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Moving towards more sustainable aquaculture practices: a meta-analysis on the potential of plant-enriched diets to improve fish growth, immunity and disease resistance

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Abstract

Aquatic animal diseases are one of the major limiting factors in aquaculture development, with disease emergence forecast to increase with global change. However, in order to treat increasing diseases in a context of global emergence of antimicrobial resistance and strengthening regulations on antimicrobial use, sustainable alternatives are urgently needed. The use of plant supplements to increase fish immunity and disease resistance has gained much popularity within the last decades. The use of functional supplements, such as plants, can also improve growth and feed assimilation, contributing to a better optimization of aquaculture resources (e.g. fish meal inclusion). We conducted a systematic review and meta-analysis in order to identify the research gaps in the use of plant-enriched diets in fish aquaculture and estimate, for the first time, the overall efficacy of plant-enriched diets on fish growth, immunity and disease resistance as well as the effect of intrinsic parameters (fish trophic level, type of plant material, dosage, treatment duration and pathogen species) on the treatment efficacy. We found that plant-enriched diets significantly enhanced growth, immunity and disease survival of treated fish, regardless of the fish trophic level, treatment duration and type of material used. We also show that plant supplements are a versatile alternative that can benefit different aquaculture sectors (from small-scale fish farmers to intensive productions). Finally, we observed that studies need to improve the information reported about the plant material used (e.g. origin, identification, chemical composition), in order to allow the comparison of different experiments and improve their repeatability.

Key words: disease prevention, fish aquaculture, immunostimulant, medicinal plants, plant supplements, sustainable aquaculture.

Introduction

Aquaculture is forecast to increase by 62% between 2010 and 2030, in order to supply the increasing fish and seafood demand derived from a steadily growing population and changing consumption patterns, providing over two thirds of total fish and shellfish consumed worldwide (Worldbank 2013; FAO 2018). Aquaculture also contributes significantly to the economy of many households, with an estimation of over 100 million people relying on aquaculture for a living

(FAO 2018). In fact, despite some controversy, evidence suggests that aquaculture plays an essential role in global food security and poverty alleviation, which are central to the 2030 UN Agenda of Sustainable Development Goals (Béné *et al.*, 2016; Belton *et al.* 2018; UN 2018). Aquaculture does not only provide an important source of protein and income but can also furnish ecosystem services such as wastewater treatment, bioremediation, habitat restoration and replenishment of wild populations (Troell *et al.* 2014; Froehlich *et al.* 2017). However, in order to provide social

and environmental benefits, sustainable aquaculture practices are required. Otherwise, aquaculture can contribute to increasing stress on water resources, overfishing of wild stocks for feed production, introduction of invasive species, pathogen transmission between reared and wild organisms and selection and dissemination of antimicrobial resistance (Troell *et al.* 2014).

Despite the important role of aquaculture, the sector faces numerous challenges that hamper its expansion. Aquatic animal diseases are considered to be one of the major limiting factors for aquaculture development (Stentiford *et al.* 2012, 2017), with increasing global trade, intensification of systems and climate change contributing to the emergence of infectious diseases (Karvonen *et al.* 2010; Perry *et al.* 2013; Reverter *et al.* 2020). With high culture densities, intensified systems of production facilitate the evolution and spread of more virulent pathogens and the occurrence of disease outbreaks due to stressed and immuno-compromised animals (Bondad-Reantaso *et al.*, 2005; Pulkkinen *et al.*, 2010). Weather events such as storms, droughts and high temperatures negatively affect the water quality (e.g. causing salinity changes, introducing pollutants into aquaculture systems and lowering oxygen levels), causing animal stress and compromising their immune system (Weatherdon *et al.*, 2016; Dubey *et al.* 2017; Abdel-Tawwab *et al.* 2019). Changes in precipitation and temperature regimes can also increase the transmission of infectious diseases and contribute to the expansion of their geographic distribution by providing new habitats for the pathogens or by increasing the contact time between pathogens and hosts (Vezzulli *et al.*, 2016; Polgreen & Polgreen 2018).

Despite the efforts deployed in improving disease surveillance and management, economical losses related to disease outbreaks in aquaculture are estimated at over US\$9.5 billion per year (Shinn *et al.* 2015). Most of these losses occur in developing countries (> 90% of the world's aquaculture), where aquaculture is mostly rural and diseases are often not appropriately diagnosed or treated (Brummett *et al.*, 2014; FAO 2018). In order to prevent and mitigate the economic losses that can threaten their livelihood, farmers regularly administer antibiotics and other veterinary drugs such as disinfectants to reared aquatic animals (Rico *et al.* 2013; Cabello *et al.* 2016; Miranda *et al.* 2018). However, the recurrent use of such chemicals does not only present side effects on the aquaculture system by decreasing animal immune system (Yang *et al.* 2017) and selecting for more virulent strains (Azzam *et al.* 2017), but is also a global health threat due to the selection and emergence of antibiotic-resistant bacteria (Marti *et al.* 2011; Cabello *et al.* 2016). Several alternative strategies have been proposed to prevent disease outbreaks and limit the use of veterinary drugs in aquaculture such as vaccination and the use of

functional feed supplements. Vaccination has proven an excellent tool in reducing the use of antibiotics in some aquaculture sectors such as the Norwegian salmon (Brudeseth *et al.* 2013). However, it is a highly specific technique that requires a clear disease diagnosis and a costly vaccine development, which are often not available for tropical emergent diseases (Brudeseth *et al.* 2013). Furthermore, vaccines are often too expensive for a widespread use among small-scale fish farmers and they present limited efficacy in multiagent infections (Pridgeon 2012). In fact, coinfections with homologous or heterologous pathogens are very common in aquaculture (Kotob *et al.* 2017), and therefore, a more holistic approach is required to reduce chemotherapeutant usage in disease prevention and treatment in aquaculture (Caruso 2016; Lieke *et al.* 2019). Since disease outbreaks are intimately related to the physiological state of the animals, the use of feed supplements that maximize fish fitness and promote their immune systems such as medicinal plants and probiotics has gained considerable attention over the last decade (reviewed in Reverter *et al.* 2014; Hoseinifar *et al.*, 2018b; Dawood *et al.* 2020). The use of functional feed supplements is especially interesting because it is a relatively inexpensive practice that can also provide benefits on fish growth and feeding efficiency (Encarnação 2016; Guerreiro *et al.*, 2018; Abdel-Latif *et al.* 2020). Since a better use of aquaculture resources, including fish feed, is required for the sustainable development of aquaculture (Naylor *et al.* 2009; Alhazaa *et al.* 2019), the incorporation of plants or probiotics in fish diets could contribute at the same time to better disease prevention and better feed assimilation (Hoseinifar *et al.*, 2018a; Dawood *et al.* 2019).

Research on the use of medicinal plants and their derived extracts in aquaculture has exploded during the last years, with nowadays, hundreds of articles studying the effects of oral plant administration on fish growth, health and immunity (reviewed in Reverter *et al.* 2017; Suttili *et al.* 2018). Plant-enriched diets have been reported to increase growth, improve feeding efficiency, improve haematological parameters, enhance immune parameters (e.g. lysozyme, complement activity, phagocytic activity, total protein, immunoglobulin) both in blood serum and in fish mucus, display antioxidant effects and confer better disease resistance against different fish pathogens (e.g. Awad & Awaad 2017; Suttili *et al.* 2018; Zhu 2020; Abdel-Latif *et al.* 2020b). Although the specific mechanisms behind the observed physiological effects in fish (e.g. enhancement of certain immune parameters) are still poorly described, some research suggests that plant extracts could activate Toll-like receptors (type I of transmembrane proteins involved in innate immune response), which in turn activate several pathways involved in cell signalling cascade activation, promoting a pro-inflammatory response (e.g. upregulating

pro-inflammatory cytokine expressions such as TNF- α and IL-1 β) and ultimately modulating both the innate and adaptive immune response (Vallejos-Vidal *et al.* 2016; Hoseinifar *et al.* 2020).

Despite the relevance of the previous narrative reviews in the advancement of the topic (e.g. Reverter *et al.* 2014; Van Hai 2015; Awad & Awaad 2017; Stratev *et al.*, 2018; Sutuli *et al.* 2018), the overall efficacy of plant-enriched diets and the effect of intrinsic parameters (e.g. type of plant material, dosage, duration of the treatment) on the treatment efficacy have never been quantified. In this article, we have (i) performed a systematic review in order to investigate the current state of research in the use of plant-enriched diets and identify research gaps and (ii) performed a meta-analysis (a quantitative method to combine and analyse data) to study the efficacy of plant-enriched diets on stimulating growth, immunity and disease resistance in cultured fish. We have also analysed the effect of several variables (fish trophic level, type of plant material used, duration of treatment, dosage and type of pathogen for survival data) on the treatment efficacy of each of the studied parameters (weight gain, specific growth rate, feeding conversion ratio, haemoglobin, serum total protein, immunoglobulin, lysozyme, complement activity, phagocytic activity and disease survival).

Materials and methods

Data collection

We systematically searched all peer-reviewed journal articles and theses that investigated the effects of enriched-plant diet administration on growth, immunity or disease resistance of reared fish using the Web of Science, up to the 15 May 2019. Literature search was performed following the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines (Moher *et al.* 2009, Fig. S1), and the following keyword combination was used: (plant OR herb OR phyto*) AND (aquaculture* OR farm* OR rear*) AND fish AND (growth* OR immun* OR disease*) AND (supplement* OR oral*).

Articles were reviewed to determine whether they met the following criteria: (i) at least one of the following parameters was reported for both fish fed with a control diet and fish fed with plant-enriched diet: weight gain (g), specific growth rate (SGR %), feeding conversion ratio (FCR), haemoglobin (g dL⁻¹), serum total protein (g dL⁻¹), immunoglobulin (mg mL⁻¹), lysozyme activity (U mL⁻¹), phagocytic activity (%), complement activity (ACH₅₀, U mL⁻¹) or disease survival (%) (ii) mean, number of replicates and standard deviation or standard error were reported either numerically or graphically for each of the parameters and (iii) type of extract (type of solvent use for the extraction if any), inclusion rate, quantity of feed

administered and treatment duration were clearly identified. Only studies investigating the effects of plants as supplement (bioactive or medicinal plants) and not as a main diet constituent were included. Studies that evaluated the effect of more than one plant species at a time (mixed herbs) were not included.

When a study investigated the use of several plant species or dosages, we considered them as distinct observations. When a study reported parameters at different time points, only the final time point was considered, to minimize codependency between observations. For each observation, we extracted the following data: year of publication, geographic region of the study (as defined by Worldbank), country of the study, income level of the country (as defined by Worldbank), fish habitat (freshwater, marine or euryhaline), fish taxonomy (species, family), substance type (plant, algae, fungi), plant taxonomy (species, family and order), type of plant extract used (powder, aqueous, ethanol, methanol, essential oil and other), treatment duration (weeks), inclusion rate (g plant kg⁻¹ feed), quantity of feed administered (% fish weight day⁻¹) and type of pathogen used for the infection (only for the survival data, since for all other datasets, parameters were measured from healthy fish). Fish trophic levels were obtained from Fishbase (www.fishbase.se). Dosages were then calculated by multiplying the inclusion rate and the quantity of feed administered and expressed as mg of plant/100g fish*day.

From each study, we also extracted information in order to study the drivers behind the plant choice and evaluate how much information regarding the plant material was provided. We classified the studies depending on the author's drivers: (i) known medicinal properties only, (ii) known medicinal properties and local availability (iii) known medicinal properties and previous studies on aquatic species and (iv) known medicinal properties, local availability and previous studies on aquatic species. We also collected information on the origin of the plant (collected from natural habitat, bought as a whole plant in a local market, bought as a manufactured commercial preparation or unreported). Finally, we recorded whether the following information regarding the plant material was provided: (i) geographic origin, (ii) period of sampling, (iii) plant voucher or expert identification and (iv) chemical composition.

Data analysis

Effect size

The standardized difference in means (Hedges's *g* (g), Hedges & Olkin 1985) was used as the effect size to assess the efficacy of plant-enriched diets in enhancing growth, immune parameters or survival of treated fish and was calculated for each individual observation as the difference

between the mean of the experimental treatment and the control divided by their pooled standard deviation and multiplied by a correction term to reduce bias from small sample sizes (package *esc* in R version 3.6). Strong outliers ($Q1 - 3 \times IQR$ or $Q3 + 3 \times IQR$, where $Q1$ and $Q3$ are the first and third quartile and IQR is the interquartile range) were removed from the datasets to decrease heterogeneity.

Publication bias

Rosenberg's fail-safe number was calculated to test for publication bias in the datasets using the package *metafor* for R (Rosenberg 2005). This number, which is a weighted extension of Rosenthal fail-safe number, indicates the number of studies, of the same weight (average weight of those already being used), needed to change the result from significant to nonsignificant. If this number is sufficiently high ($>5n + 10$, where n is the number of studies analysed), the results can be considered robust with regard to publication bias (Rosenthal 1979).

Random-effect model

Since studies often included several experiments (e.g. several plant dosages tested), whose data were collected as different observations, we fitted three-level meta-analytic models and investigated the distribution of variance over the three levels using R code adapted from Assink and Wibbelink (2016), where level 2 and level 3 were included as random-effects. Level 1 represented the replicates within an experiment, level 2 the experiments within a study and level 3 the different studies used. Full (3-level) and reduced (2-level) models were then compared using the Akaike's information criterion (AIC), and the most parsimonious model (with lower AIC) was retained for each dataset. The magnitude of the effect size was considered significant when the confidence interval did not include zero (Gurevitch & Hedges 1993). All models were constructed using the *rma.mv* function from the *metafor* package for R.

Meta-regression

Contribution of the explanatory variables (fish trophic level, treatment duration, type of extract, dosage and type of pathogens) was assessed by adding them one at a time as fixed-effects in the previously selected model. Since dose responses and duration effects are often not linear, a quadratic term of dosage and duration was also added as fixed-effect in the corresponding models and the two models (with and without quadratic dose term) were compared using the *anova* function in *metafor* to select for the more parsimonious model (lower AIC). A test of moderators (omnibus test) was used to evaluate whether the explanatory variables explained significantly some of the heterogeneity observed. All models were constructed using the *rma.mv* function from the *metafor* package for R.

Since dosage was the only continuous variable that contributed significantly to the heterogeneity observed, we calculated the optimum dosage (for each type of material) that would yield the highest effect size for each of the parameters. This was done by predicting the effect size estimates using the previously selected model (either with or without quadratic dose term), with the function *predict* from the *metafor* package for R and selecting the largest effect size.

Results and discussion

Research trends

After screening the literature, we retained 137 articles published between 2004 and 2019 that investigated the *in vivo* effects of dietary plant supplements on fish growth, immune-related parameters and disease survival (Fig. 1a, Fig. S1, Table S1). Literature investigating *in vivo* effects of plant supplementation on fish has grown exponentially during the last decade, with over half of the articles being published during the last five years (Fig. 1a). Most of the research was performed in low- and middle-income countries (LMICs), with Middle East and North Africa, and South Asia (particularly Iran, India and Egypt) being the major contributors (Fig. 1b–d). The increasing interest in the use of medicinal plants in aquaculture coincides with directives on the regulation of antimicrobial use and the reduction in antimicrobial resistance at national and international level such as the Global Action Plan established by the World Health Organization (WHO 2015) or the National Action Plan against drug resistance for the period 2013–2020 in Vietnam (Binh *et al.* 2018; Lulijwa *et al.* 2019). Antimicrobials have been traditionally used in aquaculture both as prophylaxis and as disease treatment, but following banning in most countries of prophylactic treatments (Henriksson *et al.*, 2015), medicinal plants have arisen as an affordable prevention alternative available to small-scale fish farmers in LMICs.

Twenty per cent of the research (29 studies) was, however, performed in high-income countries, showing a worldwide interest in the use of plant-enriched diets (Fig. 1c). A change in the consumption patterns towards organic food and more sustainable food production systems has been observed especially in high-income countries, and therefore, the use of more environmental-friendly practices for the prevention and treatment of fish diseases is highly requested, bringing along an increase in the product value (Carlucci *et al.* 2015; Vittersø & Tangeland 2015). Our results show, that despite over half of the research is performed on freshwater species, mainly tilapia (Cichlidae) and carps (Cyprinidae), a significant number of studies explored the effect of plant-enriched diets on high-

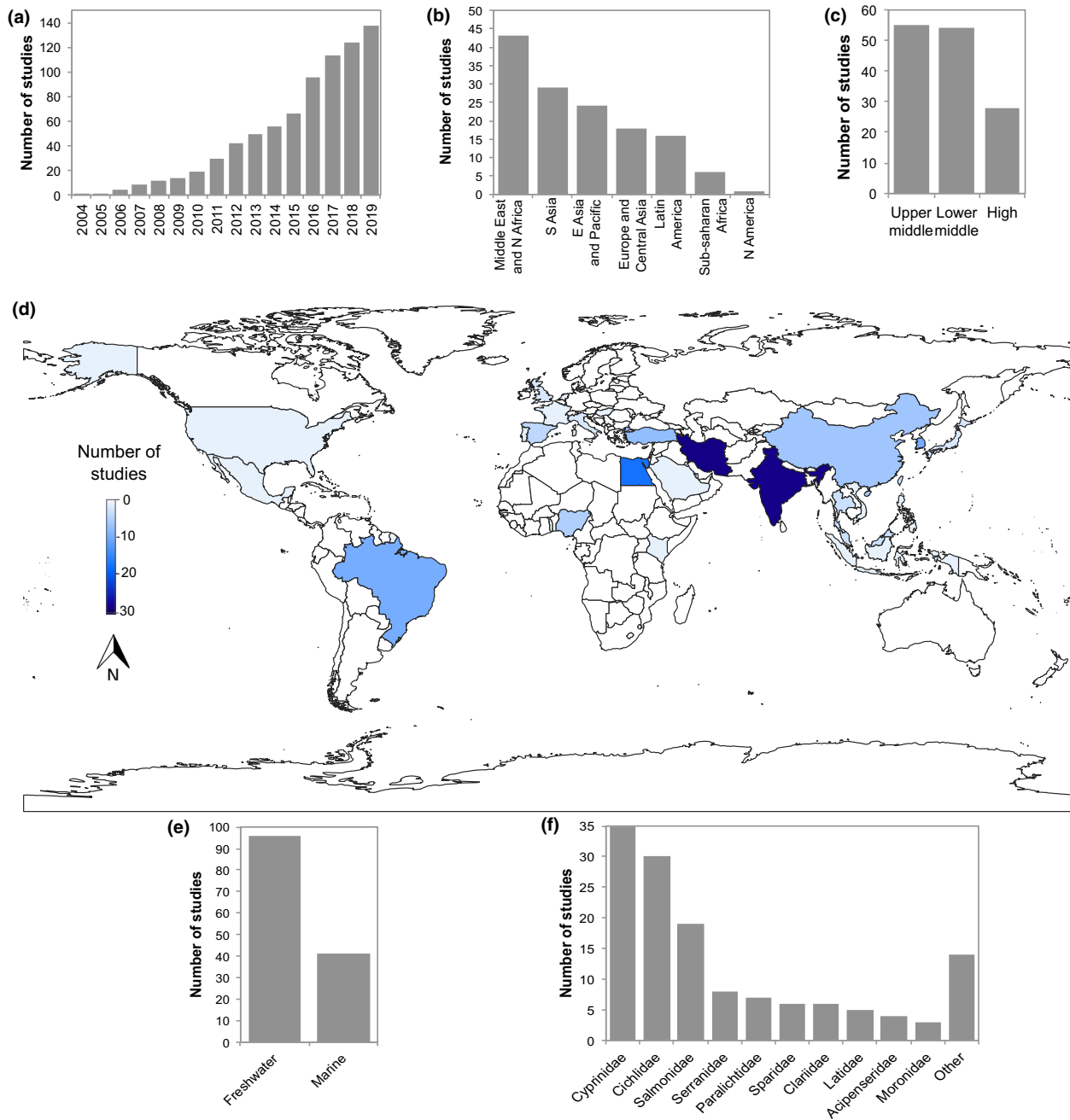


Figure 1 Research trends on the use of plant-enriched diets in fish aquaculture. (a) cumulative number of published articles by year, (b) number of published articles by Worldbank geographic region, (c) number of published articles by Worldbank income group, (d) number of published articles by country (map created using QGIS version 3.4. Madeira), (e) number of published articles by species habitat and F: number of published articles by fish species.

value species such as salmonids or marine species such as groupers (Serranidae), flounders (Paralichthyidae), sea breams (Sparidae) and sea basses (Moronidae) (Fig. 1e,f). Altogether, these results suggest the use of bioactive plants can benefit different farming systems, from small-scale rural farmers seeking inexpensive disease prevention

alternatives, to intensive farms exploring more sustainable alternatives to meet consumer demands and strengthening antimicrobial use regulations. Therefore, interest in the use of plants as functional feed supplements in fish aquaculture will likely keep increasing in the near future. However, usage recommendations are needed for their widespread

and safe use and to control long-term plant toxicity effects on both cultured species and consumers.

Plant use and reporting

Plant choice

The literature collected explored the effect of 98 terrestrial plant, algae and fungi species, belonging to 53 families and 34 orders, with Lamiales being the order most studied, followed by Zingiberales and Asparagales (Fig. 2a,b). Despite terrestrial plants being the most studied group by far, studies evaluating the *in vivo* effect of algae-enriched diets have increased in recent years, with over 8 of the 10 studies being published after 2015 (Fig. 2a). Most ethnoveterinary studies show use of herbal therapy in farmed animals is tightly linked to plant traditional use in human medicine (Ghirotti 1996; Souto *et al.* 2011; Caruso *et al.* 2013), which explains the fact all studied plants possessed known medicinal properties (Fig. 2c), and also the higher number of terrestrial plants. However, with increasing access to aquatic resources and increasing number of studies reporting algal bioactivities, algae arise as an inexpensive and available alternative for disease control and prevention in aquaculture (Shanmughapriya *et al.* 2008; Vatsos & Rebours 2015; Thanigaivel *et al.*, 2016). For example, some algal genera with the broadest antimicrobial activities reported to date include *Sargassum* and *Asparagopsis* (Genovese *et al.* 2012; Tanniou *et al.* 2014; Marino *et al.* 2016; Telles *et al.* 2018). Previous studies have shown that *Sargassum*-enriched diets increased immune parameters and disease survival in Asian seabass (*Lates calcarifer* Bloch, 1790) (Yangthong *et al.* 2016) and improved growth, immune parameters, hepatic antioxidant status and expression of immune-related genes in black sea bream (*Acanthopagrus schlegelii* Bleeker 1854) (Shi *et al.* 2019), whilst *Asparagopsis*-enriched diet promoted batfish (*Platax orbicularis* Forsskal, 1775) growth and expression of immune-related genes (Reverter *et al.* 2016). Biomass valorization of these algae, often considered proliferative or invasive, would not only benefit the aquaculture sector by providing cheap alternative to antimicrobials, but would contribute towards the mitigation of environmental and economic costs related to their proliferations (Balboa *et al.* 2015; Milledge & Harvey 2016; Milledge *et al.* 2016).

The combination between reported medicinal activities and local availability was the most common reason for plant selection (Fig. 2c), suggesting an important role of local pharmacopeia in the selection of plants, which would partly explain the high diversity of plants studied. Similar results were obtained from a field study performed in a rural fish farmer community in Indonesia, highlighting the importance of ethnological knowledge on the selection and

use of plants in aquaculture (Caruso *et al.* 2013). Our results also show that nearly half (47%) of the studies used plants collected from their natural habitats (Fig. 2d). This result not only confirms the importance of local ethnobotanical knowledge, but indicates use of medicinal plants could be in line with an ecosystem-based approach to aquaculture (EAA), which promotes greater consideration of ecosystem functions and services in production systems, further integrating aquaculture into its economical and biophysical context (Aubin *et al.*, 2019). Twenty-nine per cent ($n = 39$) of the plants were bought whole at local markets, whereas 11% ($n = 15$) bought commercial preparations of plants (powder or extracts). Overall these results show the dichotomy between studies interested in inexpensive and easily accessible materials (collected or bought at a local market) and studies exploring the effect of more manufactured and thus more expensive products (commercial extracts), probably more oriented towards high-price cultured species. Finally, a significant quantity of studies (12%) did not provide any indication on where the plant material studied was obtained, which is a cause of concern since plant origin can be related to the plant bioactivity (Fig. 2d).

Reporting of plants used

Chemical composition of plants and algae and their associated bioactivities are largely variable and depend on several factors such as stage of maturity, season and geography (Stengel *et al.* 2011; Pavarini *et al.* 2012). However, very few of the articles revised in this work provide relevant information to assess this variability, which makes it difficult to compare the studies even when investigating the same plant species and drawing usage recommendations. For instance, only 22 studies (16%) analysed the chemical composition of the plants, 6 of which reported very coarse results (<3 compounds studied) (Fig. 2e). Geographic origin (country region) was reported in 42% of the articles but only 8% of the studies gave details about the time or period of collection (Fig. 2e). Finally, plant identification by an expert and conservation of a voucher was only performed in 24% of the studies. Even though some plants are easily identified, some herbs or algae can be highly cryptic and expert identification and voucher repository are necessary as means to ensure traceability of plants studied (Hedberg 1993; Culley 2013). Altogether, these results suggest reporting of plants used in aquaculture studies needs to improve, since lack of relevant information could hamper the transfer of the knowledge acquired by research to fish farmers. Chemical characterization of all studied plants would be ideal, but if not possible, reporting of geographic location and time of collection should be reported and a voucher kept in a recognized facility.

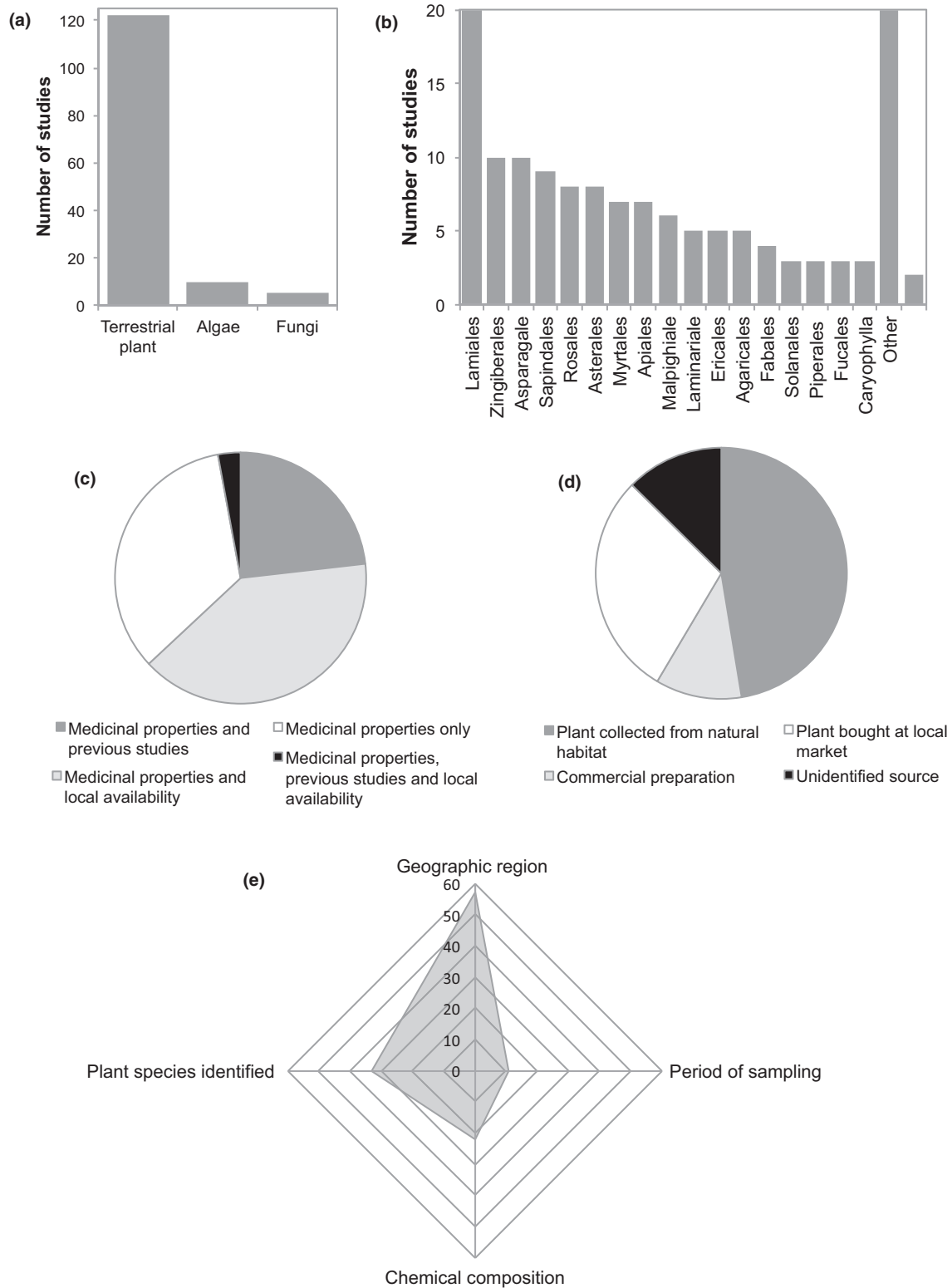


Figure 2 Research trends on the plant use and reporting in studies evaluating plant-enriched diets in fish. (a) number of studies by type of plant (terrestrial plant, algae and fungi), (b) number of studies by plant order, (c) reason for plant choice, (d) origin of plants and E: information reported on used plants.

Efficacy of plant-enriched diets on growth, feeding efficiency, immune parameters and survival

After screening the literature and extracting the data, we obtained 10 different datasets for each of the parameters (weight gain, specific growth rate, feeding conversion efficiency, haemoglobin, total protein, lysozyme activity, ACH50 activity, phagocytic activity, immunoglobulin and survival) with a total of 1,647 observations. After removal of strong outliers, we obtained 1,522 observations (Table S2).

Rosenberg's fail-safe number was largely higher than the critical value for all the parameters; therefore, we can consider the observed results as a reliable estimate of the true effect size (Table 1).

Both level 2 (different observations within a study) and level 3 (different studies) accounted for a significant amount of variance, and after comparing AIC derived from both models, three-level models were chosen for the 10 parameters studied (Table S3). Three-level random-effect models showed that all parameters were significantly enhanced ($P < 0.001$) in fish fed with plant-enriched diets compared with the control fish, confirming plant supplementation as an effective tool to enhance growth, improve feeding efficiency, increase immune parameters and improve disease resistance in fish (Fig. 3). Despite many individual articles having previously shown plant supplementation enhanced growth, immune parameters and improved disease survival (e.g. Nguyen *et al.* 2016; Yunis-Aguinaga *et al.* 2016; Hoseini-far *et al.* 2019; Mehrabi *et al.* 2019; Abdel-Latif *et al.* 2020b), this is the first meta-analysis confirming the efficacy of plant supplementation on fish on a broad scale.

However, although average effect size (g) was significantly different from 0 in all parameters, high heterogeneity levels were observed, and some effect sizes of individual studies

Table 1 Rosenberg's fail-safe number calculated for each of the parameters analysed. A study is considered robust when the Rosenberg's fail-safe number is bigger than the robust fail safe ($5 * \text{number of observations} + 10$)

| Parameter | Number of observations (N) | Rosenberg's fail safe N^* | Robust fail safe ($5N + 10$) |
|---------------------|--------------------------------|-----------------------------|--------------------------------|
| Weight gain | 241 | 201,533 | 1,215 |
| SGR | 247 | 183,158 | 1,245 |
| FCR | 223 | 38,180 | 1,125 |
| Haemoglobin | 122 | 3,842 | 620 |
| Total protein | 121 | 4,745 | 615 |
| Lysozyme | 183 | 46,238 | 925 |
| Phagocytic activity | 108 | 20,087 | 550 |
| ACH50 | 84 | 20,087 | 430 |
| Immunoglobulin | 77 | 4,112 | 395 |
| Survival | 114 | 9,698 | 580 |

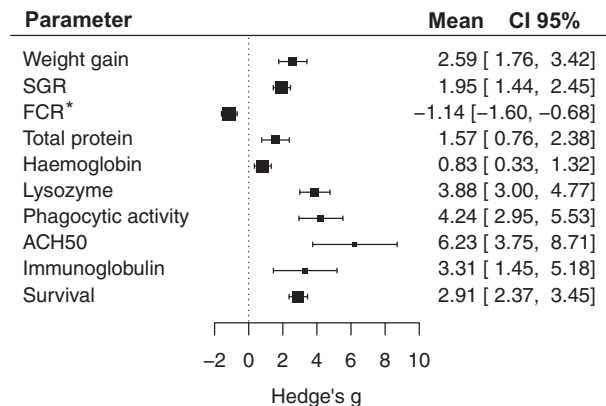


Figure 3 Forest plot reporting the effect size (Hedge's g) for the ten parameters studied (weight gain (g), SGR (%), FCR, total serum protein ($g\ dL^{-1}$), haemoglobin ($g\ dL^{-1}$), lysozyme ($U\ mL^{-1}$), phagocytic activity (%), complement activity ACH50 ($U\ mL^{-1}$), immunoglobulin (g/dL) and survival of infected fish (%). Mean and 95% confidence interval (CI) effect sizes are reported. *Lower FCR ratio indicates better feed assimilation.

were negative (Table S4). None of the plant-enriched diets analysed here significantly decreased the fish survival (significantly negative Hedge's g), indicating their probable safe use, but some treatments decreased growth, feeding efficiency as well as some immune parameters studied (Table S4). For example, whilst the aqueous extract of the Chinese herb *Lycium barbarum* enhanced the specific growth rate in grass carp (*Ctenopharyngodon idella* Valenciennes in Cuvier and Valenciennes, 1844) at doses of 40 and 4 mg plant kg^{-1} fish*day ($g_{40} = 3.83 \pm 0.77$, $g_4 = 12.45 \pm 2.1$) and in Nile tilapia (*Oreochromis niloticus* Linnaeus, 1758) at 4 mg extract/kg fish*day ($g = 5.39 \pm 0.99$), a dose of 40 mg plant kg^{-1} of fish*day resulted in a decreased specific growth rate in Nile tilapia ($g = -4.59 \pm 0.88$) (Mo *et al.* 2016). Similarly, the ethanolic extract of *Ginkgo biloba* increased the lysozyme levels in rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) when administered at 3 and 6 mg extract/kg fish*day ($g_3 = 5.54 \pm 0.71$, $g_6 = 6.92 \pm 0.85$) but induced a reduction in lysozyme when administered at 12 mg extract/kg fish*day ($g = -7.89 \pm 0.95$) (Hajirezaee *et al.* 2019). In order to investigate and determine which explanatory variables (fish trophic level, duration of the treatment, type of extract used, dose and type of pathogen for the survival data) influenced significantly the effect size, we performed meta-regressions.

Effect of different variables on the efficacy of plant-enriched diets on fish

Fish trophic level. Dietary habits are strong determinants of the gastrointestinal (GI) morphology (Wagner *et al.* 2009; Karachle & Stergiou 2010), and recent research suggests that fish trophic level is also highly related to the gut

microbiota composition and enzyme activities (Liu *et al.* 2016). Therefore, fish trophic level could influence the assimilation of medicinal plants and its efficacy on enhancing growth, immunity and improving disease resistance.

The literature we analysed in this article covered fish with varied trophic levels, with high abundance of low (<2.5, mainly cichlids such as tilapia) and high (3.5–4, mainly salmonidae such as rainbow trout and marine species such as the bastard halibut *Paralichthys olivaceus* (Temminck and Schlegel, 1846) trophic level species (Fig. 4a). We analysed the effect of trophic level on the 10 parameters studied, and we found that fish trophic level was only significantly correlated to feed conversion ratio (FCR) (Fig. 4b, Table S5). Since trophic level is negatively correlated to intestinal length, a pattern that seems to reflect the higher digestive times required to digest plant tissues compared with animal tissues (Wagner *et al.* 2009; Karachle & Stergiou 2010), better FCR (lower values) in fish with lower trophic levels is not unexpected. However, it is interesting that even though higher trophic level species might present lower plant assimilation due to faster digestions, plant efficacy on their immunity and disease resistance is not affected.

Plant material. Generally, the efficacy of medicinal plants is tightly related to the abundance of bioactive compounds. Combinations of different molecules can display additive or synergetic effects and as such chemical composition is a major determinant of the observed bioactivity. This means that different plant species will inherently possess different compositions and thus the effect they exert on fish can

vary, but different materials of a same plant species (e.g. dried whole plant or extract) might also display different effects (Vági *et al.* 2005). For example, the use of dried whole plants is cheap but they often contain large amounts of indigestible and antinutritional compounds that could interfere with the plant efficacy (Francis *et al.* 2001; Lech & Reigh 2012). Extracts on the other side, whose composition greatly depends on the mode of extraction and the polarity of the solvents used, are much more concentrated with bioactive molecules. However, high concentrations of bioactive metabolites can sometimes display toxic effects on fish. For example, 50% of goldfish (*Carassius auratus* Linnaeus, 1758) died (LC_{50}) when exposed to baths of 50.3, 31.4 and 35.2 mg/L of chloroform, ethyl acetate and methanolic extracts of *Bupleri chinensis* (Wu *et al.* 2011).

Despite the chemical variability between different plant and algae species studied, we aimed to review the most commonly used plant materials in aquaculture and to investigate whether some types were generally more effective. Powdered plants were the most used material (45%), probably due to the low associated costs, easy use and relative safety (Fig. 5). Ethanolic extracts were the second most used material (24%), followed by essential oils (12%) and aqueous and methanolic extracts (9%) (Fig. 5). The high use of essential oils is probably linked to the high abundance of studies investigating plants from the Lamiales order, which are recognized for essential oil production. We also found two studies that used specific material to extract targeted components from the plants or algae species studied. For example, del Rocio Quezada-Rodríguez

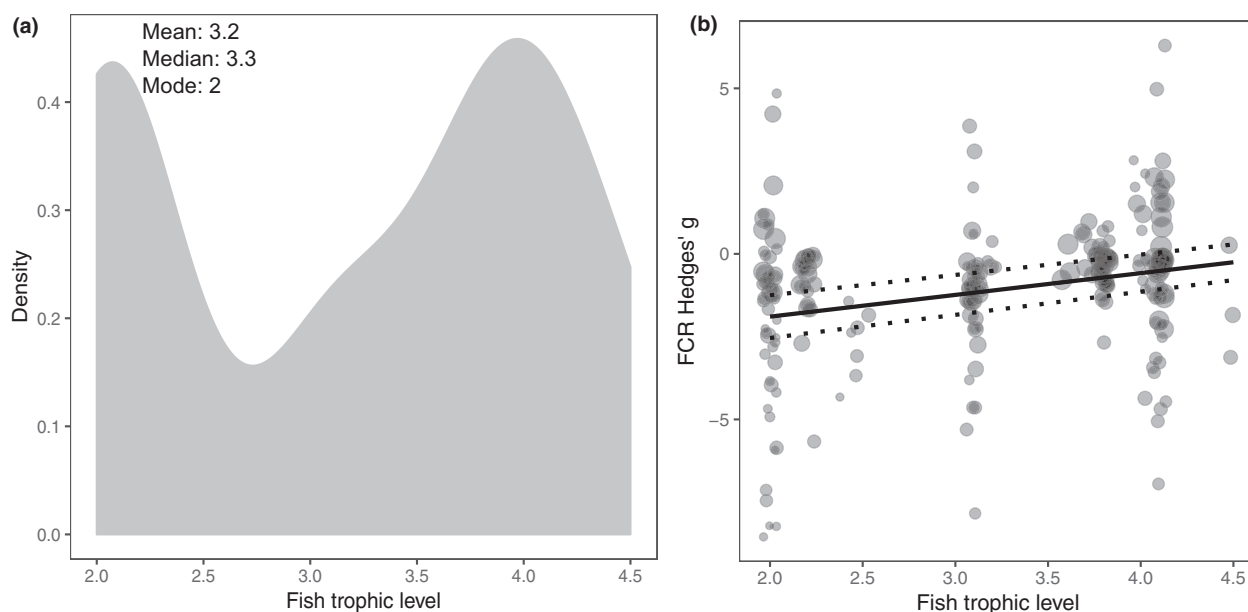


Figure 4 Density plot showing the distribution of fish trophic level from the observations included in the meta-analysis (a) and meta-regression of fish trophic level with feed conversion ratio (FCR) (P -value < 0.05) (b).

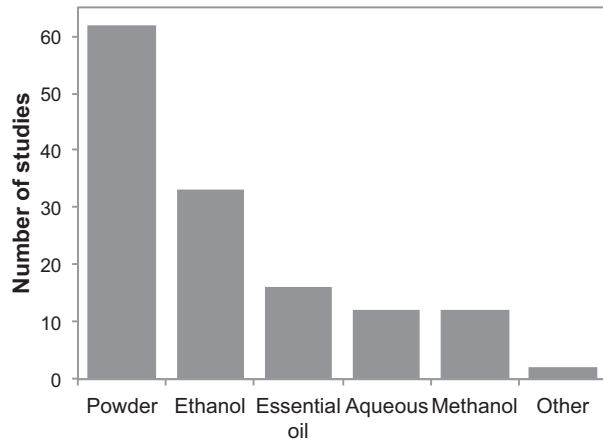


Figure 5 Number of articles studying each type of plant material (powdered plant, ethanol extract, essential oil, aqueous extract, methanol extracts and other).

and del Rocío and Fajer-Ávila (2017) used an acidic extraction (HCl) in order to maximize extraction of ulvan, a recognized immunostimulant from the green algae *Ulva clathrata*. And Mones and Angeles (2017) used fermentation in order to break down complex organic compounds present in banana peel and increase its digestibility. Interestingly, the type of material alone did not affect the effect size (g) in any of the parameters studied (Fig. S2,

Table S6), suggesting the important contribution of other parameters such as dosage in the effect size.

Dosage (related to plant material). Dosage, which is intimately related to the material used, is one of the most important factors in treatment efficacy and safety. Whereas too low dosages might not display the desired effect on fish, too high dosages can be toxic and have negative effects on fish growth, immune system and survival (e.g. Talpur & Ikhwanuddin 2012; Mo *et al.* 2016). For example, diet supplementation with dried garlic (*Allium sativum*) at 5 mg/100g fish*day did not enhance lysozyme levels ($g = 0.22 \pm 0.63$) in Asian sea bass, but supplementation at 15 mg/100g fish*day increased significantly lysozyme in treated fish ($g = 2.31 \pm 0.85$) (Talpur & Ikhwanuddin 2012). On the other hand, black sea bream fed with an enriched diet in freeze-dried *Sargassum horneri* at 240 mg/100g fish*day had significant higher weight gain than the control ($g = 3.29 \pm 0.74$), but *S. horneri* supplementation at 360 mg/100g fish*day induced a significant decrease in black sea bream weight gain ($g = -1.53 \pm 0.54$) (Shi *et al.* 2019).

The dosages used in the literature reviewed varied depending on the material (Fig. 6). Studies using powdered plant material used the highest dosages (0.1–420 mg/100 g fish*day), with a mean dosage of 69.3 mg/100g fish*day and a median dosage of 40 mg/100g fish*day. Ethanol

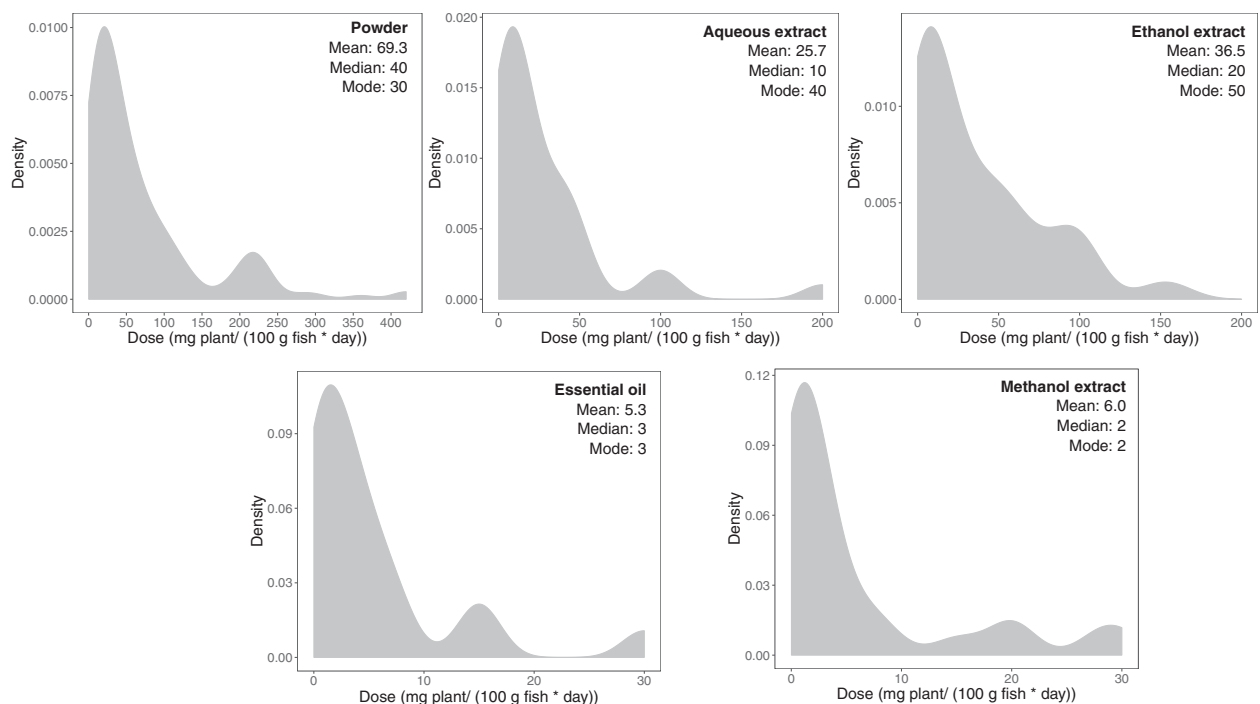


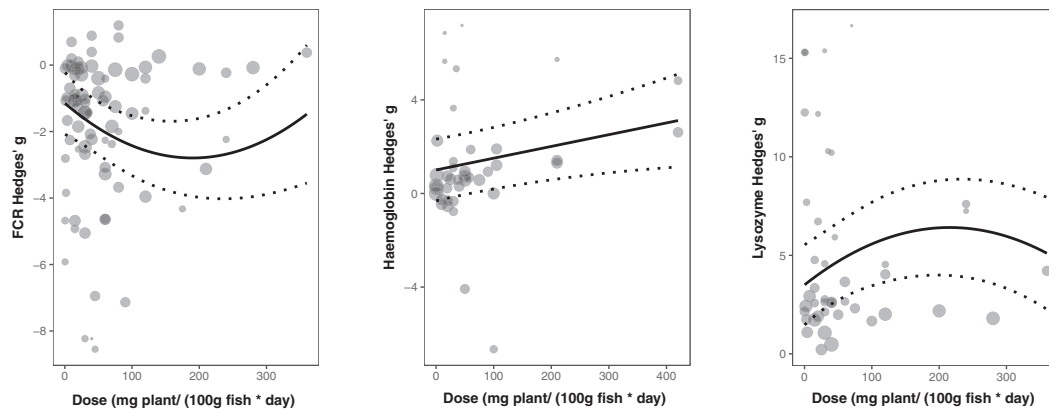
Figure 6 Density plot showing the distribution of dosages (mg plant/100 g fish*day) for each type of plant material. Dosage mean, median and mode are shown for each type of plant material.

(0.2–160 mg/100 g fish*day) and aqueous dosages (0.03–200 mg/100g fish*day) were similar, with average dosages of 36.5 and 20 mg/100g fish* day, respectively. Finally, studies investigating the effects of essential oils (0.005–30 mg/100g fish*day) and methanol (0.01–30 mg/100g fish*day) extracts used the lowest doses, with mean doses of 5.3 and 6 mg/100g fish*day, respectively (Fig. 6). Comparison of meta-regression models showed that dosage effect was not always linear, and in these cases, the quadratic dosage term was included accordingly (Table S7). Results from meta-regressions show that dosage of powdered plants significantly affected the effect size of FCR, haemoglobin and lysozyme (Table S8, Fig. 7a). Better FCR and higher lysozyme levels were observed with increasing doses powdered of plants until around 200 mg plant/100g fish*day, when the effect size started decreasing (Fig. 7a). Haemoglobin levels increased with increasing dosages of powdered plants, although these results should be interpreted carefully since most of the studies analysed dosages under 200 mg/100g fish*day (Fig. 7a). Similarly, complement activity and phagocytic activity levels increased with

higher dosages of ethanol extracts until around 100 mg plant/100g fish*day, when the effect size started decreasing (Fig. 7b). Both weight gain and SGR started to decrease with increasing dosages of ethanol extracts (Fig. 7b). We did not observe any significant effect on any of the parameters for the essential oils, aqueous and methanol extracts, although these results could be a consequence of lower number of studies for these types of materials (Table S9).

Since dosages often had a significant effect on the effect size, we have calculated the best effect size (optimum dose) obtained for each type of material, in order to investigate 1) whether similar optimum doses were obtained for the different parameters (growth, immunity and survival) for the same type of material and 2) whether some types of material (at the optimum dose) displayed significant higher effects on most parameters. Despite some variability, we observed that optimum doses for powdered plant material were the highest (140–420 mg plant/100 g fish*day), followed by ethanol extracts (20–160 mg plant/100 g fish*day) and aqueous extracts (2–20 mg plant/100 g fish*day). Methanol extracts and essential oils, which normally display the highest

(a) Powdered plants



(b) Ethanol extracts

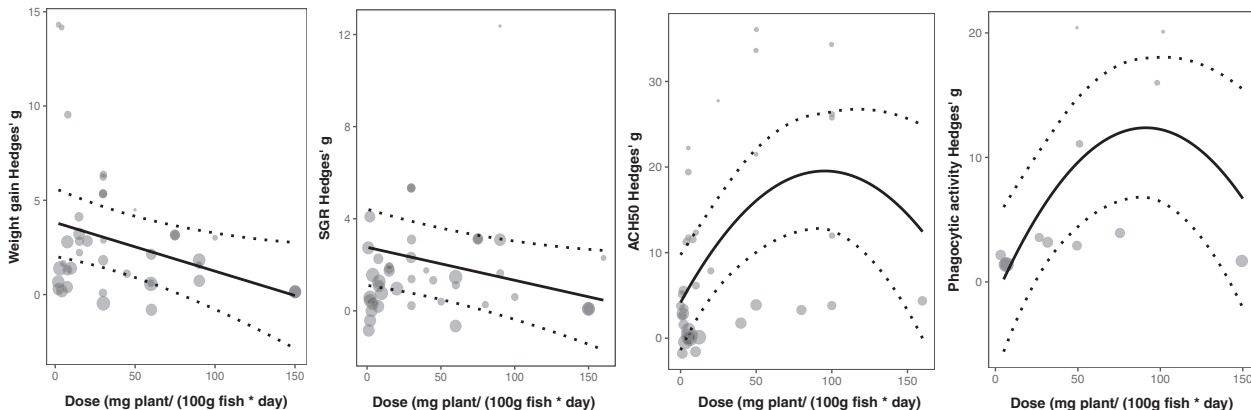


Figure 7 Significant meta-regressions (P -value < 0.05) between dosage of powdered plants and FCR, haemoglobin and lysozyme (a) and between dosage of ethanol extracts and weight gain, SGR, complement activity ACH50 and phagocytic activity (b).

toxicities, presented the lowest optimum doses at 0.5–20 mg plant/100 g fish*day and 0.005–15 mg plant/100 g fish*day, respectively (Fig. 8). In our study, the lower optimum doses

of aqueous extracts (thus higher activity) compared with ethanol extracts diverge from previous works that showed higher antimicrobial *in vitro* activities of alcoholic

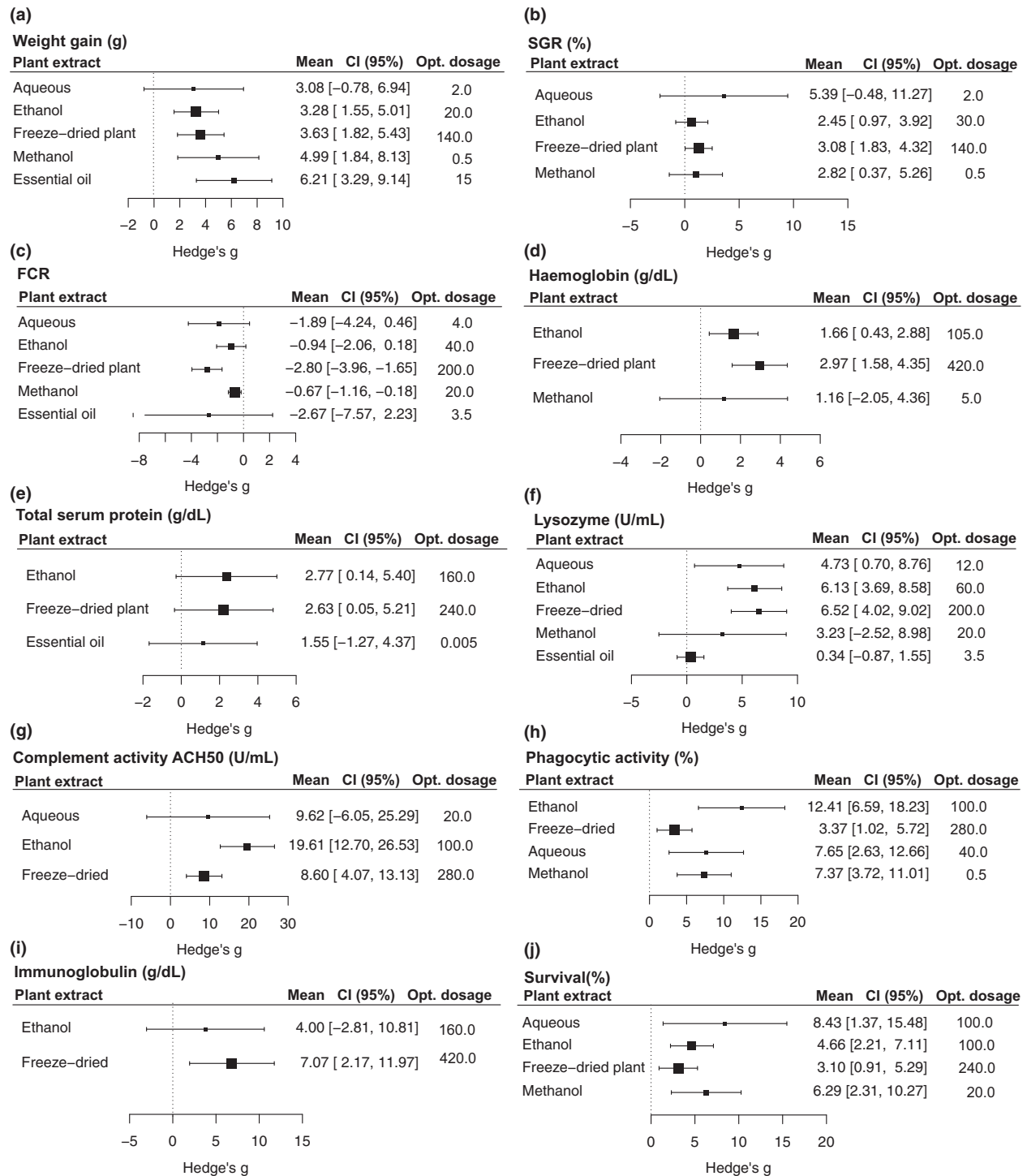


Figure 8 Forest plots reporting the best effect size (Hedge's *g*) observed (optimum dosage) for each parameter for each type of material. Mean and 95% confidence interval (CI) effect sizes are reported. (a) weight gain (g), (b) SGR (%), (c) FCR, (d) haemoglobin (g dL⁻¹), (e) total serum protein (g dL⁻¹), (f) lysozyme (U mL⁻¹), (g) complement activity ACH50 (g/dL), (h) phagocytic activity (%), (i) immunoglobulin (g dL⁻¹) and (j) survival (%).

(methanol, ethanol) extracts (e.g. Eloff 1998). However, underlying mechanisms involved in immunomodulation and disease resistance of orally administered plants in animals, despite not being studied in depth, are probably very different than *in vitro* tests. Therefore, in order to evaluate the full potential of plant materials on fish health and disease resistance, *in vivo* tests need to be performed to study their immunomodulatory and disease resistance effects. We would also like to highlight that results from our analyses draw general trends, but different dose-effects are expected from plants with different chemical compositions associated with either inter- or intraspecific (spatial and temporal) variabilities. Therefore, in order to establish the optimum dosages and the most adequate plant material, individual studies are still required.

Interestingly, we did not observe any significant difference in the size effect of the different type of materials at their optimum dose; powdered plants, extracts and essential oils being equally effective when used at the appropriate dosages. These results suggest that plant supplementation in fish is extremely versatile and can be implemented in a wide range of aquaculture systems, the choice of material depending on the type of systems and resources available. For example, whilst manual inclusion of dried plants might be the best option for rural farmers, encapsulated essential oils, which display better stability (Yang *et al.* 2015), might be a better-adapted alternative for large intensive facilities.

These results, however, also raise the question whether some type of materials producing toxic by-products (such as solvent residues like methanol) should be discouraged since equally effective greener alternatives exist (e.g. aqueous, ethanol extracts or supercritical fluids).

Treatment duration. Treatment duration is one of the parameters regarded as vital for the treatment efficacy. Choosing the right treatment duration is not only important to observe the maximum effect but has also economic implications. Therefore, determining the optimum treatment duration in which plant-enriched diets display the maximum effects on fish immunity and disease survival has been the object of many studies (e.g. Kaleeswaran *et al.* 2011; Binaii *et al.* 2014; Ngugi *et al.* 2015). Previous research has found that 1 week of plant enrichment increased lysozyme and immunoglobulin levels in fish, but other haematological (white blood cells, haemoglobin and haematocrit) and immune parameters (total protein, phagocytic activity, respiratory burst activity and complement activity) were only enhanced after 2 and 4 weeks of plant supplementation (Harikrishnan *et al.* 2011, 2012a, 2012b, 2012c). However, another study found no significant difference in the survival of diseased fish fed with plant-enriched diets for 4 and 16 weeks (Ngugi *et al.* 2015).

The treatment duration of the literature analysed here ranged from one to 16 weeks, 4 weeks being the most

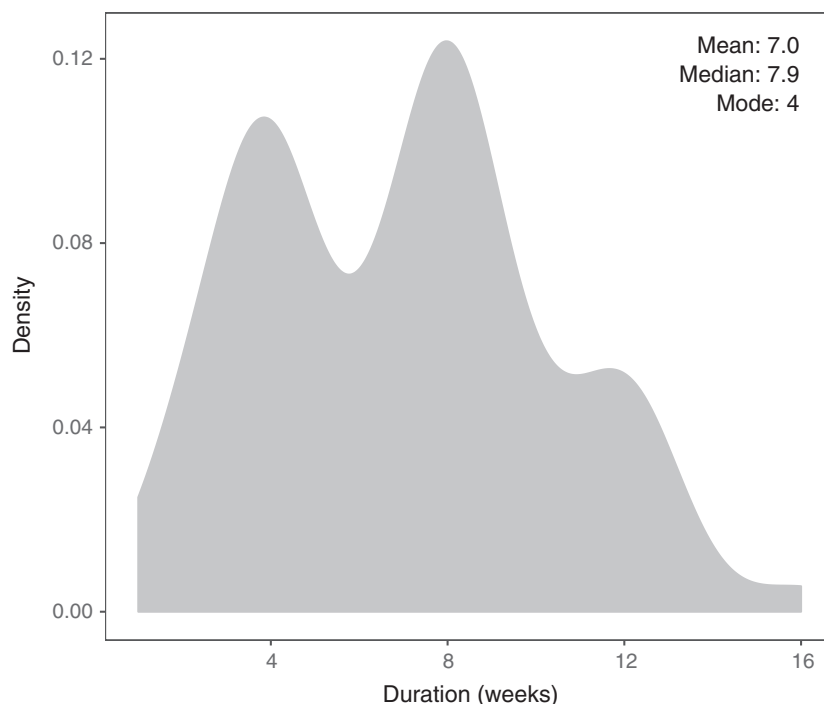


Figure 9 Density plot showing the distribution of treatment duration (weeks) from the observations included in the meta-analysis. Treatment mean, median and mode are shown.

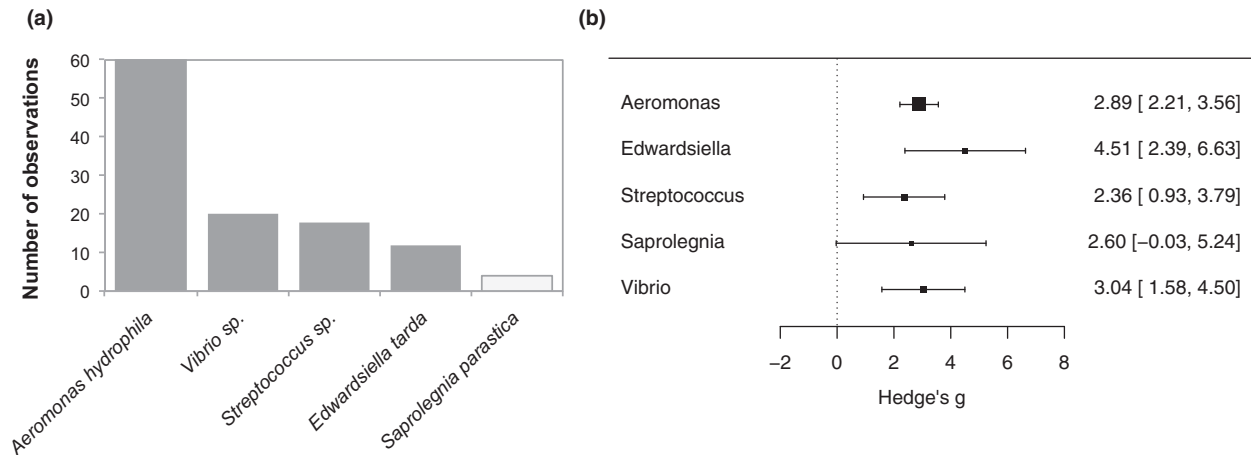


Figure 10 Pathogens used in the observations from the survival dataset (a) and forest plot reporting the effect size (Hedge's *g*) for the different pathogen genus studied (b). Mean and 95% confidence interval (CI) effect sizes are reported.

common treatment duration, 7 weeks the average treatment duration and 7.9 weeks the median treatment duration (Fig. 9). We built two meta-regression models to analyse the effect of duration on the effect size, one with only linear duration as fixed-termed effect and one with linear and quadratic duration terms. Comparison of both models resulted in selection of the reduced models (only linear duration as fixed-termed effect) for all parameters studied (Table S10). Interestingly, none of the 10 parameters was significantly correlated to the treatment duration, suggesting that administration of plant-supplemented feed for relatively short periods (e.g. 2–4 weeks) was as effective as longer supplementations (≥ 8 weeks) (Table S11).

Type of pathogen. *In vitro* tests (e.g. antimicrobial, antifungal and antiviral) have shown different microorganisms display different sensitivities to medicinal plants (Wei *et al.* 2008; Turker & Yildirim 2015). Therefore, we decided to analyse whether the type of pathogen involved in the challenge trials significantly explained some of the heterogeneity observed in the effect size. Since dose of infection is often chosen according to preliminary DL_{50} tests (median lethal dose) and is tightly related to the virulence of the specific strain used, we decided not to include it as a parameter in our models. Over 96% of the articles included in this work investigated the survival of fish infected with bacteria, with only four studies (3.5%) evaluating survival of fish with a fungal infection (*Saprolegnia parasitica*) (Fig. 10a). Over half of the studies evaluated the effect of plants on the survival of fish infected with pure cultures of *Aeromonas hydrophila*, with *Vibrio* (*Vibrio harveyi*, *V. anguillarum* and *V. alginolyticus*), *Streptococcus* (*Streptococcus agalactiae* and *S. iniae*) and *Edwardsiella* (*E. tarda*) being the other bacterial genus used (Fig. 10a). Our results show

that the type of pathogen used in the challenges did not contribute significantly to the heterogeneity observed in the survival effect size (test of moderators: P -value = 0.13) (Fig. 10b, Table S11). These results confirm the increasing interest in the use of plant-enriched diets as an alternative to antibiotics and show their efficacy in reducing mortality of fish infected by major bacterial and fungi pathogens.

Concluding remarks

The interest of plants as functional feed supplements in aquaculture has grown exponentially during the last decade, and it will probably keep increasing as worldwide antimicrobial use regulations strengthen and antimicrobial resistance is recognized as a global health emergency (IACG 2019). Our results show that use of plant-enriched diets is highly versatile and can benefit different aquaculture sectors with a wide variety of cultured species. Plant-enriched diets can provide LMICs and small-scale rural farmers with preventive measures and an inexpensive alternative to antibiotics, but also with more sustainable alternatives for disease management in high-income countries. Since plants studied were most often collected from their natural habitats, such practice could bring further local benefits to rural communities, such as higher income and lower dependency of external products and promotion of their traditional knowledge and local biodiversity. However, studies would need to evaluate whether a recurrent collection could be sustained or if local production should be organized.

Plant-enriched diets effectively enhanced growth, immunity and disease survival of treated fish, regardless of the trophic level of the fish species studied, the duration of the treatment and the type of material used. These results suggest that relatively short treatments (e.g. 2–4 weeks) can be

as effective as longer treatments (>8 weeks), which could allow to reduce treatment-associated costs. Secondly, since all types of materials proved similar efficacy, selection of the most appropriated material (powdered plant or type of extract) should be case-specific, based on the aquaculture system, technology and resources available. However, environmental-friendly materials (e.g. powdered plant or extracts with low-toxicity solvents) should be encouraged. The dosage of the plant administered arouses as an important parameter and thus needs to be appropriately studied according to the plant and the type of material chosen. Finally, aquaculture studies need to improve the information reported about the plant material used, in order to allow comparison of different experiments and their repeatability. Furthermore, more applied research and structuration are needed to transpose the knowledge acquired through basic research (e.g. laboratory experiments) into the field, where conditions are more variable and different challenges might arise.

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Data Availability Statement

See Appendix S1 for a full list of research articles included in this study.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 References included in the quantitative meta-analysis.

Table S2 Number of observations collected from 137 articles for each of the parameters studied, number of

observations after extreme outlier removal ($Q1 - 3 \times IQR$, $Q3 + 3 \times IQR$) and upper and lower fences (Hedges g') used for outlier removal.

Table S3 Distribution of variance over the three levels of the meta-analytic models.

Table S4 Observations that yielded negative effect sizes (expressed as mean \pm standard error) in at least one of the parameters studies

Table S5 Results of the meta-regression models with trophic level of the fish species used in the experiment as fixed-term effects.

Table S6 Results of the meta-regression models with type of material as fixed-term effects.

Table S7 Comparison of full (dosage and quadratic term of dosage as fixed-effect terms) and reduced (dosage as fixed-effect term) models.

Table S8 Results of the meta-regression models with dosage and quadratic dosage (depending on the previously selected model, Table 8) as fixed-term effects.

Table S9 Comparison of full (duration and quadratic term of duration as fixed-effect terms) and reduced (duration as fixed-effect term) models.

Table S10 Results of the meta-regression models with duration of the treatments as fixed-term effects.

Table S11 Results of the meta-regression model for survival data with type of pathogen as fixed-term effects.

Figure S1 PRISMA diagram showing the process for locating and including studies in the meta-analysis studying the effect of plant-enriched diets on growth, immunity and survival of cultured fish.

Figure S2 Forest plots reporting the effect-size (Hedge's g) for each parameter for each type of material.

Appendix S1 List of 137 articles included in systematic review and meta-analysis (currently only 128!).