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RESEARCH ARTICLE

Effectiveness of natural compounds against *Gyrodactylus turnbulli* parasites: Apex® Branchia

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Despite continued growth in aquaculture, infectious diseases remain a critical concern causing annual losses of up to £10 billion. Many chemical treatments, widely used in the past, have now been banned due to their toxic effects on humans, fish and the environment. This sparked a shift towards natural compounds and herbal remedies that can reduce fish pathogens when incorporated in host diets. The challenge though is to find that 'needle in the haystack' - which of these compounds are most effective. Here, a reliable and consistent method was used that allows us to test the anti-parasitic activities of a botanical compound in multiple rounds, using the guppy-*Gyrodactylus turnbulli* system. Our blind feed trials confirm the efficacy of a commercially available product, Apex® (a mixture of organic acids, inactivated yeast and yeast extracts, herbal extracts and essential oils) significantly reduced parasite burden and increased host survival rate. Even though efficacy of natural compounds is both dose- and source-dependent, they can be adjusted according to host-parasite species and method of application. Additionally, feeding prior to parasite exposure likely enhances fish immune function, working synergistically with the direct effect that therapeutants have on the parasite. Concluding, Apex is a promising health-supporting natural functional feed additive, and botanicals can provide significant health benefits, urgently needed to protect the increasing global demand for suitable fish food.

Introduction

In 2023, global fish production reached approximately 185 million tonnes, with 94 million tonnes derived from aquaculture production, accounting for 51% of the total, a significant increase from 25.7% in 2000 (FAO 2023). Human fish consumption increased at an average annual rate of 3.1% from 1961 to 2017 reaching 150 million tonnes with production reliance on fishmeal and fish oil remaining stable at about 19%. So, aquaculture is a rapidly growing food-producing sector, aquatic products are highly traded, with trade boosted by increased consumption, improved storage, preservation, transportation and liberalization policies. Fish production, however, faces numerous challenges, including climate change, poor water quality, high stocking densities, and infectious diseases (Martos-Sitcha et al. 2020). These issues are further compounded by stringent environmental regulations and decreasing availability of optimal production sites (FAO

2023). By 2050, the global population is projected to reach 9.7 billion (UNDESA 2024), but with 10% undernourished or suffering from chronic hunger (FAO 2023). Simultaneously, rising socio-economic and environmental concerns have fuelled growing interest in fish welfare, aiming to enhance production and minimize disease-related mortality. Parasites have a major impact in aquaculture, affecting farm production, sustainability, and economic viability due to the costs associated with control and management of infection and stock loss (Shinn et al. 2005); they are the 'weeds of aquaculture' (Cable and Harris 2002).

Traditional preventative and effective chemicals extensively used in the past, such as malachite green and formalin, were banned due to their detrimental impact on humans, animals and the environment (Srivastava, Sinha, and Roy 2004). Anti-helminthics, like praziquantel, have other adverse effects including altering food fish palatability (Hirazawa et al. 2004; R. Williams et al. 2007). Even though anti-parasitic drugs administered orally with food, such as praziquantel and trichlorfon, can also be applied as bath applications (Dunn et al. 1990), they too are linked with environmental and host toxicity, development of host resistance and low palatability (Hirazawa et al. 2004; R. Williams et al. 2007). Vaccination can significantly reduce disease outbreaks in aquaculture, typically administered via intraperitoneal injection, immersion or oral delivery (Komar et al. 2004; Muktar, Tesfaye, and Tesfaye 2016). However, developing fish vaccines that are effective with long-lasting immunity, cost-efficient to produce and license, and easy to administer often proves commercially unviable, especially for helminths (Muktar, Tesfaye, and Tesfaye 2016). Hence, only a few fish vaccines have been developed for major diseases (Harikrishnan et al. 2012; Pasnik, Evans, and Klesius 2005).

As a result, the demand for alternative control strategies has grown, leading to increased research on the potential use of plant extracts as sustainable feed supplements to support health management and reduce the severity of parasite infections when used in preventive mode (Immanuel et al. 2009; Wunderlich et al. 2017). Many studies have highlighted the potential application of phytochemicals such as alkaloids, flavonoids, phenolics, terpenoids and essential oils (EOs) for treating parasitic infections within aquaculture (Anthony, Fyfe, and Smith 2005; Reverter et al. 2014; Tavares-Dias 2018). We previously tested *in vitro* and *in vivo* efficacy of herbal compounds using the guppy-*Gyrodactylus turnbulli* model in bath treatments (Schelkle, Snellgrove, and Cable 2013). Here, we used the same system to assess the effectiveness of the functional feed additive Apex® Branchia, marketed as a health solution to reduce the severity of infections by monogenean gill parasites (Adisseo 2024).

Materials and Methods

Host and parasite origins and maintenance

Wild-type guppies (*Poecilia reticulata*) originated from the Lower Aripo River, Trinidad, in 2012, but were then maintained in the lab first in Exeter University, and then from 2018 in Cardiff University Aquatics Lab, where they were kept for breeding. Ornamental guppies used in the experiments were purchased from a wholesaler immediately before the start of experiments and were treated once with the anti-bacterial and anti-fungal drug Binox as a precautionary step (Russo and Dillehay 2005). Stocks were housed in 70 L tanks of dechlorinated water (70 fish per tank) in 24 ± 0.5 °C and a 12:12 h light: dark photoperiod. Fish were being fed daily with tropical fish flakes (Aquarian®) supplemented with live *Daphnia magna* and live freshly hatched *Artemia* nauplii cultured at Cardiff University Aquatics Lab.

The parasites used for experimental infections were the *Gt3* strain of *Gyrodactylus turnbulli*, which originated from an ornamental stock of guppies purchased in Nottingham in 1997. A culture of parasites was created by isolating a single parasite from an infected ornamental guppy and infecting a naive fish. By infecting multiple fish, a culture was created, which has since been maintained in multiple 1 L containers at 24 ± 1 °C under a 12:12 hr light: dark photoperiod. In brief, each container houses a maximum of four fish, infected collectively with approximately 40 worms. The culture is maintained by screening fish every 72 hr, so any heavily infected fish is replaced with naive, in order to keep the parasite population viable (Stewart et al. 2017). Naive fish came from specific pathogen free cultures, where fish have never been exposed to parasites and no immune response has been initiated.

Experimental method

For this study, a common experimental approach was established to provide consistency across multiple feed trials. Even within the feed trial, treatments were tested in fish of different strains and age, allowing for a greater sample size and consistency of the results. All feed trials were conducted blind, during both experimental procedures and data analyses. Overall, 40 treatments produced by Adisseo were tested here, in one or multiple rounds (Adisseo 2024). Due to confidentiality, specifics about any products or ingredients incorporated into the feeds could not be disclosed, with the exception of one commercial solution Apex® Branchia which we compared to a diet without the functional feed additive (Control). Apex® Branchia is a mixture of organic acids, inactivated yeast and yeast extracts, herbal extracts and essential oils on a mineral carrier that aims to reduce the impact of monogenean gill parasites on productivity and survival of fish, by promoting fish immune competency (Adisseo 2024).

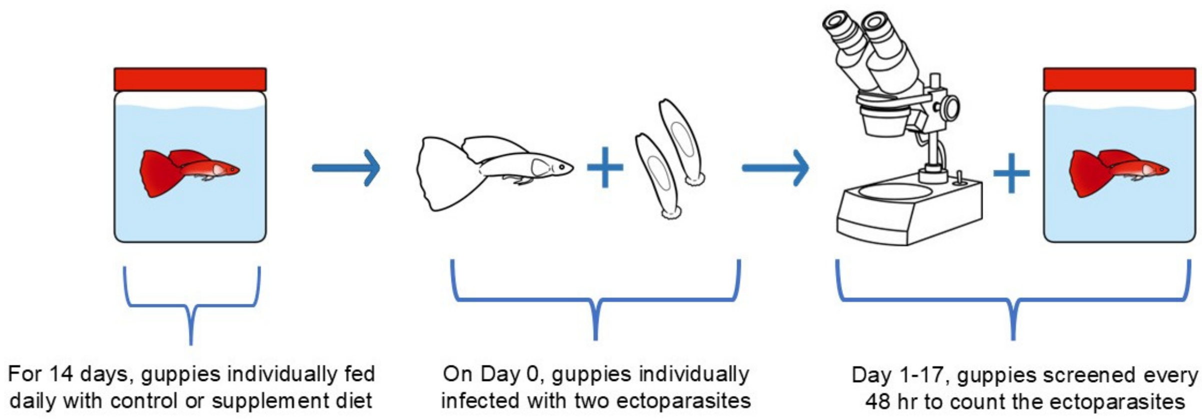

Figure 1. The experimental procedure of the feed trials for Rounds 1-3.

For 14 days guppies were fed once daily (15:00 pm) with their respective diet (2% of their body weight; Levy et al. 2015) and their water changed every 48 hr. After 14 days, guppies were anaesthetised with 0.2% MS222 and infected with two *Gyrodactylus turnbulli* worms under a dissecting microscope with fibre optic illumination (Day 0 of infection). Guppies were then returned to their individual 1 L pots to recover and screened again the next day (Day 1 of infection) to ensure the infection was successful. Subsequently every 48 hr, the parasite number was monitored and recorded for 17 days. During that period, they were still fed daily according to their diet.

In brief, for each feed trial, fish were anaesthetised with 0.2% tricaine methanesulfonate (MS222), size-matched and placed individually in 1 L pots of dechlorinated water. Once assigned into treatments, fish were fed once daily (15:00 pm) with their respective diet (2% of their body weight; Levy et al. 2015) and their water was changed every 48 hr for 14 days. After two weeks of feeding, fish were anaesthetised and infected with two *G. turnbulli* worms (Day 0 of infection). For experimental infections, a heavily infected fish, the donor, was overdosed with MS222 and then sacrificed by pithing. By placing the donor close to the anaesthetised recipient, direct contact allowed for transfer of *G. turnbulli* worms from the caudal fin of donor to that of the recipient fish. All infections and subsequent screening were observed under a dissecting microscope with fibre optic illumination. The recipient fish was returned to its 1 L pot to recover. Fish body and fins were screened again the next day (Day 1 of infection) to ensure the infection was successful and subsequently every 48 hr, so that the parasite number was monitored and recorded for 17 days (Figure 1). During that period, they were still fed daily according to their diet. In the meantime, all feeds were stored in the dark at room temperature when not in use.

Apex® Branchia

The feed trial focused on the efficacy of the commercial solution Apex® Branchia, where fish were provided with a commercial feed formula that is supplemented with Apex® Branchia (0.5%) against the Control (no feed additives), in three different rounds. Round 1 tested Apex® Branchia against the Control diet in naive wild juvenile guppies (n=17 per treatment; mean standard length 8.8 ± 0.6 mm). The efficacy of Apex® Branchia was then tested on naive wild-type (n=18 per treatment, 13.5 ± 0.6 mm) and ornamental (n=14 per treatment; 24.75 ± 4.14 mm) female adult guppies in Round 2 and in Round 3, respectively. After the 17-day trial in Round 2, surviving fish retained in their 1 L containers continued to be fed with their respective diets, and then one month after the first experimental infection, remaining fish (n=10 per treatment) were re-infected (Day 45), to investigate how the treatment, in combination with the host's immune response, would impact re-infection (Round 4).

Once, experimental trials concluded, infected fish were treated with the anti-helminthic drug Levamisole (0.1%) to eliminate any parasites and were screened clear three consecutive times to ensure that fish were parasite free (Schelkle et al. 2009).

Statistical analysis

For statistical analyses, the parasite metrics examined were mean parasite number, maximum parasite number over the 17-day infection trajectory and parasite count of each fish on each individual day. Using the 'lme4' library (Bates et al. 2015), two Generalised Linear Models (GLMs) were constructed to assess which treatment had the best efficacy, therefore treatment was the independent term. For mean parasite number, the models were fitted with a 'Gamma' family and 'logit' link function, whereas for maximum parasite number, the model was fitted with 'quassipoisson' family and 'identity' link function. Additionally, two GLMs fitted with a 'binomial' family and 'logit' link function, were then used to determine whether the survival percentage of the fish and the percentage of fish that cleared their infection were significantly affected by treatment with yes/no responses. Finally, a Generalised Linear Mixed Model (GLMM), was used to examine the parasite count on each fish on each day individually to determine if there was a significant difference in parasite count between treatments. The model included fish ID as a random term to account for repeated measures. As fish for all treatments were size-matched at the start of the feed trails, fish size variability was not taken into account. For all models, error families were chosen based on the lowest AIC values and the robustness of the models was assessed with visual examination of model plots to check standardised residuals, for normal distribution and homogeneity of variance (Bates et al. 2015; Pinheiro and Bates 2000). In all tests, the level of significance was taken as $p < 0.05$. All statistical analyses were performed in the R statistical software version 4.1.1 (R Core Team 2019). Data is only reported on the most effective treatment.

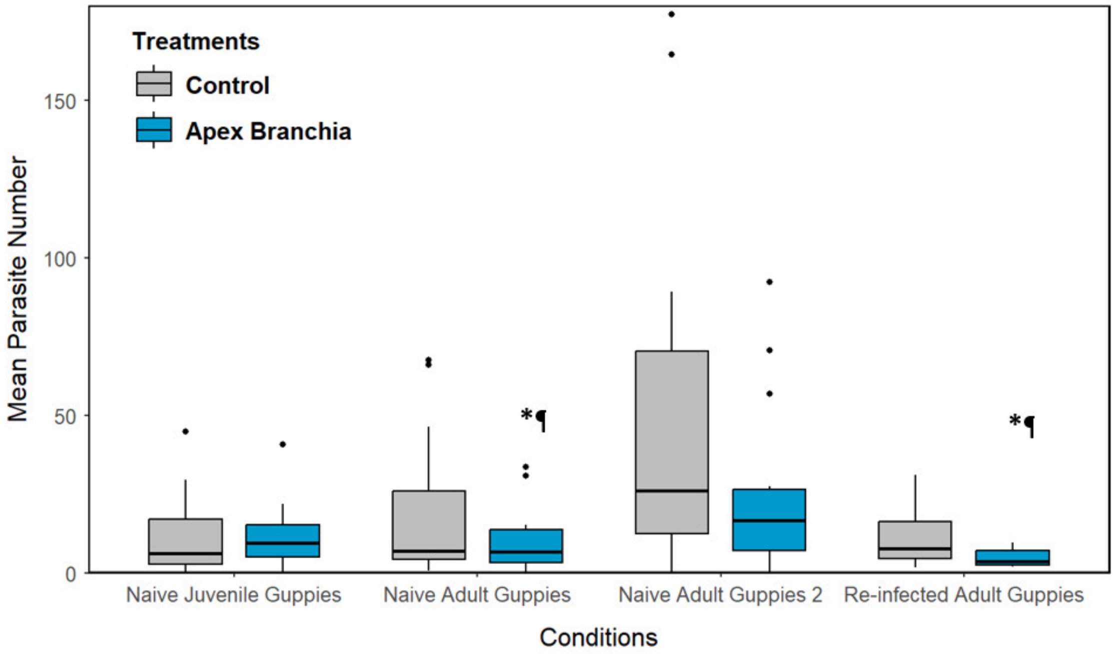

Figure 2. Box and whisker plots of mean parasite number of guppies infected with *Gyrodactylus turnbulli* in four different experimental rounds: naive juvenile wild-type guppies (n=17); naive adult wild-type and naïve adult (2) ornamental guppies (n=18 and n=14) respectively; and re-infected adult wild-type guppies from Round 1 (n=10).

In all experiments, fish fed with supplemented diet Apex® Branchia (blue) had a lower mean parasite number than the Control (grey) in all conditions. Black dots represent outliers; bars the upper and lower limits; the box the first and third quartile with the horizontal line showing the median. Asterisks represent significant difference (*$p < 0.05$) between Control and Apex® Branchia.

Results

Apex® Branchia had a significantly lower mean parasite number across multiple experimental rounds, indicating the effectiveness of the in-feed supplementation in reducing parasite load (Figure 2). In both naïve and re-infected adult guppies, the maximum parasite number was significantly lower for Apex® Branchia fed fish compared to the controls.

Validation of Apex® Branchia

I. ROUND I - NAIVE JUVENILE WILD-TYPE GUPPIES

Using naïve juvenile wild-type guppies, fish survival following infection with *Gyrodactylus turnbulli,* was almost significantly affected by treatment, with treatment Apex® Branchia increasing fish survival compared to the Control (GLM; $p = 0.06$). Even though mean and maximum parasite number (Figure 3a) as well as number of fish that cleared their infection were not significantly different, fish on Apex® Branchia generally performed better compared to the Control, with lower parasite burden and increased host survival (Table 1).

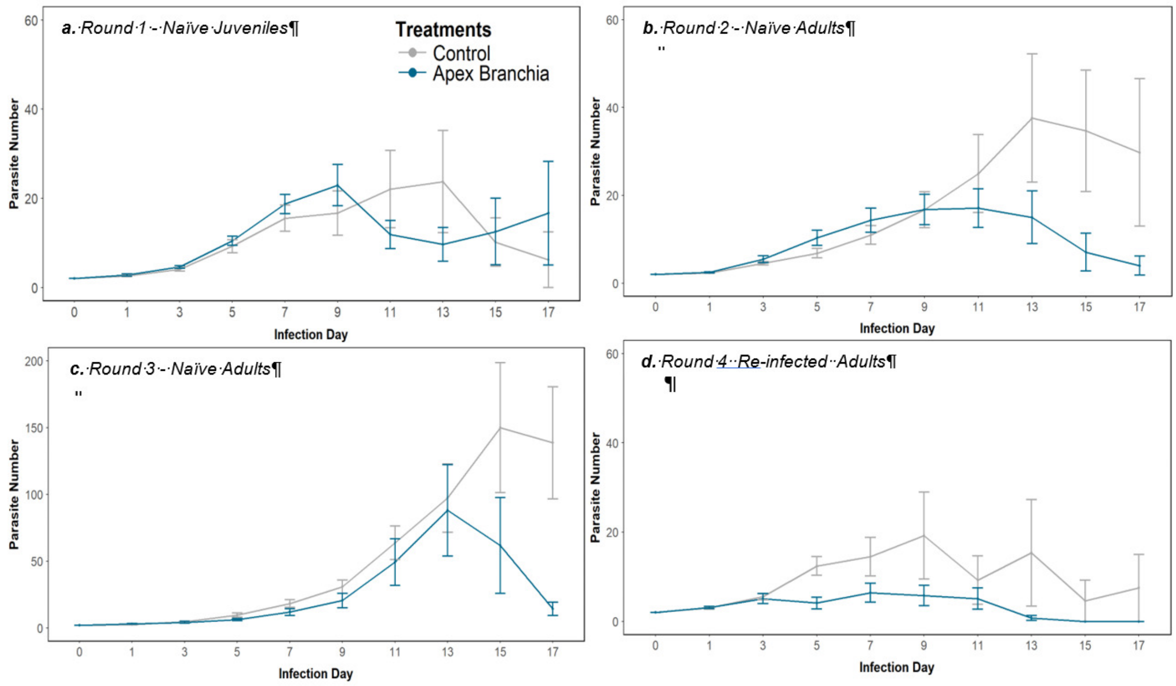

Figure 3. Infection trajectories of naïve guppies infected with *Gyrodactylus turnbulli* parasites; grey showing the Control and blue showing the treatment Apex® Branchia, with standard error bars.

a. In juvenile wild-type guppies (n=17 per treatment), treatment Apex® Branchia had a lower parasite count than the control, during a 17-day infection trajectory. b. In adult wild-type guppies (n=18), mean infection trajectory of treatment Apex® Branchia was lower than the control over a 17-day period. c. Similarly, in adult ornamental guppies (n=14), average infection trajectory of treatment Apex® Branchia was lower than the control over a 17-day period. d. In previously infected adult wild-type guppies (n=10) average infection trajectory of Apex® Branchia was more promising, with lower parasite burden than the control over a 17-day period.

II. ROUND 2 - NAIVE ADULT WILD-TYPE GUPPIES

In Round 2, fish fed Apex® Branchia had a lower mean (GLM; $p = 0.095$) and maximum parasite number than the Control (GLM; $p < 0.001$; Figure 3b). When looking at parasite count across all individuals on each screening day (every 48 hr), treatment Apex® Branchia was also lower than the Control. Even though, fish survival as well as number of fish that cleared their infection were not affected by treatment, percentages were still higher than the Control (Table 1).

III. ROUND 3 - NAIVE ADULT ORNAMENTAL GUPPIES

Naive adult ornamental guppies fed Apex® Branchia had lower mean (GLM; $p = 0.112$) and maximum parasite numbers than the Control (GLM; $p < 0.001$; Figure 3c) and a lower parasite count over the 17-day infection trajectory, despite a lower survival rate exhibited in ornamental fish (Table 1).

Table 1. Feed trial results of treatment Apex® Branchia against the Control across multiple rounds.

Round	Fish strain (n per treatment)	Treatment	Mean Parasite number	Maximum parasite number	Fish Survival (%)	Fish Cleared (%)
1	Naïve juvenile wild (n=17)	Control	12.2	36.5	58.8	52.9
		Apex Branchia	11.45	37.1	88.2	52.9
2	Naïve adult wild (n=18)	Control	17.4	59.6	88.9	38.9
		Apex Branchia	9.8	25.1	94.4	44.4
3	Naïve adult ornamental (n=14)	Control	47.9	160.8	94.8	15.8
		Apex Branchia	25.6	96.1	57.1	7.1
4	Re-infected adult wild (n=10)	Control	11.8	35.9	80	70
		Apex Branchia	5.0	10.1	90	80

Four variables were measured: mean parasite number, maximum parasite number, percent of fish that cleared the infection and percent of fish that survived. Overall, treatment Apex® Branchia performed better than the Control across all variables, with lower mean and maximum parasite number across the infection trajectories.

iv. Round 4 – Re-infected adult wild-type guppies

Previously infected adult fish fed Apex® Branchia performed better compared to those fed the Control diet (Table 1). Specifically, the Apex® Branchia group had a lower mean (GLM; $p = 0.022$), maximum parasite number (GLM; $p = 0.018$) and parasite count than the Control (GLMM; $p = 0.052$; Figure 3d; Table 1).

Discussion

The commercial product Apex® Branchia is an effective functional feed additive, promising in reducing the severity of *Gyrodactylus turnbulli* infection on guppies. It increased survival rate of infected juveniles by at least 30% (Round 1) and reduced parasite load by 40-50% in adult guppies, during primary (Rounds 2 and 3) and secondary infections (Round 4). As illustrated by Schelkle, Snellgrove, and Cable (2013), this host-parasite model provides a reliable and simple testing model for treatments against infectious diseases, generating consistent results across multiple feed trials.

Medicinal plant products have been used as potential therapeutic measures for modulating the immune response and, specifically, the use of herbs to prevent and control fish diseases (Galina et al. 2009; Ji et al. 2012). Efficacy of essential oils is influenced by both time and dose (Soares et al. 2016; Mukherjee et al. 2014; C. F. Williams and Lloyd 2012). Prolonged exposure can improve fish growth rate and food conversion ratio (Talpur and Ikhwanuddin 2012), as well as reduce parasite burden on the host (Abd El-Galil and Aboelhadid 2012). However, there is also potential for overdosing with adverse effects (Yin et al. 2006). For instance, histological examination of fish fed a diet supplemented with 20% garlic powder revealed elevated muscle dystrophy (Fridman, Sinai, and Zilberg 2014). Consequently, dosage optimization is essential to minimize negative outcomes (Galina et al. 2009). Other factors influencing the effectiveness of botanical compounds include the source, geographical origin, strain, age of the product, seasonality, climate and preparation methods, all of which can alter the concentration of active components (Burt 2004; Lawson, Wood, and Hughes 1991). Preparation processes, such as freeze drying and mincing, can significantly alter the effectiveness of products (Lemar, Turner, and Lloyd 2002; Schelkle, Snellgrove, and Cable 2013). For example, the number of parasites was significantly reduced on infected fish exposed to garlic from different sources, treatments with minced and granule forms reduced worm burdens by 66% and 75% after three doses, whereas Chinese freeze-dried garlic and allyl disulphide were 95% effective after a single application (Schelkle, Snellgrove, and Cable 2013). Therefore, botanical compounds need to be adjusted according to the host-parasite system, method of application and nature of the ingredients (Chitmanat, Tongdonmuan, and Nunsong 2005; Schelkle et al. 2009).

In-feed prophylactic supplementation is generally preferable to bath treatments (e.g., Hirazawa et al. 2004; Sutili et al. 2016) as oral administration is non-stressful and non-invasive (Dunn et al. 1990). Even though fish may not consume the entire feed delivered, less product is wasted and released to the environment, minimising any negative environmental impacts (Dunn et al. 1990). In bath applications, natural compounds directly target ectoparasites, potentially affecting their membrane permeability and fluidity, leading to cell death, similar to the mode of action of pesticides and bactericides (Cox et al. 2001; Horn and Duraisingh 2014; Reddy and Kim 2015). In contrast, in-feed essential oils can enhance host immunity over a conditioning period, typically of at least 14 days (Nya, Dawood, and Austin 2010; C. F. Williams and Lloyd 2012). For example, garlic and allicin supplements, in the form of oral treatment, increase production of phagocytic, lysozyme, and anti-protease activities, strengthening the host's immune system to combat infections (Cristea et al. 2012; Jones 2001; Militz et al. 2013). The current study involving a minimum 14-day in-feed supplementation significantly reduced *Gyrodactylus* parasite load on guppies and increased host survival, indicating that botanicals can be a strategy to support health management and reduce the severity of parasite infections.

In summary, in-feed supplementation of functional feeds additives formulated to reduce the severity of parasite infections is a sustainable solution to minimising infection (Reverter et al. 2014; Tavares-Dias 2018). Apex® Branchia, a commercial product, is promising against *Gyrodactylus* ectoparasites, with wider potential use in aquaculture.

Ethical standards

All applicable institutional guidelines for the care and use of animals were followed (Kilkenny et al. 2014). Procedures and protocols were conducted under UK Home Office licence (PPL 303424) with approval by the Cardiff University Animal Ethics Committee.

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Conflicts of Interest

WGNO and MMIS are employees of Adisseo.

Author Contribution Statement

EA, JC, WGNO and MMIS conceived and designed the study. EA and JC carried out the experimental feed trials, analysed the data and wrote the manuscript. All authors approved the final manuscript.

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