparticles. Alternative products were tested mainly on Nile tilapia (*Oreochromis niloticus*), rainbow trout (*Oncorhynchus mykiss*), rohu (*Labeo rohita*), and common carp (*Cyprinus carpio*).

Mostly, alternative products were tested for their preventive and protective effects. Some studies have also evaluated the curative effect of alternatives like probiotic or triherbal extract-enriched diets (Harikrishnan et al. 2010), aqueous methanolic extracts of tetra (*Cotinus coggygria*) (Bilen and Elbeshti 2019), and therapeutic phages (Imbeault et al. 2006; Kim et al. 2015). All investigations presented a comparative study in the present review which in the test groups, fish were treated with the alternative candidates and in the control/negative group, fish were not treated. Moreover, alternative products efficacies were sometimes compared with antibiotics (oxytetracycline) (Park et al. 2017; Won et al. 2017; Lee et al. 2016b).

To evaluate the preventive efficacy of functional feed alternative against *Aeromonas* spp. infections, pathogen was injected by the intraperitoneal (IP) route but fish were also exposed to *Aeromonas* spp. by immersion (Bandyopadhyay and Das Mohapatra 2009; Liu et al. 2013b), by cohabitation (Irianto and Austin 2003; Hoque et al. 2018; Menanteau-Ledouble et al. 2017), or by oral intubation (Ngamkala et al. 2010; Dong et al. 2018). *A. hydrophila* was mainly used to infect freshwater fish, with the exception of rainbow trout mainly infected with *A. salmonicida*. Various infection doses were investigated in challenge experiments that depended mainly on bacterial strain, fish species, administration routes and the survival rate required by the authors. Indeed, different infectious doses could lead to a same RPS. For example, *A. hydrophila* infection dose at 10^3 and 10^9 CFU ml^{-1} injected by IP route in Mozambique tilapia induced a RPS of 10% (Rajeswari et al. 2016; Suguna et al. 2014). In contrast, a similar infectious dose could lead to very different RPS (*A. salmonicida* doses at 2.4 10^7 and 2.10^7 CFU ml^{-1} induced a RPS of 80 and 12%, respectively, in rainbow trout (Kim and Austin 2006; Park et al. 2017). The post-infection day duration after *Aeromonas* challenge should be also taken to account for the mortality rate records which might vary from hours to weeks, depending on the investigation conditions.

Probiotics

In an expert consensus document, the definition of a probiotic has been recently clarified as: "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al. 2014). In the interest of probiotics use in aquaculture, it was proposed to extend the definition to "living microbial additives that benefit the health of hydrobionts and therefore increase productivity" (Martínez Cruz et al. 2012). In aquatic species, the microbial community in gastrointestinal tract depends on the external environment including water and

feed. Potential probiotic bacteria need to tolerate the temperature of pond water in addition to the bile salts and low pH detected in fish intestines. Potential probiotics must also improve feed utilization and growth by considering their viability under processing conditions when added to fish feed (Irianto and Austin 2002; Lacroix and Yildirim 2007). Moreover, other essential properties are defined relative to safety as a non-pathogenic microorganism and to the absence of plasmid-encoded antibiotic resistance (Martínez Cruz et al. 2012). The mechanistic basis and beneficial activities of probiotics previously were explained as being due to a modification of intestinal microbiota, production of antibacterial or antitoxin substances (bacteriocins and organic acids), modulation of the immune system and competition with pathogens for nutrients, and adhesion to intestines (Myers 2007).

The efficacy of potential probiotic bacteria has been extensively studied in which lactic acid bacteria (*Lactobacillus* spp., *Lactococcus* spp.) and *Bacillus* spp. were the most commonly used probiotic (Table 1). *Saccharomyces cerevisiae* yeasts have also a great promise as a potential probiotic substance (Abdel-Tawwab et al. 2008; Abdel-Tawwab 2012; Ran et al. 2015, 2016; Abass et al. 2018).

Among lactic acid bacteria and *Bacillus* spp., a large diversity of bacterial species and strains were evaluated. For example, for *Bacillus* spp., 11 strains belonging to 7 species have been investigated in this review (Table 1). Potential probiotic bacteria were provided from various sources, either bacteria isolated from fish in a local laboratory, commercial strains that were directly purchased like feed additives as *Lactococcus lactis* (Suprayudi et al. 2017) or even, final commercial product as "Organic green" composed of *Lactobacillus acidophilus*, *Bacillus subtilis*, *Saccharomyces*, and *Aspergillus oryzae* (Aly et al. 2008).

Probiotic products were administered orally as a feed supplement except some cellular components of probiotic bacteria which were injected intraperitoneally (Ramesh et al. 2015; Giri et al. 2015a, b, c; Ramesh and Souissi 2018). They were administered in a very wide range of dosages and durations, from milligrams to grams per kilogramg of feed, and for days to months before the infectious challenge. Generally, for *S. cerevisiae* yeasts, the optimal probiotic dose was proposed to be 1 to 2 g kg^{-1} diet from 56 to 84 days to protect Nile tilapia (*O. niloticus*) against *A. hydrophila* infections (Abdel-Tawwab et al. 2008; Abdel-Tawwab 2012; Ran et al. 2015, 2016) but increased to 70 g kg^{-1} under stress condition (Abass et al. 2018). For potential probiotic bacteria, the optimal dose varied between 10^7 and 10^{10} cfu g^{-1} diet for 2 to 3 months, depending on the species and strain of probiotic and the fish species (Table 1).

The increase of the survival rate and protection effect in the probiotic feeding group compared with the control group was a result of the preventive effect of these probiotics against *Aeromonas* spp. However, the amplitude of the survival rate between the probiotic and control groups varied greatly and depended on the probiotic species, the feeding dosages and durations, and the experimental infection (dose of bacteria, administration route, duration) (Table 1). In *Catla catla*, the effect of *B. circulans* depended on the probiotic dosage: the survival rate was 96.7% with 2×10^5 CFU 100 g^{-1} feed whether 40.0% with 2×10^6 CFU 100 g^{-1} feed and 6.7% in the control group (Bandyopadhyay and Das Mohapatra 2009). Besides dose-dependent effects, the duration of feeding fish with probiotics seemed to be an important matter to achieve a higher protection. For example, the relative level of protection against *A. hydrophila* in Nile tilapia for each probiotic agent *Bacillus pumilus* or mixture of *Lactobacillus acidophilus*, *Bacillus subtilis*, *Saccharomyces*, and *Aspergillus oryzae* showed to be higher at the end of the 2nd month than at the end of the 1st month of the feeding trial (Aly et al. 2008).

Table 1 Summary of *in vivo* studies in three freshwater fish species for probiotics and bacterial secondary metabolites or enzymes

Substances	Infectious challenge Species – dose - route	Fish species	Substances administration Dose - Route- Duration	(D): SR in test groups vs control	Reference
Saccharomyces cerevisiae yeast	*Aeromonas hydrophila* 2×10^8 cfu ml^{-1}/fish-IP	Nile tilapia *(Oreochromis niloticus)*	30-70 g/kg diet-PO-84d optimal dose: 70g/kg diet under stress condition	D14: 97 % vs 87%	Abass et al. 2018
	Aeromonas hydrophila 5×10^5 cell ml^{-1}/fish-IP		0.50 - 5.0 g/kg diet -PO-84d optimal dose: 2 g/kg diet	D10: 35-55 % vs 20%	Abdel-Tawwab 2012
	Aeromonas hydrophila 5×10^5 cell ml^{-1}/fish-IP		0.25 - 5.0 g/kg diet -PO-84d optimal dose: 1 g/kg diet	D10: 35-55 % vs 25%	Abdel-Tawwab et al. 2008
commercial preparation of live & heat-inactive *Saccharomyces cerevisiae* yeast	*Aeromonas hydrophila* 10^8 cell ml^{-1}/fish-IP		1 g/kg diet (107 cfu/g diet)-PO-56d optimal preparation: live yeast	NS	Ran et al. 2015
commercial preparation of live & heat-inactive *Saccharomyces cerevisiae* yeast	*Aeromonas hydrophila* 10^8 cell ml^{-1}/fish-IP		1 g/kg diet -PO-56d	NS	Ran et al. 2016
commercial preparation of yeast	*Aeromonas sobria* 1.5×10^7 cell ml^{-1}/fish-IP		50 - 250 g/kg diet -PO-30d optimal dose: 250 g/kg diet	D14: 55-70% vs 25%	Reda et al. 2018
Bacillus licheniformis Dahb1 (HM235407.1)	*Aeromonas hydrophila* 10^7 cell ml^{-1}/fish-IP	Mozambique tilapia (*Oreochromis mossambicus*)	10^5,10^7 cfu/g diet-PO-28d optimal dose: 10^7 cfu/g diet	D10: 65-55% vs 14%	Gobi et al. 2018
B. licheniformis KADR5 ; *B. pumilus* KADR6 (separately): live cell (lv) & subcellular components (cp) isolated from rohu gut	*Aeromonas hydrophila* 10^5 cells ml^{-1}/fish-IP	rohu (*Labeo rohita*)	10^8 cfu/g diet -IP(cp)/PO(lv)-14d	D10: 60-80% (cp); 67-77% (lv) vs 20%	Ramesh et al. 2015
B. subtilis KADR1: live cell (lv)& subcellular components (cp) isolated from rohu gut			10^6- 10^{10} cfu/g diet –PO (lv)/IP (cp)-28d optimal dose:10^8 cfu/g diet	D10: 39- -80%(lv);77%(cp) vs18%	Ramesh and Souissi 2018
B. aerophilus KADR3 isolated from rohu gut			10^7 - 10^9 cfu/g diet-PO- 42d optimal dose: 10^8 cfu/g diet	D10: 41-72% vs 20%	Ramesh et al. 2017
B. subtilis VSG, Pseudomonas aeruginosa VSG2; *Lactobacillus*	*Aeromonas hydrophila* 10^7 cell ml^{-1}/fish-IP		0.1 mg (cp)/fish-IP (21d) optimal species :Lp & Pa	D21: 50-83 vs 20 %	Giri et al. 2015a

Table 1 (continued)

Substances	Infectious challenge Species – dose - route	Fish species	Substances administration Dose - Route- Duration	(D): SR in test groups vs control	Reference
plantarum VSG3(cp)(separately) from rohu gut					
Bacillus spp. MVF1 (KP256503) isolated from freshwater fish (*Mystus vittatus*) gut			10^5 10^9 cfu/g diet-PO-70d optimal dose: $10^{7\text{-}9}$ cfu/g diet	D10: 30-75%	Nandi et al. 2017
B. amyloliquefaciens CCF7	*Aeromonas hydrophila* 10^7 cell ml^{-1}/fish-IP		10^5 - 10^9 cfu/g diet-PO-70d optimal dose: $10^{7\text{-}9}$ cfu/g diet	NS	Nandi et al. 2018
B. subtilis isolated from *Cirrhinus mrigala* gut	*Aeromonas hydrophila* 10^6 cell ml^{-1}/fish-IP		0.5×10^7- 1.5×10^7 cfu/g diet-PO-15d	D3: not mentioned	Kumar et al. 2008
B. circulans PB7 isolated from the intestine of Catla	*Aeromonas hydrophila* 10^5&10^7cfu/ml-Immersion 1h	Catla (*Catla* catla)	2×10^4 - 2×10^6 cell/100g diet -PO- 60d optimal dose: 2×10^5 cell/100g diet	D10 :40-96% vs 6%	Bandyopadhyay and Das Mohapatra, 2009
B. subtilis	*Aeromonas hydrophila* 8 × 10^9cell ml^{-1}/fish-IP	grass carp (*Ctenopharyngodon idellus*)	2.4×10^7 cfu/g diet-PO-42d	NS	Tang et al. 2018
B. coagulans MTCC9872; *B. licheniformis* MTCC 6824;*Paenibacillus polymyxa* MTCC 122 (separately)	*Aeromonas hydrophila* 10^6 cfu ml^{-1}/fish-IP	common carp *(Cyprinus carpio)*	10^9 cfu/g diet -PO-80 d optimal species: *Paenibacillus polymyxa*	D5 :36-50% vs 20%	Gupta et al. 2014
Bacillus velezensis V4 isolated from marine recirculation aquaculture systems	*Aeromonas salmonicida* 10^4 cfu ml^{-1}-IP	rainbow trout (*Oncorhynchus mykiss*)	10 - 50 g/kg diet -PO-60d	99-93% vs 91%	Gao et al. 2017
B. subtilis (Bs) ; *B. licheniformis* (Bl) & *(Bs + Bl)*	*Aeromonas salmonicida* 2×10^7 cfu ml^{-1}/fish-IP		5 g/kg diet-PO- 56 d	D15: 50% vs 12% oxytetracycline: 55%	Park et al. 2017
B. subtilis AB1 isolated from rainbow gut	*Aeromonas* sp. ABE1 2.3×10^6 cfu ml^{-1}/fish-IP		10^4 - 10^9 cfu/g diet; -PO-14d optimal dose: 10^7 cfu/g diet in all forms	65-100% vs 5-15 %	Newaj-Fyzul et al. 2007
Lactobacillus plantarum isolated from Persian sturgeon gut	*Aeromonas hydrophila* 2.1×10^7 cfu ml^{-1}/fish-IP	common carp *(Cyprinus carpio)*	0.56×10^6 cfu/g diet (0.3g/kg diet) - 1.2×10^6cfu (0.7g)-PO- 80d optimal dose: 1.2×10^6 & 0.9×10^6 cfu/g diet	D14: 35-50% vs 25 %	Soltani et al. 2017

Table 1 (continued)

Substances	Infectious challenge Species – dose - route	Fish species	Substances administration Dose - Route- Duration	(D): SR in test groups vs control	Reference
Lactobacillus plantarum strains	*Aeromonas hydrophila* 10^8 cfu mL^{-1}/fish-IP		10^8 cfu/g diet -PO-14d	D14: 35% vs 15 %	Kazuń et al. 2018
Lactobacillus rhamnosus or L. sporogenes commercial product	*Aeromonas hydrophila* 1.8 × 10^6cells ml^{-1}/fish-IP		1 g/kg diet	D30: 65-55% vs 15%	Harikrishnan et al. 2010
Lactococcus lactisQ-8, Lactococcus lactisQ-9,and Lactococcus lactisZ-2	*Aeromonas hydrophila* 5 × 10^6cfu ml^{-1}/fish-IP		5 × 10^8 cfu/g diet -PO-56d	48h: 88-90% vs 82%	Feng et al. 2019
Lactobacillus plantarum VSG3 isolated from rohu gut	*Aeromonas hydrophila* 10^7 cfu ml^{-1}/fish-IP	rohu (*Labeo rohita*)	10^6 - 10^{10}cfu/g diet -PO-60d optimal dose: 10^8 cfu/g diet	D10: 37-77% vs 14%	Giri et al. 2013
Lactobacillus plantarum SM16 & SM33, *L. fermentum* SM51, *L. brevis* SM56, *Pediococcus pentosaceus* SM64 (together) isolated from rohu gut	*Aeromonas hydrophila* 10^7 cfu ml^{-1}/fish-IP		10^9 cfu/g diet-PO- 30d	D30: 90%,D50:60 %, D70:40% vs 30%	Maji et al. 2017
Lactobacillus plantarum; L. delbrueckii subsp. *bulgaricus* isolated from Shabout *(Barbus grypus)* gut *; L. casei PTCC 1608* as a commercial positive control (separately)	*Aeromonas hydrophila* 3.7 × 10^8cfu mL^{-1}/fish-IP	Shabout (*Barbus grypus*)	5×10^7 cfu/g diet-PO- 60d optimal species: autochthonous probiotics	D15: 63-76% vs 30%	Mohammadian et al. 2016
Lactobacillus casei	*Aeromonas hydrophila*	Shabout (*Tor grypus*)	5×10^6 - 5×10^8 cfu/g diet-PO- 60d optimal dose: $5\times10^{6\text{-}7}$cfu/g diet	NS	Mohammadian et al. 2019
Lactobacillus plantarum; L. delbrueckii subsp. *bulgaricus* isolated from Shabot *(Barbus grypus)* gut *L. casei PTCC 1608* as a commercial positive control (separately)	*Aeromonas hydrophila* 5 × 10^8 cfu mL−1/fish-IP		5×10^7 cfu/g diet-PO- 60d optimal species: autochthonous probiotics	NS	Mohammadian et al. 2018
Lactococcus lactis D1813 (commercial product)	*Aeromonas hydrophila* 10^6 cells ml^{-1}/fish-IP	Nile tilapia *(Oreochromis niloticus)*	0.25 - 1.0 g /kg diet -PO-154d optimal dose: 0.5 g/kg diet	D14: 85-100% vs 55	Suprayudi et al. 2017
Lactobacillus rhamnosus GG	*Aeromonas hydrophila* 0.5 ml bacteria pellets/fish-oral intubation		10^{10} cfu/g diet -PO-14 days	D21: 95% vs 85	Ngamkala et al. 2010

Table 1 (continued)

Substances	Infectious challenge Species – dose - route	Fish species	Substances administration Dose - Route- Duration	(D): SR in test groups vs control	Reference
marine *Lactobacillus plantarum* AH 78	*Aeromonas hydrophila* 5 × 10^7 cells ml^{-1}/fish-IP		5 - 20 g/kg diet (3.4 ×10^8 - 1.3 × 10^9 cfu/g diet)-PO-40d	D14: 66-87% vs 20	Hamdan et al. 2016
Lactobacillus brevis JCM 1170(Lb) *; L. acidophilus* JCM 1132(La) (separately)	*Aeromonas hydrophila* 10^8 cell/g ; 14-d immersion	hybrid tilapia (*O.niloticus*×*Oreochromis aureus*)	10^5 - 10^9 (La) or (Lb)/g diet -PO- 35d optimal dose and species :10^9 (Lb) cfu/g diet	D14: 15-50% vs 10 %	Liu et al, 2013b
Lactococcus lactis 16-7, isolated from crucian carp gut	*Aeromonas hydrophila* 4 × 10^8 cfu mL^{-1}/fish-*orogastric intubation*	Crucian carp (*Carassius carassius*)	10^9 cfu/g diet-PO- 42d	NS	Dong et al. 2018
Lactococcus lactis CLFP 100 and *Leuconostoc mesenteroides* CLFP 196	*Aeromonas salmonicida*	Brown trout (*Salmo trutta*)	Not available	Significant	Balcázar et al. 2009
Lactobacillus acidophilus (MTCC 10307)	*Aeromonas hydrophila* 4× 10^6cell ml^{-1}-IP	catla (*Catla catla*)	10^7 cfu/fish -IP	NS: induced Catla thymus macrophage cells	Patel et al. 2016
Enterococcus faecalis	*Aeromonas hydrophila* 10^7 cfu ml^{-1}/fish-IP	javanese carp (*Puntius gonionotus*)	10^7cell /g diet-PO-15d	48h: 53% vs 0 %	Allameh et al. 2017
Paenibacillus ehimensis NPUST1 isolated from water samples of tilapia culture pools	*Aeromonas hydrophila* 10^6 cfu ml^{-1}-IP	Nile tilapia *(Oreochromis niloticus)*	10^6,10^7 cfu/g diet-PO-60d optimal dose: 10^7 cfu/g diet	D7: 40-59% vs 20%	Chen et al. 2019
Rummelii bacillus stabekisii	*Aeromonas hydrophila* 10^{5-6} cfu ml^{-1}-IP		10^6,10^7 cfu/g diet-PO-60d optimal dose: 10^7 cfu/g diet	D7: 56-60% vs 33%	Tan et al. 2019
Carnobacterium maltaromaticum B26; C. divergens B33(separately) isolated from Rainbow trout gut	*Aeromonas salmonicida* 2.4 × 10^7 cfu ml^{-1}-IP	rainbow trout (*Oncorhynchus mykiss*)	10^7cfu/g diet-PO-14d	D14: 80% vs 20%	Kim and Austin 2006
Dead cells preparation of unidentified Gram-positive *coccus* A1-6, *V. fluvialis* A3-47S, *Aeromonas hydrophila* A3-51 and *Carnobacterium BA211* separately	*Aeromonas salmonicida* 10^6 cfu ml^{-1}-IP & cohabitation		10^7 cfu/g diet -PO-14d	92-100% vs 40 %	Irianto and Austin 2003

Table 1 (continued)

Substances	Infectious challenge Species – dose - route	Fish species	Substances administration Dose - Route- Duration	(D): SR in test groups vs control	Reference
Aeromonas sobria (GC2) and *Brochothrix thermosphacta* (BA211)	*Aeromonas bestiarum* 10^5 cfu ml^{-1}-IP at the base of the dorsal fin		GC2: 10^8; BA211:10^{10} cfu/g diet-PO-14d optimal species: *BA211*	D14: 76-88% vs 22%	Pieters et al. 2008
Pseudomonas aeruginosa VSG-2 isolated from rohu gut	*Aeromonas hydrophila* 10^7 cell ml^{-1}/fish-IP	rohu *(Labeo rohita)*	10^5 - 10^9 cfu/g diet -PO-60 days optimal dose:10^7 cfu/g diet	D10: 34-66% vs 11%	Giri et al. 2012
Pseudomonas aeruginosa FARP72 isolated from the skin mucus of freshwater catfish *Clarias batrachus*	*Aeromonas hydrophila* 1.5 × 10^5 cfu ml^{-1}-cohabitation		10^7 cfu ml^- with or without *A. hydrophila*-cohabitation-15 min	D10: 64-77% vs 25%	Hoque et al. 2018
mixture of *Saccharomyces cerevisiae*(Sc), *Bacillus subtilis* (Bs) & *Aspergillus oryzae*(Ao)	*Aeromonas hydrophila* 2 ×10^6 cfu ml^{-1}-IP	Nile tilapia *(Oreochromis niloticus)*	5g/kg((Bs):1.5×10^9,(Sc):10^9,(Ao):2×10^9) or 10 g/kg ((Bs):3×10^9,(Sc):2×10^9,(-Ao):4×10^9cfu/g) diet-PO-28d	D21: 22-24 % vs 6 %	Iwashita et al. 2015
Bacillus pumilus (Bp); commercial product (1kg: 10^{11} cells of *Lactobacillus acidophilus, Bacillus subtilis, Saccharomyces* and *Aspergillus oryzae)*	*Aeromonas hydrophila* 10^8 cell ml^{-1}-IP		10^6,10^{12}(Bp)cell/g diet; commercial product at 1&2 g/kg diet- PO- 30& 60d-	D56(after 30&60 feeding days):74-84% vs 68 %	Aly et al. 2008
Saccharomyces cerevisiae(Sc), *Bacillus subtilis* (Bs) and/or *Lactococcus lactis* (Ll)	*Aeromonas hydrophila* 10^6 cfu ml^{-1}-IP	rohu (*Labeo rohita*)	10^8 cfu/g diet -PO- 60d optimal combination: Bs+Ll+Sc	D7:60-85 vs 40 %	Mohapatra et al. 2014
B. subtilisVSG1(Bs), *L.plantarum VSG3*(Lp); *and/or P.aeruginosaVSG2* (Pa*)* isolated from rohu gut	*Aeromonas hydrophila* 10^7 cell ml^{-1}-IP		10^8 cfu/g diet- PO- 60d-optimal combination: Bs+ Lp+ Pa	D15: 46-86% Vs 13	Giri et al. 2014
1-Deoxynojirimycin (DNJ) from *Bacillus subtilis*	*Aeromonas hydrophila* 10^8cfu ml^{-1}-IP	*Yoshitomi tilapia* (*Oreochromis* Spp.*)*	DNJ: -5 mg/L incorporated into the diet – viable cells: 0.2×10^{10} - 4.23×10^{10} cfu/kg diet -PO- 56 d optimal dose: viable cells : 2.5 ×10^{10} cfu/kg diet or more; DNJ:5 mg/L	Viable cells D7: 26-60% vs 24% DNJ D7:14-49 % vs 12%	Tang et al. 2017

Table 1 (continued)

Substances	Infectious challenge Species – dose - route	Fish species	Substances administration Dose - Route- Duration	(D): SR in test groups vs control	Reference
Active Cyclo-(Phe–Tyr) or Cyclo-(Phe–Gly) from *Bacillus Licheniformis* XY-52	*Aeromonas hydrophila* 3.5 × 10^7cfu ml^{-1}-IP	common carp *(Cyprinus carpio)*	5 - 20 g/kg diet-PO-21d optimal dose :20g/kg diet	D15: 69% vs 32%	Chen et al. 2015
Phospholipopeptide biosurfactant from *Staphylococcus hominis*	*Aeromonas hydrophila* 10^9 cell ml^{-1}-IP(on day 8)	Mozambique tilapia (*Oreochromis mossambicus*)	2 - 200 mg kg^{-1} body weight-IP optimal dose :200 mg kg^{-1} BW	D7: 40-70% vs 10%	Rajeswari et al. 2016
poly-b hydroxybutyrate ehydroxyvalerate from *Bacillus thuringiensis* B.t.A102	*Aeromonas hydrophila* 10^3 cell ml^{-1}-IP		10-50 g/kg diet-PO-28d optimal dose :50g/kg diet	D14: 25-62% vs 10%	Suguna et al. 2014
Bacillus amyloliquefaciens or xylanase-expressing *B. amyloliquefaciens R8*	*Aeromonas hydrophila* 2 × 10^6 cfu ml^{-1}-IP	Nile tilapia (*Oreochromis niloticus)*	10^6,10^7 cfu/g diet-PO-60d optimal dose: 10^7 cfu/g diet	D7: 40-59% vs 20%	Chen et al. 2019
Lactobacillus plantarum strain JCM1149 and/or AHL lactonase AIO6	*Aeromonas hydrophila* 10^5cfu ml^{-1}-immersion	tilapia (*Oreochromis niloticus*♀ × *O. aureus* ♂)	10^8 cfu/g diet-PO-14d	NS	Liu et al. 2016

Note: *NS*: not studied, *PO*: oral administration; *IP*: intraperitoneal injection; *d*: days of treatment; *SR*: Survival rate; *D*: day post- infection

Due to the influence of many different factors on experimental results, it is difficult to compare the preventive effect of the different probiotics tested against *Aeromonas* spp. infection. However, some publications compared several probiotics under the same experimental conditions. *P. polymyxa MTCC122* seemed to have a better protective effect against *A. hydrophila* than *B. coagulans MTCC9872* or *Bacillus licheniformis MTCC6824* in common carp (Gupta et al. 2014). Similarly, *Lactobacillus brevis JCM1170* had a better efficacy than *L. acidophilus JCM1132* against *A. hydrophila* in tilapia (Liu et al. 2013b). Furthermore, it has been demonstrated that the incorporation of multispecies probiotics of *Saccharomyces cerevisiae*, *B. subtilis*, and *Lactococcus lactis* (Mohapatra et al. 2014) or *B. subtilis*, *L. plantarum*, and *P. aeruginosa* (Giri et al. 2014) improves health status more effectively than the incorporation of a monospecies probiotic in the diet.

The preventive effect of probiotics against *Aeromonas* spp. could be explained in part by their immunostimulant effect. *Paenibacillus polymyxa* had a better immunostimulant effect than *Bacillus coagulans* MTCC9872 or *Bacillus licheniformis* MTCC 6824, which could explain the better protective efficacy of *P. polymyxa* against *A. hydrophila* (Gupta et al. 2014). However, in contrast with the survival rates, a combination of several probiotics did not seem to significantly increase the immunostimulant effect compared with a single probiotic (Park et al. 2017; Aly et al. 2008).

The duration of time that fish are fed probiotics seemed to be also an important factor on influencing the immunological parameters in fish. Several immunological parameters measured in mucus and serum were improved after 28 days but not after 14 days of *B. licheniformis* Dahb1 feeding (Gobi et al. 2018). Similarly, administering *Bacillus aerophilus* KADR3 over a 6-week period resulted in a slightly higher immunostimulant effect than over a three-week period (Ramesh et al. 2017). However, some studies have concluded that immunostimulation can be observed after a 30-day period of probiotic feeding, which is then followed by a declining trend (Giri et al. 2012, 2013, 2014; Mohammadian et al. 2016).

In addition to an immunostimulant effect, the administration of probiotics might protect against tissue lesions induced by *Aeromonas*. Histological analysis demonstrated that the severity of lesions in intestines and gills was less in rohu fish (*L. rohita*) fed with *B. subtilis*, *L. lactis*, and *S. cerevisiae* after the *A. hydrophila* challenge (Mohapatra et al. 2014). In addition, the intestines of Nile tilapia (*O. niloticus*) exposed orally to *L. rhamnosus GG* showed an increased inflammatory cell infiltration and reduced intestinal damages from *A. hydrophila* (Ngamkala et al. 2010). *L. lactis* 16-7 could also reduce intestinal mucosal barrier damage and inflammation induced by *A. hydrophila* by antagonizing the colonization of *A. hydrophila* in crucian carp intestine (Dong et al. 2018). Probiotics could also fortify the intestinal structure. Live baker's *S. cerevisiae* yeast and *Lactobacillus plantarum* AH 78 increased microvilli length of fish intestine (Ran et al. 2015, 2016; Hamdan et al. 2016) and *L. plantarum* JCM1149 and AHL lactonase enzyme had a synergistic effect on the microvilli density (Liu et al. 2016).

Some studies have found that probiotics could also modify freshwater fish microbiota (Carnevali et al. 2017; Akhter et al. 2015; Dimitroglou et al. 2011). Dietary administration of the grass carp (*C. idella*) with *Shewanella xiamenensis* A-1, *Aeromonas veronii* A-7, and *Bacillus subtilis* for 28 days or Nile tilapia with *Rummeliibacillus stabekisii* for 8 weeks, induced benefic alteration of intestinal microbiota by increasing the abundance of *Cetobacterium* genus with potential immunity function, by reducing the abundance of the potential pathogenic bacteria and by promoting the reproduction of potential probiotics (Hao et al. 2017; Tan et al. 2019). In contrast, feed supplementation by either heat-inactivated or live

commercial preparation of the baker's yeast *S. cerevisiae* did not influence Nile tilapia (*O. niloticus*) gut microbiota markedly (Ran et al. 2016).

In addition, probiotics or their secondary metabolites might increase the health status of fish by increasing feed conversion and growth performance (Table 1). Among the different studies analyzed in this review which resulted to higher growth performance after probiotic feeding, there is only one report mentioned that administration of a *S. cerevisiae*, *Bacillus subtilis*, and *Aspergillus oryzae* mixture had no significant effect on growth rates while feed conversion was increased (Iwashita et al. 2015). Probiotic treatments can also have influence on body or organ content. A higher level of proteins and lipids was found in the carcass of fish fed with *Bacillus circulans PB7* (Bandyopadhyay and Das Mohapatra 2009). *Enterococcus faecalis* supplementation also significantly enhanced the production of digestive enzymes in Javanese carp (*Puntius gonionotus*) intestine as well as the level of propionic and butyric acids (short-chain fatty acids) while no significant difference ($P > 0.05$) in acetic acid production was observed (Allameh et al. 2017).

Finally, probiotics could participate to stress control. *S. cerevisiae*-exposed Nile tilapia showed greater tolerance to stress induced by elevated water temperature (40 °C for 48 h) or by a 24-h hypoxia exposure compared with the control group (Abass et al. 2018).

Prebiotics

Prebiotics are non-digestible fibers that are selectively utilized by host microorganisms to confer health benefits and enhance growth performance due to the byproducts generated from their fermentation by gut commensal bacteria, such as changing the composition of the microbiota, inhibiting pathogens, stimulating immune responses and improving stress resistance (Gibson and Roberfroid 1995; Gibson et al. 2017, Ringø et al. 2010, 2014a, b; Patel and Goyal 2012). Prebiotics are defined by three criteria: (a) resistance to gastric acidity, hydrolysis by host enzymes and gastrointestinal absorption; (b) fermentation by intestinal microbiota; and (c) selective stimulation of the growth and/or activity of intestinal bacteria (Gibson et al. 2004). Prebiotics can be classified according to their molecular size or degree of their carbohydrates polymerization into oligosaccharides (inulin, fructooligosaccharides (FOS), mannanoligosaccharides (MOS)) or polysaccharides such as β-glucans (Ringø et al. 2010, 2014a, b; Patel and Goyal 2012).

Among prebiotics investigated to prevent disease in freshwater fish species by *Aeromonas* spp., β-glucan (β-1,3-glucan or β-1,6-glucan) have been paid attention extendingly (Anjugam et al. 2018; Ji et al. 2017; Douxfils et al. 2017; Falco et al. 2012; Barros et al. 2014; Ngamkala et al. 2010; Zheng et al. 2011), which is mostly isolated from the cell wall of the yeast *S. cerevisiae*. Commercial products which consisted of a mixture of β-glucan and MOS were also tested (Gupta et al. 2008; Yarahmadi et al. 2014, 2016; Ebrahimi et al. 2012). MOS (Liu et al. 2013a) and microbial levan as a fructan-polysaccharide (Rairakhwada et al. 2007; Gupta et al. 2008) have also been studied.

Generally, β-glucan products were administered orally and added to the basal diet as a feed supplement and seemed to be efficient in preventing the mortality associated with *Aeromonas* spp. infection, as represented by significant differences ($p \leq 0.05$) in survival rate and protection effect between the prebiotic and control groups (Table 2). However, as seen with probiotics, the level of preventive effects depends on several factors such as dose and duration. However, feeding fish with β glucan at 1 to 2 g kg^{-1} diet for at least 2 weeks seemed to be optimal to high protection and immune response in different *Aeromonas* infected freshwater

Table 2 Summary of *in vivo* studies in three freshwater fish species for prebiotics

Substances	Infectious challenge Species – dose - route	Fish species	Substances administration Dose - Route- Duration	(D):SR in test groups vs control	Reference
β-1,3 glucan binding protein based zinc oxide nanoparticles (Ppβ-GBP--ZnO NPs)	*Aeromonas hydrophila* 10^7 cell ml^{-1}-IP	Mozambique Tilapia (*Oreochromis mossambicus*)	0.01- 0.04 g/kg diet-PO-30d optimal dose :0.04g /kg diet	D10: 55-90% vs 15%	Anjugam et al. 2018
β-1, 3-glucan produced by *Saccharomyces cerevisiae*	*Aeromonas salmonicida* 3×10^5 cell ml^{-1}-IP	rainbow trout (*Oncorhynchus mykiss*)	0.5-1-2 g/kg diet-PO-42d optimal dose :2 g/kg diet	D7: 42-68% vs 32%	Ji et al. 2017
commercial product: β-1,3/ 1,6-glucans produced by *Saccharomyces cerevisiae*	non-lethal *Aeromonas hydrophila* 10^8 cfu ml^{-1} -IP		1- 5 g/kg diet-PO-15&30d optimal dose :2 g/kg diet for 15d	NS	Douxfils et al. 2017
	non-lethal *Aeromonas salmonicida* 4×10^8 cell ml^{-1}-IP	common carp *(Cyprinus carpio)*	1g/kg diet-PO-14d	NS	Falco et al. 2012
β-glucan (85% glucan) from *S. cerevisiae* and Vit C	*Aeromonas hydrophila* 10^5 cfu ml^{-1}-IP	Nile tilapia (*Oreochromis niloticus)*	1g β-glucan /kg diet &600 mg Vit C/kg diet -PO-7,15,30,45d; optimal duration : at least 15d	D15: 64-68% (15,30,4-5d) vs 45% (7d)	Barros et al. 2014
Purified glucan powder	*Aeromonas hydrophila* -0.5 ml bacterial pellets/fish-oral endotracheal intubation		10g/kg diet-PO-14d	D21:100% vs 85%	Ngamkala et al. 2010
commercial product, a mixture of partially autolyzed	*Aeromonas hydrophila* 5×10^5 cfu ml^{-1}-IP		4-12 g/kg diet-PO-56d optimal dose:8-12 g/kg diet	D21: 60-73 % vs 53%	Zheng et al. 2011

Table 2 (continued)

Substances	Infectious challenge Species – dose - route	Fish species	Substances administration Dose - Route- Duration	(D):SR in test groups vs control	Reference
brewer's yeast including glucan, dairy ingredient components and dried fermentation products					
mannan oligosaccha-ride (MOS)	*Aeromonas hydrophila* 10^8 cell ml^{-1}-IP	crucian carp (*Carassius auratus gibelio*)	60 - 240, 480mg/kg diet -PO-70d; optimal dose: 240-480 mg/kg	D7:23-60% vs 20%	Liu et al. 2013a
microbial levan	*Aeromonas hydrophila* 1.8× 10^8 cfu ml^{-1}-IP	common carp *(Cyprinus carpio)*	1-10 g/kg diet-PO-75d optimal dose:5 g/kg diet	D10: 66-100% vs 0%	Rairakhwada et al. 2007
		rohu *(Labeo rohita)*	2.5-12.5 g/kg diet-PO-60d optimal dose: 12.5 g/kg diet	D10: 20-60% vs 0%	Gupta et al. 2008
commercial product (mainly includes β-glucan and MOS)	*Aeromonas hydrophila* 1.5× 10^8 cfu ml^{-1}-IP	rainbow trout (*Oncorhyn-chus mykiss*)	2 g/kg diet-PO-42d	D14: 44% vs 7%	Yarahmadi et al. 2016
	Aeromonas hydrophila 4.9× 10^7 cfu ml^{-1}-IP		2 g/kg diet-PO-45d	D10: 64% vs 24%	Yar Ahmadi et al. 2014
	Aeromonas hydrophila 10^8cell ml^{-1}-IP	common carp *(Cyprinus carpio)*	0.5-2.5 g/kg diet-PO-56d optimal dose :1-1.5 g/kg diet	D10: 50-67% vs 44%	Ebrahimi et al. 2012

Note: *PO*: oral administration; *IP*: intraperitoneal injection; *d*: days of treatment; *SR*: Survival rate; *D*: day post- infection; *NS*: not studied

fish species including rainbow trout (*O. mykiss*), common carp (*C. carpio*), and Nile tilapia (*O. niloticus*) (Douxfils et al. 2017; Falco et al. 2012; Barros et al. 2014; Ji et al. 2017). In addition, combination of β-glucan and MOS (commercial product) resulted also in high disease resistance against *A. hydrophila* in rainbow trout (*O. mykiss*) (Yarahmadi et al. 2014, 2016) and common carp (*C. carpio*) (Ebrahimi et al. 2012); however, application of β-glucan and MOS alone has not been demonstrated by the authors.

The immunostimulant effect of prebiotics is well-known and many studies have indicated that immunosaccharides as β-glucan FOS, MOS, or inulin are beneficial to aquatic animals (Das et al. 2017; Ringø et al. 2010, 2014a, b; Merrifield et al. 2010; Song et al. 2014). However, the underlying mechanisms of prebiotics in enhancement of fish immunity need to be further explored. Some studies shown that a diet supplemented with β-glucan could display the gene expression levels of some immune and inflammation-related cytokines in *Aeromonas* spp. infected fish but the response depends on the organ, with an upregulation in the spleen and head kidney but a downregulation in the gut (Ji et al. 2017; Douxfils et al. 2017; Falco et al. 2012; Yarahmadi et al. 2014). Furthermore, in some investigations, no significant effect of dietary β-glucan on immune parameters (leucocyte subpopulations, lysozyme activity, ACH50) assessed in serum of rainbow trout and Nile tilapia has been proved despite a preventive effect against *Aeromonas* infection (Barros et al. 2014; Ji et al. 2017; Douxfils et al. 2017); even more, overdoses and/or prolonged of β-glucan (0.5% for 30 days rather than 2% for 15 days)) led to a poor immune response (Douxfils et al. 2017).

The preventive effect of β-glucan could also be explained by promoting a rapid healing of the intestinal damage and increasing neutrophil infiltration induced by *Aeromonas* spp. (Ngamkala et al. 2010). The improvement of intestinal morphology has been demonstrated with supplementation of β-glucan and MOS by increasing villi height and *tunica muscularis* thickness as well as gut protease and lipase activities resulting to higher trout (*O. mykiss*) growth and feed efficiency (Khodadadi et al. 2018). In addition, higher intestinal villi and improvement of intestinal morphology were observed in MOS-fed (1.5–2 g/kg diet) rainbow trout fish (Yilmaz et al. 2007; Dimitroglou et al. 2009).

Synbiotics

Synbiotics are nutritional supplements, combining a mixture of probiotics and prebiotics in the form of synergism as health-enhancing functional ingredients (Gibson and Roberfroid 1995). In aquaculture, synbiotics can be used in supplementation form or external bath in order to improve growth performance and feed utilization as well as increasing disease resistance, digestibility, and stimulation of the immune system of aquatic organisms (Cerezuela et al. 2011; Ringø and Song 2016; Das et al. 2017). In this paper, synbiotics beneficial effect intended to protect freshwater fish against *Aeromonas* infections have been reviewed like *L. plantarum* JCM1149 and scFOS (Liu et al. 2017), *B. subtilis* and MOS (Kumar et al. 2018), inactivated *E. faecalis* and MOS (Rodriguez-Estrada et al. 2013), *Bacillus* spp. (*B. coagulans* or *B. subtilis*) and Chitooligosaccharide (COS) (Lin et al. 2012; Devi et al. 2019), *L. rhamnosus* GG, and natural source of oligofructose-enrich inulin from Jerusalem artichoke or Kantawan (*Helianthus tuberosus*) (Sewaka et al. 2019) (Table 3). Prior studies revealed that dietary administration of synbiotic induced higher immune modulation (Sewaka et al. 2019; Devi et al. 2019; Kumar et al. 2018; Rodriguez-Estrada et al. 2013; Lin et al. 2012) and disease protection (Sewaka et al. 2019; Devi et al. 2019; Kumar et al. 2018; Rodriguez-Estrada et al. 2013; Liu et al. 2017; Lin et al. 2012), as well as growth rate (Sewaka et al. 2019; Rodriguez-

Table 3 Summary of in vivo studies in three freshwater fish species for synbiotics

Substances	Infectious challenge			Fish species	Substances administration			Day post-infection: SR in test groups vs. control	Reference
	Species	Dose	Route		Dose	Route	Duration (days of treatment)		
Short chain fuctooligosaccharides (scFOS) and *Lactobacillus brevis* JCM1170 and/or *Lactobacillus plantarum* JCM1149	*Aeromonas hydrophila*	10^8 cell g^{-1}	14 days immersion	Hybrid tilapia	1 g scFOS kg^{-1} diet; 10^8 CFU g^{-1} diet; optimal preparation: synbiotics of Lp JCM1149 and scFOS	PO	35 days	Day 28, 30–55 vs. 15%	Liu et al. (2017)
Mannan oligosaccharide (MOS) and *Bacillus subtilis*	*Aeromonas hydrophila*	2×10^7 CFU ml^{-1}	IP	Indian Major Carp (*Cirrhinus mrigala*)	2 levels of probiotic: high ($15\% \times 10^7$ CFU ml^{-1}) and low ($5.0\% \times 10^7$ CFU ml^{-1}) probiotic and 2 levels of prebiotic: high (0.6%) and low (0.2%) prebiotic; optimal dose: high level of synbiotic	PO	60 days	Day 15, 35–80 vs. 20%	Kumar et al. (2018)
Mannan oligosaccharide (MOS) and/or *Enterococcus faecalis* (Ef)	*Aeromonas salmonicida*	2.4×10^3 CFU ml^{-1}	IP	Rainbow trout (*Oncorhynchus mykiss*)	2.5–5 g kg^{-1} Ef; 2.5–5 g kg^{-1} MOS; optimal dose, 5 g kg^{-1} Ef + 5 g kg^{-1} MOS	PO	84 days	Day 14, 40–75 vs. 15%	Rodriguez-Estrada et al. (2013)
Chitooligosaccharide (COS) and/or *Bacillus coagulans* (Bs)	*Aeromonas veronii*	2.4×10^8 CFU ml^{-1}	IP	Koi (*Cyprinus carpiokoi*))	10^9CFU g^{-1} BS; 2 g kg^{-1} diet COS	PO	56 days	Day 14; 60–64 vs. 33%	Lin et al. (2012)
oligofructose-enrich inulin from Jerusalem artichoke (*Kantawan* (*Helianthus tuberosus*) (JA) and/or *Lactobacillus rhamnosus* GG (LGG))	*Aeromonas veronii*	10^7 CFU ml^{-1}	IP	Juvenile red tilapia (*Oreochromis* spp.)	10^8CFU ml^{-1} LGG and 10 g JA kg^{-1}; optimal preparation: synbiotic	PO	30 days	Day 15, 85–95 vs. 44%	Sewaka et al. (2019)
Chitooligosaccharide (COS) and/or *Bacillus subtilis* (Bs)	*Aeromonas hydrophila*	10^7 CFU ml^{-1}	IP	Rohu (*Labeo rohita*)	1 g kg^{-1} diet of COS or Bs; optimal preparation: synbiotic	PO	30 days	90–95 vs. 20%	Devi et al. (2019)

Note: *PO*, oral administration; *IP*, intraperitoneal injection; *SR*, survival rate

Estrada et al. 2013; Lin et al. 2012) compared with probiotic or prebiotic diets in singular preparations. However, administration of 2 g COS kg^{-1} diet and *B. coagulans* 10^9 CFU g^{-1} separately for 56 days resulted to identical protection in *A. veronii*-infected koi (*C. carpio* koi) in comparison with the combination preparation (survival rate, 60–64% in all treatment groups vs. 33% in control) (Lin et al. 2012).

Synbiotic preparations could have the effects on fish intestinal morphometry. *B. licheniformis* and FOS could improve microvilli length of triangular bream (Zhang et al. 2013) and *L. rhamnosus* GG and oligofructose-enriched inulin increased absorptive area in juvenile red tilapia (*Oreochromis* spp.) intestine fish probably leading to higher absorption of available nutrients and better growth performance (Sewaka et al. 2019).

Plants

Medicinal plants and their secondary metabolites, phytochemical compounds, fractions, and plant extracts have attracted much attention as substitutes for antibiotics in controlling the outbreak of diseases in aquaculture due to their eco-friendly and cost-effectiveness benefits. Plant products have a natural origin and most of these medicinal plants do not represent a hazard for human health, animal health, or the environment (Stratev et al. 2018). Medicinal plants can produce various favorable effects due to their active principles such as alkaloids, terpenoids, tannins, saponins, and flavonoids. They can be used for their anti-stress and antioxidant properties, for their growth performance and appetite stimulation enhancement as well as their immunostimulation effect against fish diseases. They also can have antibacterial, antiviral, antifungal, and antiparasitic activities against fish and shellfish pathogens (Reverter et al. 2017).

In this review, phytochemical compounds included a wide range of medicinal plant families which were purchased or collected locally. Whole plants, parts of plants (leaf, seed, fruit), or secondary metabolites extracted with different solvents (water, methanol, chloroform, ethyl acetate) were tested (Table 4).

Plant products generally were added to feed in a wide range of dosages and durations depending on various phytochemical substances tested in different fish species in previous studies. However, in some studies, plant extracts were injected intraperitoneally (Divyagnaneswari et al. 2007; Alexander et al. 2010; Devasree et al. 2014; Kirubakaran et al. 2016) or fish were immersed in plant extract (Rather et al. 2017). Investigations demonstrated a significant preventive effect of the majority of herbal extracts against *Aeromonas* spp., but the effect depends on the phytochemical products and their administration. For example, the survival rate in Mozambique tilapia (*O. mossambicus*) was higher in fish fed with a chloroform form of *Nyctanthes arbortristis* seed extract at 1 g kg^{-1} diet for 21 days (Kirubakaran et al. 2010) than in fish injected intraperitoneally at 20 mg kg^{-1} with a methanol form of the same seed (Kirubakaran et al. 2016), around 70 and 55%, respectively. However, some plant extracts seem to have no protective effect against *Aeromonas* spp. infection as methanolic extract of black cumin (*Nigella sativa*) (Celik Altunoglu et al. 2017) and oyster mushroom (*Pleurotus ostreatus*) in feeding trials (Bilen et al. 2016a, b) or the mixture of propolis and *Aloe barbadensis* (aloe) (Dotta et al. 2018).

Some combinations of herbal extracts showed a synergistic effect. For example, combination of two Chinese herbs (*Astragalus membranaceus*; *Lonicera japonica*) and boron (Ardó et al. 2008), *Astragalus radix* Chinese herb and *Ganoderma lucidum* fungi (Yin et al. 2009), or *Satureja khuzestanica* Iranian herb mixed with *Oliviera decumbens*

Table 4 Summary of *in vivo* studies in three freshwater fish species for phytochemical compounds

Substances	Infectious challenge Species – dose - route	Fish species	Substances administration Dose - Route- Duration	(D): SR in test groups vs control	Reference
Guava (*Psidium guava*) & mango *(Mangifera indica*) ethanolic leaf extract alone or together	*Aeromonas hydrophila* 10^7 cells ml^{-1}/fish-IP	rohu *(Labeo rohita)*	5, 10 g/kg diet -PO-35d;optimal dose & preparation: 5g/kg diet of each plant	D7: 60-80% vs 35%	Fawole et al. 2016
guava (*Psidium guajava* L.) leaves	*Aeromonas hydrophila* 10^7 cells ml^{-1}/fish-IP		1,5, 10 , 15, g /kg diet-PO-60 d ;optimal dose:5 g/kg diet	D14: 40- 66% vs 23%	Giri et al. 2015b
Magnifera indica (mango) kernel	*Aeromonas hydrophila* 2× 10^6 cells ml−1/fish-IP		1,5, 10 g /kg diet-PO-60 d ; optimal dose:5 g/kg diet	D10: 74-98% vs 50%	Sahu et al. 2007
ginger *(Zingiber officinale)* extract	*Aeromonas hydrophila* 10^7 cells ml^{-1}/fish-IP		2, 4, 6, 8, 10 g /kg diet-PO-60d ;optimal dose:8 g/kg diet	D15: 10-65% vs 19%	Sukumaran et al. 2016
Achyranthes aspera seed	*Aeromonas hydrophila* 3× 10^6 cells ml^{-1}/fish-IP		1, 10, 50 g /kg diet-PO-14d ;optimal dose:50 g/kg diet	D9: 30-70% vs 20%	Rao et al. 2006
Ashwagandha *(Withania somnifera) root powder*	*Aeromonas hydrophila* 10^7 cells ml^{-1}/fish-IP		1, 2, 3 g /kg diet-PO-42d ;optimal dose:2 g/kg diet	D14: 9-42% vs 2%	Sharma et al. 2010
Hybanthus enneaspermus aqueous extract (Violaceae)	*Aeromonas hydrophila* *10^7 cells ml^{-1}/fish-IP*		1, 2, 3, 4 g /kg diet-PO-42 d ;optimal dose:3 g/kg diet	D14: 30-70% vs 10%	Giri et al. 2017
Chlorophytum borivilianum root polysaccharide	*Aeromonas hydrophila*		1, 2, 3,4 g /kg diet-PO-42d ;optimal dose:4 g/kg diet	D30: 36-73% vs 26%	Giri et al. 2015c

Table 4 (continued)

Substances	Infectious challenge Species – dose - route	Fish species	Substances administration Dose - Route- Duration	(D): SR in test groups vs control	Reference
	10^7 cells ml^{-1}/fish-IP				
banana peels *(Musa acuminate)*	*Aeromonas hydrophila* 10^7 cells ml^{-1}/fish-IP		10, 30, 50,70 g /kg diet-PO-60d ; optimal dose:50 g/kg diet	D14: 26-70% vs 20%	Giri et al. 2016
grass *Cynodon dactylon* ethanolic extract (Poaceae)	*Aeromonas hydrophila* 10^6 cells ml^{-1}/fish-IP	Catla (*Catla catla*)	0.5,5, 50 g/kg diet-PO-7, 14, 21,28d optimal dose & duration: 50 g/kg for 7d	D28: 21-73% vs10-18%	Kaleeswaran et al. 2011
oyster mushroom (*Pleurotus ostreatus*) or nettle (*Urtica dioica*) methanolic extracts	*Aeromonas hydrophila* 10^8 cells ml^{-1}/fish-IP	rainbow trout (*Oncorhynchus mykiss*)	0.1, 0.5 g/kg diet-PO-30 d optimal extract &doses: 0.1 & 0.5 g nettle /kg diet	D14: 10-60% vs 0%	Bilen et al., 2016a
household garlic (*Allium sativum*) press (Amaryllidaceae)	*Aeromonas hydrophila* 10^7 cells ml^{-1}/fish-IP		0.5, 1, 5, 10 g/kg diet-PO-14d optimal dose: 0.5,1 g/100g diet	D14: 88-96% vs12%	Nya and Austin 2009a
Oven-dried garlic bulbs	*Aeromonas hydrophila* 10^6 cells ml^{-1}/fish-IP		5, 10 g/kg diet-PO-14d optimal dose: 1 g/100g diet	D14: 54-90% vs 18-20%	Nya and Austin 2011
black cumin (*Nigella sativa*) methanolic extrac(Ranunculaceae)	*Aeromonas hydrophila* 10^7 cells ml^{-1}/fish-IP		0.1, 0.5 g/kg diet--P30 d	D14: 50% in all treated & control groups	Celik Altunoglu et al. (2017
ginger *(Zingiber officinale)*	*Aeromonas hydrophila* 10^7 cells ml^{-1}/fish-IP		0.5, 1,5,10 g/kg diet-PO-14 d optimal dose: 0.5 g/100g diet	D14: 84-100% vs 36%	Nya and Austin 2009b

Table 4 (continued)

Substances	Infectious challenge Species – dose - route	Fish species	Substances administration Dose - Route- Duration	(D): SR in test groups vs control	Reference
aqueous methanolic extracts of tetra (*Cotinus cog-gygria*)	*Aeromonas hydrophila* 10^8 cells ml^{-1}/fish-IP		4, 8, 12 mg/100μl twice a day-PO-10 d optimal dose: 8, 12 mg/100μl	D10: 55-74% vs 53%	Bilen and Elbeshti 2019
Aloe vera powder, (Aloeaceae)	*Aeromonas salmonicida* (Formalin--killed)		5 g/kg diet-PO-42d	NS	Zanuzzo et al. (2015
caper (*Capparis spinosa*) methanolic extract	*Aeromonas hydrophila* 10^8 cells ml^{-1}/fish-IP		0.1, 0.5 g/kg diet-PO-30 d	D14: 70--80% vs 50%	Bilen et al. 2016b
Lupin (*Lupinus perennis)*, mango *(Mangifera indica*) or stinging nettle (*Urtica dioica*)	*Aeromonas hydrophila* 10^7 cells ml^{-1}/fish-IP		10 g/kg diet-PO-14 d	D10: 96--100% vs 32%	Awad and Austin 2010
Polysaccharide of *Ficus carica* (FCPS), *Radix isatidis* (RIPS)*& Schisandra chinensis* (SCPS) alone	*Aeromonas hydrophila* 6× 10^7 cells ml^{-1}-IP	crucian carp (*Carassius carassius*)	500 mg/kg diet-PO-21 d optimal Polysaccharide: FCPS	D14: 42-57% vs 5%	Wang et al. 2016
Leaves from banana (*Musa nana*) or maize (*Zea mays*)	*Aeromonas hydrophila* 10^8 cells ml^{-1}-IP	grass carp (*Ctenopharyngodonid-ella*)	Pellets + banana/maize leaves-PO-	D10: 74-90% vs 90%	Mayrhofer et al. 2017
Bioactive Compound from *Dryopteris crassirhizoma*	*Aeromonas hydrophila* 10^7 cells ml^{-1}-IP		Immunised with 1-50 μg/ml per fish -21d	D14: 56-73% vs 23%	Chi et al. 2016
Peperomia pellucida leaf extract ; (Piperaceae)	*Aeromonas hydrophila*	red hybrid Tilapia (*Oreochromis sp.*)	25 – 100 mg/kg diet -PO-7 d	D28: 82-83% vs 17%	Lee et al. 2016a

Table 4 (continued)

Substances	Infectious challenge Species – dose - route	Fish species	Substances administration Dose - Route- Duration	(D): SR in test groups vs control	Reference
	(10^9 cfu ml^{-1}/fish-IP)				
Miers (*Tinospora cordifolia*) leaf Water soluble fraction (Menispermaceae)	*Aeromonas hydrophila* 10^8 cells ml^{-1}-IP on day 7	Mozambique tilapia (*Oreochromis mossambicus*)	6 - 600 mg kg^{-1}BW—IP (Day 1& 4) optimal dose: 6 mg kg^{-1}BW double dose	D15: 50-90 % vs 20 %	Alexander et al. 2010
Solanum trilobatum water (WSF) or hexane soluble (HSF) fractions (Solanaceae)			4 - 400 mg kg^{-1}BW-IP (Day 1& 4); optimal dose:400 mgkg^{-1}BW(WSF) single dose or 4 mg kg-1BW (HSF) double dose	D15: 35-84% vs 20%	Divyagnaneswari et al. 2007
Eclipta alba leaf aqueous extract (Asteraceae)	*Aeromonas hydrophila* 10^8 cells ml^{-1}-IP		0.1 - 10 g/kg diet-PO- 7-21d optimal dose & duration: 10g/kg diet for 14 d	D15: 30-80% vs 20%	Christybapita et al. 2007
Guava (*Psidium guava*) aqueous or ethanol leaf extracts (Myrtaceae)	*Aeromonas hydrophila* 10^7 cells ml^{-1}-IP		1 - 10 g/kg diet -PO-30 d optimal dose: 10 g/kg	D10: 35-97% vs 15%	Gobi et al. 2016
Wormwood (*Artemisia afra*) leaf powder (Asteraceae)	*Aeromonas hydrophila* 10^6- 4×10^6cfu ml^{-1}-IP		30 - 120 g/kg diet-PO-45 d optimal doses:90 & 120 g/kg diet	D10: 40-90% vs 30-60%	Mbokane et al. 2018a
			30 - 120 g/kg diet-PO-45 d optimal dose: 120 g/kg diet	D10: 30-90% vs 20-50%	Mbokane et al. 2018b
Moringa oleifera powdered leaves	*Aeromonas hydrophila* 10^6- 4×10^6cfu ml^{-1}-IP				
Nyctanthes arbortristis leaf water soluble fraction (Oleacea)	*Aeromonas hydrophila* 10^3 cells ml^{-1}-IP on day 7		3.2 - 400 mg kg^{-1}BW-IP(Day 1& 4) optimal dose: 400 mg kg^{-1}BW	96h:30-60% vs 10%	Devasree et al. 2014

Table 4 (continued)

Substances	Infectious challenge Species – dose - route	Fish species	Substances administration Dose - Route- Duration	(D): SR in test groups vs control	Reference
Nyctanthes arbortristis seeds Chloroform extract (Oleacea)	*Aeromonas hydrophila* 10^8 cells ml^{-1}-IP		0.1 - 10 g/kg diet-PO-7-21 d optimal dose& duration:1g/kg diet for 21 d	D15: 22-70% vs 15%	Kirubakaran et al. 2010
Nyctanthes arbortristis seeds Methanol extract (Oleacea)			2-200 mg kg^{-1}BW-IP optimal dose: 20 mg kg^{-1}BW	D15: 40-55% vs 25%	Kirubakaran et al. 2016
Toona sinensis Roem.(Meliaceae) hot-Water extract	*Aeromonas hydrophila* 5 $\times 10^9$ cells ml^{-1}-IP		4-8 mg/kg diet-PO-45 d optimal dose: 8 mg /kg diet	D7: 63-70% vs 43%	Wu et al. 2010
Cucurbita mixta (L.) seed	*Aeromonas hydrophila* 3.1×10^7 cells ml^{-1}-IP		2-6 g/kg diet-PO-28 d optimal dose: 4&6 g/kg diet	D30: 80-90% vs 10%	Saiyad Musthafa et al. 2017
Mucuna pruriens (L.) seed	*Aeromonas hydrophila* 3.1×10^7 cells ml^{-1}-IP		2-6 g/kg diet-PO-28 d optimal dose: 4&6 g/kg diet	D30: 80-90% vs 10%	Saiyad Musthafa et al., 2018
cinnamon (*C. zeylanicum*) nanoparticles	*Aeromonas hydrophila* 5×10^5 cells ml^{-1}-IP		0.25-10 g /kg diet-PO-56 d optimal dose:3g /kg diet	D7: 80-100% vs 34%	Abdel-Tawwab et al. 2018
crude Propolis or Propolis -ethanolic Extract (PEE)	*Aeromonas hydrophila*	Nile tilapia (*Oreochromis niloticus)*	10 g/kg diet-PO-28 days optimal extract :ethanolic extract	D15: 55-58% vs 15%	Abdel-Tawwab and Ahmad 2009
propolis and aloe (*Aloe barbadensis*) Miller mixture	*Aeromonas hydrophila* 5 $\times 10^5$ cells ml^{-1}-IP		10 g/kg diet-PO-15 days	D7: 55% vs 44%	Dotta et al. 2018
Turmeric powder (*Curcuma longa*) (Zingiberaceace)	*Aeromonas hydrophila*		50-200 mg /kg diet-PO-84 d optimal dose:50 mg /kg diet	D15: 80-95% vs 70%	Mahmoud et al. 2017

Table 4 (continued)

Substances	Infectious challenge Species – dose - route	Fish species	Substances administration Dose - Route- Duration	(D): SR in test groups vs control	Reference
	1.5×10^8 cells ml^{-1}-IP				
Chinese herbs (*Astragalus membranaceus*; *Lonicera japonica*) and/or boron (Fabaceae& Caprifoliaceae)	*Aeromonas hydrophila* 5×10^7 cells ml^{-1}-IP		1 g/kg diet for each herb; 0.5 g/kg of boron-PO-28 d;optimal preparation: both herbs with Boron	D10: 25-70% vs 15%	Ardó et al. 2008
Echinacea purpurea or Garlic (*Allium sativum*) (Asteraceae or Amaryllidaceae resp.)	*Aeromonas hydrophila* 10^8 cells ml^{-1}-IP		30g/kg(E)diet or 1.0 ppt (G)-P-PO-30,60,90d optimal condition: (E) or (G) for 60 & 90d resp.	D7: 15-50% vs 5-10%	Aly and Mohamed 2010
Withania somnifera root powder	*Aeromonas hydrophila* 10^8 cells ml^{-1}-IP		25, 50 g /kg diet-PO-42 d optimal dose:50g /kg diet	D14: 63-80% vs 30%	Zahran et al. 2018
dry leaf powder or dried leaf ethanol extract of guava (*Psidium guajava)*	*Aeromonas hydrophila* 3.44×10^6 cells ml^{-1}-IP		0.1 mg^{-1}ml added to diet-PO-6 d	D14: 90% vs 50%	Pachanawan et al. 2008
Aqueous extract of *Azadirachta indica* (neem) or Green synthesis of silver nanoparticles (G-AgNPs) of neem; (Meliaceae)	*Aeromonas hydrophila*	mrigal carp (*Cirrhinus cirrhosus*)	immersion in 50 μL of treatments daily for 20 d optimal preparation: (G-AgNPs) of neem	D20: 61-74% vs 10%	Rather et al. 2017
stinging nettle *(Urtica dioica)*	*Aeromonas hydrophila* 10^7 cells ml^{-1}-IP	ningu (*Labeo victorianus*)	10 - 50 g/kg -PO-112 d;optimal dose:5% diet	D18: 95% vs 0%	Ngugi et al., 2015
Hairy willow herb *(Epilobium hirsutum)* ethanolic extract (Onagraceae)	*Aeromonas hydrophila* 3×10^8 cells ml^{-1}-IP	common carp *(Cyprinus carpio)*	5,- 30 g/kg diet -PO-56 d;optimal dose:30 g/kg diet	D30: 77-96% vs 75%	Pakravan et al. 2012

Table 4 (continued)

Substances	Infectious challenge Species – dose - route	Fish species	Substances administration Dose - Route- Duration	(D): SR in test groups vs control	Reference
Mixture of *Ocimum basilicum*, *Cinnamomum zeylanicum*, *Juglans regia* &*Mentha piperita* extracts	*Aeromonas hydrophila* 10^8 cells ml^{-1}-IP	common carp *(Cyprinus carpio)*	0.5 - 1.25 g/kg diet -PO-45d optimal dose:1 g/kg diet	D10: 60-91% vs 48%	Hajibeglou and Sudagar et al. 2010
Basil leaf (*Ocimum basilicum*) ethanolic extract (Lamiaceae)			100 - 1600 mg/ kg diet-PO-60 d optimal dose: 400 mg/kg diet	D10: 49-88% vs 51%	Amirkhani and Firouzbakhsh 2015
Astragalus radix and/ or *Ganoderma lucidum,* Chinese herbs and/or fungi (Faboideae, Ganodermataceae resp.)	*Aeromonas hydrophila* 10^6 cells ml^{-1}-IP		5 g/kg -PO-35d;optimal preparation combination of herbs	D6: vaccinated group: 50-60% vs 40%; non-vaccinated group: 40% vs 10%	Yin et al. 2009
Oliviera decumbens and/or *Satureja khuzestanica* , Iranian herbs (Apiaceae & Lamiaceae)	*Aeromonas hydrophila* 10^6 cells ml^{-1}-IP		5 g/kg -PO-35d;optimal preparation: *S. khuzestanica*	D10: vaccinated group:55-64% vs 50%; non-vaccinated:5-31% vs 0%	Alishahi et al. 2016
fibrous root of *Rhizoma Coptidis* (FRC) and its main alkaloids	*Aeromonas hydrophila* 10^7cells ml^{-1}-IP		12.5-50 g/kg FRC; 0.78 g/kg total alkaloids (TA), 0.78 g/kg berberine (BBR), 0.78 g/kg coptisine (Cop) diet-PO-21 d optimal treatment: FRC-25, FRC-50, TA, BBR and Cop	D10: 55-80% vs 40%	Zhou et al. 2016
Aegle marmelos leaf extract (Rutaceae)	*Aeromonas hydrophila* 1.5×10^4 cells ml^{-1}-IP		5-50 g/kg diet -PO-50 d optimal dose: 5 g/kg	D20: 83-96% vs 60%	Pratheepa et al. 2010
triherbal leaf extract of neem (*Azadirachta indica*), tulsi *(Oscimum sanctum)* & turmeric(*Curcuma longa*)	*Aeromonas hydrophila* 1.8×10^6 cells ml^{-1}-IP		On Day 6 post-infection: 1 g/kg diet-PO-28d	D30: 50% vs 15%	Harikrishnan et al. 2010

Note: *NS*: not studied; *PO*: oral administration; *IP*: intraperitoneal injection; *d*: days of treatment ; *SR*: Survival rate; *D*: day post- infection

(Alishahi et al. 2016) were more efficient in controlling *Aeromonas* infection than applying each plant alone. However, guava or mango ethanolic leaf extract alone resulted in a higher protection of rohu (*L. rohita*) against *A. hydrophila* than feeding them with both at the same level (Fawole et al. 2016).

The protective effect of phytochemical products could be due to their immunostimulant effect. Indeed, in all publications presenting a protective effect of the products, immune responses and oxidative status were enhanced significantly compared with the control groups. In contrast, the lack of protective effect of black cumin (*Nigella sativa*) methanolic extract could be linked to the absence of an immunostimulant effect (Celik Altunoglu et al. 2017). Herbal extracts enhanced fish immunity through different patterns. For example, a higher humoral immune responses of Mozambique tilapia (*O. mossambicus*) was noticed after 3 weeks of *Eclipta alba* leaf aqueous extract feeding in contrast with no significant modulation in the cellular immune responses (Christybapita et al. 2007). Two Chinese herbs (*Astragalus membranaceus*; *Lonicera japonica*) enhanced blood phagocytic cell functions but had a moderate effect on the plasma lysozyme level and no effect on plasma total protein and total immunoglobulin level (Ardó et al. 2008). As result of immunocompetence is increased by plant products, their applications were also studied to enhance the efficacy of some vaccines in farmed fish. *Astragalus radix* Chinese herb could be used in order to obtain higher survival rate in vaccinated common carp (*C. carpio*) after an *A. hydrophila* infection (Yin et al. 2009). However, *Aloe vera* powder did not enhance immune responses against a formalin-killed atypical *A. salmonicida* in rainbow trout (Zanuzzo et al. 2015).

Furthermore, the consumption of a diet containing *Rehmannia glutinosa* RG led to the accumulation of more beneficial microorganisms while inhibiting the growth of potential pathogens as *Aeromonas sp.* in the intestine of common carp (*C. carpio*) and which could have positive effects on the immune response of carp (Chang et al. 2018).

Essentials oils

Essential oils (EOs) are volatile, lipophilic, odoriferous, and liquid substances derived from plants for the food, hygiene, cleaning products, perfumery, and also pharmaceutical industries for their potential therapeutic effects (Edris 2007). Over the past two decades, several studies have evaluated the application EOs as a dietary additive in aquaculture due to their diverse properties (e.g., anesthetic, antioxidant, and antimicrobial) that can improve health, growth, and welfare of fish (Souza et al. 2019). The main biochemical compounds of some EOs may play a major role by acting as an anti-pathogen (Perricone et al. 2015). It has been reported that EOs can protect fish from pathogens by enhancing fish immunity, improving fish growth and feed utilization (Vaseeharan and Thaya 2014), and gut bacterial community modulation (Sutili et al. 2017; Ngugi et al. 2017; Al-Sagheer et al. 2018).

In this paper, the application of EOs to protect freshwater fish from *Aeromonas* infection were analyzed in Table 5 including EOs of lemongrass (*Cymbopogon citratus*) or geranium (*Pelargonium graveolens*) (Al-Sagheer et al. 2018), bitter lemon (*Citrus limon*) (Ngugi et al. 2017), *Litsea cubeba* leaf (Nguyen et al. 2016), and a commercial product (encapsulated oregano, anise, and citrus EOs) (Menanteau-Ledouble et al. 2015) which demonstrated effective protection against *Aeromonas* spp. infection in Nile tilapia (*O. niloticus*), ningu (*L. victorianus*), common carp (*C. carpio*), and rainbow trout

Table 5 Summary of *in vivo* studies in three freshwater fish species for essential oil

Substances	Infectious challenge Species – dose - route	Fish species	Substances administration Dose - Route- Duration	(D): SR in test groups vs control	Reference
lemongrass (*Cymbopogon citratus*) or geranium (*Pelargonium graveolens*) alone	*Aeromonas hydrophila* 1.5×10^8 cfu mL^{-1}-IP	Nile tilapia (*Oreochromis niloticus)*	200, 400 mg/kg diet-PO-84 d optimal dose & essential oil: 200mg lemongrass/kg diet & 400mg geranium/kg diet	D14: 85-95 % vs 70 %	Al-Sagheer et al. 2018
bitter lemon (*Citrus limon*) fruit peels (Rutaceae)	*Aeromonas hydrophila* 10^7cell/fish-IP	ningu(*Labeo victorianus*)	10- 80 g/kg diet-PO-28 d optimal dose: 50g/kg diet	D18: 50-80 % vs 0 %	Ngugi et al. 2017
Litsea cubeba leaf (linalool-rich chemotype)	*Aeromonas hydrophila* 10^7 cfu mL^{-1}-IP	common carp (*Cyprinus carpio*)	20 - 80 g/kg diet-PO-21 d optimal dose: 80g/kg diet	D14: 37-63 % vs 27%	Nguyen et al. 2016
Satureja thymbra (Lamiaceae)	*Aeromonas salmonicida* 1.5×10^8 cfu mL^{-1}-IP	rainbow trout (*Oncorhynchus mykiss*)	10 – 800 μg μL^{-1}-IP	0%	Okmen et al. 2012
commercial product (encapsulated Oregano + anise +citrus)	*Aeromonas salmonicida* IP: 7×10^3 cfu mL^{-1}; Immersion: 10^5 CFU ml^{-1} 2h; Cohabitation		0.2g/kg diet-PO-175 d	D35: 82% vs 63%	Menanteau-Ledouble et al. 2015

Note: *PO*: oral administration; *IP*: intraperitoneal injection; *d*: days of treatment ; *SR*: Survival rate; *D*: day post- infection

(*O. mykiss*) respectively by improving immunological response, oxidative status, or growth performance.

Satureja thymbra EO was also tested in rainbow trout (*O. mykiss)* against *A. salmonicida* but effective doses of *S. thymbra* EO determined in vitro caused toxic effects and total mortality shortly after injection and doses with low or no toxic effect did not increase the bactericidal activity of fish blood (Okmen et al. 2012). All of the EOs tested in this paper were administered orally as a feed additive except *Satureja thymbra* EO, which was injected intraperitoneally (Okmen et al. 2012).

Algae

Algae, including both macroalgae (seaweed) and microalgae (unicellular), are fast growing photosynthetic organisms which are potentially good sources of energy because of their high lipid content. They also contain amino acids, minerals, vitamins, chlorophyll, and some substances that have antioxidant effects (Sirakov et al. 2015; Kent et al. 2015). Several advantages of algae as an additive in aquaculture have attracted much attention, such as the positive effect on growth performance, increased triglycerides and protein deposition in muscle, protection of fish from disease, decreased nitrogen output into the environment, increased fish digestibility, physiological activity, starvation tolerance, and carcass quality (Halima 2017; Becker 2004; Mustafa and Nakagawa 1995).

In this review, the efficacy of microalgae as green algae (*Chlorella vulgari*) or blue-green algae (*Spirulina platensis*) were revealed in Nile tilapia (*O. niloticus*) (Abdel-Tawwab and Ahmad 2009; Fadl et al. 2017) (Table 6). The efficacy of polysaccharide fraction of a marine macroalga (*Caulerpa scalpelliformi* or *Padina gymnospora*) was also presented in Nile tilapia (*O. niloticus*) and common carp (*C. carpio*) (Rajendran et al. 2016; Yengkhom et al. 2018). In addition, the favorable protective efficacy of microencapsulated seaweed extracts was revealed against *A. salmonicida* in *O. mossambicus* (Thanigaivel et al. 2019) (Table 6). Algae treatments were administered orally as a feed supplement except the polysaccharide fraction of a marine macroalga (*Caulerpa scalpelliformi*), which was injected intraperitoneally (Yengkhom et al., 2018). All treatments demonstrated significant differences ($p \leq 0.05$) in survival rate and protection effect between algae groups and control groups against *Aeromonas* infection. In addition, a significant increase of non-specific immune responses has been showed in *Aeromonas* challenge due to algal alternatives (Abdel-Tawwab and Ahmad 2009; Rajendran et al. 2016; Fadl et al. 2017; Yengkhom et al. 2018; Thanigaivel et al. 2019). Furthermore, *Chlorella* and *Spirulina* could improve growth performance of fish, and the proteins and lipids contents in Nile tilapia (*O. niloticus*) (Abdel-Tawwab and Ahmad 2009; Fadl et al., 2017).

Bacteriophages

Use of phages, virulent virus which infect and destroy bacteria, would be a highly promising option to control diseases. However, it has not yet been fully investigated in aquaculture (Oliveira et al. 2012). In the present review, few studies evaluated the efficacy of bacteriophage in treating *Aeromonas* infection in farmed freshwater fish. It was seen that bacteriophage HER 110 can protect 90% of brook trout (*S. fontinalis*) in comparison with total mortality in the control group after 4 days of *A. salmonicida* infection (Imbeault et al. 2006). In addition, *Aeromonas* Phage PAS-1 can be

Table 6 Summary of *in vivo* studies in three freshwater fish species for algae

Substances	Infectious challenge Species – dose - route	Fish species	Substances administration Dose - Route- Duration	(D): SR in test groups vs control	Reference
microalgae-enriched fodder: green algae (*Chlorella vulgari*) or/and blue-green algae (*cyanobacterium Spirulina platensis*)	*Aeromonas hydrophila* 3 × 10^8 cfu ml^{-1}-IP	Nile tilapia (*Oreochromis niloticus)*	150 g/kg diet-PO-56d	D14:100% vs 61%	Fadl et al. 2017
live Spirulina (*Arthrospira platensis*)	*Aeromonas hydrophila* 5 × 10^5 cfu ml^{-1}-IP		1.25, -10 g/kg diet -PO-84d optimal dose: 5-10 g/kg diet	D10:30-90% vs 20%	Abdel-Tawwab and Ahmad 2009
polysaccharide fraction of a marine macroalga (*Caulerpa scalpelliformi)*	*Aeromonas hydrophila* 5 × 10^5 cfu ml^{-1}-IP		2-200 mg / kg—IP- 7 and 21d	7d post treatment D15: 90-95% vs 55% 21d post treatment D15: 45-65% vs 35%	Yengkhom et al. 2018
methanolic extract of the marine macroalga, *Caulerpa scalpelliformis*			2- 200mg / kg—IP-7d	D15: 36-72% vs 55%	Yengkhom et al. 2019
microencapsulated seaweed (*Gracilaria foliifera or Sargassum longifolium*) extracts	*Aeromonas salmonicida* 10^{3-7} cfu mL^{-1}		10- 50 μl in diet -1d on challenge	D15: 30-85% vs 5-20%	Thanigaivel et al. 2019
polysaccharide fraction of a marine macroalga *(Padina gymnospora)*	*Aeromonas hydrophila* 2.1 × 10^9 cfu ml^{-1}-IP	common carp *(Cyprinus carpio)*	0.1- 10g polysaccharide/kg diet or 10g Macrogard™/kg diet -PO- 7, 14, and 21d optimal dose & duration:10g polysaccharide/kg diet for 14 days	D15: 35-90% vs 25-30%	Rajendran et al. 2016

Note: *PO*: oral administration; *IP*: intraperitoneal injection; *SR*: Survival rate

applied as a biological control of *A. salmonicida* subsp. *salmonicida* infection with increased survival rates and mean times to death in rainbow trout (*O. mykiss*) (Kim et al. 2015).

Others functional products

As mentioned previously, many research studies have focused on the development of functional feed alternatives, examining probiotics, prebiotics and plant-derived compounds or extracts to maintain fish health and performance. There is also a growing interest in nanoparticles due to their antimicrobial effects and as drug delivery systems (Shaalan et al. 2016). For example, 100 μl intraperitoneal injection of fucoidan-coated (marine polysaccharide) gold nanoparticle (Fu-AuNPs) resulted to higher survival rate in treatment group in comparison with control group after 72 h (70 vs. 10%) against *A. hydrophila* in Mozambique tilapia (*O. mossambicus*) (Vijayakumar et al. 2017) (Table 7). However, its mode of action has not been studied in vivo while the synthesized Fu-AuNPs at 100 μg ml^{-1} showed effective inhibition of *A. hydrophila*, which is much higher than that of chloramphenicol in vitro assay (Vijayakumar et al. 2017).

The incorporation of rare earth elements such as azomite, mineral ore (Musthafa et al. 2016) and minerals such as yellow loess (sedimentary deposit of mineral particles) (Lee et al. 2016b; Won et al. 2017) in fish feed has been assessed as a means to control *Aeromonas* infection (Table 7). The efficacy of yellow loess against *A. salmonicida* in rainbow trout represented an improved growth performance, non-specific immune responses, and a furunculosis resistance (Lee et al. 2016b; Won et al. 2017).

Furthermore, the utilization of organic acids has attracted considerable attention recently due to their antimicrobial properties and role in enhancing nutrient availability in aquaculture (Ng and Koh 2017). It has been found that a commercial product which contains formic, propionic, and lactic acids and cinnamaldehyde, may be effective as an alternative method to control the impact of furunculosis in rainbow trout. However, significant difference was not found in the feed conversion ratio with the control group in this assay (Menanteau-Ledouble et al. 2017).

Main perspective

In this review, the efficiency of functional alternative products against *Aeromonas* infection and their potential mechanisms of action in freshwater fish were analyzed and compared. The selected studies tested highly diverse products with wide ranges of doses and durations of administration in different species of freshwater fish which were experimentally infected by *Aeromonas.* Furthermore, the experimental design of *Aeromonas* infection was also varied by the species and the strains of *Aeromonas* bacteria, the infectious doses, and the administration routes. It consequently was almost impossible to compare the studies or to determine whether one product is more effective than another. However, most of these alternatives were added to the basal diet as a feed supplement and were effective in inducing a preventive effect against mortality caused by *Aeromonas* spp. and in increasing growth performance. First, immunostimulation was the main mechanism of action investigated in the studies reviewed; nevertheless, in some studies, the protective effect of the product is clearly linked to the immunostimulant effect, but in other studies, a protective effect was observed without an increase of fish immunocompetence. Second, products feeding could also induce modifications of the gut microbiota (e.g., increase of the beneficial micro-organisms and decrease of the pathogen bacteria) as well as of the intestine morphometry (e.g., beneficial effects on the structure and decrease of tissue lesions induced by bacteria). All these mechanisms of action need to be described and explained in fish because they are clearly gaps that need to be filled in order

Table 7 Summary of *in vivo* studies in three freshwater fish species for non-classified group

Substances	Infectious challenge Species – dose - route	Fish species	Substances administration Dose - Route- Duration	(D): SR in test groups vs control	Reference
Fucoidan (marine polysaccharide) coated gold nanoparticle	*Aeromonas hydrophila* 6×10^8 cfu ml^{-1}-IP	Mozambique Tilapia (*Oreochromis mossambicus*)	100 µl -IP	72h :70% vs 10%	Vijayakumar et al. 2017
Azomite (mineral ore)	*Aeromonas hydrophila*		2- 6 g/kg diet-PO-30d optimal dose: 4 g/kg diet	D30: 80-90% vs 10%	Musthafa et al. 2016
Shilajit, a natural mineral original from India	*Aeromonas hydrophila* 3.1×10^7 cells ml^{-1}-IP		2-6 g/kg diet--PO-28d optimal dose: 4 & 6 g/kg	D14:82-92% vs 10%	Saiyad Musthafa et al. 2018
natural mineral materials: yellow loess, SG (commercial product), Mk (commercial product) or barley stone	*Aeromonas salmonicida* 2×10^7 cfu ml^{-1}-IP	rainbow trout (*Oncorhynchus mykiss*)	4 g/kg diet-PO-56d optimal preparation: yellow loess or SG	D15:30-45% vs 12% oxytetracycline :45%	Won et al. 2017
yellow loess (sedimentary deposit of mineral particles)			5-20 g/ kg diet-PO-84d	D14: 15% vs 0% oxytetracycline :15%	Lee et al. 2016b
commercial product (combination of formic, Propionic, lactic acids and cinnamaldehyde)	*Aeromonas salmonicida* 10^5 cfu ml^{-1} (2h) IP: 2×10^7 cfu ml^{-1} cohabitation		0.8 g/kg diet-PO-175d	D35;IP :70% vs 25% Immersion: all 70% cohabitation: 100% vs 90%	Menanteau-Ledouble et al. 2017

Note: *PO*: oral administration; *IP*: intraperitoneal injection; *d*: days of treatment; *SR*: Survival rate; *D*: day post- infection

to draw conclusions concerning their role in the protective effect of the products. Furthermore, alternative-to-antibiotics researches need to benefit from greater access to expertise in pharmacokinetics and pharmacodynamics, formulation and toxicology, for example, by creation of partnerships with biotechnology companies.

Although there are numerous clinical trials on alternative products in experimental conditions in order to reduce antibiotic use in aquaculture, there is a clear need for careful clinical trial designs in experimental conditions with relevant endpoints: primary endpoints such as reduction of morbidity and mortality but also secondary or surrogate endpoints such as changes in cytokine levels or changes in imaging of infections. Finally, in our knowledge, no evaluation of the functional feed alternative efficiency has been carried out in fish farms, where *Aeromonas* infection could be heterogeneous between fish, in contrast with the experimental conditions and where the environmental bacterial flora and the quality of water could influence the effect of the product. So, there is also a clear need for careful clinical trial designs in fish farm conditions, especially in order to ensure their benefits and their technical feasibility but also to improve the economic models.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

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EXPERIMENTAL STUDIES

Chapter 2: Widespread transmission of *Aeromonas* antibiotic-resistant bacteria and their genes in aquatic ecosystems

Aeromonas is a ubiquitous bacterium in aquatic environments and is well-known for its antimicrobial resistance profiles (Janda and Abbott, 2010; Piotrowska and Popowska, 2014). However, *Aeromonas* infections are considered as less important public health problem or even, and so are underestimated; therefore, their clinical and environmental epidemiology are not very well known (Ghenghesh et al., 2008). Even though, *Aeromonas* spp. are opportunistic pathogens of humans and causative agents of fish diseases but their distribution in aquatic ecosystems are become more and more as a major public health problem due to possessing different antibiotic resistance genes which are being detected within this genus. The main targets of *Aeromonas* are fish, which are exposed to these natural pathogens and therefore, sometimes antibiotic treatment, mainly medicated feed distributed in water, is the only solution to overcome these diseases (Zhang et al.2009; Piotrowska and Popowska, 2014).

Increasing the release of antibiotics, antibiotic resistant bacteria, and resistance genes to the aquatic environment and therefore, the evolution and dissemination of horizontal transfer of resistance genes within this genus and other environmental/opportunistic bacteria could become a serious problem around the world (Patil et al, 2016; Watts et al., 2017). It is important to examine the aquatic environment as a whole, including fish and sediments as well as effluents from fish farming facilities to evaluate the transmission risk of antibiotic resistant bacteria and resistance genes from the environment to humans. Hence, in the research article below, antimicrobial susceptibility profiles and resistance genes in *Aeromonas* isolated from environment (water and biofilm) and rainbow trout of two fish farms in France were evaluated. This study was followed for 7 months including summer, an optimal season for furunculosis out breaks in order to compare

Aeromonas strains in fish detected with furunculosis, isolates from fish, pond water and biofilm before and after antibiotic treatment.

A- Article N° 2: Antimicrobial susceptibility profiles and resistance genes in *Aeromonas* isolated from environment and rainbow trout of two fish farms in France

Antimicrobial susceptibility profiles and resistance genes in *Aeromonas* isolated from environment and rainbow trout of two fish farms in France

(submitted to journal of Microorganisms)

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Abstract

This study presents the occurrence and abundance of *Aeromonas* antibiotic-resistant bacteria (ARB) and genes (ARGs) isolated from water, biofilm and fish in two commercial trout farms before and one week after flumequine treatment. Wild (WT) and non-wild (NWT) strains were determined for quinolones (flumequine, oxolinic acid and enrofloxacin), oxytetracycline (OXY), florfenicol (FFN), trimethoprim-sulfamethoxazole (TMP) and colistin (COL), and pMAR (presumptive multi-resistant) strains were classified. Forty-four ARGs for the mentioned antibiotics, beta lactams and multi-resistance were quantified for 211 isolates. *Bla*SHV-01, *mex*F and *tet*E were the dominant ARGs. A greater occurrence and abundance of *tet*A2, *sul*3, *floR*1, *blaSHV*-01 and *mex*F were observed for NWT compared to WT. The occurrence of pMAR and NWT *Aeromonas* for quinolones, OXY, FFN, TMP and COL and ARGs depended on the *Aeromonas* origin, antibiotic use and the presence of upstream activities. Our results revealed the impact of a flumequine treatment on *Aeromonas* present on a fish farm through an increase in NWT and pMAR strains. The link between fish and their environment was shown by the detection of identical ARB and ARGs in the two types of samples. There appears to be a high risk of resistance genes developing and spreading in aquatic environments.

Introduction

A rise in antibiotic-resistant bacteria (ARB) and antibiotic-resistant genes (ARGs) has been reported in pathogenic, commensal and environmental bacteria over the last few years as a consequence of the wide use of antimicrobial agents to control human and animal infections (Marti et al., 2014 Berendonk et al., 2015; Graham et al., 2019). The overuse of antimicrobial agents is a major source of antibiotic pollution in the environment (Cabello et al., 2013; Watts et al., 2017; Santos and Ramos 2018). Like other farmed species, aquatic animals may play a role in the selection and spread of resistant environmental and pathogen bacteria (Jacobs and Chenia, 2007; Penders and Stobberingh, 2008; Naviner et al., 2011; Piotrowska and Popowska, 2014; Patil et al., 2016).

In global aquaculture production, the most widely used antibiotics are from the trimethoprim/sulfonamide, quinolone and tetracycline families, which have been found to be related to the development of ARB and ARGs, and more often multi-drug resistance strains (Lulijwa et al., 2020; Sun et al., 2020). Approximately 80% of antibiotics used in aquaculture, commonly applied as a feed supplement in pond water, enter the environment with their activity intact (Romero et al., 2012; Watts et al., 2017; Santos and Ramos 2018). The overuse of antimicrobial agents is a major source of ARGs and antibiotic pollution in the environment (Cabello et al., 2013; Watts et al., 2017; Santos and Ramos 2018). Some of the antibiotics administered in fish are excreted unchanged in feces and urine and discharged into rivers. This may lead to contamination of surface water, and sometimes of water intended for human use such as drinking water supplies (Romero et al., 2012; Ranjan et al., 2017; Rasu and Majumdar, 2017; Talagrand-Reboul et al. 2017; Watts et al., 2017; Santos and Ramos 2018).

The prolonged presence of antibiotics in raceway water, combined with high numbers of bacteria in the polybacterial matrices of biofilms and potential contamination of aquatic environments by pathogens of human and animal origin, could stimulate selective pressure on the exchange of genetic information between aquatic and terrestrial bacteria, and creates the potential risk of the development and spread of antibiotic-resistant bacteria and genes between fish, their environment and humans (Muziasari et al., 2016; Watts et al. 2017). The passage of antimicrobial resistant bacteria and resistance genes from fish and their environment to terrestrial livestock and humans could favor the survival and maintenance of ARB and widespread emergence of drug-resistant pathogens in environmental reservoirs. Moreover, upstream aquaculture activities should be considered as a reservoir and at the origin of ARB and ARGs in downstream animal and human facilities (Rasul and Majumdar 2017; Santos and Ramos 2018).

Aeromonas is a genus of gram-negative bacteria belonging to the *Aeromonadaceae* family, and consists of a group of opportunistic environmental pathogens, with some species able to cause disease in humans, fish, and other aquatic animals (Lamy, 2012a; Watts et al., 2017; Figueras, 2020). They are autochthonous to aquatic environments and have been isolated easily from different kinds of water such as rivers, lakes, ponds, estuaries, drinking water, groundwater, wastewater, and sewage in various stages of treatment, and they may persist attached to biofilms on biotic or abiotic surfaces in environment ecosystems (Kirov et al., 2004; Naviner et al., 2011; Cai et al., 2019). *Aeromonas* outbreaks are currently a common phenomenon in freshwater farmed fish. Some *Aeromonas* species, such as *Aeromonas salmonicida* sub *salmonicida,* are a pathogen agent of furunculosis, one of the most common diseases in salmonid farmed fish worldwide that causes important financial losses in the aquaculture industry (Bebak et al., 2015; Mzula et al., 2019). In France, one of Europe's biggest aquaculture producers of freshwater fish (39,500 tonnes

in 2019), notably rainbow trout (*Oncorhynchus mykiss*), furunculosis has been fairly well controlled. However, recurring clinical cases recently have been reported, particularly in the late spring and summer (ANSES 2015; FranceAgriMer 2019; CIPA 2019).

Previous studies have indicated the presence of *Aeromonas* in aquaculture systems with high levels of resistance to antibiotics and gene resistance determinants (Jacobs and Chenia, 2007; Penders and Stobberingh, 2008). Some studies assessed the antimicrobial sensitivity of *Aeromonas* species isolated from farmed rainbow trout and their environment in which they were resistant to quinolones and fluoroquinolones, streptomycin, oxytetracycline, chloramphenicol, florfenicol, sulfamethoxazole-trimethoprim and β-lactams (Saavedra et al., 2004; Naviner et al., 2011;Vega-Sánchez et al., 2014). Multidrug resistant *Aeromonas* gene-harboring strains like *sul*1, *tet*A, and *floR* also have been detected in different species of farmed fish (Patil et al., 2016; Duman et al., 2020). However, an analysis of the high diversity and abundance of *Aeromonas* ARGs and their antimicrobial sensitivity profiles due to antibiotic treatments has not yet been carried out in fish farms and their environment. Furthermore, studies on *Aeromonas* antimicrobial susceptibility remain rare, and no epidemiological cut-off values are currently available from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) to interpret minimum inhibitory concentrations (MIC) of *Aeromonas* spp. (Baron et al., 2017; Duman et al., 2020; EUCAST, 2021). To understand the extent of ARG transmission in aquatic ecosystems, this study focused on the evolution of antimicrobial susceptibility (MIC) and resistance genes in environmental and fish *Aeromonas* isolated from two rainbow trout fish farms during a seven-month period that included episodes of furunculosis and an administration of antibiotics.

Material and methods

Ethics statement

This study was approved by the members of the Animal Experiment Ethics Committee of at Oniris, in France (CERVO-2020-6-V).

Description of the farms

This study was carried out on two commercial rainbow trout fish farms (fish farms A and B) in France over seven months (February to August 2020). Both farms are fed by river water through an open water circuit system. These fish farms are composed of upstream ponds for juvenile trout and downstream ponds for the growth of fish until they reach the commercial weight (around 1 to 2.5 kg). The farms were chosen due to their recent history of furunculosis and antibiotic use. In August 2018, furunculosis had been observed and treated on farms A and B by trimethoprim/sulfonamide and florfenicol antibiotics, respectively. Earlier, farm B had experienced furunculosis outbreaks that were treated with the same antibiotics and enrofloxacin (in July 2016 and 2017). Furthermore, Farm B also administered the furunculosis autovaccine from the end of 2018 through February 2020, with the last dose given 10 days before the start of the study. No vaccination was carried out in farm A.

These two fish farms also were selected based on their different environmental areas and biosecurity practices. Farm A, with 320 tonnes of production per year, was situated near other animal farms, including two other fish farms and several pig and cattle breeding sites located upstream of Farm A. Farm B, with 110 tonnes of production per year, was situated in an isolated area without any other farms nearby.

Sampling

Fish farms A and B were monitored monthly over seven months to survey the health of the fish and the administration of antibiotics in the case of disease outbreaks. Monthly samples of fish, pond water and biofilm were taken from two existing downstream raceways close to the end section of the rearing ponds on each farm. These ponds were dedicated to this study and no fish were added to the pond water during the study period. Sampling on farm A was realized from February to August while sampling on farm B was carried out from February to July (fish were slaughtered commercially in August). On farm A, two additional clinical samples also were taken following furunculosis episodes, one in May and another in July, which were collected one day before the start of the antibiotic treatment in July. One monthly sampling in August then was carried out one week after the end of the antibiotic treatment. The sampling schedule for April was cancelled on both farms due to health regulations related to the worldwide COVID pandemic.

All of the samples collected were transported for further bacteriological analysis under proper cold transport conditions on the day of the visit within 2-3 hours of being taken.

Fish samples

In total, 18 fish were sampled from each fish farm monthly and during the additional visits. The studied population was selected based on the maximum probability of isolating the *Aeromonas* bacteria from fish farms which were recently infected with furunculosis. The fish sample size for each pond (≤ 22500 fish per raceway) was determined by considering the frequency of furunculosis to be 30% in the studied farms based on the analysis of Cannon & Roe (Cannon and Roe, 1982; Thrusfield, 2007). Fish samples were autopsied and clinical lesions were recorded for each case. The samples of spleen, mucus from the posterior digestive tract, gills and skin mucus

were dissected and cultured on Agar GSP (Merck KGaA, Germany), the selective medium for detecting *Aeromonas* spp. (Palumbo et al., 1992).

Water samples

One liter of pond water was collected from each study pond using a sterile water bottle. Each water sample was filtered in 10 parts (10x100 ml) using the filtration manifold system (Millipore, Germany) through a cellulose nitrate membrane filter, 47-mm diameter, 0.22 µm pore size (Sartorius, Germany). The filter membrane then was placed in a petri dish into which 1ml of sterile normal saline solution was added. The bacteria were detached from the filter membrane by pipetting the sterile water on the membrane (Dufour et al., 1981). The solution then was diluted at 10^{-1} and 10^{-2}. Then 100 µl of each dilution were inoculated and thinly spread on Agar GSP (Merck KGaA, Germany).

Biofilm samples

Prior to the start of the study, biofilm experimental surfaces were created on a plastic structure and installed in each study pond. Each month, two biofilms were removed from each pond and taken for analysis. One biofilm surface was taken for the bacteriological analysis of cumulative previous months, and another biofilm surface was also collected from a previous month and then replaced with the biofilm surface of the following month. Each biofilm plate was placed in a sterile bottle filled with the corresponding pond water. The plastic biofilm surface (5 x 5 cm) was detached from the plate and put into the filtered sterile stomacher bag (177×302 mm) into which 10 ml of sterile normal saline solution was added. After being put in the mini-mixer (stomacher) (Lab-Blender 400, UK), which operated at a speed of 230 rpm for 15 min, attached cells were removed from the biofilm surface into the stomacher bag. Using a sterile pipette, the

filtered cells were aspirated from the stomacher bag and placed in a sterile tube (Baribeau et al., 2005). The samples were diluted at 10^{-2} and 10^{-3}, and then 100µl of solution was inoculated and thinly spread on Agar GSP (Merck KGaA, Germany).

Aeromonas isolation and identification

All seeded samples isolated from fish, water and biofilm were incubated at 22 °C for 48 h (Palumbo et al., 1992). Then, up to five yellow colonies often surrounded by yellow zone (depigmentation of the GSP medium) were removed per fish organ, and two colonies from biofilm and water samples. Each isolated colony was subcultured in Agar GSP for 48 h at 22 °C in order to obtain the pure colonies (Palumbo et al., 1992). After 48 h, the pure colony was inoculated in liquid medium (TSB) (Biokar, France) for 24 h at 22°C. *Aeromonas* spp. were identified at the genus level by polymerase chain reaction (PCR) (Khan et al., 2009), and cultures were conserved in a cryopreservation tube at -80 °C. By considering the origin and morphology of colonies in order to avoid the cluster-forming units, up to three *Aeromonas* isolates per sample were selected for antimicrobial susceptibility testing. Then, up to two *Aeromonas* isolates per sample, depending on the isolate's antimicrobial susceptibility profiles, were selected for antimicrobial resistance gene study.

Among these strains, some *Aeromonas* were considered as healthy environmental and fish isolates when no episode of furunculosis or antibiotic treatment had been observed. Other *Aeromonas* were isolated from furunculosis fish and some *Aeromonas* were considered as treated environmental and fish isolates when these strains were isolated following an antibiotic treatment.

Antimicrobial susceptibility test

The broth micro-dilution method (document M49-P, CLSI, 2006) was used to determine the MIC values of seven antimicrobial agents, namely flumequine, acid oxolinic, enrofloxacin, oxytetracycline, florfenicol, sulfamethoxazole-trimethoprim and colistin for *Aeromonas* isolates. In this study, the antimicrobial agents were chosen based on their use in veterinary medicine, mainly in aquaculture, and human medicine against *Aeromonas* infections and the consideration of antimicrobial resistance profiles (Lamy 2012b; Watts et al., 2017). To prepare the antibiotic solutions, each antimicrobial agent at 20X concentration first was prepared with the solvent recommended. The solutions of antimicrobial then were diluted 1:10 in BMH (Oxoid, UK) adjusted. Afterwards, a series of doubling dilutions of each antimicrobial agent was prepared in BMH to obtain final concentrations of flumequine (0.016- 256 µg/ml), acid oxolinic (0,016-64 µg/ml), enrofloxacin (0,016-64 µg/ml), oxytetracycline (0.016-512 µg/ml), florfenicol (0.25-32 µg/ml), trimethoprim-sulfamethoxazole (0.015/0.3-64/1216 µg/ml) and colistin (0.781-400 µg/ml) before the bacterial strains inoculation. Fifty microliters of each solution were distributed in 96-well microplates (Corning® 3367; 96 Wells, Costar, NY). The positive and negative control wells received 50 µl of BMH used for the preparation of dilutions and then the microplates were stored at −20 °C. For all MIC assays, *Escherichia coli* ATCC 25922 and *A. salmonicida* subsp *salmonicida* ATCC 33658 were used as reference controls (document M49-P, CLSI, 2006).

Briefly, the overnight TSA (BIOKAR ref. BK047HA; France) cultures of *Aeromonas* spp. were incubated in BMH broth at 22 °C for 24 h. Then *Aeromonas* spp. cultures were re-incubated for about 3-6 hours in BMH broth at 22 °C with continuous agitation. These cultures were diluted in BMH at 1% to obtain a final concentration at approximately 10^6 CFU/ ml. The calibration of inoculum was verified by bacterial enumeration. Fifty microliters of inoculum suspension of each

bacterial strain were mixed with 50 µl of each dilution of antimicrobial agents in U- bottom assay microplate (Corning® 3367- 96 Wells, USA). The positive and negative control wells received 50 µl of BMH used for the inoculum suspension. Microplates were incubated under aerobic conditions at 22 °C for 24 hours. MIC values were determined at the lowest concentration where no bacterial culture was observed after 24 hours of incubation in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI) , document M49-P (CLSI, 2006).

MIC and presumptive epidemiological cut-off values (COWT) analysis

From the distribution of the MIC values, the minimum inhibitory concentration required to inhibit the growth of 50% (MIC50) or 90% (MIC90) of the strains, and presumptive epidemiological cut-off values (COWT) were calculated.

COWT were calculated using two methods (Baron et al., 2017), namely the Kronvall and Turnidge methods (Kronvall, 2010; Turnidge et al., 2006). For the Kronvall method, a fully automated and freely available Excel spreadsheet calculator (updated version, 2019) was used to apply the normalized resistance interpretation (NRI) [available at http:// www.bioscand.se/nri/]). The Turnidge method was applied through an updated version (2020) of the ECOFFinder tool [available from the EUCAST website at https://www.eucast.org/mic_distributions_and_ecoffs/, ECOFF95%, SOP10.1). In this study, the determination of COWT (Kronvall and/or Turnidge) depended on the distribution of MIC values for each antibiotic for *Aeromonas* isolates.

Following the CLSI guidelines, microbial populations were separated into two interpretive categories: a wild-type population (WT), those with no mechanisms of acquired resistance or reduced susceptibility for the antimicrobial agent, and a non-wild-type population (NWT), those with presumed or known mechanisms of acquired resistance and reduced susceptibility for the

antimicrobial agent. The number and percentage of NWT were calculated by considering all Kronvall and/or Turnidge results. Multidrug resistance was defined as the absence of susceptibility to at least one agent in three or more antimicrobial categories (Magiorakos et al., 2012; Harnisz and Korzeniewska, 2018; Sweeney et al., 2018). In this study, the number of presumptive multi-antibiotic resistant *Aeromonas* (pMAR) was calculated for all environmental and clinical studied samples among five antimicrobial categories including quinolone, tetracycline, sulfonamide, polymyxin and phenicol.

Detection and relative abundance of *Aeromonas* ARGs

DNA extraction was performed following the protocol of isolating Genomic DNA Gram Negative Bacteria (Promega Corporation, 2019) using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions, with added enzymatic and mechanical cell lysis steps. Afterwards, DNA was quantified using Thermo Scientific™ Spectrophotometers NanoDrop™ 2000/2000c (Fisher Scientific SAS, France) and then stored at -80 °C until use.

The presence of common ARGs was studied in relation to the antibiotic classes frequently used in both veterinary medicine, mainly in aquaculture, and human medicine against *Aeromonas* infections (Lamy 2012b; Watts et al., 2017), including *qnr* and *aac(6')-Ib* for fluoroquinolone; *dfr*A, *sul* and *str* for sulfonamide-trimethoprim; *mcr* for polymyxin; *tet*A, *tet*B, *tet*C, *tet*D, *tet*E, *tet*G and *tet*M for tetracycline; *floR* and *cat*A for phenicol; *bla*-CTX-M, *bla*-ACC, *bla*-DHA, *bla*-IMP, *bla-KPC*, *bla*SHV, *bla*CMY for β-lactams and *mex*F for multidrug ARGs.

In total, a set of 44 specific primer pair genes and three housekeeping genes including *16S*-1, *16S*-2 rRNA and *rpo*B genes (Table 1) were selected to target sequence diversity within a gene (Liu et al., 2016; Xavier et al., 2016; Muziasari et al., 2017; Borowiak et al., 2017; Helsens et al.,

2020). A negative control (no DNA) was also considered in each quantitative PCR (qPCR) run. The qPCR amplification was performed by the "Human and Environmental Genomics" Platform (Rennes, France) using the Takara SmartChip Real-time PCR system (Takara, USA), which runs a high-throughput, nanoliter-scale real-time PCR. The 5184-well plates with a reaction volume of 100 nl were filled with the SmartChip MultiSample NanoDispenser (Takara, USA). The SmartChip MyDesign Kit (Takara, USA) was used and the PCR cycling conditions were as follows: denaturation at 95 °C for 5 min followed by 42 cycles included denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s. A final round of denaturation-annealing was performed. The specificity of amplification was assessed through the analysis of the melting curve of each PCR product. The detection limit of amplification was set at a threshold cycle (C_T) of 27 (Karkman et al., 2016; Muziasari et al., 2017; Zhu et al., 2013; Helsens et al., 2020). The relative abundance of each detected gene was calculated proportionally to the 16S-1 rRNA gene in each sample using the $2^{-\Delta CT}$ method, in which ΔC_T = (C_T detected gene – C_T 16S-1 rRNA gene) (Muziasari et al., 2017; Zhu et al., 2013; Helsens et al., 2020).

Statistical analysis

Statistical analyses were performed using R Studio software (version 1.4.1103), R Markdown package (R Core Team, 2021). The statistical analyses to compare the distribution of MIC values for antimicrobial agents and relative abundance of ARGs in *Aeromonas* strains isolated from healthy, furunculosis and antibiotic-treated fish and their environment on two fish farms were realized using the Kruskal-Wallis test. The statistical analyses to compare the occurrence of NWT *Aeromonas* strains and the presence of ARGs between the groups studied were realized using a

logistic regression test for each antimicrobial agent or ARG. A significant difference was expressed by a p-value below the 5% confidence interval.

Table 1: Primers used for PCR amplification. F: Forward; R: reverse.

Primer Name	Sequence (5' → 3')	Primer Name	Sequence (5' → 3')
***qnrA* F**	AGGATTTCTCACGCCAGGATT	*floR*-01 F	ATTGTCTTCACGGTGTCCGTTA
***qnrA* R**	CCGCTTTCAATGAAACTGCAA	*floR*-01 R	CCGCGATGTCGTCGAACT
***qnr*B F**	GCGACGTTCAGTGGTTCAGA	*cat*A1 F	GGGTGAGTTTCACCAGTTTTGATT
***qnr*B R**	GCTGCTCGCCAGTCGAA	*cat*A1 R	CACCTTGTCGCCTTGCGTATA
***aac(6')-Ib*-01 F**	GTTTGAGAGGCAAGGTACCGTAA	*bla*ACC F	CACACAGCTGATGGCTTATCTAAAA
***aac(6')-Ib*-01 R**	GAATGCCTGGCGTGTTTGA	*bla*ACC R	AATAAACGCGATGGGTTCCA
***aac(6')-Ib*-02 F**	CGTCGCCGAGCAACTTG	*bla*CMY F	CCGCGGCGAAATTAAGC
***aac(6')-Ib*-02 R**	CGGTACCTTGCCTCTCAAACC	*bla*CMY R	GCCACTGTTTGCCTGTCAGTT
***dfr*A1-01 F**	GGAATGGCCCTGATATTCCA	*bla*CTX-M-01 F	GGAGGCGTGACGGCTTTT
***dfr*A1-01 R**	AGTCTTGCGTCCAACCAACAG	*bla*CTX-M-01 R	TTCAGTGCGATCCAGACGAA
***dfr*A1-02 F**	TTCAGGTGGTGGGGAGATATAC	*bla*DHA F	TGGCCGCAGCAGAAAGA
***dfr*A1-02 R**	TTAGAGGCGAAGTCTTGGGTAA	*bla*DHA R	CCGTTTTATGCACCCAGGAA
***dfr*A12 F**	CCTCTACCGAACCGTCACACA	*bla*IMP-01 F	AACACGGTTTGGTGGTTCTTGTA
***dfr*A12 R**	GCGACAGCGTTGAAACAACTAC	*bla*IMP-01 R	GCGCTCCACAAACCAATTG
s*ul*1 F	CAGCGCTATGCGCTCAAG	*bla*IMP-02 F	AAGGCAGCATTTCCTCTCATTTT
***sul*1 R**	ATCCCGCTGCGCTGAGT	*blaI*MP-02 R	GGATAGATCGAGAATTAAGCCACTCT
***sul*2 F**	TCCGATGGAGGCCGGTATCTGG	*bla*IMP-03 F	GGAATAGAGTGGCTTAATTC
***sul*2 R**	CGGGAATGCCATCTGCCTTGAG	*bla*IMP-03 R	GGTTTAACAAAACAACCACC
***sul*3 F**	GCCGATGAGATCAGACGTATTG	*bla*KPC-02 F	CAGCTCATTCAAGGGCTTTC
***sul*3 R**	CGCATAGCGCTGGGTTTC	*bla*KPC-02 R	GGCGGCGTTATCACTGTATT
***str*A F**	AATGAGTTTTGGAGTGTCTCAACGTA	*bla*KPC-03 F	GCCGCCGTGCAATACAGT
***str*A R**	AATCAAAACCCCTATTAAAGCCAAT	*bla*KPC-03 R	GCCGCCCAACTCCTTCA
***str*B F**	GCTCGGTCGTGAGAACAATCT	*bla*SHV-01 F	TCCCATGATGAGCACCTTTAAA
***str*B R**	CAATTTCGGTCGCCTGGTAGT	*bla*SHV-01 R	TTCGTCACCGGCATCCA
***mcr*-1 F**	CGGTCAGTCCGTTTGTTC	*mex*F F	CCGCGAGAAGGCCAAGA
***mcr*-1 R**	CTTGGTCGGTCTGTAGGG	*mex*F R	TTGAGTTCGGCGGTGATGA
***mcr*-2 F**	TGTTGCTTGTGCCGATTGGA	*16S*-01 R	GGGTTGCGCTCGTTGC
***mcr*-2 R**	AGATGGTATTGTTGGTTGCTG	*16S*-01 F	ATGGYTGTCGTCAGCTCGTG
***mcr*-3 F**	TTGGCACTGTATTTTGCATTT	*16S*-02 R	CCTACGGGAGGCAGCAG
***mcr*-3 R**	TTAACGAAATTGGCTGGAACA	*16S*-02 F	ATTACCGCGGCTGCTGGC
***mcr*-4 F**	ATTGGGATAGTCGCCTTTTT	*rpoB* F	CGAACATCGGTCTGATCAACTC
***mcr*-4 R**	TTACAGCCAGAATCATTATCA	*rpoB* R	GTTGCATGTTCGCACCCAT
***mcr*-5 F**	ATGCGGTTGTCTGCATTTATC		
***mcr*-5 R**	TCATTGTGGTTGTCCTTTTCTG		
***tet*A-01 F**	GCTGTTTGTTCTGCCGGAAA		
***tet*A-01 R**	GGTTAAGTTCCTTGAACGCAAACT		
***tet*A-02 F**	CTCACCAGCCTGACCTCGAT		
***tet*A-02 R**	CACGTTGTTATAGAAGCCGCATAG		
***tet*B-01 F**	AGTGCGCTTTGGATGCTGTA		
***tet*B-01 R**	AGCCCCAGTAGCTCCTGTGA		
***tet*B-02 F**	GCCCAGTGCTGTTGTTGTCAT		
***tet*B-02 R**	TGAAAGCAAACGGCCTAAATACA		
***tet*C-01 F**	CATATCGCAATACATGCGAAAAA		
***tet*C-01 R**	AAAGCCGCGGTAAATAGCAA		
***tet*C-02 F**	ACTGGTAAGGTAAACGCCATTGTC		
***tet*C-02 R**	ATGCATAAACCAGCCATTGAGTAAG		
***tet*D-01 F**	TGCCGCGTTTGATTACACA		
***tet*D-01 R**	CACCAGTGATCCCGGAGATAA		
***tet*D-02 F**	TGTCATCGCGCTGGTGATT		
***tet*D-02 R**	CATCCGCTTCCGGGAGAT		
***tet*E F**	TTGGCGCTGTATGCAATGAT		
***tet*E R**	CGACGACCTATGCGATCTGA		
***tet*G-01 F**	TCAACCATTGCCGATTCGA		
***tet*G-01 R**	TGGCCCGGCAATCATG		
***tet*G-02 F**	CATCAGCGCCGGTCTTATG		
***tet*G-02 R**	CCCCATGTAGCCGAACCA		
***tet*M-01 F**	CATCATAGACACGCCAGGACATAT		
***tet*M-01 R**	CGCCATCTTTTGCAGAAATCA		
***tet*M-02 F**	TAATATTGGAGTTTTAGCTCATGTTGATG		
***tet*M-02 R**	CCTCTCTGACGTTCTAAAAGCGTATTAT		
***tet*M-03 F**	GCAATTCTACTGATTTCTGC		
***tet*M-03 R**	CTGTTTGATTACAATTTCCGC		

Results

Fish farms follow-up and clinical observations

Two fish farms were surveyed for the presence of furunculosis outbreaks and antibiotic treatment from February to August 2020. On farm A, two episodes of furunculosis were confirmed by the veterinarian in May and July with a mortality rate of around 2.1 % and 3.4%, respectively. Bacteriological analysis showed the presence of dark-brown bacterial colonies typical of *Aeromonas salmonicida* on TSA agar (BIOKAR ref. BK047HA; France). Clinical signs, such as lesions on the skin, hemorrhagic intestinal tract and splenomegaly, were observed in sampled fish more in July than May. In June, the mortality rate had decreased compared to May at 1.8% and no clinical signs were found in sampled fish. Fish were treated with flumequine at 12g/kg feed for eight days in late July 2020 and almost no mortality was observed thereafter (0.1%). Therefore, two additional clinical samplings from moribund or fish with furuncle (boil or lesion) were realized on farm A in May and July. Therefore, sampling in July, was performed one day before starting the antibiotic treatment. Afterward, in August, one monthly sampling was carried out one week after the end of the antibiotic treatment. No episodes of furunculosis and no antibiotic treatments were observed on farm B.

Antimicrobial susceptibility

A total of 257 *Aeromonas* spp. were selected for antimicrobial susceptibility tests from farms A and B, including 189 *Aeromonas* from fish samples and 68 from environmental strains (49 and 19 isolates from water and biofilm, respectively). Among these *Aeromonas* strains, 153 isolates were considered as healthy environmental and fish isolates, including 58 and 98 strains isolated from farms A and B, respectively, when no episode of furunculosis and no antibiotic treatment

have been observed. Fifty-four isolates were collected from fish with furunculosis signs after the confirmation of furunculosis on farm A. Fifty isolates were considered as treated environmental and fish isolates with the *Aeromonas* strains isolated following an antibiotic treatment.

For each antimicrobial susceptibility test, the MIC results obtained for the reference strains were in accordance with CLSI guidelines (data not shown) (CLSI, 2006). MIC value distributions of the seven antimicrobial agents and the corresponding MIC50 (median) and MIC90 (90th percentile) for 257 *Aeromonas* isolates are showed in Table 2. MIC values below the tested ranges varied, but were all less than 12% for most of the antimicrobials tested, while MIC values above the tested ranges were not observed for any of the isolates and antimicrobials tested in this study. The differences between the MIC50 and MIC90 values were at least four dilutions for oxytetracycline (OXY), enrofloxacin (ENRO), florfenicol (FFN) and colistin (COL). Oxolinic acid (OA) and flumequine (FLUQ) showed five and six dilutions, respectively, and trimethoprim-sulfamethoxazole (TMP) presented the most difference with seven dilutions between MIC50 and MIC90 values.

COWT values were calculated for seven antimicrobial agents for all isolates using the Kronvall and/or Turnidge methods (Table 2). Similar COWT values were obtained by Kronvall and Turnidge methods for all antimicrobials except for OA and OXY. The difference in the COWT values was only one dilution for OA (Kronvall lower than Turnidge method) but five dilutions for OXY. For OA, due to its MIC values distribution, a COWT value at 0.064 µg/ml by the Turnidge method seemed to be more appropriate to calculate the NWT isolates in this study. The COWT value was computed for OXY at 1 µg/ml using the Kronvall method while it was calculated at 32 µg/ml by the Turnidge method, which was greater than the highest MIC (16 µg/ml) for isolates tested in this study. Therefore, in this study, the COWT value was considered at 1 µg/ml for OXY

to calculate the number of non-wild-type (NWT) isolates or the isolates which presented MIC values higher than the COWT values.

The percentages of NWT *Aeromonas* ranged from 13% (FFN) to 60% (OXY). After oxytetracycline, the quinolone compounds (FLUQ, OA and ENRO) displayed the highest percentages, from 45% to 52% (Table 2). Among all of the isolates tested in this study, 109 (42%) isolates were considered as NWT strains for all of the three quinolone compounds (FLUQ, ENRO and OA).

Table 2: Distribution of MIC values (µg/ml) in 257 isolates of *Aeromonas* spp.

MIC (µg/ml)	0.008	0.016	0.032	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	HR (%)	MIC50	MIC90	COWT Kronvall (K) & Turnidge (T)	NWT (%)
Flumequine		1	37	40	16	14	39	28	13	3	2	2	15	16			31 (12%)	0. 5	32	0.25 (K&T)	118 (45%)
Oxolinic acid	29	47	17	10	10	13	33	27	9	5	22	9	1				25 (9%)	0.25	8	0.032 (K) 0.064 (T)	139 (53%) (T)
Enrofloxacin	12	49	33	23	48	29	28	24	5	1							5 (2%)	0.125	1	0.064 (K&T)	135 (52%)
Oxytetracycline					19	29	18	26	13	30	82	30					10 (3%)	4	16	1 (K) 32 (T)	155 (60%) (K)
Florfenicol					11	50	68	28	35	8	9	18					32 (12%)	0.5	8	2 (K&T)	35 (13%)
MIC (µg/ml)	**0.007/ 0.15**	**0.015/ 0.3**	**0.03/ 0.6**	**0.06/ 1.18**	**0.125/ 2.38**	**0.25/ 4.75**	**0.5/ 9.5**	**1/ 19**	**2/ 38**	**4/ 76**	**8/ 152**	**16/ 304**	**32/ 608**				**HR (%)**	**MIC50**	**MIC90**	**Kronvall (K) & Turnidge (T)**	**NWT (%)**
Trimethoprim-Sulfamethoxazole	35	56	43	32	23	3	6	8	15	12	14	7	3				-	0.03/0.6	4/ 76	0.06/ 1.18 (K&T)	91 (35%)
MIC (µg/ml)	**0.39**	**0.781**	**1.56**	**3.12**	**6.25**	**12.5**	**25**	**50**	**100**	**200**							**HR (%)**	**MIC50**	**MIC90**	**Kronvall (K) & Turnidge (T)**	**NWT (%)**
Colistin	7	57	56	41	13	12	6	32	14	**5**							15 (5%)	3.12	50	3.12 (K&T)	83 (32%)

Note: Gray color represents the selected range of dilutions for MIC values study. HR: number (percentage) of isolates for which the MIC value was below the range test (no isolate had an MIC value above the range test) in this study. COWT: Epidemiological Cut-Off Values were calculated using two methods Kronvall (K) and Turnidge (T). Green color represents the MIC > COWT values or the isolates of non-wild-type (NWT) resulting from Kronvall and/or Turnidge method. NWT (%): number (percentage) of isolates for which the MIC values were above the COWT value considered in this study.

Patterns of antimicrobial susceptibility in *Aeromonas* spp. isolated from healthy, furunculosis and antibiotic-treated fish and their environment

The distributions of antimicrobial susceptibility of 257 *Aeromonas* strains isolated from healthy, furunculosis and FLUQ antibiotic-treated fish and their environment for the antibiotics tested on fish farms A and B are presented in Figure 1. MIC distributions appeared to have a similar pattern for the quinolone compounds, showing three distinct populations for FLUQ, ENRO and OA, while OXY, FFN, TMP and COL presented a bimodal pattern. For all isolates, MIC values were distributed in greater antimicrobial agent concentrations (more than COWT) on farm A than on farm B ($p < 0.05$), except for FFN MIC values which were distributed similarly on both farms, mostly less than COWT. In healthy *Aeromonas* strains, greater MIC values were observed only for OXY and OA on farm A compared to farm B ($p < 0.05$).

On farm A, no significant differences were observed in MIC values between furunculosis *Aeromonas* strains and healthy isolates for all of the antibiotics tested ($p > 0.05$). Among the antibiotics tested, FLUQ, OA, ENRO (quinolone compounds), COL and TMP showed significantly higher MIC values for FLUQ-treated isolates compared to healthy *Aeromonas* strains on farm A ($p < 0.05$) (Figure 1).

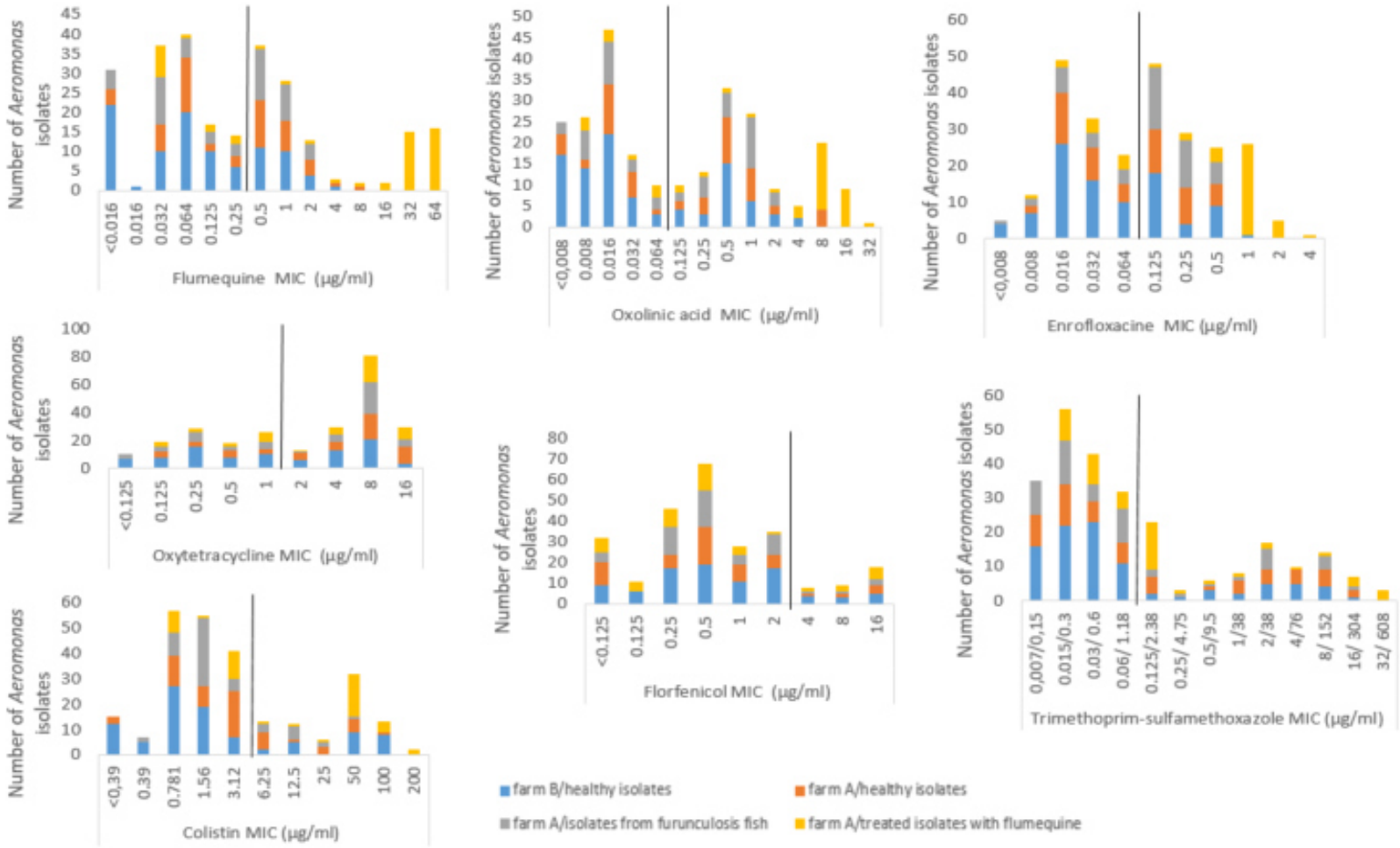

Figure 1: The distributions of antimicrobial susceptibility of 257 *Aeromonas* strains isolated from healthy, furunculosis and flumequine antibiotic treated fish and their environment for antibiotics tested in two fish farms (A and B). The calculated epidemiological cut-Off value (COWT) were showed with a bar to define the wild type (before the bar) and non-wild type (after the bar) populations in this study.

Distribution of NWT *Aeromonas* and pMAR in healthy, furunculosis and antibiotic-treated fish and their environment

NWT *Aeromonas* isolates for each antibiotic tested in this study originated from different sample collections, namely environmental (water and biofilm) samples and fish samples, with or without a furunculosis episode or flumequine treatment, on each farm (Figure 2). With regard to the occurrence of NWT *Aeromonas*, no significant differences were found between the environmental and fish samples studied on both farms for all antibiotics tested in healthy NWT *Aeromonas* ($p > 0.05$). Comparing the two farms, the occurrence of NWT *Aeromonas* for quinolone compounds (FLUQ, OA and ENRO), OXY and TMP was greater on farm A than on farm B for healthy strains ($p < 0.05$), but for FFN and COL, no significant differences were observed between farms A and B ($p > 0.05$). On farm A, furunculosis fish did not show a greater occurrence of NWT *Aeromonas* compared to healthy fish for all antimicrobial agents ($p > 0.05$). To study the presence of NWT *Aeromonas* for the healthy and FLUQ-treated strains studied, no differences were observed for OXY (healthy= 66% vs FLUQ-treated =68%). In contrast, the occurrence of treated NWT *Aeromonas* was greater rather than in healthy strains ($p < 0.05$) for COL (25% vs 58%), FLUQ (48% vs 76%), AO (52% vs 74%), ENRO (57% vs 78%), TMP (36% vs 54%) and FFN (10% vs 22%) (Figure 2). Nevertheless, these FLUQ-treated strains isolated from farm A were found with more NWT *Aeromonas* in fish rather than in environmental samples for all antibiotics tested ($p < 0.05$).

In this study, approximately 36% (93 out of 257) isolates of *Aeromonas* strains were determined as presumptive multi-antibiotic resistant bacteria (pMAR). With regard to the occurrence of pMAR *Aeromonas* for healthy strains, the presence of pMAR *Aeromonas* was greater on farm A than on farm B (37% vs 24%) ($p < 0.05$). However, the distributions of pMAR

Aeromonas for healthy isolates showed no significant differences between environmental and fish samples on both farms ($p > 0.05$)

On farm A, furunculosis fish did not show a greater occurrence of pMAR *Aeromonas* compared to healthy fish ($p > 0.05$). With regard to the presence of pMAR *Aeromonas* for the healthy and FLUQ-treated strains studied, a greater occurrence of treated pMAR *Aeromonas* rather than healthy ones were found (healthy= 32% vs FLUQ-treated =68%) ($p < 0.05$) and more pMAR *Aeromonas* were found in fish than in environmental samples (46% vs 28%) ($p < 0.05$) (Figure 2).

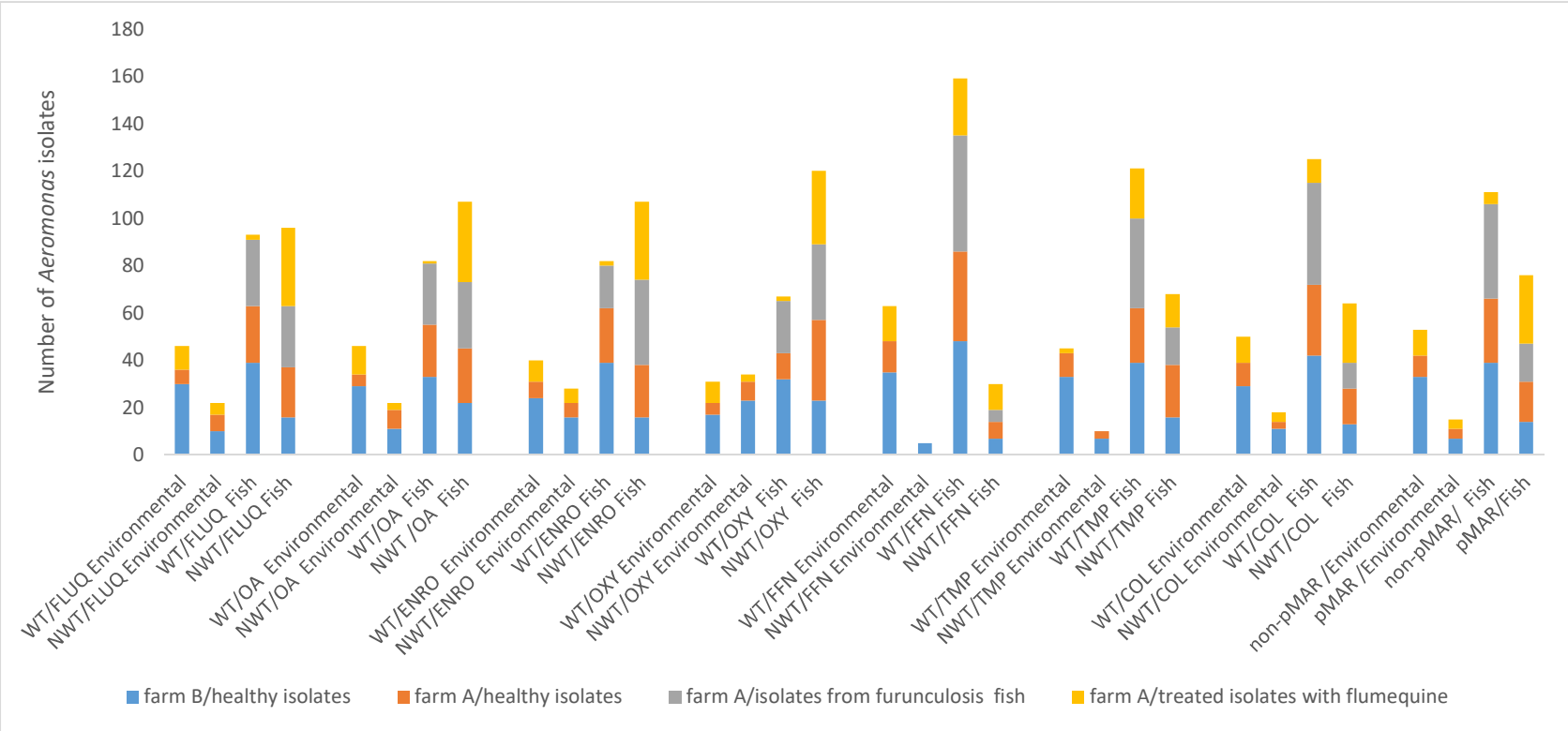

Figure 2: The distribution of wild type (WT) and non-wild type (NWT) *Aeromonas* isolated from environmental (water pond and biofilm) and fish samples for each antimicrobial agent on two fish farms (A and B). *Aeromonas* strains isolated from healthy, furunculosis and antibiotic treated fish and their environment. Note: no episode of furunculosis or antibiotic treatment were observed on farm B; FLUQ: flumequine; OA: oxolinic acid; ENRO: enrofloxacin; OXY: oxytetracycline; FFN: florfenicol; TMP: Trimethoprim-sulfamethoxazole; COL: colistin; pMAR: presumptive multi-antibiotic resistant *Aeromonas*.

Occurrence and abundance of *Aeromonas* antibiotic-resistant genes

Among 257 *Aeromonas* spp., 211 isolates were selected for ARG analysis by considering their origin and antimicrobial susceptibility profiles. The occurrence, number of strains which express the gene, and abundance estimated by the relative abundance (RA) of *Aeromonas* ARGs were studied for 44 specific genes including quinolones, tetracycline, sulfonamide-trimethoprim, phenicol, polymyxin, beta-lactam and multidrug resistance genes. Among these genes, 30 primers were detected and quantified in WT and NWT *Aeromonas* strains (Table 3).

For ARGs involved in quinolone resistance, four genes were expressed; *qnr*A, *qnr*B, *aac6Ib*01 and *aac6Ib*02. The occurrence of *qnr*A and *aac6Ib*02 was significantly greater in WT than in NWT *Aeromonas,* but no differences were found in terms of the average RA ($p>0.05$). For *qnr*B and *aac6Ib*01 genes, only one strain expressed these genes. This strain was a NWT *Aeromonas* isolated from a fish treated with flumequine from farm A, with a very high MIC for ENRO, FLUQ and OA (4, 8 and 32 µg/ml respectively) and the highest abundance for *qnr*B, *aacb6Ib*01 and *aac6Ib*02 genes (0.655, 0.640 and 0.512, respectively).

Among the ARGs tested that are involved in tetracycline resistance, no differences between NWT and WT strains were observed for occurrence and abundance of *tet*B2, *tet*C-02, *tet*D-02, *tet*G-02 and *tet*M1. For *tet*G01, although there was a difference between the RA of NWT and WT ($p<0.05$), the RAs were very low and no difference for occurrence was observed ($p>0.05$). *Tet*E was the dominant antibiotic-resistant gene in 72% (152/211) of *Aeromonas* studied. The occurrence of *tet*E gene was significantly greater in NWT than in WT *Aeromonas* (123 and 29 respectively) *(*$p <0.05$) but no significant differences were found for the RA between NWT and WT *Aeromonas*

(0.06 and 0.02, respectively) ($p > 0.05$) (Table 3). Finally, significant differences of occurrence and abundance between NWT and WT strains were only observed for tetA2 gene ($p < 0.05$).

Eight ARGs for sulfonamide-trimethoprim were expressed in *Aeromonas* strains. No differences between NWT and WT strains were observed for *str*A (occurrence and RA, $p > 0.05$). The occurrence, but not the RA, of *dfr*A1-1, *dfr*A1-2, *sul*1and *str*B was significantly greater in NWT than in WT *Aeromonas* ($p < 0.05$). Conversely, the RA, but not the occurrence, of sul2 was significantly greater in NWT than in WT *Aeromonas* ($p < 0.05$). For *dfr*A12 gene, only six NWT strains expressed this gene. These six strains were all isolated on farm A from fish treated with flumequine and had high MIC (2-38/32-608 µg/ml for TMP). Occurrence and RA were significantly greater for NWT than WT strains only for sul3 ($p < 0.05$) (Table 3).

For florfenicol, *floR*-1 was detected with a higher occurrence and RA for NWT *Aeromonas* than for WT strains ($p > 0.05$). Finally, for the polymyxin family, only *mcr*2 and *mcr*3 genes were expressed but at a very low level and there were no differences in their occurrence or RA between the NWT and WT strains (not applicable and $p > 0.05$, respectively) (Table 3).

Among the ARGs tested, *tet*A2, *sul*3 and *floR*1 were detected with a higher significant occurrence and abundance in NWT than in WT *Aeromonas* ($p < 0.05$). The distribution of these ARGs in WT and NWT *Aeromonas* is displayed among healthy, furunculosis and antibiotic-treated fish and their environment on the two fish farms (Figure 3). To compare the occurence of ARGs in "healthy" *Aeromonas* isolated from the two farms (fish and environment), no significant differences were found for the three ARGs ($p > 0.05$). Similarly, no significant differences were found between healthy environmental and fish strains isolated from both farms ($p > 0.05$). On farm A, more *tet*A2, but not *sul*3 and *floR*1genes, were detected for strains isolated from furunculosis

fish compared to healthy ones. For the three ARGs, no significant differences were seen between FLUQ-treated *Aeromonas* and healthy fish and environmental strains ($p > 0.05$) (Figure 3).

MexF genes were observed in 136 out of 211 studied isolates including 67 and 69 pMAR and non-pMAR strains, respectively. The occurrence and abundance of *mex*F were compared between these strains. This gene showed a higher average RA in pMAR *Aeromonas* than in non-pMAR *Aeromonas* (0.069 vs 0.005, $p< 0.05$). Similarly, the occurrence of *mex*F genes were significantly greater in pMAR rather than in non-pMAR *Aeromonas* (67/91:73% vs 69/120: 57% respectively) *(*$p <0.05$). No significant differences were found for *mex*F in healthy *Aeromonas* strains between the two farms ($p > 0.05$) (Figure 3). However, more *mex*F genes were found in fish samples than in environmental samples on farm A ($p < 0.05$), while on farm B, the occurrence of *mex*F genes in water and biofilm samples was higher than in fish samples ($p < 0.05$) in healthy *Aeromonas*. On farm A, no significant differences were seen between furunculosis and healthy strains for *mex*F ($p >0.05$). *Mex*F genes were more present in FLUQ-treated *Aeromonas* than in healthy isolates from fish samples, but not in environmental samples ($p < 0.05$) (Figure 3).

Three ARGs for beta lactams were expressed in *Aeromonas* strains including *bla*SHV-01, *bla*-IMP2 and *bla*-KPC3 genes. *Bla*-IMP2 and *bla*-KPC3 genes were expressed but at a very low occurrence and abundance. In contrast, *bla*SHV-01 genes were observed in 144 out of 211 *Aeromonas* strains with an RA at 0.0056. To analyze the occurence of *bla*SHV-01 genes in "healthy" *Aeromonas* isolated from the two farms (fish and environment), no significant differences were found between the two farms ($p >0.05$). However, more blaSHV-01 genes were found in fish samples than in environmental samples on farm A ($p < 0.05$), while on farm B, the occurrence of *blaSHV*-01 was higher in water and biofilm samples than in fish samples ($p < 0.05$) in healthy *Aeromonas*. On farm A, more *bla*SHV-01 genes were detected for strains isolated from

furunculosis fish compared to healthy fish ($p < 0.05$). A higher presence of *bla*SHV-01 in FLUQ-treated *Aeromonas* than in healthy ones was observed in both fish and environmental strains ($p < 0.05$) (Figure 3).

Table 3: The relative abundance (RA) [in proportion to the 16S rRNA gene] and distribution of antibiotic resistance genes (ARGs) in wild-type (WT) and non-wild-type (NWT) *Aeromonas* isolated from healthy, furunculosis and antibiotic-treated fish and their environment on two fish farms; FLUQ: flumequine; OA: oxolinic acid; ENRO: enrofloxacin; OXY: oxytetracycline; FFN: florfenicol; TMP: Trimethoprim-sulfamethoxazole; COL: colistin; pMAR: presumptive multi-antibiotic resistant *Aeromonas*; NS*:* not significant; NA*:* not applicable.

	***Aeromonas* spp (n=211)**		**ARGs**	**NWT**			**WT**				
	NWT (n)	**WT (n)**		**n**	**mean (RA)**	**sd**	**n**	**mean (RA)**	**sd**	**p-value (n)**	**p-value (RA)**
3 Quinolones	105	106	*qnr*A	34	0,00016	0,00027	47	0,00011	0,00014	p < 0.05	NS
FLUQ	113	98	*qnr*B	1	0,65586		0			NA	NA
OA	123	88	*aac(6')-Ib*-01	1	0,64029		0			NA	NA
ENRO	128	83	*aac(6')-Ib*-02	11	0,03037	0,12418	20	0,00018	0,00016	p < 0.05	NS
			*tet*A2	17	0,15126	0,30516	5	0,00013	0,00010	p < 0.05	p < 0.05
OXY	143	68	*tet*B2	74	0,00017	0,00029	40	0,00019	0,00025	NS	NS
			*tet*C-02	27	0,00048	0,00045	10	0,00087	0,00092	NS	NS
			*tet*D-01	6	0,00015	0,00010	0			NA	NA
			*tet*D-02	2	0,00003	0,00001	1	0,00019	0,00019	NS	NS
			*tet*E	123	0,05969	0,09113	29	0,02540	0,05679	p < 0.05	NS
			*tet*G-01	38	0,00039	0,00046	21	0,00077	0,00069	NS	p < 0.05
			*tet*G-02	9	0,00007	0,00008	4	0,00006	0,00004	NS	NS
			*tet*M1	13	0,00116	0,00381	7	0,00007	0,00002	NS	NS
			*tet*M2	1	0,01930		0			NA	NA
			*tet*M3	1	0,00031		0			NA	NA
TMP	81	130	*sul*1	41	0,05938	0,02253	5	0,02947	0,04186	p < 0.05	NS
			*sul*2	26	0,03711	0,07584	32	0,00046	0,00114	NS	p < 0.05
			*sul*3	45	0,13263	0,05046	14	0,02422	0,06013	p < 0.05	p < 0.05
			*dfr*A1-1	25	0,16801	0,06140	3	0,13359	0,11725	p < 0.05	NS
			*dfr*A1-2	18	0,12146	0,05408	4	0,05189	0,06499	p < 0.05	NS
			*dfr*A12	6	0,06721	0,00377	0			NA	NA
			*str*A	32	0,00042	0,00072	45	0,00023	0,00049	NS	NS
			*str*B	16	0,16930	0,23542	2	0,07267	0,12576	p < 0.05	NS
FFN	31	180	*floR*-1	16	0,06315	0,04174	24	0,00375	0,01542	p < 0.05	p < 0.05
COL	78	133	*mcr*2	0			1	0,00020		NA	NA
			*mcr*3	1	0,00004		3	0,01849	0,01841	NS	NS

	ARGs	**n**	**mean(RA)**	**sd**
Beta-lactams	*bla*SHV-01	144	0.0056534	0.0104554
	bla-KPC3	8	5,45E-05	4.6e-05
	bla-IMP2	1	6,68E-05	NA

			ARGs	**pMAR**			**non-pMAR**				
	pMAR (n)	**non-pMAR (n)**		**n**	**mean (RA)**	**sd**	**n**	**mean (RA)**	**sd**		**p-value**
Multi-resistance	91	120	*mex*F	67	0.0699265	0.0706432	69	0.0055260	0.0089965	p < 0.05	p < 0.05

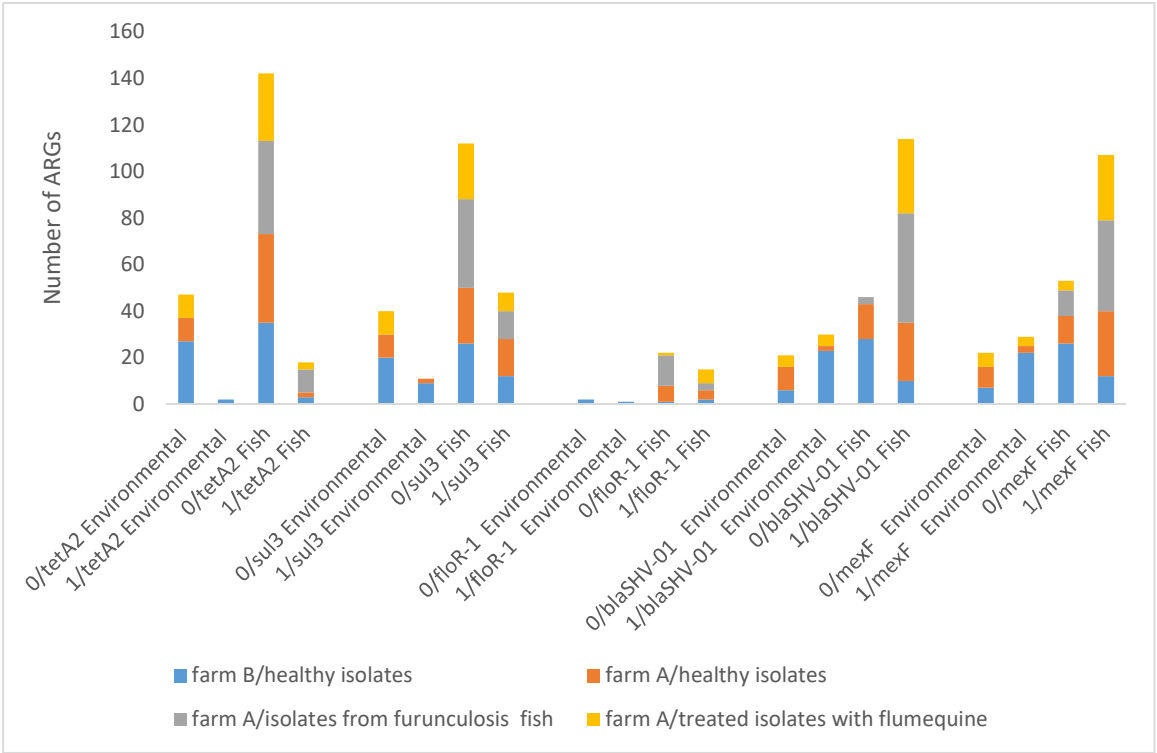

Figure 3: The distribution of antibiotic resistance genes (ARGs) in *Aeromonas* isolated from environmental and fish samples in fish farm A and B. *Aeromonas* strains isolated from healthy, furunculosis and antibiotic treated fish and their environment. Note: no episode of furunculosis and antibiotic treatment have been observed in farm B; 0: ARG has not been detected;1: ARG has been detected.

Discussion

In this study, MIC distributions of 257 *Aeromonas* strains isolated from fish, water and biofilm on two rainbow trout farms over seven months were determined for antibiotics commonly used against *Aeromonas* infections. MIC distributions and the MIC50 and MIC90 values calculated showed a few differences compared to a previous study (Baron et al., 2017), with our study finding three distinct populations for quinolones and much higher values for quinolones and OXY. The greatest difference was found in the MIC50 value for TMP (0.03/0.6 µg/ml) which was lower than those observed in four different studies relative to *Aeromonas* (Kämpfer et al., 1999; Goñi-Urriza et al., 2000; Lamy et al., 2012b; Baron et al., 2017). These differences can be explained by differences between Aeromonas species, in the sources (environmental or fish) and locations (isolated or farming area) where the strains were isolated, and in the occurrence of diseases with antibiotic treatments.

Aside from the methods used to calculate COWT (Kronvall or Turnidge) and a few differences in COWT values for some antimicrobial agents as OA and OXY, our results were in accordance with the results obtained by Baron et al., (2017) and Duman et al., (2020), although the origins of the *Aeromonas* strains were different between these two studies (freshwater of different rivers or cultured fish) and our study (water, biofilm and fish samples from fish farms including an episode of furunculosis and FLUQ treatment).

Few epidemiological cut-off values for *Aeromonas* could be found in the reference reports of antimicrobial susceptibility testing. CLSI has proposed the epidemiological cut-off values of *Aeromonas salmonicida* for FFN (4 µg/ml) and ormetoprim-sulfadimethoxine (0.5/9.5 µg/ml), and the clinical break point for OXY (susceptible: ≤ 1 µg/ml) and OA (susceptible: ≤ 0.12 µg/ml) (CLSI, 2020). In addition, EUCAST has determined the clinical break point of *Aeromonas* spp. for TMP (susceptible: ≤ 2 µg/ml) (EUCAST, 2021). Aside from

Aeromonas species, our COWT were close to these values indicated by CLSI and EUCAST. It has been argued that in the absence of a clinical break point for various antimicrobial agents, especially in *Aeromonas* spp., epidemiological cut-off values could be used to detect and monitor resistance (Smith et al., 2007). Although interpretative criteria change over time, determining COWT and delineating WT (susceptible) from NWT (not susceptible) populations allowed us to evaluate antibiotic resistance profiles in *Aeromonas* spp.

On both farms A and B, NWT and pMAR isolates were detected in healthy *Aeromonas* strains from fish and environment samples, but the occurrence of NWT for FLUQ, OA, ENRO, OXY and TMP and pMAR *Aeromonas* was higher on farm A than farm B. The detection of antibiotic-resistant and MAR *Aeromonas* spp. on rainbow trout farms and in other various freshwater environments was previously reported by several authors revealing that the presence of ARB could be due to the history of diverse antibiotic administrations on fish farms and/or to various animal and human activities in upstream areas (Saavedra et al., 2004; Naviner et al., 2011; Vega-Sánchez et al., 2014). On both farms studied, antibiotic treatments in fact had been prescribed two, three and four years previously to our knowledge. The higher presence of NWT and pMAR *Aeromonas* strains on farm A may be due to the input river water being contaminated by various human activities and by effluents from the other fish, pig and cattle breeding sites located upstream of farm A, while farm B was situated in an isolated area. Naviner et al., (2011) observed *Aeromonas* quinolone-resistant strains prior to an antibiotic treatment on a trout farm where the water was contaminated by effluents of farm activities upstream of the fish farm.

One week after FLUQ treatment, we found that the occurrence of NWT for quinolones (FLUQ, AO and ENRO) and also for other antimicrobial classes as COL, TMP and FFN as well as pMAR *Aeromonas* in FLUQ-treated isolates was greater than in heathy isolates.

Similarly, Naviner et al., (2011) observed more *Aeromonas* quinolone-resistant strains after FLUQ treatment compared to prior antibiotic exposure on a rainbow trout farm. They also presented the resistance profiles of other antimicrobial classes like OXY, TMP and FFN in FLUQ-treated isolates. The increase of *Aeromonas* spp. resistant to quinolones and other antimicrobial classes may be associated with FLUQ treatment for which genetic determinants responsible for the resistance are frequently carried on mobile genetic elements like plasmids, transposons and integrons borne on specific transposons or plasmids (Arattoli, 2001; Gordon et al., 2007; Naviner et al., 2011). The occurrence of NWT and pMAR *Aeromonas* increased quickly (one week in our study) and then could persist at least 22 days (Naviner et al., 2011) after the FLUQ treatment on the fish farm. Similarly, Guardabassi et al. (2000) already found in the water of a trout farm the persistence of antibiotic-resistant *Acinetobacter* up to six months after the end of the OA treatment.

A high presence of NWT *Aeromonas* for OXY (67% of *Aeromonas*) was observed in this study, which could be explained mainly by the predominant occurrence of *tet*E efflux pump gene in NWT rather than in WT *Aeromonas* (COWT: 1µg/ml; 123 vs 29 isolates). However, the gene *tet*A2 showed significant differences between NWT and WT strains for both occurrence and abundance. Previous research showed the high occurrence of *tet*E gene in NWT *Aeromonas* (43 vs 22 isolates; COWT: 2 µg/ml) (Duman et al. 2020). Similarly, *tet*E and/or *tet*A have been detected as a common *tet* gene studied (A-E) in motile *Aeromonas* strains from Danish and Turkey fish farms and environments (Agersø et al., 2007, Duman et al. 2020). In our study, the occurrence of efflux pump genes (*tet*A-G) was greater than ribosomal protection protein *tet* gene (*tet*M) in *Aeromonas*. However, Muziasari et al. (2017) found high abundances of *tet* M in intestinal DNA from farm-raised salmonid fish.

Although 105 out 211 *Aeromonas* strains (49%) were consider as NWT for the three quinolones (FLUQ, OA and ENRO), all four plasmid-mediated quinolones resistance genes studied including *qnr* (A and B) and *aac6Ib* (01 and 02) genes did not seem to be involved in this quinolone resistance. Indeed, the occurrence of *qnr*A and *aac6Ib*02 was significantly greater in WT than in NWT *Aeromonas* (no differences were found between the average RA). For *qnr*B and *aac6Ib*01 genes, only one strain expressed these genes. This may be explained by the potential presence of other quinolone ARGs that have not been studied, such as *qnr*S and *aac-6'-Ib-cr* (Fang et al., 2014; Chenia, 2016). Our findings are in accordance with a previous study in which neither the *qnr*A nor the *qnr*B gene was detected in any of the 40 resistant *Aeromonas hydrophila* strains isolated from aquatic animals, and only two strains were detected with *aac6Ib,* while all the enrofloxacin-resistant isolates harbored *qnr*S plasmid-mediated quinolone resistance genes (Fang et al., 2014). Conversely, Chenia (2016) showed no *aac-6'-Ib-cr* but a high prevalence of *qnr*B and *qnr*S (41% and 24% respectively) for *Aeromonas* spp. isolated from South African freshwater fish. However, in our study, a strain highly resistant to the three tested quinolones also expressed the highest abundance for *qnr*B and *aac6Ib* (01 and 02) genes, showing the importance of these genes in the resistance to quinolones.

Around 38% of *Aeromonas* spp. (91 out of 211 isolates) were determined to be NWT isolates for TMP, which can be linked to the presence of eight studied ARGs, mainly sulfonamides resistance genes like *sul*1, *sul*2, *sul*3 and *str*B, and trimethoprim resistance genes (dihydrofolate reductase) such as *dfr*A1-1 and *dfr*A1-2, which were expressed significantly more in NWT than in WT *Aeromonas* (occurrence or abundance). However, only *sul*3 showed a higher occurrence and RA in NWT than in WT strains. Of the TMP resistance genes studied, *sul*3 gene therefore may play a greater role in the spread of *Aeromonas* resistant bacteria in aquatic environments. Duman et al. (2020) and Capkin et al. (2017) reported *sul*1 as the most common TMP resistance gene in *Aeromonas* species, but Piotrowska and Popowska (2015)

indicated a higher presence of *sul*2. The differences observed between studies can be attributed to the regional diversity of the isolates.

Although 78 out of 211 strains (36%) were determined as NWT for colistin, only a few polymyxin genes such as *mcr*2 and *mcr*3 genes (1 and 4 strains respectively) were detected, while *mcr* 1 to 5 were the resistance genes most found among *Aeromonas* species and other gram-negative bacteria such as *E. coli* (Gharaibeh and Shatnawi, 2019). In our study, resistance to florfenicol (COWT: 2 µg/ml) was found for 31 out 211 isolates (14%), which can be associated with the higher occurrence and abundance of *floR*-1 efflux pump gene in NWT than in WT isolates. Our results are in line with previous authors who considered that most fish pathogenic bacteria, including *Aeromonas* spp., mediate florfenicol resistance through *floR* (Tekedar et al., 2020; Duman et al. 2020). Although β-lactam antibiotics are not used in aquaculture, *bla*SHV-01 was detected in 144 out of 211 (68%) *Aeromonas* strains in our study. Indeed, *Aeromonas* strains seem to be intrinsically resistant to this antibiotic family (Bakken et al., 1988). Two previous studies showed the low sensitivity of *Aeromonas* strains to β-lactams and an unexpected imipenem and the presence of *bla*CphA/IMIS *intI*1 and *bla*SHV (ESBL genes with class 1 integron) in *Aeromonas* from farmed rainbow trout (Saavedra et al., 2004; Vega-Sánchez et al., 2014). Therefore, resistance to β-lactams in ubiquitous *Aeromonas* bacteria can be a great concern for public health due to the frequent administration of these antibiotics in human medicine (Saavedra et al., 2004).

The mex systems were associated with multidrug resistance genes like *Mex*AB-OprM, *Mex*CD-OprJ, *Mex*EF-OprN and ect. In our study, 136 of the 211 *Aeromonas* studied carried *mex*F genes with occurrence and RA greater in pMAR *Aeromonas* than in non-pMAR *Aeromonas* for five antimicrobial classes. To our knowledge, it is the first description of a mex

system detected for *Aeromonas spp*. Only *AheABC* multidrug efflux pump was expressed in *A. hydrophila* at a low level involving an intrinsic multidrug resistance (Hernould et al., 2008).

By comparing the *bla*SHV-01 and *mex*F distributions on the two fish farms, we found the same profiles of antimicrobial resistance among *Aeromonas*. The occurrence of these genes was significantly higher in fish than in water and biofilm collected from farm A, while farm B showed the inverse. This may be explained by the different location of each farm and their distance from other animal and human facilities which may result in the spread of ARB and ARGs. As farm B was situated in an isolated area, the ARGs could have come from a long distance away. Previous studies found that antibiotic resistance bacteria and resistance genes may be transferred by the water current and persisted even over a long distance (20 km downstream) (Sabri et al., 2020).

Furthermore, a greater occurrence of pMAR and quinolone resistant bacteria on the one hand, and *bla*SHV-01 and *mex*F genes on the other, were detected in *Aeromonas* spp. isolated from FLUQ-treated fish and their environment than in healthy strains. Previous findings revealed that a two-component regulatory system of two proteins (an inner membrane histidine kinase and a cytoplasmic response regulator) interconnects resistance to polymyxins, (fluoro)quinolones and β-lactams in *Pseudomonas aeruginosa*. The mechanisms of resistance for these antimicrobial agents such as an altered permeability, an increased drug efflux and a reduced porin pathway of the bacterial membrane could be integrated through an overexpression of the *mex* efflux system in gram-negative bacteria such as *Pseudomonas aeruginosa* (Muller et al., 2011). Similarly, *Aeromonas* spp. as a gram-negative bacteria might harbor the multidrug resistance mechanisms for quinolone and beta-lactam antimicrobial agents mainly after a FLUQ treatment.

Conclusion

This study demonstrates that aquaculture farms may be considered as a huge environmental reservoir of multidrug resistance bacteria and ARGs, and suggests that *Aeromonas* may be used as an indicator of antimicrobial susceptibility for aquatic ecosystems. Our findings clearly show that human and animal husbandry activities on the one hand, and antibiotic treatments administered on fish farms on the other, impact the presence and dissemination of ARB and ARGs in fish and their environment. There is thus a high risk that resistance genes may develop and spread between fish, their environments and humans. Future research should focus on screening and quantifying plasmids and other mobile genetic elements involved in antimicrobial resistance from *Aeromonas* isolates in aquatic systems and their persistence in the environment also should be studied. Moreover, the maintenance and dissemination of ARB and ARGs associated with antibiotics that are mainly applied in aquaculture and also are used in human medicine need to be examined. Our findings point out that the increase and persistence of ARB and ARGs on mobile genetic elements after an antibiotic treatment on a fish farm might have a great impact on human, animal and environment health. Furthermore, sustainable aquaculture practices investing in new approaches to reduce the spread of antibiotic resistance need to be established.

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Data availability statement

Data sharing not applicable - no new data generated.

Conflict of interest

The authors declare that they have no conflict of interest**.**

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Chapter 3: Efficacy of functional alternative additives against furunculosis (*Aeromonas salmonicida* subsp. *salmonicida*) on rainbow trout (*Oncorhynchus mykiss*) under experimental and natural conditions

According to bibliography studies, the majority of studied alternatives were carried out on probiotics, plants, and prebiotics. In addition, essential oils (EOs) derived from plants have been also evaluated against *Aeromonas* infections in aquaculture. These products have been added to a commercial fish feed to achieve a favorable effect on survival rate and immunological parameters of fish in many experimental *Aeromonas* infections. However, they have not yet been tested in natural conditions in fish farms (Article N°1). In order to select an appropriate alternative product, the applicability, feasibility and efficacy of applying these products in fish farms have to be considered. Firstly, the alternative substances have to be commercialized and compatible with the qualified presumption of safety (QPS) criteria, intended to provide a harmonized generic pre-evaluation to support safety risk assessments of biological agents performed by EFSA's (European Food Safety Authority) scientific Panels. Then, *in vitro* and *in vivo* studies as well as on farm research have to be conducted on functional alternatives products to ensure their safety and efficacy. For example, for probiotics, taxonomic identity of applied microorganisms has to be defined. Moreover, scientific data need to be sufficient to establish their safety for humans, animals or environment. Their lack of pathogenic and virulence properties and resistance plasmid have to be established and substantiated, and thereby their intended use have to be clearly described and well defined (EFSA 2017). However, poor viability and high susceptibility of probiotic bacteria strains to the environmental conditions like fish ponds, and also their survival and activity in fish intestine as well as biological safety and regulatory restrictions have encouraged scientifics to investigate innovate methods to control fish disease in aquaculture. Recently, the use of essential oils or

their bioactive compounds, for controlling diseases that compromise the production and productivity of fish has been increasing. Herbal therapy is a potentially beneficial alternative for fish farming, since it may be cheaper and more safe for environment. Among these alternatives products, commercial prebiotics (fibers and natural sugars from bacteria, yeast cell walls or plants) and commercial plant essential oils, as feed additives, seem to be more practical rather than probiotics (live microorganisms) to be manufactured in feed industries by considering the manufacturing process including high heat and pressure, biological safety and regulatory restrictions (Martinez Cruz et al. 2012). Therefore, in this project, we focused on commercial prebiotics and essential oils products to evaluate their efficacy against *Aeromonas* spp. Based on the bibliographic analysis, we have selected the most studied prebiotics alternatives against *Aeromonas* spp. (Article N° 1) including fructose and mannan oligosaccharides (FOS and MOS) and various essential oils (cinnamon, oregano, thyme, clove and etc.) and their major phytochemical compounds (cinnamaldehyde, carvacrol, thymol and etc.) by considering their antimicrobial and immunostimulant effects in aquaculture.

We have tested the selected alternatives products, including essential oils and their major phytochemical compounds, in *in vitro* studies using broth micro dilution method, against the most known *Aeromonas* pathogen bacteria in salmonid freshwater fish, *A. salmonicida* subsp. *Salmonicida*. Regarding the structure of prebiotics as a non-digestible carbohydrate, the effects of prebiotics against bacterial diseases can be explained through their immunostimulant and indirect antibacterial properties as well as through the modification of gut microbiota. Moreover, prebiotics influence the gut microbiota by inducing the expression of some immunomodulatory molecules, especially cytokines, in fish gastrointestinal tract (Ringø et al. 2010). Since, probiotics, alone, do not show direct antimicrobial effect against bacteria in *in vitro* studies. Therefore, we have assessed the *in vitro* antimicrobial effect of various commercial essential oils and of their chemical constituents on *A. salmonicida* subsp.

salmonicida, which is presented in this manuscript as a research article (Article N° 3**)**: *In vitro* antimicrobial effect of various commercial essential oils and their chemical constituents on *Aeromonas salmonicida* subsp. *salmonicida*).

A- *In vitro* studies

a) Article N° 3: *In vitro* antimicrobial effect of various commercial essential oils and their chemical constituents on *Aeromonas salmonicida* subsp. *Salmonicida*

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SPECIAL ISSUE ARTICLE

In vitro antimicrobial effect of various commercial essential oils and their chemical constituents on *Aeromonas salmonicida* subsp. *salmonicida*

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Keywords
Aeromonas salmonicida subsp. *salmonicida*, antimicrobials, aquaculture, diseases, essential oils, fish (live), phytochemicals, resistance.

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Abstract

Aims: This study aimed to evaluate *in vitro* efficacy of essential oils (EOs) and their compounds (EOCs) alone or in combination against *Aeromonas salmonicida* subsp. *salmonicida*, the causative agent of furunculosis in salmonid fish.

Methods and Results: Antimicrobial activity of 13 EOs and 16 EOCs was investigated for four *A. salmonicida* subsp. *salmonicida* strains using broth microdilution. The checkerboard assay was used to evaluate a putative synergy between the most efficient EOs and EOCs against the tested strains. Cinnamon bark, oregano, clove, and thyme oils and their major compounds cinnamaldehyde, eugenol, carvacrol and thymol showed the lower minimum inhibitory concentration and minimum bactericidal concentration values. The association of cinnamaldehyde and eugenol (V/V: 30%/70%) showed a synergistic activity against three tested strains. The combinations of cinnamon with oregano, clove or thyme EOs showed a neutral or additive activity against all the tested strains.

Conclusions: Cinnamon, oregano, clove and thyme oils and their major phytochemical compounds showed strong activities against *A. salmonicida* subsp. *salmonicida* strains.

Significance and Impact of the Study: To reduce the use of antibiotics in aquaculture, phytochemicals such as cinnamaldehyde and eugenol can be tested alone or in combination in *in vivo* studies as functional feed alternatives.

Introduction

Aquaculture has become the fastest growing sector producing food for human consumption around the world (FAO 2018). In intensive aquaculture, farmed fish can be affected by various infectious diseases (Kennedy *et al.* 2015). Furunculosis is a common infection in freshwater fish, especially salmonid species, and is caused by *Aeromonas salmonicida* subsp. *salmonicida* (Austin and Austin 2012). This disease is responsible for haemorrhagic septicaemia in the acute form, and fish depreciation due to the development of boils in the muscles in the chronic form (Austin and Austin 2012). Antibiotic treatments may therefore be prescribed to treat animals and avoid significant economic losses due to the bacterial disease (Romero *et al.* 2012). However, the emergence of resistance to multiple antimicrobial agents has become a global health issue (Ranjan *et al.* 2017; Rasul and Majumdar 2017; Santos and Ramos 2018). To decrease the putative negative impact of the use of antibiotics in fish farms, alternative strategies have been developed to improve fish health while reducing the spread of antimicrobial resistance. Phytochemical therapy seems to offer potential alternatives to antibiotic use in aquaculture (Romero *et al.* 2012; Cunha *et al.* 2018).

Over the past two decades, several studies have evaluated the efficacy of plant essential oils (EOs) against fish diseases. Due to the principal active components of their EOs (e.g. phenolics, aldehydes and terpenoids), medicinal plants can have various beneficial effects by enhancing nonspecific immune responses in fish and direct antibacterial activity (Romero *et al.* 2012; Nazzaro *et al.*

2013; Reverter *et al.* 2017; Cunha *et al.* 2018; Stratev *et al.* 2018). Some studies have investigated the antibacterial properties of phytochemicals against *Aeromonas* sp. such as cinnamaldehyde (Okolie and Chenia 2013; Kot *et al.* 2019), eugenol (Sutili *et al.* 2014; Bandeira Junior *et al.* 2018), carvacrol (Zheng *et al.* 2009; Bandeira Junior *et al.* 2018) and thymol (Zheng *et al.* 2009; Heo *et al.* 2012; Bandeira Junior *et al.* 2018), which are the main components of cinnamon, clove, oregano and thyme EOs, respectively (Cunha *et al.* 2018; Stratev *et al.* 2018). The increased interest in alternative phytochemical substances has furthermore encouraged experiments to find new medical applications by exploiting synergistic activities to increase their efficacy (Chouhan *et al.* 2017). However, synergy between EOs and/or essential oil compounds (EOCs) against *A. salmonicida* subsp. *salmonicida* has not been studied extensively. The present paper determined the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of several EOs and their major chemical constituents alone or in combination against four *A. salmonicida* subsp. *salmonicida* strains using broth microdilution *in vitro* assay. This study was undertaken to identify the EOs able to efficiently overcome antimicrobial resistance.

Materials and methods

Bacterial strains

Antimicrobial sensitivity to EOs/EOCs was tested in four *A. salmonicida* subsp. *salmonicida* bacterial strains that were isolated in France: ATCC 14174 reference strain isolated from brook trout; CAE235 and CAE258 strains isolated from rainbow trout with furunculosis; and CAE452 environmental strain. Bacterial strains were cultured in routine microbiology culture investigations and were stored in a freezer (−80°C). The cultures were prepared in Tryptone casein soy agar (TSA) (Biokar ref. BK047HA; Beauvais, France) at 22°C for 24 h.

Essential oil and their compounds

In all, 13 EOs and 16 EOCs were purchased from Aroma-Zone (Paris, France) and Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany), respectively. The EOs and their biochemical constituents are listed in Table 1. The EO compounds were chosen based on the composition of our selected EOs. They included cinnamaldehyde, citral, citronellal, eucalyptol, linalool (or linalol), geraniol, (R)-(+)-limonene, (S)-(−)-limonene, sabinene hydrate/4-thujanol, thymol, carvacrol, eugenol, (−)-menthol, (−)-borneol, α-terpineol and (−)-terpinen-4-ol.

Antibacterial effect: broth microdilution method

Each product was investigated using antimicrobial susceptibility testing. The MIC and MBC of each selected product were determined using broth microdilution methods. The protocol was based on a reports by the national committee of the Clinical and Laboratory Standards Institute (CLSI) (CLSI 2014a, 2014b, 2014c) with certain adjustments. First, each product was diluted in ethyl alcohol 60% and then in Mueller-Hinton broth (BMH) (Oxoid, UK) adjusted to calcium and magnesium at 20–25 and 10–12·5 mg l^{-1}, respectively and supplemented with 2% of Tween 20 to reach a final concentration of approximately 8000 μg ml^{-1} before bacterial strains inoculation. Similarly, the binary combinations of selected EOs/EOCs were prepared (from 10 to 90%). Afterwards, a series of doubling dilutions of EOs/EOCs were prepared in BMH (1 : 2 to 1 : 128). For each EO/EOC, the exact final concentration in μg ml^{-1} was calculated taking into account the density and/or the purity reported by the manufacturer.

The overnight TSA cultures of the four *A. salmonicida* subsp. *salmonicida* strains were incubated in BMH broth at 22°C for about 3–6 h with continuous agitation to prepare an exponential phase of culture at approximately 10^8 CFU (colony forming unit) per ml. These cultures were diluted in BMH at 1% to obtain a final concentration at approximately 10^6 CFU per ml. The number of viable bacteria of four *A. salmonicida* subsp. *salmonicida* strains was then determined by bacterial enumeration to verify the bacterial concentration of the suspension. Fifty microliters of inoculum suspension of each bacterial strain were mixed with 50 μl of each dilution of EOs/EOCs tested in U-bottom assay microplate (Corning® 3367; 96 Wells, Costar, NY). The positive and negative control wells received 50 μl of BMH used for the preparation of EOs/EOCs dilutions and 50 μl of inoculum suspension or 50 μl of BMH, respectively. Microplates were incubated under aerobic conditions at 22°C for 24 and 48 h. MICs were determined at the lowest concentration where no bacterial culture was observed after 24 and 48 h of incubation using the method for broth dilution susceptibility testing of bacteria isolated from aquatic animals in accordance with the guidelines of the CLSI, document VET04-A2 (CLSI 2014b). To determine the MBCs of EOs/EOCs against *A. salmonicida* subsp. *salmonicida* strains after 48 h, 1 μl solution of each microplate well was placed on a TSA agar rectangular Petri dish (Biokar). After 24-h incubation at 22°C, MBCs were determined at the lowest concentration of each EO/EOC where no bacterial growth was observed under the binocular microscope. Finally, the MBC/MIC ratio was

Table 1 Volatile chemical constituents of various tested essential oils according to manufacturer

Essential oils	Main biochemical constituents: gas phase chromatography (%)		
Ceylon cinnamon bark (*Cinnamomum zeylanicum/verum*)	Aldehydes: (E)-cinnamaldehyde (68·31%) Terpene esters: cinnamyl acetate (2·52%)	Phenols: eugenol (3·68%) Sesquiterpenes: β-caryophyllene (5·67%)	Monoterpenes: β-phellandrene (3·06%)
Thujanol thyme thymus (*Thymus vulgaris*)	Monoterpenols: terpinen-4-ol (11·68%), myrcen-8-ol (2·90)%, (E) -thujanol (15·44%)	Esters: myrcen-8-yl acetate (5·83%)	Monoterpenes: γ-terpinene (7·82%)
Compact oregano (*Origanum compactum*)	Phenols: carvacrol (50·07%) Thymol: (15·13%)	Sesquiterpenes: beta-caryophyllene (1·57%)	Monoterpenes: γ-terpinene (14·49%), para-cymene (7·67%)
Clove (*Eugenia caryophyllata*)	Esters: eugenyl acetate (12·38%)	Phenol: eugenol (82·06%)	Sesquiterpenes: β-caryophyllene (4·23%)
Tea tree (*Melaleuca alternifolia*)	Monoterpenols: terpinen-4-ol: (42·65%), α-terpineol:(3·16%)	Monoterpenes: γ-terpinene (20·66%), α-terpinene (9·62%), terpinolene: (3·56%), para-cymene (1·00%)	_
Geraniol thyme vulgaris (*Thymus vulgaris*)	Monoterpenols: geraniol: (25·40%)	Esters: geranyl acetate (41·43%) myrcen-8-yl acetate (3·77%)	Sesquiterpenes: β-caryophyllene (5·64%)
Oregano vulgaris (*Origanum vulgare*)	Phenols: carvacrol (64·49%) Thymol (3·35%)	Terpenic alcohol: linalol: (2·20%)	Monoterpenes: γ-terpinene (8·29%), para-cymene (12·92%)
Ravintsara (*Cinnamomum camphora*)	Terpene oxides: 1.8-Cineole (61·09%)	Monoterpenes: Sabinene (13·43%), α-pinene (4·48%), β-pinene (3·34%)	Monoterpenols: α-terpineol (6·65%)
Provence linalool thyme vulgaris (*Thymus vulgaris*)	Terpenic esters: linalyl acetate (7·33%) Monoterpenols: linalol: (73·36%)	Phenols: thymol (2·84%)	β-caryophyllene (3·62%)
Provence green oregano (*Origanum heracleoticum*)	Phenols: carvacrol (65·51%)	Sesquiterpenes: β-caryophyllene (1·72%)	Monoterpenes: γ-terpinene (6·71%), para-cymene (12·34%)
Thyme satureoides (*Thymus satureoides*)	Monoterpenols: borneol (32·33%), α-terpineol (14·23%)	Terpenic phenols: carvacrol (9·54%), thymol (2·61%)	Monoterpenes: camphene (7·24%)
Cineole rosemary (*Rosemary officinalis*)	Terpene oxides: 1.8-Cineole (49·49%)	Ketones: camphor (9·24%) Monoterpenols: borneol: (2·21%)	Monoterpenes: α-pinene (13·70%), β-pinene (4·78%)
Thymol thyme vulgaris (*Thymus vulgaris*)	Terpenic phenols: thymol (46·17%), carvacrol (4·18%)	Monoterpenols: linalool (4·23%)	Monoterpenes: para-cymene (18·81%), γ-terpinene (10·19%), myrcene (1·61%)

calculated by considering the MIC values at 48 h for each product against four tested strains to determine the tolerance to bactericidal activity of each EO/EOC.

Checkerboard assay

We performed a checkerboard synergy test (Rand *et al.* 1993; Garcia and Isenberg 2010; Souza *et al.* 2014; Bandeira Junior *et al.* 2018) through the lowest FICI (fractional inhibitory concentration index) method as described in the Clinical Microbiology Procedures Handbook (Garcia and Isenberg 2010) to determine the synergistic, antagonistic or additive effects between the most efficient EOs/EOCs for *A. salmonicida* subsp. *salmonicida* strains. The different associations (50 + 50%) of Ceylon cinnamon bark (*Cinnamomum zeylanicum/verum*) with oregano (*Origanum vulgare*), clove (*Eugenia caryophyllata*) and thyme with geraniol (*Thymus vulgaris*) EO were tested. Ceylon cinnamon bark (*C. verum*) and oregano (*O. vulgare*) with different combinations (90 and 10%) were also tested. Similarly, binary associations of cinnamaldehyde and eugenol or thymol and carvacrol (90 and 10%) were studied. The results were determined by the following calculation of the FICI:

FICI = (MIC of product A in combination/MIC of product A alone) + (MIC of product B in combination/MIC of product B alone).

Synergy effect of EO or EOC products can be observed for FICI values ≤0·5; additivity or indifference effect for FICI values between 0·5 and 4; and antagonism for FICI values >4 (Souza *et al.* 2014; Bandeira Junior *et al.* 2018).

Statistical analysis

Nonparametrical data were analysed using Kruskal–Wallis, one-way ANOVA test (PRISM 5.0) to compare the MIC and MBC values of each *A. salmonicida* subsp. *salmonicida* strain with other strains in each repeated test.

Results

Minimum inhibitory concentration and minimum bactericidal concentration for various essential oils

Among all tested EOs, Ceylon cinnamon bark (*C. zeylanicum/verum*), oregano (*O. vulgare*, *O. compactum*, *O. heracleoticum*) and clove (*E. caryophyllata*) were the most effective EOs against *A. salmonicida* subsp. *salmonicida* strains (Table 2). We observed that these EOs showed low values with a MIC ≤520 μg ml^{-1} for at least three tested strains: ATCC 14174, CAE 235 and CAE 452. Thyme with geraniol or thymol (*T. vulgaris*) and thyme satureoides (*T. satureoides*) had a moderate value with a MIC between 520 and 1840 μg ml^{-1} for at least three tested strains. Thyme with thujanol or linalool (*T. vulgaris*), tea tree (*Melaleuca alternifolia*), ravintsara (*Cinnamomum camphora*) and rosemary with cineole (*Rosemary officinalis*) showed a high value with a MIC above 3200 μg ml^{-1} for at least two strains. The MIC and MBC values of the different *A. salmonicida* subsp. *salmonicida* strains varied, but the differences were not significant ($P \geq 0{\cdot}05$) (Table 2). The difference between the MICs after 24 and 48 h of incubation was not significant for all tested EOs ($P \geq 0{\cdot}05$) (results are not shown). For the products that were tested the most extensively, the MBC/MIC ratio was comprised between 1 and 2 (Table 2).

Minimum inhibitory concentration and minimum bactericidal concentration for compounds of various EOs

Among all of the main biochemical constituents tested, cinnamaldehyde, eugenol, carvacrol and thymol showed the lowest MIC values, respectively, against *A. salmonicida* subsp. *salmonicida*, especially cinnamaldehyde (MIC: 62–125 μg ml^{-1}) (Table 3). These EOCs also demonstrated the lowest MIC value ≤688 μg ml^{-1} for at least three tested strains (ATCC 14174, CAE 452 and 258). Geraniol, (−)-terpinen-4-ol, sabinene hydrate (4-thujanol), α-terpineol, (−)-Borneol, (−)-menthol and citral had moderate values with a MIC between 870 and 2000 μg ml^{-1} for all four tested strains. (R)-(+)-limonene, linalool, citronellal, (S)-(−)-limonene and eucalyptol showed high values with a MIC ≥4000 μg ml^{-1} for at least one tested strain.

As can be seen in Table 3, the MIC and MBC values of the different *A. salmonicida* subsp. *salmonicida* strains varied after exposure to EOCs, but the differences were not significant ($P \geq 0{\cdot}05$).

Table 2 Minimum inhibitory concentration (MIC) at 24 h and minimum bactericidal concentration (MBC)/MIC ratio at 48 h of various essential oils against *Aeromonas salmonicida* subsp. *salmonicida* strains

	A. salmonicida subsp. *salmonicida* strains							
	ATCC 14174		CAE 235		CAE 452		CAE 258	
	MIC (μg ml^{-1})	MBC/MIC	MIC (μg ml^{-1})	MBC/MIC	MIC (μg ml^{-1})	MBC/MIC	MIC (μg ml^{-1})	MBC/MIC
Ceylon cinnamon bark (*Cinnamomum zeylanicum/verum*)	245	1	245	1	61	1	490	1
Oregano vulgaris (*Origanum vulgare*)	226	1	226	2	113	1	453	1
Compact oregano (*Origanum compactum*)	458	1	458	1	229	1	458	1
Provence green oregano *(Origanum heracleoticum*)	458	1	458	1	458	1	458	2
Clove (*Eugenia caryophyllata*)	520	1	520	1	520	1	520	2
Geraniol thyme vulgaris *(thymus vulgaris*)	880	1	880	1	440	1	440	1
Thymol thyme vulgaris (*thymus vulgaris*)	907	1	907	1	907	1	907	1
Thyme satureoides (*Thymus satureoides*)	1840	1	1840	1	1840	1	1840	1
Thujanol thyme thymus (*Thymus vulgaris*)	1784	1	3568	1	892	1	≥3568	1
Tea tree (*Melaleuca alternifolia*)	3624	1	3624	1	3624	1	≥3624	1
Ravintsara (*Cinnamomum camphora*)	≥3592	1	3592	1	≥3592	1	≥3592	1
Provence linalool thyme vulgaris (*Thymus vulgaris*)	3360	1	≥3360	1	≥3360	1	≥3360	1
Cineole rosemary (*rosemary officinalis*)	≥3628	1	≥3628	1	≥3628	1	≥3628	1

Table 3 Minimum inhibitory concentration (MIC) at 24 h and minimum bactericidal concentration (MBC)/MIC ratio at 48 h of various compounds of EOs against *Aeromonas salmonicida* subsp. *salmonicida* strains

	A. salmonicida subsp. *salmonicida* strains							
	ATCC 14174		CAE 235		CAE 452		CAE 258	
	MIC (μg ml^{-1})	MBC/MIC	MIC (μg ml^{-1})	MBC/MIC	MIC (μg ml^{-1})	MBC/MIC	MIC (μg ml^{-1})	MBC/MIC
Cinnamaldehyde	125	1	125	2	62	1	125	2
Eugenol	250	2	250	1	125	2	250	2
Carvacrol	344	2	688	1	344	1	344	1
Thymol	500	2	500	1	500	1	500	2
Geraniol	870	1	870	2	870	1	870	1
(−)-Terpinen-4-ol	500	2	1000	1	500	2	1000	1
Sabinene hydrate: 4-thujanol	1000	1	1000	1	500	2	1000	1
α-Terpineol	2000	1	500	2	500	2	1000	1
(−)-Borneol	2000	1	1000	1	1000	2	2000	1
(−)-Menthol	2000	2	1000	2	1000	1	2000	2
Citral	1740	1	1740	1	1740	1	1740	1
(R)-(+)-Limonene	3334	1	3334	1	1667	1	3334	1
Linalool	≥3445	1	≥3445	1	≥3445	1	3445	1
Citronellal	≥3256	1	≥3256	1	≥3256	1	≥3256	1
(S)-(−)-Limonene	≥3342	1	≥3342	1	3342	1	3342	1
Eucalyptol	≥3647	1	≥3647	1	≥3647	1	≥3647	1

The difference between the MICs after 24 and 48 h of incubation was not significant for all tested EOCs ($P \geq 0{\cdot}05$) (results are not shown). Our results indicate an MBC/MIC ratio of 1 or 2 for the most extensively tested products.

Synergy test of various EOs and their major compounds

In the light of our results, the antibacterial effect of the most effective EOs against four A. *salmonicida* subsp. *salmonicida* strains, namely Ceylon cinnamon bark (*C. zeylanicum/verum*) oil in combination with oregano (*O. vulgare*), clove or geraniol thyme, were evaluated. The FICI of the associations of oregano (*O. vulgare*), clove or geraniol thyme EOs with cinnamon bark (50 + 50%) were comprised between 0·5 and 4 (data not shown), suggesting an additive effect between EOs. None of the combinations tested had an antagonist or a synergistic effect. The associations of the two strongest EOs, Ceylon cinnamon bark (*C. verum*) and oregano (*O. vulgare*), with different quantities (from 10 to 90%) demonstrated a similar pattern. Given our results, we studied the antibacterial effect of cinnamaldehyde and eugenol biochemical compounds of the most effective EOs (Ceylon cinnamon bark: cinnamaldehyde (68·31%) and eugenol (3·68%); Table 1) against four *A. salmonicida* subsp. *salmonicida* strains. Only the combination of cinnamaldehyde (30%) and eugenol (70%) against CAE235, CAE235, CAE452 and CAE258 showed a synergistic effect with a FICI = 0·47 (Table 4). For all of the other tested combinations of cinnamaldehyde and eugenol, there was an additive effect. The different associations (90 and 10%) of carvacrol and thymol, which are the main biochemical constituents of oregano (*Origanum vulgare*) EO (carvacrol (64·49%) and thymol (3·35%); Table 1), also demonstrated an additive activity against all tested strains (data not shown).

Discussion

Previous investigations demonstrated the antibacterial activity of phytochemicals against various bacterial cells (Dorman and Deans 2000; Vaseeharan and Thaya 2014; Perricone *et al.* 2015; Chouhan *et al.* 2017). Their activity can be classified as strong, moderate and weak based on their MIC value (Sartoratto *et al.* 2004; Souza *et al.* 2017). According to our results, a strong activity against the four *A. salmonicida* subsp. *salmonicida* bacterial strains, with MIC and MBC values ≤520 μg ml^{-1}, was determined for cinnamon (*C. verum*), oregano (*O. vulgare*, *O. compactum*, *O. heracleoticum)* and clove (*E. caryophyllata)* oil. Similar to our work, a strong activity for cinnamon (*C. cassia)*, oregano (*O. vulgare*), thyme (*T. vulgaris*) (Starliper *et al.* 2015) and clove (*E. caryophyllata)* (Kot *et al.* 2019) EOs have been shown against *A. salmonicida* subsp. *salmonicida*. EOs such as thyme with geraniol (*T. vulgaris*), thyme with thymol (*T. vulgaris*) and thyme satureoides (*T. satureoides*) had a moderate activity with a MIC between 520 and 2000 μg ml^{-1}. Our results are in accordance with the work of Navarrete *et al.* (2010), who found MIC values of *T. vulgaris* ranging from 80 to 1280 μg ml^{-1} against different bacterial

Table 4 Fractional inhibitory concentration (FIC) and fractional inhibitory concentration index (FICI) of cinnamaldehyde in association with different combinations of eugenol

	ATCC 14174		CAE235		CAE452		CAE258	
	FIC	FICI	FIC	FICI	FIC	FICI	FIC	FICI
Cinnamaldehyde 90%	3·61	3·66	1·79	1·84	0·89	0·91	1·79	1·84
Eugenol 10%	0·05		0·05		0·02		0·05	
Cinnamaldehyde 70%	1·40	1·47	0·69	0·76	1·40	1·55	0·69	0·76
Eugenol 30%	0·07		0·07		0·15		0·07	
Cinnamaldehyde 50%	2·01	2·26	1	1·25	1	1·25	1	1·25
Eugenol 50%	0·25		0·25		0·25		0·25	
Cinnamaldehyde 30%	0·59	0·76	0·29	0·47*	0·29	0·47*	0·29	0·47*
Eugenol 70%	0·17		0·17		0·17		0·17	
Cinnamaldehyde 10%	0·80	1·7	0·40	1·3	0·40	1·3	0·40	1·3
Eugenol 90%	0·90		0·90		0·90		0·90	

MIC 48 h is considered for FIC calculation.
*Indication of a synergistic effect.

fish pathogens. However, thyme with thujanol or linalool (*T. vulgaris*), ravintsara (*C. camphora*), rosemary with cineole (*R. officinalis*) and tea tree (*M. alternifolia*) showed a weak activity with MIC values above 3200 μg ml^{-1}. Similarly, Kot *et al.* (2019) showed a low sensitivity to *M. alternifolia* oil, with MIC values of tea tree for *A. salmonicida* subsp. *salmonicida* strains in the range of 780–3120 μg ml^{-1}.

In parallel with EOs, major compounds of EOs such as cinnamaldehyde, eugenol, carvacrol and thymol also had a strong activity, with MIC and MBC values ≤520 μg ml^{-1}. Phytochemicals such as geraniol, (−)-terpinen-4-ol, sabinene hydrate (4-thujanol), α-terpineol, (−)-Borneol, (−)-menthol and citral had a moderate activity, respectively, with MIC values between 520 and 2000 μg ml^{-1}. However, limonene, linalool, citronellal and eucalyptol showed a weak activity with MIC values above 3200 μg ml^{-1}. The antimicrobial activity of the major compounds of EOs resembled that of the EOs themselves. EOCs of EOs with strong activity also demonstrated strong activity, while those of EOs with a moderate to weak activity likewise demonstrated weak efficacy against the *A. salmonicida* subsp. *salmonicida* strains. Hence, the major components of EOs have the most critical antibacterial responsibility.

In the present study, cinnamaldehyde was the most effective phytochemical with a MIC value from 62 to 125 μg ml^{-1} against the four *A. salmonicida* subsp. *salmonicida* strains. The efficacy of cinnamaldehyde against bacteria can be explained by perturbation of cytoplasmic enzymes, increase in the membrane permeability of the bacterial cell and alteration of the fatty acids structure of bacterial cell membrane (Wendakoon and Sakaguchi 1995, Dorman and Deans 2000, Andrade-ochoa *et al.* 2015). It has been seen that the MIC value of cinnamaldehyde (MIC: 10 μg ml^{-1}) can be comparable with MIC values of antibiotics such as oxytetracycline and gentamicin (MIC: 10 and 50 μg ml^{-1}, respectively) for treatment of fish infections caused by *A. salmonicida* subsp. *salmonicida* (Kot *et al.* 2019). The efficacy of the other compounds with high activity, such as eugenol, carvacrol and thymol, can be related to their phenol group, which may increase membrane permeability by acting on the outer membrane of bacteria at low concentrations (Tiwari *et al.* 2009; Nazzaro *et al.* 2013).

The concentrations of the main components of an EO should be taken into account when the EO is used. The concentrations of carvacrol and thymol in EOs play an important role in their antibacterial activity. Higher MIC values have been determined for oregano oils from *O. compactum* (mainly carvacrol at 50·07% and thymol at 15·13%) and *O. heracleoticum* (mainly carvacrol at 65·51%) compared with oregano oil from *O. vulgare*, which mainly contains both carvacrol and thymol at 64·49 and 3·35%, respectively. The efficacy of cinnamon bark oil can also be attributed to the concentration of its major aldehyde component (cinnamaldehyde). For example, Starliper *et al.* found MBC values from 0·01 to 0·03 v/v% against *A. salmonicida* subsp. *salmonicida* strains for three commercial sources of Cassia cinnamon bark oils (*C. cassia*) containing 61–99% cinnamaldehyde (Starliper *et al.* 2015). In our study, similar MBC values from 61 to 490 μg ml^{-1}, equivalent to 0·006–0·05 v/v%, were obtained with Ceylon cinnamon bark oil (*C. zeylanicum/verum*) containing 68% cinnamaldehyde, showing that the cinnamaldehyde concentration was more important than the cinnamon variety (Cassia *vs* Ceylon).

In addition to the major components present in EOs (from 20 to 70% in comparison to other trace components), minor components in EOs should also be

considered as playing a contributing role due to their antibacterial properties (Nazzaro *et al.* 2013; Chouhan *et al.* 2017). Antibacterial activity of EOs may depend on the concentrations of their major and minor bioactive compounds due to potential synergistic activity between these components. In our study, oregano (*O. vulgare*) showed lower MIC values (113–453 μg ml^{-1}) compared to its main constituents (MIC from 344 to 688 μg ml^{-1} for carvacrol, and MIC of 500 μg ml^{-1} for thymol). However, the association of carvacrol and thymol (10 and 90%) did not show a synergistic activity (FICI values between 0·5 and 4), and the antibacterial role of linalol (2·20%), γ-terpinene (8·29%) and para-cymene (12·92%), minor constituents in oregano, should be considered. In contrast, the association of cinnamaldehyde (30%) and eugenol (70%), which were both bioactive components of cinnamon oil, showed a lower MIC value than cinnamon oil, cinnamaldehyde alone or eugenol alone (FICI values ≤0·5). It has been suggested that a eugenol and cinnamaldehyde combination can interact with different proteins by combining the hydroxyl group on eugenol and preventing enzyme action, while the carbonyl group on cinnamaldehyde adheres to proteins to prevent the action of amino acid decarboxylases (Wendakoon and Sakaguchi 1993; Yena and Chang 2008). Due to the synergistic effects of EOCs, lower concentrations of each EOC in a combination can achieve the same or even greater efficacy than when the EOC is used alone (Kon and Rai 2012; Kot *et al.* 2019). Furthermore, associations of EOs/EOCs with antibiotics have been investigated to increase EOs/EOCs efficacy and decrease antibiotic doses. Synergistic association has been demonstrated for *Aloysia triphylla*, *Lippia alba* EOs and florfenicol, as well as for linalool in combination with florfenicol or oxytetracycline (Cordeiro *et al.* 2017; Bandeira Junior *et al.* 2018).

In vitro activity of EOs and compounds may also depend on the bacterial strains studied. Indeed, Kot *et al.* (2019) and Heo *et al.* (2012) showed lower MIC values for trans-cinnamaldehyde and thymol against *A. salmonicida* subsp. *salmonicida* strains than those in our study. The MIC and MBC values of some EOs and EOCs varied slightly for each *A. salmonicida* subsp. *salmonicida* strain, but the lower MIC results were obtained for the environmental strain compared to the clinical ones (our study, Table 2). Similarly, *A. hydrophila* isolated from fish (clinical strain) and milk (environmental strain) showed different MIC and MBC values for each tested phytochemical (Bandeira Junior *et al.* 2018). These *A. hydrophila* strains could resist differently to a specific phytochemical by having different virulence factors (Bandeira Junior *et al.*, 2018). It has been demonstrated that the virulence genes (*vapA*, *aexT*, *ascV* and *ascC*) of *A. salmonicida* subsp. *salmonicida* isolated from turbot (*Psetta maxima*) were clearly different from those of environmental *A. salmonicida* strains (Lago *et al.* 2012).

Finally, a MBC/MIC ratio of 1 or 2 was found for all of the phytochemicals alone or in combination against our four *A. salmonicida* subsp. *salmonicida* strains. A phytochemical is considered bacteriostatic when the MBC/MIC ratio is above 4, while it is bactericidal when this ratio is lower or equal to 4 (Soro *et al.* 2010; Kot *et al.* 2019). Therefore, cinnamon, oregano, clove, thyme and their major compounds (cinnamaldehyde, eugenol, carvacrol and thymol) exhibited the strongest inhibitory and bactericidal effects against *A. salmonicida* subsp. *salmonicida* strains, suggesting their potential use as antibacterial agents to prevent and control furunculosis caused by *A. salmonicida* subsp. *salmonicida* in aquaculture. A previous *in vivo* study showed that cinnamon oil (*C. verum*) and trans-cinnamaldehyde protected tilapia (*Oreochromis niloticus*) and catfish (*Ictalurus punctatus*) from some fish pathogens (Rattanachaikunsopon and Phumkhachorn 2010; Abdelhamed *et al.* 2019). Zheng *et al.* (2009) also showed that oregano oil (*O. heracleoticum*) could protect channel catfish (*I. punctatus*) from *A. hydrophila* infection by enhancing an antioxidant effect and improving growth performance. Moreover, Sutili *et al.* (2014) reported that eugenol promoted the survival rate in *A. hydrophila* infected silver catfish (*Rhamdia quelen*), despite the high *in vitro* MBC values (3200 μg ml^{-1}) against the four tested *A. salmonicida* subsp. *salmonicida* strains.

In conclusion, cinnamon, oregano, clove and thyme oil and their main compounds, cinnamaldehyde, carvacrol, eugenol and thymol, alone or in association, were found to be effective against *A. salmonicida* subsp. *salmonicida*. The application of phytochemicals could be interesting in furunculosis treatment by exploiting their synergistic antibactericidal activity. Furthermore, *in vivo* and on-farm research is needed to ensure the use of phytochemicals as functional alternative products to reduce antibiotic prescriptions in aquaculture.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

This article does not contain any studies with animals performed by any of the authors.

Data Availability Statement

Data sharing not applicable—no new data generated.

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b) The selection of commercial functional alternative product

In vitro studies on some commercial Eos (essential oils) found that cinnamon, oregano, clove and thyme oil and their main compounds, cinnamaldehyde, carvacrol, eugenol and thymol, alone or in association, can be effective against *A. salmonicida* subsp. *salmonicida* (Article N° 3). According to bibliography studies, prebiotics, including fructose and mannan oligosaccharides (FOS and MOS) which are naturally occurring polysaccharides part of the bacteria (yeast cell walls or plants) can also be used in farm fish against furunculosis (Article N° 1). However, these studies have not completely evaluated the mechanism action of alternatives substances. Moreover, they are not tested in on-farm conditions in natural exposure to *A. salmonicida* subsp. *salmonicida.* Therefore, this *in vitro* study aimed to select an appropriate commercial functional feed additive product "alternative to antibiotic" in order to use this product further in *in vivo* experimental studies and then, on farm field.

In this project, the selection of commercial feed additive products for further *in vivo* and on-farm studies to control furunculosis in rainbow trout (*O. mykiss*) was based on the biographic research, on the *in vitro* study, on some practical experiences from various feed additive producer companies, aquaculture veterinarians and fish farmers by considering the French regulation on feed additives for animal use. As a result, we have tested the efficacy of three commercial feed additive products containing phytochemicals and/or prebiotics from three different aquaculture feed companies against *A. salmonicida* subsp. *salmonicida,* according to the protocol described in the *in vitro* study using broth micro dilution method (explained in Article N° 3). The results revealed that all products showed a bactericidal effect against four *A. salmonicida* subsp. *salmonicida* strains in the same range. Almost the minimum inhibitory concentration has been detected at 0.5 µl ml^{-1} for all products and no significant difference has been found between the tested products ($P \geq 0.05$) (Kruskal–Wallis, One-Way ANOVA test) (**Table 1**).

Based on the results and collaboration of “Le Gouessant Aquaculture” company with Oniris-INRAE to achieve this project on fish farms with fish farmers, the functional alternative product in this thesis work was provided from “Le Gouessant Aquaculture” in France.

We have conducted a confidential contract with “Le Gouessant Aquaculture” to know how they process and formulate their feed additive in order to associate the *in vitro* (as described previously) and *in vivo* findings. Prior to on-farm research in fish farms, we evaluated the efficacy of the functional alternative feed product providing by “Le Gouessant Aquaculture” on an *in vivo* assay in controlled experimental conditions against *A. salmonicida* subsp. *salmonicida* in rainbow trout (*O. mykiss*) in aquaculture experimental station at Oniris-INRAE-BIOEPAR 1300.

Table 1. Minimum Inhibitory Concentration (MIC) at 24 h and Minimum Bactericidal Concentration (MBC)/MIC ratio at 48 h of various commercial alternative feed additives against *A. salmonicida* subsp. *salmonicida* strains.

	***A. salmonicida subsp. salmonicida* strains**							
	ATCC 14174		**CAE 235**		**CAE 452**		**CAE 258**	
	MIC μl ml^{-1}	**MBC/MIC**	**MIC** μl ml^{-1}	**MBC/MIC**	**MIC** μl ml^{-1}	**MBC/MIC**	**MIC** μl ml^{-1}	**MBC/MIC**
Product 1	0.5	2	0.5	2	0.5	2	0.5	2
Product 2	0.25	2	0.5	2	0.5	2	0.5	2
Product 3	0.5	2	0.5	2	0.5	2	0.5	2

B- *In vivo* studies

a) **Article N° 4: The effect of prebiotics and plant essential oils-enriched diet on immune response and disease resistance of vaccinated/non-vaccinated rainbow trout (*Oncorhynchus mykiss*) against *Aeromonas salmonicida* infection**

The effect of prebiotics and plant essential oils-enriched diet on immune response and disease resistance of vaccinated rainbow trout (*Oncorhynchus mykiss*) against *Aeromonas salmonicida* infection

(will be submitted to journal of Fish and shellfish immunology after being *completed by further immunological analyses)*

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Abstract

The present study examined the effects of a commercial prebiotic and essential oils additive (PEA) on the growth performance, disease resistance and immunological parameters in vaccinated and non-vaccinated rainbow trout against *Aeromonas salmonicida* subsp. *salmonicida* (ASS). Eight groups of fish (+/- PEA, +/- vaccine and +/- ASS inoculation) were studied. Mortalities were recorded daily meanwhile clinical and bacteriological investigations were also carried out. Body weight and immune parameters as lysozyme activity, alternative hemolytic complement (ACH50) activity and anti-ASS antibody rate in serum were measured. Before ASS inoculation, mortality was very low (< 3%) and no alteration of fish health status was detected in all studied groups ($p > 0.05$) resulting in a safety of PEA and vaccine. A humoral immune response was induced 4 weeks after vaccine injection but no differences were observed between fish fed with or without PEA. However, there were fish whose vaccination induced only a very low production of anti-ASS antibodies in both groups but the number of these fish was lower in the PEA-fed group than in the PEA-free group. After ASS inoculation, there were no significant differences in mortality (12 to 28 %) and morbidity between challenge groups. ASS inoculation induced a decrease of lysozyme activity but increase of ACH50 and antibody-ASS production at 3rd week post-inoculation. The number of fish with important increase of anti-ASS antibody rate was higher in inoculated group fed without PEA than fed with PEA. This suggests that there are significantly fewer infected fish in PEA than in non-PEA group. The immunostimulant effect of PEA used in this study and its role on resistance against furunculosis is under-discussed.

Keywords

Aeromonas salmonicida subsp. *salmonicida*, prebiotics-essential oils functional feed alternative, vaccination, rainbow trout, disease resistance, immune response

1. Introduction

Aquaculture remains the fastest growing food-producing sector for human consumption around the world. The production of higher-value freshwater fish species such as salmon and trout also projected to grow further (FAO, 2018). An intensive farming of salmonids could be threatened on a high mortality and economic loss due to infectious diseases as furunculosis by *Aeromonas. salmonicida* subsp. *salmonicida*. This disease is responsible for haemorrhagic septicaemia in the acute form, and fish depreciation due to the incidence of boils in the muscles in the chronic form in salmonids (Austin B and Austin D, 2012). Treatment practices to control these infectious diseases, mainly furunculosis, are associated with the regular use of large quantities of antibiotics that resulting the increase of economic losses as well as the problems of antibiotic resistance bacteria which are incompatible with sustainable aquaculture (Van Boeckel et al., 2015, Watts et al., 2017).

Despite the routine of vaccination in aquaculture, its application against furunculosis has been considered by some limitations due to controversial efficacy, intra-abdominal adhesions after vaccine injection and difficulty of vaccinating young animals individually because of their small size (Assefa, 2018; Adams, 2019). In salmonids against *Aeromonas salmonicida* subsp. *salmonicida*, an acceptable level of protection can only be achieved by immunization with oil-adjuvanted bacterins delivered by injection (Muktar and Tesfaye, 2016; Villumsen et al., 2017; Assefa, 2018). Moreover, the development of commercial vaccines is limited by economic considerations, biological problems and regulatory restrictions in aquaculture which leads fish farm sectors to a wide use of autovaccines initially (Sudheesh and Cain, 2017; Ma et al., 2019). Autogenous vaccine (autovaccine) are prepared from cultures of microorganisms obtained from an individual in a farm and then used to immunize that all individuals in the same farm against further spread and progress of the same microorganisms. These vaccines may suggest a solution to emerging pathogens of interest, when no

commercially licensed product is available, or when commercially licensed products have not provided adequate protection (Yanong, 2011; Adams, 2019).

In recent years, the application of the functional feed alternative inclusive of natural antimicrobial effects and immunostimulant properties like plant extracts and prebiotics as β-glucan and mannan oligosaccharides (MOS) has been paid attention to control aquaculture diseases and reduce the potential negative impacts of antibiotics on public health and the environment (Nazzaro et al., 2013; Ringø et al., 2014; Mastan, 2015; Cunha et al., 2018). Previously, antibacterial activities of phytochemical alternatives such as cinnamon, thyme or clove have been shown against *Aeromonas salmonicida* (Heo et al., 2012; Kot et al., 2019; Hayatgheib et al., 2020). Particularly, *in vivo* studies on the favorable effects of functional alternatives to control *Aeromonas* spp. including infections in freshwater fish were analyzed and focused on their capacities to enhance the immunocompetence and disease resistance of fish (Hayatgheib et al., 2020). However, few studies investigated plant extracts to increase efficacy of a vaccine against *Aeromonas salmonicida sub salmonicida* infection. Yin et al., (2009) showed the induced immunocompetence of herbal extracts on vaccinated-carp (*Cyprinus carpio*) like the increase of lysozyme activity and serum antibody titer against *Aeromonas hydrophila*.

In this study, the effects of a commercial feed additive based on a mixture of essential oils and yeast extract composed of β-glucan and mannan oligosaccharides (MOS) prebiotics have been evaluated for enhancement of immunity and increase of resistance to *A. salmonicida* subsp. *salmonicida* in rainbow trout (*O. mykiss*). To our knowledge, this research reports the first study on the application of prebiotics and essential oils association in vaccinated and non-vaccinated rainbow trout to control furunculosis. This study also evaluates the increase of

vaccine efficacy by adding alternative treatments to protect fish disease and therefore reduce the need for antibiotics treatments.

2. Materials and methods

2.1. Ethics statement

This study was approved by the members of the Animal Experiment Ethics Committee of Pays-de-la-Loire and the Ministry of Higher Education, Research and Innovation in France (N° APAFIS 21481).

2.2. Animals

Six hundred healthy rainbow trout (*O. mykiss*) (80 ± 5g) were purchased from National Institute of Agricultural Research (INRAE) Experimental Fish Farm of the Monts d'Arrée (PEIMA) located in Brittany, France. Fish were transferred to Antibiotic resistance - Pathogenicity - fish infectiology INRAE department (UMR 1300, APPIfish) at Oniris in Nantes, France. They were acclimatized to the experimental conditions in three fiber glass tanks (600 L), with recirculating water system for three weeks. The water temperature, O_2 and pH were maintained at 16 ± 0.5 °C, 8.8 ± 0.5 mg/L and 7± 0.5 respectively. During adaptation period, fish were fed twice a day with a commercial diet (Le Gouessant Aquaculture, France) at the rate of 1% of their body weight (BW).

2.3. Diet formulation and feeding regime

An industrial preparation of fish feed (Le Gouessant Aquaculture, France) (Table 1) with feed additives mainly including cinnamaldehyde, eucalyptol (1,8-cineol), eugenol and thymol, among others phytochemicals in combination with β-glucan and MOS prebiotics at 2 kg/ton feed, called AQUABOOST® was purchased from "Le Gouessant Aquaculture" in France.

Table 2: Formulation of basal diet from Le Gouessant Aquaculture®

Diet formulation*	
Component	%
Proteins	40
Lipids	23
Cellulose	2.5
Ash	6.8
Phosphor	0.95

Note: Diet fortified with vitamins and fatty acids.

2.4. Experimental design

A schema of the experimental design is shown in Figure 1.

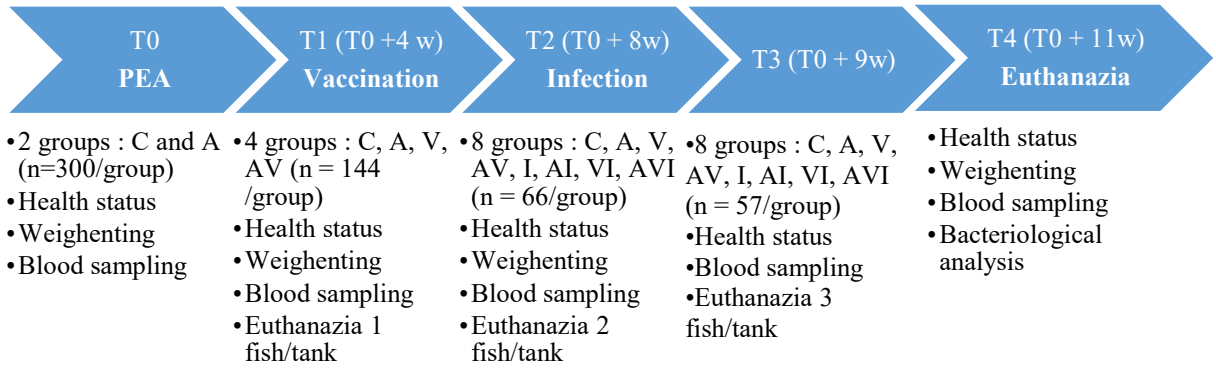

Figure 1: A schema of the experimental design
(C = control; A = additive, V = vaccinated; AV = additive vaccinated; I = inoculated challenge control; AI = additive inoculated; VI = vaccinated inoculated; AVI = additive vaccinated inoculated)

After the adaptation period (T0), fish were anesthetized (2-phenoxyethanol at 0.3 mL/L) and then checked for their health status and tagged with Passive Integrated Transponder (PIT). They were distributed randomly in 24 tanks (each tank with 200 L volume) at density of 25 fish per tank. Thereafter, fish were fed in 2 groups (300 fish per group) corresponding to two feed regimens, basal diet or basal diet enriched with prebiotics and essential oils additives (PEA) twice daily (1.5% BW). On 4th week of feeding (T1), each of these 2 groups were divided into two additional groups including vaccinated and non-vaccinated. In vaccinated

group, 100 µL auto vaccine per fish (developed against *A. salmonicida* subsp. *salmonicida* strain CAE1414 at 10^9 CFU/mL) were injected intraperitoneally (IP) (CEVA-BIOVAC, France). Non-vaccinated fish were injected intraperitoneally (IP) with 100 µl of sterile normal saline solution (sodium chloride, 0.9% w/v). Eight weeks after feeding and 4 weeks after vaccination (T2), fish were injected intramuscularly (IM) with 100 µL *A. salmonicida* subsp. *salmonicida* CAE1414 at 1.5×10^4 CFU/mL (equivalent to a lethal dose 20 (LD_{20}) at 1.5×10^3 CFU per fish) (inoculated fish) or 100 µL of normal physiological saline solution (sodium chloride, 0.9% w/v) only (non-inoculated fish). The experimental treatments and fish groups are shown in Table 2. At the end of the experiment on 3th week post-infection (T4), fish in all groups were euthanized (2-phenoxyethanol at 0.6 mL/L). During the experiment, at three time points (T1, T2 and T3), three, six and nine fish were euthanized (2-phenoxyethanol at 0.6 mL/L) and spleen and the end of the posterior intestine were dissected for further immunological and histological investigations (this section is of no concern my doctoral thesis).

Table 2: Experimental treatments and fish groups. Note: *A. salmonicida subsp. salmonicida* (ASS); control= C, additive= A, vaccine= V; additive vaccinated= AV, inoculated challenge control= I, additive inoculated= AI, vaccinated inoculated= VI, additive vaccinated inoculated= AVI.

Experimental group	PEA	Vaccine	ASS inoculation
C	-	-	-
A	+	-	-
V	-	+	-
AV	+	+	-
I	-	-	+
AI	+	-	+
VI	-	+	+
AVI	+	+	+

2.5. Sampling and experimental observation

At five time points T0 to T4, fish were kept starved 24h prior to the sample collection and then were anesthetized by immersion in an anesthetic bath containing 2-phenoxyethanol

at 0.3 mL/L. Fish were weighted (at T0, T1, T2 and T4) and observed for health status. Blood samples were taken from the caudal vein for further immunological analysis at each time point. Blood samples were aliquoted into non-heparinized tubes and left to clot for 12 h (at 4 °C), prior to centrifugation at 3000 g for 15 min to isolate the serum. Sera were stored at −80 °C for further analysis.

Twice a day and over the course of experiment, fish have been observed and mortality and clinical signs such as inappetance, altering of swimming behavior, slight darkening of skin, presence of lesion, furuncle or boil have been recorded. Dead and moribund fish were collected and then euthanized (2-phenoxyethanol at 0.6 mL/L) for bacteriological analysis; thereby furuncle in case of presence, spleen and the end of the posterior intestine were dissected. For the detection of *A. salmonicida*, tissues samples were cultured in tryptone casein soy agar (TSA) (BIOKAR ref. BK047HA; France) at 22°C for 48h and 96h prior to the bacteria identification (occurrence of colonies surrounded by dark-brown pigment on TSA).

2.6. Immune parameters

2.6.1. Serum lysozyme activity

The lysozyme activity protocol was adjusted from Ellis et al. (Ellis, 1990) and Milla et al. (Milla et al., 2010). The lysozyme activity of sera samples was measured using a method based on the ability of lysozyme to lyse the bacterium *Micrococcus lysodeikticus*. In a 96-well microplate, 5 µL of fish serum were mixed with 15 µL of phosphate buffered saline (PBS) (0.05 M, PH 6,2) and then with 130 µL of 0.6 mg/mL suspension of *M. lysodeikticus* (Sigma-Aldrich M3770-5G, USA). In addition, a control serum corresponding to a mixture of fish serum was tested in each plate. Optical density (OD) at 450 nm (Thermo Scientific™ Multiskan™ Spectrophotometer) was monitored every five minutes for 20 minutes and used to calculate lysozyme activity in units. Lysozyme concentrations for samples were converted to µg/mL

using the reference curve from 6.25 to 150 µg/mL established with hen egg white lysozyme (Sigma). The ratio between the lysozyme activity of fish serum sample and the one of control serum was determined in percentage.

2.6.2. Alternative hemolytic complement activity (ACH50)

The serum alternative hemolytic complement activity (ACH50) was determined by the hemolytic assay with the rabbit red blood cells (RRBC, Clinisciences) (Yano, 1992; Danion et al., 2011). To this end, the rainbow trout serum samples and control serum as previously described, diluted to 1/32 in Veronal buffer (IDvet, France) were added in increasing amounts, from 10 to 100 µL in each well on the microplate. Then, the wells were filled with 50 µL of 2% RRBC suspension in veronal buffer. Control values of 0% and 100% hemolysis were obtained using, respectively 100 µL veronal buffer and 100 µL distilled water. Each mixture was incubated at 20°C for 60 min. The microplates were centrifuged (400 g, 5 min, 4◦C) and 75 µL of supernatant from each well were transferred into a 96-well flat-bottom microplate. The absorbance was read in a Thermo Scientific™ Multiskan™ Spectrophotometer at 405 nm. The ACH50 value was defined as the reciprocal of the serum dilution inducing the haemolysis of half the RRBC population.

2.6.3. *Aeromonas salmonicida* subsp. *salmonicida* specific antibody

The enzyme-linked immunosorbent assay (ELISA) was conducted according to previously established protocol (Erdal and Reitan, 1992; Romstad et al., 2012) with some adjustments. Briefly, microplates (NUNC Maxisorp® flat-bottom 96-well) were coated with proteins of sonicated whole cells of *A. salmonicida* subsp. *salmonicida* (5 µg /mL) in 0.1 M carbonate buffer (pH 9.6) at 4°C overnight. After 3 washes with PBS 0.1 % Tween 20 (PBST), 150 µL of gelatin 2% were added to each well and incubated for 1h at room temperature (RT). A fish serum sample and positive and negative serum diluted at 1/100 were added to each well

and incubated over night at 4°C. After washing step, 100 µL mouse-anti-salmonid Ig monoclonal antibody, clone 5F12 (Bio-Rad Laboratories Inc, USA) diluted with PBSt at 1/1000, were added to each well, and left at RT for 1h. After wash, 100 µL of horseradish peroxidase (HRP) conjugate (EMD Millipore, Chemicon©, USA), diluted with PBSt at 1/10000 were added, and left at RT for 1h. After washing step, each well was finally received 100 µL TMB (Tetramethylbenzidine Liquid Substrate, Supersensitive, for ELISA) (Sigma-Aldrich, USA) and incubated at RT for 15 min. Then, 100 µL 1 M HCl were added to each well and the plate was analyzed in an ELISA plate-reader (Thermo Scientific™ Multiskan™ Spectrophotometer) at 450 nm. The ratio between the optical density (OD) of fish serum sample and the ones of the control positive and negative controls was calculated in percentage.

2.7.Statistical analysis

Data from the hematological parameters and body weight were presented as mean values ± SD. Statistical analysis was performed by use of R Studio software (version 3.6.1). Antibody, ACH50, lysozyme and body weight between the groups were compared by analysis of deviance (Anova; random effect, paired series) and when p values were < 0.05, Turkey´s test was used. For comparing mortality, morbidity and ASS infected fish between the groups, linear regression model has been applied. p values < 0.05 were considered significant.

3. Results

3.1. Challenge experiment (mortality, morbidity and bacteriological analysis)

Before *A. salmonicida* subsp. *salmonicida* (ASS) inoculation (T2), no clinical signs have been detected in experimental groups. A very low mortality (up to 3%) has been observed in experimental groups (Table 3). Therefore, mortalities were not significantly different in groups fed with PEA or in vaccinated groups from the control group (p values > 0.05). Bacteriological analysis showed no presence of pathogen in these dead fish.

After ASS inoculation (from T2), results of mortality, morbidity (clinical presentation of furunculosis) and bacteriological analysis were really similar (Table 3). No mortalities and no morbidities have been observed in non ASS inoculated groups (C, V, A and AV) after T2 and the bacteriological analysis of these fish were negative for ASS at T4. ASS inoculated fish that developed furunculosis died and bacteriological analyses have confirmed the presence of dark-brown bacterial colonies typical of ASS on TSA agar (BIOKAR ref. BK047HA; France). Thirty-seven out of 224 dead fish showed the presence of furuncle or boil on their skin. Three out of 224 dead fish did not show any external lesions but the post-mortem observation presented other clinical signs of furunculosis as septicemic hemorrhage, mainly in intestine, and splenomegaly and bacteriological analysis confirmed ASS infection. Clinical signs such as inappetance, altering of swimming behavior, slight darkening of skin, lesion or furuncle have been appeared at day 2 post-challenge in all fish inoculated groups (groups I, VI, AI and AVI). In parallel, mortality started 3 days after ASS inoculation in all fish challenged groups and subsided by day 10 post-challenge. Thereafter, minor mortality was observed until the end of experiment at 3-week post-challenge. At the end of the experiment, mortality rates were 16.3% for group A, 28% for group AI, 14.2% for group VI and 12.2% for group AVI. Mortalities and morbidities in challenge groups with additive and/or vaccination (AI, VI and AVI) were not significantly different from the challenge control group (group I) (Table 3, p values > 0.05).

Table 3: Results various parameters studied on experimental fish groups at each studied time point (T0-T4). For each parameter, results are given as number of fish with studied parameter/ number of all fish studied (percentage). Bacterio: Bacteriological analysis considered a positive when at least one sample from spleen, intestine or lesion was detected with ASS. Vaccine/Ab: number of fish which have antibody titer greater than 80 % at T2. Infection/Ab: number of fish which have a 2-fold increase in antibody titer between T2 and T4 and greater than 33% at T4. * indicates significant differences between groups fed with or without PEA (prebiotic and essential oils additive).

Note: control= C, additive= A, vaccine= V; additive vaccinated= AV, inoculated challenge control= I, additive inoculated= AI, vaccinated inoculated= VI, additive vaccinated inoculated= AVI. At three time points (T1 to T3), one, two and three fish per tank were euthanized for further analysis.

	T0	**T1 (PEA)**		**T2 (Vaccine)**			**T4 (Inoculation)**				
group	Mean BW (g) (SD)	Mean BW (g) (SD)	Mortality (%)	Mean BW (g) (SD)	Mortality (%)	vaccine/Ab (%)	Mean BW (g) (SD)	Mortality (%)	Morbidity (%)	bacterio (%)	infection/Ab (%)
C	93.58 (11.59)	132.94 (16.68)	0/300 (0)	200.40 (29.38)	4/144 (2.78)	0/139	244.26 (48.15)	0/55	0	0/5	0/55
A	92.98 (11.98)	131.51 (13.98)	3/300 (1)	198.01 (20.91)	2/143 (1.39)	0/139	234.59 (28.78)	0/55	0	0/5	0/55
V				198.47 (30.31)	2/144 (1.39)	126/137 * (91.97)	242.49 (46.49)	0/56	0	0/5	
AV				200.01 (20.95)	1/142 (0.71)	133/137 * (97.08)	234.5 (37.65)	0/57	0	0/5	
I							247.68 * (49.31)	9/55 (16.3)	8/55 (14.5)	8/55 (14.5)	13/46 * (28.3)
AI							264.59 * (19.71)	16/57 (28)	16/57 (28)	16/57 (28)	3/41 * (7.3)
VI							251.16 (29.82)	8/56 (14.2)	7/56 (12.5)	7/56 (12.5)	
AVI							255.20 (26.60)	7/56 (12.2)	6/56 (10.7)	6/56 (10.7)	

3.2. Growth performance

In all experimental groups, fish have gained an average of 153.1 g of weight during the study (from T0 to T4) ($p < 0.05$). Vaccination and inoculation challenge have no effect on the body weight. There were no differences between vaccinated group V and inoculated group I with the control group C ($p > 0.05$).

There were also no significant differences in body weight among fish fed with or without PEA in non-vaccinated fish (group A and C respectively), in vaccinated fish (group V and AV) and in vaccinated and inoculated fish (group VI and AVI) at all-time points ($p > 0.05$). In contrast, inoculated fish fed with PEA group (AI) showed a significant increase in values of body weight compared to group inoculated challenge control (I) at T4 (difference of 16.9 g, $p < 0.05$) (Table 3).

3.3. Humoral immune response

During the 11 weeks of experiment, there were no evolution of lysozyme activity and anti-ASS antibodies production in non-vaccinated and non-inoculated fish fed with or without PEA (group C and in group A) and there were no differences between these two groups ($p > 0.05$) (Figure 2A and 2C). Regarding to ACH50 parameters, its activity was higher from T1 than T0 in group A ($p < 0.05$) while no significant difference was shown for fish in group C ($p > 0.05$) (Figure 2B). No difference between group A and group C was observed, except at T0 where the ACH50 of group C was higher than the ACH50 of group A ($p < 0.05$).

One month after vaccination (T2), a significant increase of the three immunological parameters studied was observed in the vaccinated fish (groups V and AV) (Figure 3). There were no differences between vaccinated fish fed with or without PEA for lysozyme activity and antibody production (Figure 3A and 3C). However, in group V, ACH50 activity was higher at T4 when compared to AV group ($p < 0.05$) (figure 4B). Although at T2 there was no difference

between the V and AV groups for antibody rate, the number of fish showing an increase of more than 80% was lower in the V group than in the AV group (126/137 vs 133/137, Table 3).

After the ASS challenge at T2, lysozyme activity did not change between T2 and T3 but decreased significantly at T4 in inoculated fish, fed or not with PEA (group I and group AI [Figure 4A]). On the contrary, ACH50 increases 1 week after the challenge, from T3 in both groups I and AI. Furthermore, at T3 and T4, ACH50 is higher in group AI than in group A ($p<0.05$) (Figure 4B). Concerning the production of anti-ASS antibodies (Figure 4C), a significant increase was observed in the inoculated groups I and AI 3 weeks after the challenge, at T4. At this time, the antibody level was higher in group I than in group AI. However, the number of fish with an important increase of anti-ASS antibodies production (a 2-fold increase in antibody titer between T2 and T4 and greater than 33% at T4) was higher in group I than in group AI (13/46 vs 3/41 respectively, Table 3) ($p < 0.05$). Anti-ASS antibody rate above than 33% corresponds to antibody threshold for confirming ASS infection according to outcomes in this study (sensitivity = 0.39 and specificity = 0.98).

Finally, no differences have been found among fish fed with or without PEA in vaccinated and inoculated fish (AVI and VI) for all humoral immune responses in terms of lysozyme, ACH50 activity and antibody titers ($p > 0.05$) (data not shown).

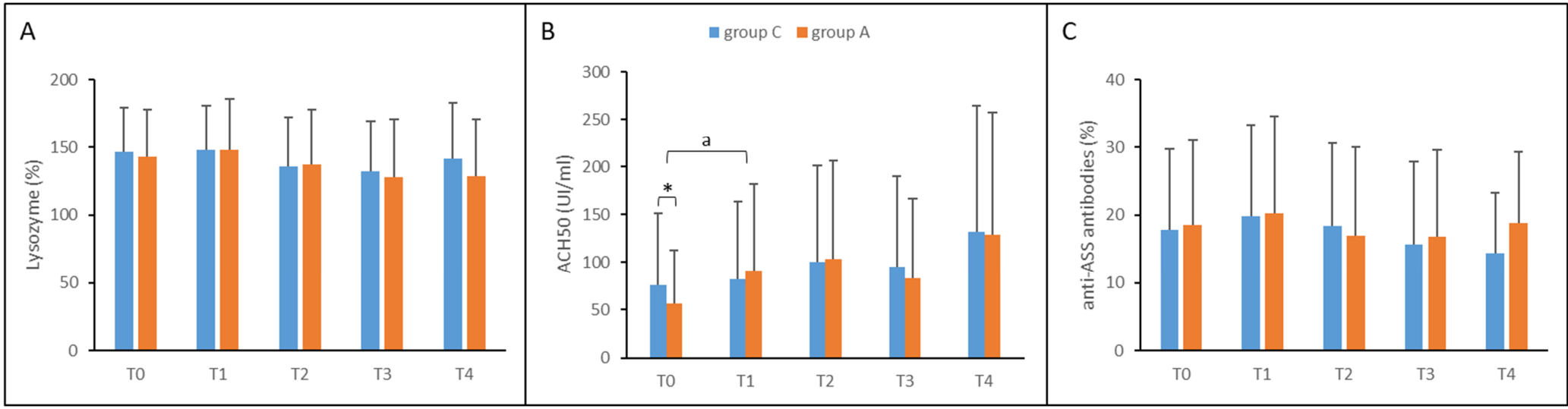

Figure 2: Effect of prebiotics and essential oils additive (PEA) on immunological parameters of non-vaccinated and non-infected fish at each time point experiment. A: lysozyme activity; B: ACH50; C: anti-ASS antibodies by ELISA. Group A: fish fed with PEA; group C: fish fed without PEA. (T0: 0 week of feeding; T1: 4 weeks of feeding; T2: 8 weeks of feeding; T3: 9 weeks of feeding; T4: 11 weeks of feeding). * indicates significant differences between group A and C ($p<0.05$), a indicates significant differences between 2 time points for group A ($p < 0.05$).

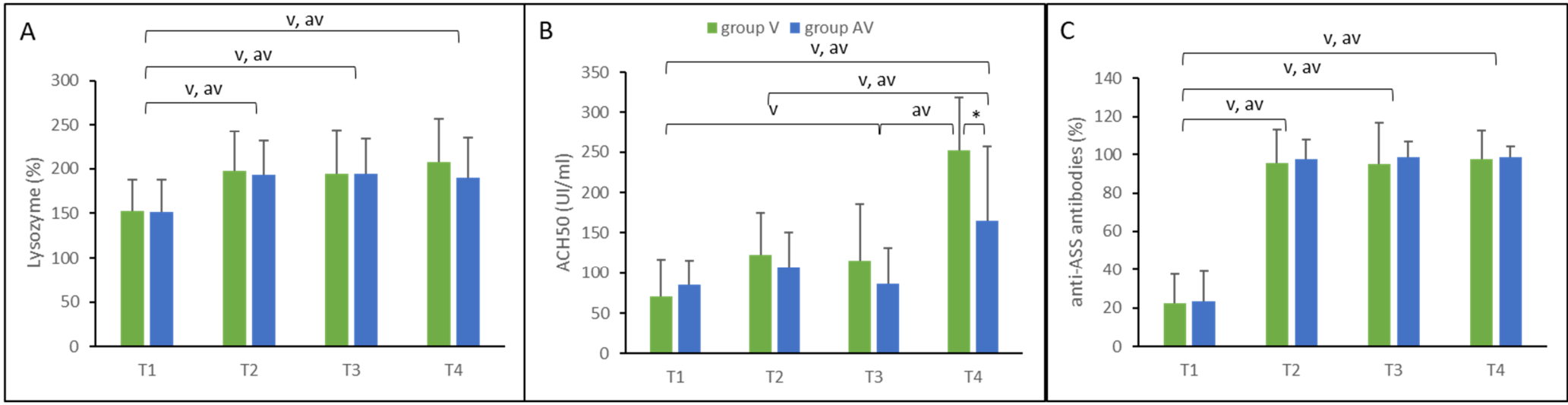

Figure 3: Effect of prebiotics and essential oils additive (PEA) on immunological parameters of vaccinated and non-infected fish at each time point experiment. A: lysozyme activity; B: ACH50; C: anti-ASS antibodies by ELISA. Group AV: vaccinated fish fed with PEA; group V: vaccinated fish fed without PEA. (T1: 4 weeks of feeding and 0 week of vaccination; T2: 8 weeks of feeding and 4 weeks of vaccination; T3: 9 weeks of feeding and 5 weeks of vaccination; T4: 11 weeks of feeding and 7 weeks of vaccination). * indicates significant differences between group AV and V ($p < 0.05$), av and v indicates significant differences between 2 time points for group AV and group V respectively ($p < 0.05$).

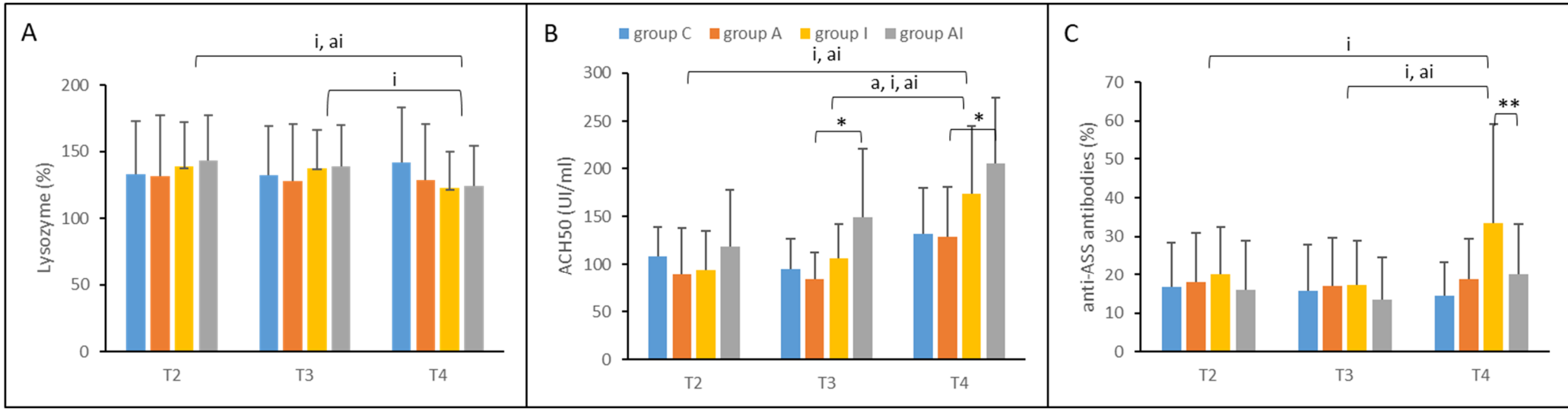

Figure 4: Effect of prebiotics and essential oils additive (PEA) on immunological parameters of inoculated fish with ASS. A: lysozyme activity; B: ACH50; C: anti-ASS antibodies by ELISA. Group A: non-inoculated fish fed with PEA; group C: non-inoculated fish fed without PEA; Group AI: inoculated fish fed with PEA; group I: inoculated fish fed without PEA. (T2: 8 weeks of feeding and 0 week of inoculated challenge; T3: 9 weeks of feeding and 1 week of inoculated challenge; T4: 11 weeks of feeding and 3 weeks of inoculated challenge). * indicates significant differences between group A and AI and ** indicates significant differences between group I and AI ($p < 0.05$), a, i and ai indicates significant differences between 2 time points for group A, group I and group AI respectively ($p < 0.05$).

4. Discussion

Immunostimulant including phytochemicals or prebiotics showed the improvement of fish immunity and disease resistance as well as bodyweight against bacterial infection including *Aeromonas* spp. (Chakraborty and Hancz, 2011; Hoseinifar et al., 2015; Hayatgheib et al., 2020). β-glucan has been shown to activate leucocyte migration and macrophage polarization and therefore, the production of pro-inflammatory cytokines and chemokines which may result in faster neutralization of pathogens including *Aeromonas* (Chadzinska et al., 2008; Brogden et al., 2014) ; more over, MOS stimulates mannose binding lectin that binds to bacteria and triggers the complement cascade (Moran, 2004). Furthermore, herbal derived-extracts presented antibacterial activities against fish and shellfish pathogens especially against *Aeromonas* species including ASS (Reverter et al., 2017; Hayatgheib et al., 2020). Several products supplemented diet as dietary β-glucan, curcumin, thyme oil showed to be beneficial in rainbow trout for promoting growth and enhancing some nonspecific immune parameters, including lysozyme activity, prior to infection challenge and as well as enhancing resistance against *Aeromonas spp* after experimental challenge (Ji et al., 2017; Yonar et al., 2019; Zargar et al., 2019). Some other studies on phytochemical or its combination with organic acids (Menanteau-Ledouble et al., 2015; 2017) had an effective protection against *A. salmonicida* subsp. *salmonicida.* Regarding to our result after ASS challenge, even though, the differences of mortality rate between challenged groups (I, AI, VI and AVI) were not significantly different but the differences of immunological parameters like anti-ASS antibody rate, ACH50 and lysozyme activity as well as growth performance among these groups were remarkable.

Firstly, during the eleven weeks of study, the dietary intake of the PEA, has shown few immunostimulant properties as the increase of ACH50 activity after 4 weeks of feeding; however,

this increase did not continue thereafter and reached at its initial level and no significant changes were observed for lysozyme activity. In accordance with our results, Douxfils et al., (2017) did not observe an alteration in the level of lysozyme and ACH50 activity in β-glucan-feed-treated rainbow trout compared to control fish. Whereas, previous studies showed higher activities of lysozyme and ACH50 in rainbow trout after β-glucan administration (Engstad et al., 1992; Yar Ahmadi et al., 2014). These disagreements could be related to the dose and duration to develop changes initiated by the PEA intake, while the modulation was mild enough to present the significant changes. On contrary, higher dose or longer duration of β-glucan administration may led to an immunosuppression and an exhausted fish immune system (Douxfils et al. 2017). Moreover, regarding to plants extracts associated with PEA, the source, location and substance with variations in herbal chemical composition, extract preparation and incorporation into the feed as well as quantity of each phytochemical or their association with other kinds of immunostimulants could alter its efficacy on fish immunity (Van Hai, 2015; Hayatgheib et al., 2020).

Secondly, mortality rate at 16.3 %, morbidity (presence of furuncle) and ASS confirmation rate through bacteriological analysis both at 14.5 %, were all correlated with the initial lethal dose of inoculated ASS at 20% (LD20). No differences for these parameters have been observed between fish fed with or without PEA (group I vs group AI). However, in both groups, a significant increase in antibody production was observed 3 weeks after ASS inoculation. Interestingly, an enhancement of anti-ASS antibody production (2-fold increase in antibody titer between T2 and T4 and greater than 33% at T4) occurred only for 7.3 % of inoculated fish fed with PEA (group AI: 3/41 fish) versus 28.3% of inoculated fish fed without PEA (group I, 13/46 fish). This important enhancement of anti-ASS antibody could be attributed to an ASS infection i.e. due to a colonization, a multiplication and a persistence of ASS in inoculated fish resulting to the

stimulation of anti-ASS antibody. In contrast, inoculated fish without increase of antibody production at T4 could be considered uninfected, with a very rapid elimination of the bacteria, and therefore, without stimulation of the adaptive response. Hence, the number of ASS infected fish was significantly lower in group fed with PEA (group AI) than in group fed with PEA (group I) which could explain the higher average of anti-ASS antibody rate in group I than AI (33 % vs 20 %) (figure 4C). Furthermore, inoculated fish fed with PEA (group AI) showed enhancing of growth performance and therefore increased bodyweight compared to challenge control group (group A).

Thirdly, ACH50 activity has significantly increased in inoculated groups compared with control group and it was higher in group AI than I after one and three weeks of challenge. The complement system inclusive of ACH50 activity comprises of large number of plasma proteins achieving lysis of pathogen and opsonization/tagging of foreign organisms which plays an important role in host innate immune defense (Oriol Sunyer et al., 1998; Nayak et al., 2018). It has been reported that mannan-oligosaccharide and β-Glucan prebiotics could enhance ACH50 activity and decrease mortality following *Aeromonas hydrophila* challenge in rainbow trout (Yarahmadi et al., 2016). Likewise, when *Cyprinus carpio* were fed with β-Glucan and challenged with a non-lethal dose of *A. salmonicida*, an improve in the complement activity was observed from 96 h to 120 h post-challenge (Pionnier et al., 2013).

Fourthly, in contrast with ACH50 activity, lysozyme activity decreased in both inoculated with/without PEA treatment groups (I and AI) compared to control fish after three weeks of ASS inoculation (T4). The decrease of lysozyme activity after bacterial challenge may be related to lysozyme antibacterial activity by attacking, hydrolyzing and breaking glycosidic bonds of peptidoglycans present in the cell wall of bacteria, which is less severe in gram-negative than gram-positive pathogens (Magnadóttir, 2006). In addition, secretion system (T3SS, T2SS and T6SS)

virulence factor in *Aeromonas* species, like *A. salmonicida*, could modulate/escape the host immune response to promote bacterial virulence (Sapkota et al., 2008; Bergh et al., 2013; Rosenzweig and Chopra, 2013; Menanteau-Ledouble et al., 2016; Soto-Dávila et al., 2019). Therefore, we suggested that *A. salmonicida* may overcome lysozyme activity in this study resulting to lower lysozyme production in group AI and I compared to control fish. Similar to our results, Yarahmadi et al., (2016) reported that the level of lysozyme decreased in *A. hydrophila*-challenged groups including prebiotic-treated/infected rainbow trout compared to control fish. However, Douxfils et al., (2017) did not observe an alteration, neither in the level of lysozyme production, nor in the ACH50 activity in *A. hydrophila*-inoculated groups including β-Glucan-treated rainbow trout compared to control fish. Furthermore, previous A. *salmonicida* infection experiment showed levels of lysozyme significantly increased in sera from fish with symptoms of acute furunculosis and high mortality from 4 to 9 days post-infection and then decreased compared to control fish (Møyner et al., 1993; Du et al., 2015). In addition, Chen et al, (2020) presented the increase of lysozyme production 3 days after infection which decreased 7 days after *A. hydrophila* challenge in common carp (Chen et al., 2020).

Finally, the efficacy of vaccination against *A*. s*almonicida* is controversial and depends on many factors as reaching sufficient immunity, labor intensive, costly and not feasible for large numbers of small fish (Plant and Lapatra, 2011). Few studies revealed the favorable effect of immunostimulant phytochemicals and/or prebiotics on vaccinated fish in order to increase the vaccine efficacy (Yin et al., 2009; Dash et al., 2014; Salah et al., 2016). The autovaccine against *A. salmonicida* used in our study could not protect fish compared to control challenge fish (mortality rate: 14.2 % vs 16.3 %) while a strong humoral response with all immunological parameters (lysozyme, ACH50 and anti ASS-antibody) significantly increased in vaccinated fish compared to

control group after four weeks of vaccination until the end of the study. These results are in contradiction with previous studies which revealed the correlation between increased levels of ASS-antibody and increased survival in the vaccinated salmonid fish. Whereas, the level of ASS-antibody production depended on different types of vaccines (inactivated, live-attenuated, recombinant and etc.), different composition of adjuvant studied (natural/herbal, mineral oil, nucleotide, liposome and etc.) and/or vaccine administration (injection or immersion) (Rømer Villumsen et al., 2012; Romstad et al., 2012; Marana et al., 2017; Braden et al., 2019).

In our study, we did not found the significant differences in immune parameters and disease resistance between challenged vaccinated rainbow trout with or without PEA (VI and AVI) but the lowest mortality was observed in PEA vaccinated group (12.2%) that may present the beneficial effect of PEA when associated with vaccination. Furthermore, while ASS-antibody average detected in V group was similar to this in AV group after 7 weeks of vaccination, the number of fish with an important increase of anti ASS-antibody rate (above 80%) were significantly higher in fish fed with PEA than in fish fed with basal diet (AV: 133/137 vs V: 126/137). Interestingly, PEA could therefore reduce the number of fish that do not develop a strong humoral response after vaccination.

5. Conclusion

Our results revealed the safety of both PEA and autovaccine used during eleven weeks of study. We have shown that PEA used in this study can only have few immunostimulating effects but certain protective effets against ASS such as better resistance to ASS infection by decreasing the number of infected fish, better vaccine uptake with a higher number of vaccinated fish with a significant increase in antibodies. Indeed, in addition to its effect on anti-ASS antibody production in infected or vaccinated fish, PEA increased the level of ACH50 activity after 4 weeks of feeding.

However, the PEA did not reduce the mortality and the morbidity rate among ASS challenged fish nor could it increase the efficacy of the autovaccine used in our experimental conditions. However, more humoral and cellular immunological parameters, like gene expression in sera or tissue etc., need to be investigated to conclude to the efficacy of PEA/vaccine on fish immunity. Moreover, dose and administration route (cohabitation, immersion and injection) of ASS have to be considered in an infection challenge as lethal dose plays a crucial role on mortality rates. Furthermore, the protocol of optimal doses and administration durations for feeding potential immunostimulant substances have to be established to avoid exhausted immune system in higher administration doses or insufficiently enhanced fish immunity because of very low administered doses. Finally, it suggested the evaluation of functional alterative products, not only in experimental conditions, but also on farm conditions to evaluate these products under natural farm conditions (water temperature and quality, farmer practices, etc.) and natural exposure to ASS pathogen.

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C- On-farm studies

In parallel to our previous research in the two fish farms (Chapter 2-A, Article N° 2), another study was conducted to evaluate the functional alternative product efficacy in rainbow trout under natural exposure to *Aeromonas salmonicida* subsp. *salmonicida* (ASS). The realization of on-farm studies is necessary to ensure an alternative's efficacy when applied into professional farming practices and diverse and uncontrolled microbial environmental conditions. Hence, the aim of this study was to evaluate the safety and efficacy of a functional alternative product on growth performance as well as health status of fish, and therefore the use of antimicrobial agents in natural exposure of ASS in a rearing situation. To our knowledge, this is the first study to evaluate antibiotic consumption as well as zootechnical and health parameters like body weight, mortalities and morbidities in fish farms during the distribution of an alternative product for seven months in order to reduce the requires for antibiotic treatments, and eventually to limit the potential risk of antibiotic resistant bacteria diffusion in aquaculture ecosystems.

Material and method

As explained previously in Article N° 2, this study has been carried out in two rainbow trout fish farms (A and B) with open water circuit system in Brittany province (France). At the beginning of the study, general information from each farm (annual tonnage of fish, pond density, etc.) as well as fish farmer's practices and biosecurity management measures like vaccine application, previous diseases and antibiotic treatments, have been gathered through a questionnaire and interview with farmers (ANNEX 1).

In each farm, two raceways were dedicated to this project: a control pond (fish were fed with basal diet previously described in Chapter 3-B, Article N° 4) and a test pond (fish were fed

with the functional feed alternative consists of prebiotics and essential oils product called AQUABOOST ® from Le Gouessant Aquaculture which was previously described in Chapter 3-B, Article N° 4. In each pond, fish were fed twice daily (from 1.5 to 3% BW) for seven months. These fish farms were monitored monthly from February to August in order to compare the nature and quantity of the antibiotics administered as well as the health and the weight of the fish. At each visit point, a questionnaire was also carried out with the farmers to follow-up the study such as fish appetite and health, fish movements (temporary change of fish raceway for cleaning and disinfection of the pond or for sorting the fish by their weight, etc.) and incidence of particular meteorological phenomena like heat wave or flood. The occurrence of a pathological episode and antimicrobial treatment prescriptions have been also surveyed (ANNEX 2). Hence, information relative to the average weight of the fish (weighting approximately tenth fish per raceway at the beginning of each month by famers), the presence of furunculosis (confirmed by the farm veterinarian) and the number of monthly death fish in the two studied pond have been gathered and recorded from farmers for further zootechnical analysis.

Statistical analysis was performed by use of R Studio software (version 3.6.1), R Markdown package) (R Core Team, 2019). Wilcoxon rank sum test with continuity correction was applied to compare body weight or mortality rate between the control and test pond.

Results

The gathered general information related to fish farms including geographical location among other farms, exploitation description, previous vaccination, diseases and treatments over the last 5 years and related to studied ponds like age, weight and number of fish are displayed in table 1.

Table 1: General information in fish farms A and B.

	Farm A	Farm B
Geographical location among other farms	Surrounded by the other farms: several pig and cattle breeding sites, 2 fish farms above the river	The only fish farm on the river
Exploitation description	- Total number of raceways: 41 - Site tonnage per year: 320 tonnes - Pond density (kg/m3): 30-45 - Rainbow trout strain: Plourin - Pond water origin: river and sometimes recirculation of water in summer though one or two pumps of 400L	- Total number of raceways: 12 - Site tonnage per year: 110 tonnes - Pond density (kg/m3): 30- 45 - Rainbow trout strain: Plourin until 2019 and then Aqualand until now - Pond water origin : river and sometimes recirculation of water in summer though one or two pumps of 400L
Disease history during the last 5 years	- August 2018: furunculosis (moderate fish loss) - 2019: hepatonephritis, enteritis, flavobacteriosis and parasites (moderate fish loss)	- July 2016 & 2017: furunculosis (very high fish loss) - August 2018: furunculosis (moderate fish loss)
Antibiotic use history during the last 5 years	2018 & 2019 : sulfonamide –trimethoprim	- 2016 & 2017: florfenicol, sulfonamide-trimethoprim), enrofloxacin and amoxicillin - 2018 : florfenicol
Vaccination history during the last 5 years	Every year against yersiniosis “ERM”. Last time: 11/2019 for small trout by dipping method	- Every year against yersiniosis “ERM”. last time: 11/2019 for small trout by dipping method - Against furunculosis on February 21 and March 27, 2018 (only once a year for batches of large fish): autovaccine by intraperitoneal route
Age, weight and number of fish in the study ponds at the start of the project	- Age: 18 months - Weight: 695 g (test pond) and 888 g (control pond) - Number: 14,352 (test pond) and 10,987 (control pond)	- Age: 21 months - Weight: 724 g (test pond) and 1165 g (control pond) - Number: 23,530 (test pond) and 11,220 (control pond)

For animal monitoring, fish in farm A underwent several sorting through fish size and weight (by exiting the larger and fat fish to another pond for being sold) during the study. This was also concerned the studied ponds but in these raceways, there was no addition of fish. Each sorting point always has been followed by cleaning the old basin with high pressure water. In fish farm B, no change in ponds and cleaning was reported during the study. Furthermore, no entry of new fish has been happened for the studied ponds in both farms.

Disease, mortality and antibiotic use during the study

Both sites encountered pathological problems during the study. The diseases occurrence, antibiotic use as well as applied vaccination from February to August 2020 is presented in table 2.

In fish farm A, the mortality rate (number of dead fish/number of fish per studied pond) gradually increased from March to May, that in May the mortality was 1.5 to 2% (Figure 1). On May 21 the veterinarian visited the farm and diagnosed a mild furunculosis. Due to limited mortality, the decision was taken initially to wait without antibiotics treatment for further disease evolution as well as antibiogram results. Mortality has been decreased on June (no disease episode) but raised up to 3.5%. in July. Therefore, all ponds including studied ponds were treated with flumequine (12 g/kg of food for 8 days at 1% of body weight per day; withdrawal period: 500d°J) in July. Finally, the administration of flumequine treatment at the end of July, overcame the furunculosis infectious problem. Totally, 45 kg of antibiotic were administered in farm A. The exact quantity of distributed antimicrobial agent for each pond including control and test raceways are not available but farmer cited that the similar quantity of antibiotic treatment has been administered in control and test pond.

In fish farm B on April 02, the veterinarian diagnosed chronic flavobacteriosis based on clinical and bacteriological parameters. Therefore, it has caused the gradual increase of mortality approximately from 1% to 3.8% from April to June and thereafter remained constant in July at the end of the study (Figure 2). Thereafter, oxytetracycline treatment (45 kg in total) has been applied for this farm but it did not concern the studied ponds due to breeder's sales schedule (Table 2). Healthy fish from these ponds were sold in August.

Finally, mortality rate between the control and test ponds did not show a significant difference in both studied fish farms A and B during this study ($p > 0.05$). For comparing the quantity of antibiotic treatment during a disease episode, no differences have been reported also.

Table 2: Vaccination, disease and antibiotic therapy during the study in fish farms A and B.

Vaccination, disease and antibiotic therapy from February to August 2020	**Farm A**	**Farm B**
Vaccination	No	Against furunculosis and yersiniosis AV BPIH RUC ASS (Autovaccin) as of February 5, 2020 (one week before the starting of study)
Disease name and date of onset of the 1st symptoms	2 episodes of furunculosis in May and July 2020: 05/25/2020 and 07/15/2020: lesions and boils on the skin and dead fish due to furunculosis (moderate fish loss: approximately 3.4%)	April 02, 2020: dead fish and presence of mild Ichthyophthirius (Costiase) parasites + puffy skin syndrome (PSD) + mild chronic septicemic flavobacteriosis (absence of bacteria in fish sampled for bacteriological examination) (moderate fish loss: approximately 3.8%)
Diseased farm pond	All farm ponds	All farm ponds
Treated farm pond	All farm ponds	All farm ponds except studied raceways (control and test ponds)
Name of treatment	Flumequine (powder)	Oxytetracycline (powder)
Dosage (dose, treatment duration) and administration route	12 g/kg of food for 8 days at 1% of body weight per day (45 kg antibiotic in total)	18 g/kg food for 10 days at 1% of body weight per day (45 kg antibiotic in total)
Treatment start date	July 21, 2020	April 22, 2020
End of treatment date	July 28, 2020	May 01, 2020

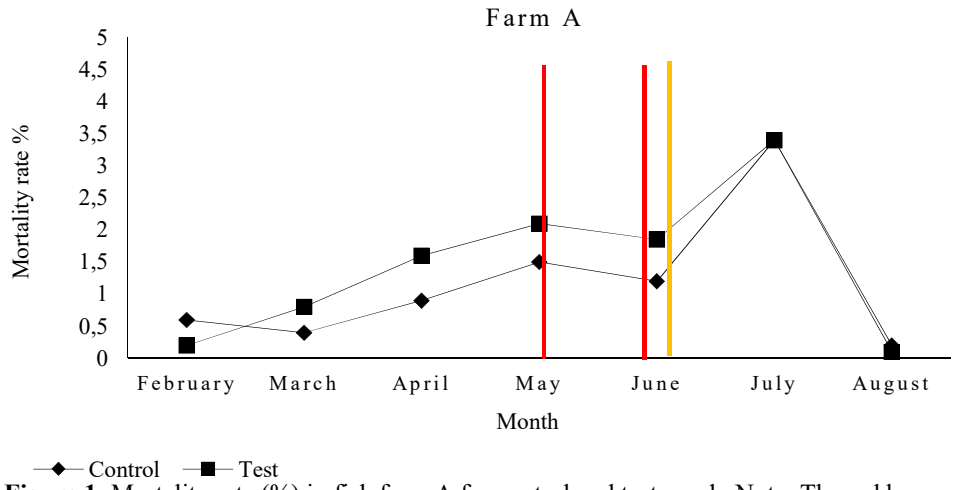

Figure 1: Mortality rate (%) in fish farm A for control and test pond. Note: The red bars represent the onset of furunculosis episode and the yellow bar indicates flumequine treatment.

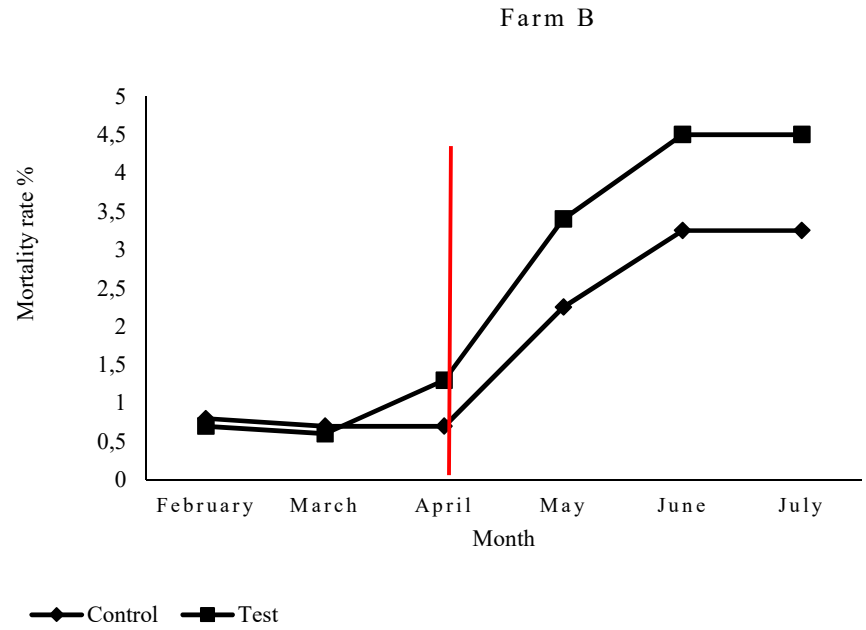

Figure 2: Mortality rate (%) in fish farm B for control and test pond. Note: The red bar represents the onset of chronic/septicemic flavobacteriosis episode. No antibiotic administered in the two studied ponds.

Growth performance

On both farms, the fish of each studied pond gained weight monthly during the seven months of study ($p < 0.05$, data not shown). On farm A, we have observed the weight gain of 1405 g and 958 g for control and test ponds respectively from February to August. However, these differences of weight gain between two studied ponds were not significant ($p > 0.05$). Similarly, on farm B, fish were grown around 900 g in both control and test ponds from the start of study in February to the end of the study in July ($p > 0.05$). By comparing the average of the body weight from the start to the end of the study, no significant differences were observed in the average of body weight between control and test ponds of each studied fish farm (A and B) ($p > 0.05$) (Table 3).

Table 3: The average of fish body weight (g) in the two studied ponds (control and test) of fish farms A and B.

	Farm A		Farm B	
	Control	Test	Control	Test
At the beginning	888	695	1165	724
At the end	2293	1653	2067	1660
Difference	1405	958	902	936
P value	> 0.05		> 0.05	

Discussion and conclusion

Previous studies have documented the health benefits of functional alternatives like probiotics as *Bacillus* spp. (Gao et al. 2017; Park et al. 2017) or *Carnobacterium* spp., (Irianto and Austin 2003;Kim and Austin 2006), prebiotic as MOS (Rodriguez-Estrada et al. 2018) or β-glucan (Ji et al. 2017), essential oils as oregano, anise and citrus (Menanteau-Ledouble et al. 2015), natural mineral materials as yellow loess (Won et al. 2017) or combination of organic acids with cinnamaldehyde (Menanteau-Ledouble et al. 2017) against ASS in rainbow trout (*Oncorhynchus mykiss*). However, all these studies have been realized under experimental controlled conditions. Due to the difficulty to obtain agreements from fish farmers and the impossibility of predicting the

natural occurrence of a bacterial disease, the effects of a functional alternative product on infectious disease are rarely addressed in field conditions on fish farms. In this study, two rainbow trout farms were surveyed for the incidence of natural ASS exposure for seven months.

Each farm presented different profiles of fish farming such as location from other farms, annual fish production or farmer practices like sorting fish by their size or conversely having larger range of fish size in a pond. In addition, other important management strategies were completely different from each holding, including vaccination practice and genetic strains of rearing rainbow trout. We have chosen these strategies to evaluate the effect of PEA under very different farming conditions. However, these differences between the two studied farms may limit our interpretation related to the health and zootechnical benefits of functional additive but it could highlight the different points between the two fish farms studied.

Thus, while in farm A, episodes of furunculosis due to ASS has been observed from May 2020, farm B have not been impacted by these bacterial disease during 2020. Historically, furunculosis has been a significant health problem on farm B, but the farmer has put measures in place to control this disease. Firstly, the strains of the raised rainbow trout were different, considering that genetic elements may have some roles in fish immunity against ASS. Secondly, fish in farm B were vaccinated with a furunculosis autovaccine recently. As reported by the fish farmer of site B, these measures resulted to less diseases and therefore less antibiotic treatments. Furthermore, environmental factors as higher clearance and flow of current water in ponds, location of farm in isolated area therefore, as well as management practices as not applying fish sorting, having smaller fish farm and therefore, less new arrival fish, all could play a role to avoid farm B from furunculosis.

Among these two different farms, results of the present study including morbidity, mortality and weight gain show that addition of AQUABOOST® did not have a positive effect. In previous experimental ASS challenges through intraperitoneal injection, MOS and β-glucan feed additive could enhance disease resistance and growth of rainbow trout when administered from 1 to 2 g/kg diet for at least 2 weeks (Ji et al. 2017; Rodriguez-Estrada et al. 2018). A commercial feed additive containing the essential oils of oregano, anise and citrus at 0.2 g/kg for almost 6 months could protect rainbow trout from ASS and enhance the increasing of fish body weight (Menanteau-Ledouble et al. 2015). Even thought, the dose and duration of used prebiotics/essential oils in this study were approximately similar to prior studies but the composition of the mix was different. Furthermore, initial size and age of fish in our study (around 700 to 1000 g) was much more than previous experimental studies (less than 100 g) while the disease resistance can also differ due to fish size and age. Indeed, peracute and acute forms leading to significant mortality are mainly observed in young fish (Menateau-Ledouble et al, 2016). In this study, only the health status of larger rainbow trout was examined for the application of alternative product due to the resurgence of furunculosis cases mainly observed in older fish in the commercial on-growing field (Avis de l'Anses, Saisine n° 2013-SA-0049C: https://www.anses.fr/fr/system/files/SANT2013sa0049-05.pdf). The antibiotic treatment of large trout also leads to significant economic losses. Finally, the challenge dose of ASS infection route and duration in natural condition remained uncertain in contrast to *in vivo* experimental studies in which dose and administration route of ASS can be concerned as critical parameters in each study to evaluate the efficacy of a functional product to control *Aeromonas* infections (Hayatgheib et al. 2020).

Furthermore, rearing and environmental conditions may have influence on disease resistance or fish gained weight which could explain also the differences in results between the

experimental and on-farm studies. Indeed, the potential impact of environmental conditions which are not constant regularly like water temperature and quality (clearance, pH, salinity, etc.) and other environmental phenomena (flood or heatwave) as well as the presence of different opportunistic environmental bacteria in each aquatic ecosystem could be important factors influencing the incidence of infectious diseases (Snieszko, 1974; Fernández-Bravo and Figueras 2020). Furthermore, farmer practices, like feeding animal in a commercial way (up to 3% of BW) and other rearing conditions that can cause stress in fish and affect their immunity system like higher density of large fish in contrast to moderate feeding of smaller fish in experimental conditions, should be underlined. For example, in this study farmer of site A, used the method of fish fasting (stop feeding the fish for two weeks) to reduce mortalities due to furunculosis in June. It has been show that rainbow trout growth rate, irrespective of feeding regime, was higher in the lower density. In addition, higher feed availability was especially needed in higher density to reduce the stress of feed competition among raised fish (Holm et al., 1990).

We should point out that the on farm studies may have some limitations due to several fish movements for commercial reasons while the follow-up of each individual among a high number of raised fish (for example 15,000 fish per raceway) has its own difficulties. Moreover, the initial differences of the average body weight were not similar in the two fish farms between test and control ponds which made not easy to compare their growth performance and food consumption may have been differed due to differences in the weight and therefore the age of the fish. A monthly follow-up in fish farm, and the record of fish body weight and mortalities needed some adaptations with farmer/commercial practices. For example, the average of body weight in each pond was based on only tenth out thousands fish. Counting the dead fish exactly at the end of the month or exiting dead fish each day from the pond were sometimes not feasible. Furthermore, the

availability of farmer to collect and transfer the data should be considered while compared to experimental practices into which selected parameters were measured precisely through a continuous surveillance and predicted situations to survey individual fish rather than large number of fish. Similarly, in order to collect information from farmers relating to previous diseases (name, time, duration, severity, diagnosis, etc.) and antibiotic treatments (name, quantity, dose, duration, etc.), more consideration and documentation seemed to be practical like a standard format of sanitary situation of farm per year over recent years.

Even thought, in this study AQUABOOST ® did not improve growth performance and did not either improve fish health. Therefore, the administration of antibiotic was necessary to control loss of fish. However, its distribution did not show any unfavorable effects and its safety was assured. Furthers studies should be conducted to test AQUABOOST ® by changing the administration modalities such as dose and duration of functional additive and evaluating more farms. Furthermore, an economic point of view to use these alternative products should be considered.

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General discussion and conclusion

Aeromonas species, including ASS, are ubiquitous bacteria in various aquatic environments and well-known for their antimicrobial resistance profiles due to antibiotic treatments and the possibility for these bacteria to exchange resistance elements among other environmental bacteria in aquatic ecosystems in relation with human or other animal farms activities (Rasul and Majumdar 2017; Santos and Ramos 2018). This may create a potential risk of the development and spread of ARB and ARG between fish, their environment and humans (Muziasari et al., 2016; Watts et al. 2017). With respect to salmonid aquaculture, one of the main source of farmed fish in France, ASS is the causative agent of typical furunculosis, one of the most important bacterial disease in salmonid (ANSES 2015; FranceAgriMer 2019). This disease often results to high mortality and antibiotic usage while vaccination frequently showed disappointing results due to insufficient and/or not long enough immunity in addition to its disadvantages relating to hardly vaccinating large number of small fish, vaccine cost, labor, etc. However, vaccination is still the main solution to prevent furunculosis (Plant and Lapatra, 2011; Assefa, 2018; Adams, 2019).

Regarding to our results of *in vivo* on 600 rainbow trout, the administration of autovaccine could not significantly reduce mortality against experimental ASS inoculation compared to challenge control fish (14.2 % vs 16.3%) but the immunity parameters (Lysozyme, ACH50 and anti-ASS antibody) were enhanced significantly 4 weeks after vaccination until the end of the study at seventh week post-vaccination. While during seven months' study on two Breton rainbow trout farms, furunculosis onset did not occurred in an auto-vaccinated farm compared to another non-vaccinated farm which had furunculosis episodes. Based on results of on-farm experiment, interview with farmers and also gathered information from the recent years, it seemed that the application of autovaccine was efficient against ASS. Hence, collective results from *in vivo* and

on-farm studies showed, on one hand the insufficient protection of vaccinated fish from ASS infection under controlled condition despite a high humoral response and on another hand the possible efficacy of vaccination in natural condition on farm. Notably, the exposure of ASS to studied farm without furunculosis outbreaks remains unanswered under natural condition while, this farm was smaller (lower annual production) and situated in an isolated area and also another rainbow trout strain was raised compared to prior farm. Furthermore, only two farms with divers characterizations were considered in this study. With respect to controlled experiment of *in vivo* assay, only one experimental design (ASS inoculation dose, ASS administration route, etc.) was studied. Therefore, many factors in experimental controlled or natural condition can play a role in order to conclude to the efficacy of vaccination. Nevertheless, additional studies in both controlled and rearing conditions are needed to determine the benefit of vaccination to prevent furunculosis.

Besides vaccination method, several studies have documented immunostimulant effects and health benefits of commercial functional feed, especially against experimental *Aeromonas* infection to limit antibiotic use in freshwater fish like probiotics (Abdel-Tawwab et al. 2008; Aly et al. 2008, Harikrishnan et al. 2010; Reda et al 2018; Suprayudi et al. 2017), prebiotics (Zheng, et al. 2011; Ebrahimi et al. 2012; Yarahmadi et al. 2014 and 2016), essential oils (Menanteau-Ledouble et al. 2015), natural mineral materials (Won et al. 2017) or combination of organic acids and essential oil (Menanteau-Ledouble et al. 2017). However, an exhaustive study including *in vitro*, *in vivo* and on-farm experiments was not reported to our knowledge. Moreover, evaluating *Aeromonas* vaccine efficacy under functional feed treatment was less studied in farmed fish (Yin et al., 2009; Salah et al., 2016). In this thesis, a commercial phytochemical/prebiotic functional product namely AQUABOOST® was selected to be studied against furunculosis in rainbow trout aiming the reduction of antibiotic use in aquaculture. Prior to product selection, through a

comprehensive literature review, potential substances mainly phytochemicals against *Aeromonas* infection in salmonid fish were selected for *in vitro* study. Our results showed that cinnamon, oregano, clove and thyme oils and their major phytochemical compounds presented strong antibacterial activities by determining lower MIC values against the four tested ASS strains through microdilution method. Based on these results, three commercial products including AQUABOOST® were also tested against ASS strains that resulting to similar antibacterial activities (MIC: 0.5 µl ml^{-1}) compared to previous experiment. According to these results, AQUABOOST® was chosen to be studied, first in *in vivo* experimental conditions through intramuscular injection of ASS to vaccinated and non-vaccinated rainbow trout, and second on two rainbow trout farms under natural exposure of ASS and rearing conditions. Meanwhile, the potential risk of ARB and ARG in fish and environment were evaluated in these two farms studied.

Regarding to *in vivo* results, AQUABOOST® seemed to be efficient in rainbow trout by enhancing some immunity parameters (ACH50 activity) prior and after challenge, improving vaccine uptake and also protecting fish from ASS infection as well as their growth performance after ASS inoculation. However, it might be interesting to study a larger number of immune parameters such as phagocytic, respiratory burst, myeloperoxidase activities as wells as further research on expression of immune-related genes induced by alternative functional feed treatments (Abo-Al-Ela, 2018; Semple and Dixon, 2020). Furthermore, the evaluations of functional feed mechanisms of action, its effects on the indigenous gut microbiota, intestinal morphology and histology studies merit further investigations. For example, prior studies demonstrated that administrated probiotic functional feed alternative resulted to lower severity of lesions in intestines and gills, increased inflammatory cell infiltration of intestine or enhanced intestinal structure by

increasing microvilli length and density after an *A. hydrophila* challenge (Ngamkala et al. 2010; Mohapatra et al. 2014; Ran et al. 2015, 2016; Hamdanetal. 2016; Liu et al. 2016; Dong et al. 2018).

Nevertheless, the increase of disease resistance was not observed by AQUABOOST®. Even thought, AQUABOOST® studied *in vitro* showed its antibacterial activity against the ASS strain studied in the *in vivo* assay but it seemed that it was not efficient in *in vivo* experiments to reduce mortality due to ASS infection. This can be explained by the concept of *in vivo* studies which provide valuable information regarding the effects of a particular substance or disease progression in a whole living organism compared to *in vitro* assays that only involves studying microorganism cells in culture. Therefore, the results of *in vivo* studies on product efficacy depend on experimental factors such as conditions into which fish are infected or into which the product is administered.

Regarding to infection challenge protocol, we have taken into account that the chosen lethal dose may be not sufficient to point out the significant differences of mortality among challenged groups in which AQUABOOST® could be more effective against higher exposure of ASS. The chosen lethal dose (LD20) in this study compared to prior studies (LD50) was more close to the mortality rate due to chronic furunculosis in natural outbreaks. While, mortality rates in chronically infected fish may be low and diseased fish may recover and become carriers. But in an acute furunculosis, mortality rate may raise up to 50% (Oidtmann et al., 2013). This issue also has been observed on our on-farm study with a mortality rate due to chronic furunculosis around 3.4%. Furthermore, the size of studied fish should also be considered in infection challenge protocol by considering that larger trout are mostly infected with chronic form of furunculosis (ANSES, 2015). In our on-farm experiment, furunculosis occurred in large rainbow trout around 1.4 kg during two episodes (each minimum one month). In the experimental *in vivo* study, a higher mortality rate (16.3%) was observed in smaller rainbow trout around 0.2 kg that started 3 days after ASS

inoculation and subsided by day 10 post-challenge. Even more, mortality rate could be much higher if a higher initial lethal dose of ASS was inoculated. Therefore, applying the initial lethal dose at 20% in ASS infection challenge is more appropriate to study the efficacy of an alternative functional feed against chronic furunculosis. Notably, chronic furunculosis in larger rainbow trout is a main concern in France salmon culture rather than acute form of this disease (ANSES, 2015). In addition, the administration route of ASS (intramuscular vs immersion or etc.) should be considered in infection challenge protocol in order to be close to natural exposure of ASS in fish farming. For example, cohabitation (Chettri et al., 2015), immersion bath (Banu et al., 1999; Bartkova et al., 2017b), feeding bacteria or skin abrasion (Banu et al., 1999) could be more similar to natural ASS infection.

Besides various experimental factors than can have influences on ASS infection challenge, the environmental elements in natural condition also should be considered. This high-lights the existing factor in farm conditions than can have influences on disease resistance such as rough hot weather and higher water temperature which is optimal for furunculosis onset or arrival of low quality water from river that received various animal and human activities (Snieszko, 1974; Fernández-Bravo and JoséFigueras 2020). We observed that among the two studied farms, the one that was closer to various animal and human activities was infected with furunculosis in summer months. Moreover, farmer sanitary practices like cleaning and disinfection of fish ponds as well as vaccination protocols or farmer managing practices as origin and strain of raised fish, loading/number of fish per pond, etc. have to be evaluated. We noticed that the infected farm with furunculosis was not vaccinated against ASS and different origin of fish was raised compared to healthy farm in this study. Even though, in healthy farm vaccination against ASS into which preventive functional feed treatment was applied, the question about the efficacy of

AQUABOOST® and/or vaccination against "potential" ASS exposure still remains unanswered. In fact, under natural conditions, we cannot be sure if ASS was introduced into this farm and if this product was efficient enough to overcome ASS infection.

Furthermore, the composition of functional feed as well as the stability of its components incorporated in feed under controlled *in vivo* study and also under natural condition like inconstant and rough environmental conditions (water pH, salinity, and etc.) have to be considered. Moreover, administration protocol of functional feed (dose, duration) have to examined in which an adequate frequency and duration of effective substances in natural conditions can provide disease resistance in ASS infected fish. For example, AQUABOOST® including phytochemicals and prebiotics like beta-glucan and MOS was administered for three constitutive months prior to furunculosis onset on farm; while longer duration of β-glucan administration (15 days compared to 30 days) showed an immunosuppression and an exhausted fish immune system in a prior study (Douxfils et al. 2017). Therefore, the efficacy of AQUABOOST® can be examined for two weeks' consecutive administration in *in vivo* studies or on farm condition especially before the start of warmer months for which conditions are optimal for furunculosis onset; AQUABOOST® can also be administered each two weeks with alteration of basal diets. However, the protocol of applying alternative functional feed additive can depend on its price and farmer technical strategies and management. Although, there can be numerous functional feed additives serving for increasing disease resistance in aquaculture, each with a different chemical composition and origin but the selection of the potential alternative feed additive has to be based on its effect, its price and its commercial availability. In overall, the application of alternative functional feed treatments is recommended to be tested on more fish farms with different rearing and environmental conditions and also to be accompanied by other prevention method like vaccination to avoid furunculosis outbreaks.

There are discussions that suggested that no one alternative will replace all uses of antibiotics, because a variety of specific and general methods are needed to both prevent and treat disease including immunostimulants, vaccines and gut microbiota modulation as most promising approaches (Cheng et al., 2014; Allen, 2017). These discussions are somehow far from ANSES report (2018), in particular within the framework of the Ecoantibio2 plan (2017-2021), into which the definition of an alternative seemed narrow and restrictive. It explained that an alternative to antibiotic can fully or partially replace the antibiotics used in curative treatment, demonstrating an equivalent or even superior efficacy to antibiotic treatments and as a part of a disease prevention approach, reducing the frequency of occurrence of certain animal diseases, thus leading to less recourse to the use of antibiotic (https://www.anses.fr/fr/system/files/ALAN2013SA0122Ra.pdf).

In addition, there is another term, "functional feed" which are feed materials as defined in Regulation (EC) No 767/2009, used in feed or drinking water to perform different functions including zootechnical performance. Therefore, there are functional feed additives for purposes of improving animal health to control diseases (Hartog et al., 2016;Watts et al., 2020). In consequence, in this manuscript, it suggested that "alternative functional feeds" are functional feed additives which provide a potential alternative to antibiotics by improving animal health and performance and therefore reducing antibiotic use. Similar to prior investigations, we observed that during the *in vivo* experimental study, the number of fish with a significant increase of anti-ASS antibodies increased among vaccinated fish fed with AQUABOOST®. However, vaccine effect on mortality or morbidity was not shown. While, in on-farm study, furunculosis did not occurred in vaccinated fish fed with AQUABOOST®. Hence, maybe insist in the drafting that the term "alternative" to antibiotics should be also "complementary" when an alternative product could be effective in association with other preventive methods like vaccination. Furthermore, maybe an

alternative product in association with/without other methods can only be efficient against certain diseases but not against all aquaculture diseases. For example, AQUABOOST® could not reduce mortality due to furunculosis in one of the studied farm but regarding to the obtained information from "Le Gouessant Aquaculture" the producer of AQUABOOST®, this product prevented the symptoms of vibriosis, a bacterial disease that affects fish and shrimp. They tested their product, *in vitro* and then *in vivo* in seabass aquaculture in Malta with satisfying results (personal communication).

Whereas, in one of the studied farm, the only solution to reduce mortality was antibiotic treatment to treat furunculosis. Therefore, we could not show that the application of functional feed alternative can reduce an antibiotic treatment and therefore decrease the potential ARB and AGR development in aquatic ecosystems. Even more, comparison between exact prescribed antibiotics during the study and exact prescribed antibiotics during previous years could not been done regarding to some missed gathered information from framers. This issue can highlight the need of precise documentations like a "standard format" of sanitary situation of farm and antibiotic use (molecule, dose, duration, total amount of used antibiotic, etc.) per year over recent years. Although, in this study the impact of a recent antibiotic treatment on the evolution of ARB and AGR was evaluated to show the adverse effect of antibiotic use on ecosystem.

Regarding to furunculosis antibiotic treatment in one of the fish farm studied, the impact of flumequine treatment on ARB and AGR of environmental *Aeromonas* species in rainbow trout and their aquatic environments as water pond and biofilm was evaluated in both farms with open circuit system. In this context, the antimicrobial susceptibility and resistance profile of *Aeromonas* strains through the micro dilution method (MIC) were determined for healthy, furunculosis and antibiotic treated samples during seven months. Moreover, resistances genes were detected and quantified

by qPCR. The results of the present study revealed the occurrence of multi-resistant, resistant/non-susceptible *Aeromonas* for quinolones and also other antibiotic families as oxytetracycline, florfenicol, trimethoprim-sulfonamide and colistin. The occurrence and abundance of ARB and AGR depended on the *Aeromonas* origin, the antibiotic use and the presence of upstream activities. We pointed out the link between fish and their environment by the detection of identical ARB and ARGs in both sample types as well as a high risk of resistance genes developing and spreading in aquatic environments. However, future research should focus on assigning the responsible resistance genes to a chromosome or plasmid and screening and quantifying espesically plasmids and other mobile genetic information which are mainly involved in dessimination of antimicrobial resistance elements from *Aeromonas* isolates in aquatic systems. Moreover, the persistence of ARB and ARGs in the environment should be studied in much more longer and much more vaste studies in relation with different aquatic ecosystems like various rivers and waterways. In addition, the maintenance and dissemination of ARB and ARGs to other enviremental or even pathogen bacteria for human and other animal species in contact to aquatic envirements are also needed to be examined.

These collective facts from *in vitro*, *in vivo* and on-farm studies are information to remind how far we still have to go in developing an effective and practical alternative functional feed and/or in complementary with other preventive method like vaccination to reduce antibiotic treatment. Moreover, sustainable aquaculture practices investing in new approaches to reduce the spread of antibiotic resistance need to be established. This thesis emphasized on the role of antibiotic treatment and lack of effective furunculosis vaccination and/or effective functional feed alternative on widespread transmission of *Aeromonas* resistant bacteria and genes in aquatic ecosystems.

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ANNEXES

Annex 1

QUESTIONNAIRE ETUDE « Thèse de doctorat projet ALTER FOR FISH »

« Evaluation de l'efficacité d'alternatif aux antibiotiques en salmoniculture Vers une diminution du développement et de la diffusion de bactéries antibiorésistantes chez le poisson, dans son environnement et chez les pisciculteurs »

Information générale de la pisciculture

Pisciculture de ……

Date de la visite : …….

Enquêteur :

Localisation de la visite (commune)

1. Coordonnées

1.1. NOM, Prénom :

1.2. Adresse du siège :

1.3. **Téléphone** :

1.4. **E-mail et web site** :

1.5. Date d'installation :

1.6. Identité des employés et personnes intervenant sur le site/bassins, et description brève de leur activité (en lien avec l'activité aquacole) :

2. Description de l'exploitation

ELEVAGE

2.1. Nombre total de bassins :

2.2. Tonnage du site par an :

2.3. Espèce élevée : ☐ TAC ☐ Truite Fario ☐ Autre : ……

Catégorie : ☐ Grossissement ☐ Alevins ☐ Géniteurs × Naissains

Génétique : ☐Aqualand ☐ Plourin

2.4. Origine des poissons :

2.5. Chargement (kg/m^3) :

2.6. Origine de l'eau des bassins (Dérivation de rivière (nom de la rivière), Circuit recirculé,)

2.7. Présence d'autres sites voisins en amont : pisciculture, autres élevages (préciser)

BASSINS TÉMOIN ET ESSAI

Numéro du bassin témoin :

Numéro du bassin essai :

	Bassin témoin	**Bassin essai**
Nature des bassins d'étude (béton, terre, …)		
Surface des bassins d'étude		
Tonnage des bassins d'étude		
Chargement (kg/m3)		
Origine des poissons		
Origine de l'eau des bassins		

3. Gestion sanitaire et historique de la prévention des maladies

3.1. Vétérinaire traitant habituel :

3.2. Historique de la situation sanitaire (des maladies) durant les 5 dernières années :

3.3. Vaccination éventuelle de tous les bassins ? ☐ Oui ☐ Non

Si oui, nom du vaccin ; comment (IP, aliment, IM, balnéation, etc)

3.4. Vaccination éventuelle des bassins témoin et essai ? ☐ Oui ☐ Non

Si oui, nom du vaccin ; comment (IP, aliment, IM, balnéation, etc)

3.5. Traitement préventif de tous les bassins (HE, prébiotiques, probiotiques…) :

☐ Oui × Non

Si oui, nom du produit…… ……Date……comment (aliment etc.) …….

3.6. Traitement préventif dans les bassins témoin et d'essai ? ☐ Oui ×Non

Si oui, nom du produit…… ……Date……comment (dans l'aliment etc.) …….

……..

3.7. Nettoyage de tous les bassins : ☐ Oui ☐ Non

Si oui, fréquence…plusieurs fois par an……. **Comment ?**

Dès que on peut (lors du tri) vider les bassins ; Surtout en été ; Vidange, nettoyage par haute pression style karcher, désinfection avec l'eau de javel et Agrigerm

3.8. Nettoyage des bassins témoin et d'essai ? : ☐ Oui ☐ Non

Si oui, fréquence…

4. Historique de la furonculose depuis 2018

4.1. Épisode de furonculose en 2018 ☐ Oui ☐ Non

Si oui, date :

Traitement :

Nom du médicament :

Traitement/ Posologie, durée du traitement :

Pourcentage moyen de mortalité lors de cette épisode clinique

4.2. Épisode de furonculose en 2019 ☐ Oui ☐ Non

Si oui, date…….

Traitement…….

Nom du médicament ……

Traitement : Posologie, durée du traitement

……

Pourcentage moyen de mortalité lors de cette épisode clinique ……

4.3. Est-ce que vous vaccinez contre la furonculose : ☐ Oui ☐ Non

Si oui, nom du vaccin, Date, **comment (IP, aliment, IM, balnéation, etc.), Protocole vaccinal : âge des truites, rappel ? si oui quand et comment ?**

Annex 2

QUESTIONNAIRE ETUDE « Thèse de doctorat : Alter for fish »

« Evaluation d'impact de l'usage d'un produit alternatif aux antibiotiques en condition naturelle d'exposition en situation d'élevage sur la santé des poissons et le recours aux antibiotiques dans les pisciculture : vers une diminution du développement et de la diffusion de bactéries antibiorésistantes chez le poisson, dans son environnement et chez les pisciculteurs »

Suivi mensuel de la pisciculture

Pisciculture de ……

Date de la visite : …….

Numéro de la visite : ……

Enquêteur :

Localisation de la visite (commune) …….

Numéro du bassin témoin …….

Numéro du bassin essai ……

1. Changement de personnel

1.1. Identité des employés et personnes intervenant sur le site/bassins dans le cas de changement depuis la dernière visite:

……

☐ Pas de changement de personnel

2. Suivi du protocole

2.1. Est-ce que vous avez rencontré des problèmes dans l'alimentation des poissons depuis la dernière visite ?

Si oui, décrire

bassin témoin ☐ Oui ☐ Non ……

bassin essai ☐ Oui ☐ Non ……

- Les poissons de bassin témoin ont-ils bien été nourris avec l'aliment de base ?

☐ Oui ☐ Non Appétit ☐ Oui ☐ Non

- Les poissions de bassin essai ont-ils bien été nourris avec l'aliment essai ?

☐ Oui ☐ Non Appétit ☐ Oui ☐ Non

2.2. Est-ce que les poissons sont entrés ou sortis des bassins d'études (témoin/ essai) depuis la dernière visite ?

Bassin témoin ☐ Oui ☐Non

Bassin essai ☐ Oui ☐ Non

Si oui,

Bassin d'études	**Pour quel motif ? Entrés ou sortis ?**	**Nombre de poissons**	**Date**	**Origine des animaux si entrés ou sortis**	**Numéro(s) bassin(s)**
Bassin témoin	……	……	…/…../….	……	……
Bassin essai	……	……	…/…../….	……	……

- Actuellement, quelle est la biomasse des bassins d'études (témoin/ essai) ?

2.3. Est-ce qu'il y a eu une modification (pompage, arrivée d'eau secondaire, etc.) dans la circulation de l'eau dans les bassins d'études (témoin/ essai) depuis la dernière visite ?

			Si oui, décrire
Bassin témoin	☐ Oui	☐ Non	…….....
Bassin essai	☐ Oui	☐ Non	…….....

			Numéro du bassin et pour quel motif
Bassin témoin	☐ Oui	☐ Non	……
Bassin essai	☐ Oui	☐ Non	……

2.4. Est-ce que les plaquettes d'étude ont été déplacées des bassins d'études (témoin/ essai) depuis la dernière visite ?

2.5. Est-ce les poissons ont été vaccinés dans les bassins d'études (témoin/ essai) ou autres bassins depuis la dernière visite ?

			Numéro du bassin	Si oui, décrire
Les bassins d'élevage	☐ Oui	☐Non	……	……
Bassin témoin	☐ Oui	☐ Non	……	……
Bassin essai	☐ Oui	☐ Non	……	……

3. Survenue d'un épisode pathologique

3.1. Est-ce qu'un épisode pathologique est survenu depuis la dernière visite dans le bassin d'étude ou dans un bassin en amont du bassin d'étude : ☐ Oui ☐Non

Si oui,

Bassin	Nom de la maladie	Date d'apparition des 1ers symptômes	Symptômes observés	Durée mortalité	kg de poissons	Mortalité observée (point)%	Numéro(s) bassin(s)
Bassins d'élevage	……	…/…../….	……	……	……	……	……
Bassin témoin	……	…/…../….	……	……	……	……	……
Bassin essai	……	…/…../….	……	……	……	……	……..

- Comment cela s'est-il propagé ? (Précisez sur le plan) …….

3.2. Avez-vous fait appel à un vétérinaire ?

☐ Oui ☐ Non Si oui, par téléphone ☐ visite ☐

3.3. Selon vous, qu'elle est l'origine de cet épisode pathologique ?

Origine de cet épisode pathologique	**Bassins d'élevage**	**Bassin témoin**	**Bassin essai**
Introduction d'animaux			
Manipulation			
Météo			
Stress (précisez)			
Passage de camion(s) piscicole(s)			
Equarrissage			
Changement de concentration en O2			
Introduction d'un aliment différent			
Visiteurs			

Autre			

3.4. Confirmation du diagnostic ☐ Oui ☐ Non Si oui précisez la date……

par : □ Clinique □ Bactério sans antibiogramme □ Bactério avec antibiogramme □ Autopsie □ Autre :

……..

Traitement (sans ou avec antibiotique)	**Bassins d'élevage**	**Bassin témoin**	**Bassin essai**
Nom du traitement			
Posologie (dose, fréquence, durée traitement)			
Voie d'administration : balnéation, voie orale, mélangé sur place à l'aliment, aliment médicamenteux, Injection			
Date du début de traitement			
Date de fin de traitement			
Numéros des bassins traités avec ce même traitement au même moment			

Résultats : …… □ Aucun

3.5. Est-ce qu'il y a eu un traitement (sans ou avec antibiotique) ? ☐ Oui ☐ Non
Si oui,

Puis-je avoir une copie de l'ordonnance ?

4. Survenue d'un épisode accidentel ou d'un évènement météorologique

4.1. Est-ce qu'il y a eu d'un épisode accidentel ou un évènement météorologique par exemple : un déversement de lisier dans le cours d'eau, une pollution accidentelle ou (orages, crues, pluies importantes) avec un impact sur la qualité de l'eau, le stress des poissons, etc ☐ Oui ☐ Non

Si oui,

Bassins d'élevage ☐ Oui ☐ Non

Bassin témoin ☐ Oui ☐ Non

Bassin essai ☐ Oui ☐ Non

Décrivez……

5. Vide sanitaire/nettoyage/désinfection/mise à sec de bassins en amont des bassins d'étude (témoin et essai)

5.1. Des/ le bassin(s) a-t-il été nettoyé/ désinfecté ? ☐ Oui ☐ Non

Si oui,

			N du bassin	Méthode de nettoyage / désinfection utilisée	Produits utilisés	Durée du vide sanitaire
Bassins d'élevage	☐ Oui	☐ Non	……..	…….	…….	…….
Bassin témoin	☐ Oui	☐ Non	……..	…….	…….	…….
Bassin essai	☐ Oui	☐ Non	……..	…….	…….	…….

Commentaire :

DOCTORAT ECOLOGIE
BRETAGNE GEOSCIENCES
LOIRE AGRONOMIE ALIMENTATION

Oniris
École Nationale Vétérinaire, Agroalimentaire et de l'Alimentation Nantes Atlantique
Santé et alimentation au cœur de la vie

Titre : Évaluation de l'efficacité d'alternatives alimentaires fonctionnelles pour lutter contre l'infection à *Aeromonas*, principalement *Aeromonas salmonicida* subsp. *salmonicida*: vers la réduction de l'utilisation d'antibiotiques pour diminuer le développement et la diffusion de bactéries et de gènes résistants aux antibiotiques chez les poissons et l'environnement

Mots clés : *Aeromonas*, bactéries résistantes aux antibiotiques, poisson, alternatives alimentaires fonctionnelles, environnement, gènes de résistance

Résumé : Des alternatives fonctionnelles à base de prébiotiques et d'huiles essentielles (PEA) contre la furonculose due à *Aeromonas salmonicida* subsp *salmonicida* (ASS) sont envisagées pour réduire l'utilisation d'antibiotiques et peut-être les bactéries résistantes aux antibiotiques (ARB) et les gènes de résistances (ARG). Les résultats *in vitro* ont montré une activité antibactérienne efficace du PEA par la méthode de microdilution. Pendant 11 semaines d'une étude *in vivo* sur 600 truites arc-en-ciel (TAC), le PEA a amélioré quelques paramètres d'immunité, la prise vaccinale, la protection des poissons contre l'infection ASS ainsi que les performances de croissance après l'inoculation ASS, mais l'augmentation de la résistance à la maladie n'a pas été observée. Une étude de sept mois sur deux fermes de TAC n'a pas montré l'effet protecteur du PEA sur l'une des fermes atteinte de furonculose dans laquelle un traitement antibiotique a été effectué. Des échantillons de poissons, d'eaux et de biofilms provenant de ces fermes ont révélé que l'occurrence et l'abondance des ARB et ARG dépendaient de l'origine d'*Aeromonas*, de l'utilisation d'antibiotiques et de la présence d'activités en amont. Le lien entre les poissons et leur environnement par la détection identique d'ARB et d'ARG ainsi qu'un risque élevé de développement d'ARB et d'ARG dans les milieux aquatiques ont été observés. Les résultats de ces études ont montré jusqu'où il convient encore aller dans le développement d'alternatives efficaces pour réduire l'utilisation d'antibiotiques ainsi que diminuer la transmission généralisée d'ARB et d'ARG dans l'écosystème aquatique en lien avec l'homme et l'animal.

Title: Evaluation the efficacy of the functional feed alternatives to control *Aeromonas* infection, mainly *Aeromonas salmonicida* subsp. *salmonicida*: towards reduction in antibiotic use to decrease the development and diffusion of antibiotic-resistant bacteria and genes in fish and environment

Keywords : *Aeromonas*, antibiotic-resistant bacteria, fish, functional feed alternatives, environment, resistance genes

Abstract : Application of functional feed alternatives based on prebiotics and essential oils additive (PEA) to control furunculosis due to *Aeromonas salmonicida* subsp. *salmonicida Aeromonas* (ASS) is considered to reduce antibiotic use and perhaps antibiotic-resistant bacteria (ARB) and genes (ARG) in fish and environment. This study evaluated the efficacy of PEA against ASS through *in vitro*, *in vivo* and on-farm studies. *In vitro* results showed efficient antibacterial activity of PEA through micro dilution method. During 11 weeks of an *in vivo* study on 600 rainbow trout, PEA enhanced few immunity parameters, improved vaccine uptake and fish protection from ASS infection as well as growth performance after ASS inoculation, but the increase of disease resistance was not observed. A seven-months study on two rainbow trout farms did not show the protection effect of PEA on one of the farm detected with furunculosis into which antibiotic treatment was done. Fish, water and biofilm samples from these farms revealed that occurrence and abundance of ARB and ARG depended on *Aeromonas* origin, antibiotic use and presence of upstream activities. The link between fish and their environment by the detection of identical ARB and ARGs as well as a high risk of resistance genes developing and spreading in aquatic environments were observed. These collective facts from *in vitro*, *in vivo* and on-farm studies showed how far we still have to go in developing an efficient and practical alternative functional feed to reduce antibiotic use as well as decrease widespread transmission of *Aeromonas* ARB and ARGs in aquatics ecosystem relating to human and various animal species.