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Phytogenic feed-additive effects on channel catfish rhamnose-binding lectin levels, and susceptibility to *Edwardsiella ictaluri*

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ABSTRACT: We investigated the effects of a phytogenic feed additive on disease susceptibility to *Edwardsiella ictaluri* in channel catfish *Ictalurus punctatus* and regulation of 6 rhamnose-binding lectin (RBL) genes. Juvenile catfish (n = 250, 13.4 ± 0.1 g) were allotted to the following treatments: control (floating diet) or EO (floating diet supplemented with essential oils; Digestarom® P.E.P. MGE). The fish were fed their respective diets for 6 wk. Following subjection to different feed treatments, all fish were exposed to pathogenic *E. ictaluri* by bath immersion. Another group of fish were not challenged (non-challenged controls, fed control feed). Mucosal tissue samples were taken to quantify gene expression levels of RBL on Days 1 and 2 post-challenge. After challenge, survival was higher (64.4 vs. 48.0%) in fish fed EO compared to controls ($p < 0.05$). Relative to non-challenged controls, gill RBL1a mRNA was higher in fish fed EO ($p < 0.05$) on Day 1 while gill RBL3b was higher in fish fed EO ($p < 0.01$) on Days 1 and 2, respectively. RBL5a in the skin and proximate small intestine did not change significantly relative to non-challenged fish on Days 1 and 2 of the disease challenge. Results demonstrate that Digestarom® P.E.P. MGE improved survival of channel catfish challenged with *E. ictaluri*. One of the mechanisms through which essential oils may improve survival is through upregulation of RBL1a and RBL3b in the gill.

KEY WORDS: Channel catfish · Essential oils · Rhamnose-binding lectin · Immune response

INTRODUCTION

The channel catfish *Ictalurus punctatus* (Rafinesque) is an important cultured food-fish species in North America. Increases in production costs have forced producers to examine ways to reduce on-farm costs, including the use of dietary supplements such as probiotics, prebiotics, synbiotics (products that contain both prebiotics and probiotics), and essential oils (aromatic compounds extracted from plants). The use of dietary supplements in aquaculture has recently been reviewed (Ringo et al. 2012, Pérez-Sánchez et al. 2013, Newaj-Fyzul et al. 2014, Song et al. 2014, Akhter et al. 2015, Huynh et al. 2017). In general, studies have examined the effect of feed additives on growth, feed conversion ratio (FCR), gut microbiota, intestinal morphology, disease susceptibility, and innate immune parameters. A recent review attempted to explain possible mechanisms of how synbiotics could improve growth and health status in aquaculture (Huynh et al. 2017). The authors suggest that synbiotics may cause intestinal epithe-

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lial cells to secrete cytokines which modulate immune functional cells such as dendritic, T-, and B-cells, and induce the ability of lipopolysaccharides to trigger tumor necrosis factor-α and toll-like receptor 2, leading to increased phagocytosis, respiratory burst activity, and nitric oxide production (Huynh et al. 2017). While these mechanisms may be species-specific, they provide an attempt to understand which regulatory pathways may be involved in regulating the observed improved production efficiencies. The use of dietary supplements to improve growth performance and reduce the incidence of disease in catfish is just beginning to be investigated.

The catfish farming industry is battling high disease loss to enteric septicemia of catfish (ESC) caused by the bacterium *Edwardsiella ictaluri*. Current methods to control this disease include vaccination, antibiotic therapy, and restricted feeding. Another method that has been examined is the addition of essential oils to the diet. Essential oils have proven beneficial in improving immune function and survival, but more studies are needed. Fish fed essential oils include channel catfish (Zheng et al. 2009, Peterson et al. 2014, 2015) and rainbow trout *Oncorhynchus mykiss* (Walbaum) (Ahmadifar et al. 2011, Giannenas et al. 2012), and results varied significantly with regard to weight gain, FCR, immunity to disease, and species. Channel catfish fed carvacrol and thymol, the 2 main active components of oregano essential oil, enhanced growth performance, hepatosomatic index, viscerosomatic index and condition factor during an 8 wk growth study (Zheng et al. 2009). These fish were then infected with *Aeromonas hydrophila* and mortality was recorded for 6 d. Mortality was reduced by 21% in catfish fed the treated diet (Zheng et al. 2009). In another study, tank-raised channel catfish were fed these aromatic compounds for 6 wk and then challenged with pathogenic *E. ictaluri* (Peterson et al. 2015). There was no difference in growth or FCR, but survival was 43% higher in catfish fed essentials oils compared to fish fed the control diet (Peterson et al. 2015).

The mechanisms through which compounds like essential oils may improve growth or disease resistance are poorly understood. One potential mechanism may be through lectin-mediated attachments. Lectins are a group of sugar-binding proteins that recognize specific carbohydrate moieties expressed by cells. Because of the ability of lectins to recognize, agglutinate, and opsonize microbial pathogens, together with their capacity to activate complement, it is plausible they may be involved in the apparent improvement in disease resistance.

Mannose-binding lectins (MBLs) have been reported in many species of fish (Vitved et al. 2000, Mitra & Das 2001, Jackson et al. 2007, Ourth et al. 2008), including channel (Zhang et al. 2012) and blue catfish *Ictalurus furcatus* (Ourth et al. 2007). MBL protein levels are higher in blue catfish, a more resistant catfish to *E. ictaluri* infection compared to other catfish (Ourth et al. 2007). MBL mRNA levels were upregulated 15-fold when channel catfish fed essential oils were challenged with pathogenic *E. ictaluri* (Peterson et al. 2015). In contrast, MBL levels were not predictive indicators of susceptibility to *Flavobacterium columnare* or *E. ictaluri* in channel catfish families with high and low susceptibility to *F. columnare* and *E. ictaluri* infection, respectively (LaFrentz et al. 2012).

Rhamnose-binding lectins (RBLs) have been reported in more than 25 species of fish (Watanabe et al. 2009), including channel catfish (Beck et al. 2012, Thongda et al. 2014). RBLs demonstrate properties and activities such as sugar-binding specificity and hemagglutinating activities (Ogawa et al. 2011). Multiple RBLs have been isolated from steelhead *O. mykiss* (Tateno et al. 2001), white-spotted charr *Salvelinus leucomaenis* (Tateno et al. 2002), and Spanish mackerel *Scomberomorous niphonius* (Terada et al. 2007) eggs. RBLs primarily recognize L-rhamnose and α-galactoside rather than β-galactoside, and do not require Ca^{+2} ions for their activity (Hosono et al. 1992, 1993). These properties distinguish RBLs from other animal lectin families such as C-type and galectin (Hosono et al. 2013). Plasma RBL from sea bass *Dicentrarchus labrax* have been shown to agglutinate and opsonize pathogenic bacteria (Cammarata et al. 2014). In a catfish study, a RBL gene (RBL 1a) was upregulated 105-fold in the gill of fish infected with *F. columnare* when compared to naïve fish (Beck et al. 2012). Beck et al. (2012) further characterized the response of RBL by showing there was robust upregulation of RBL mRNA in a susceptible family of channel catfish that was exposed to *F. columnare*. The authors also exposed catfish to different doses of RBL ligands L-rhamnose and D-galactose and found that these sugars protected catfish against columnaris disease. RBL 1a mRNA was upregulated 123-fold in fish fasted for 7 d and returned to fed control levels within 4 h of re-feeding (Beck et al. 2012). These studies demonstrate a role for RBL in innate immunity to columnaris disease and catfish nutrition.

It was surmised that essential oils may improve survival in catfish exposed to pathogens by regulating lectins such as MBL and RBL. Towards the goal

of understanding how essential oils may improve resistance to disease, we examined RBL gene expression following infection in tissues known to facilitate pathogen entry. Studies have shown that *E. ictaluri* enters through the intestines as well as through the gills and skin (Li et al. 2012, Shoemaker et al. 2012). The above-mentioned studies provide evidence that lectins are important molecules involved in the innate immune system of teleost fish. As new feed additives such as essential oils are fed to aquatic species, it will become useful to identify the mechanisms of action through which they function. The objectives of the current study were to examine disease susceptibility and the roles of 6 RBL genes in mucosal tissues of channel catfish fed essential oils and then challenged with pathogenic *E. ictaluri*.

MATERIALS AND METHODS

Fish husbandry

Fish used in the study were juvenile Delta Select catfish obtained from natural pond spawns at the USDA-ARS Warmwater Aquaculture Research Unit, Stoneville, MS, USA. Three spawns were placed in hatching baskets with well water and allowed to hatch in a clean environment with no exposure to any known pathogens. Fish were then pooled together from the 3 spawns. A total of 250 channel catfish with a mean initial weight of 13.4 ± 0.1 g were randomly assigned to ten 76 l tanks (25 fish $tank^{-1}$, 5 replicates $treatment^{-1}$), and allowed to acclimate for 2 wk under 14 h light:10 h dark photoperiod in 26.1°C flow-through well water. Water quality (pH ~8.4 and dissolved oxygen levels >5.0 mg l^{-1}) and flow rates were similar among tanks.

The acclimation period included feeding fish to apparent satiation with a commercial 32% crude protein (CP) diet (Fishbelt Feeds). After the acclimation period, fish were anesthetized with 0.1 g l^{-1} tricainemethane sulfonate (MS-222; Western Chemical) and group-weighed to the nearest 0.1 g. The fish were fed the following 2 diets for 6 wk in 5 replicate treatments: (1) control (32% CP floating diet) and essential oil (EO) (32% CP floating diet supplemented with essential oils: Digestarom® P.E.P. MGE at 200 g ton^{-1}; Fishbelt Feeds). This supplement contained the essential oils carvacrol, thymol, anethol, and limonene (Biomin). At the end of the study the fish were anesthetized, group-weighed, and placed back into their respective tanks.

Edwardsiella ictaluri challenge

All fish from the feeding study were challenged with *Edwardsiella ictaluri* 2 d after they were weighed. An *E. ictaluri* isolate (Strain 93-146) from a natural outbreak (confirmed by the Mississippi State University Aquatic Research and Diagnostic Laboratory) was used for the challenge. Fish were challenged with pathogenic *E. ictaluri* (1.9×10^7 cfu ml^{-1}; final concentration) by an *in situ* bath immersion for 30 min (Booth & Bilodeau-Bourgeois 2009). The fish were fed their respective diets during the challenge and mortality was recorded daily for 21 d. A total of 5 fish from each treatment were taken to the diagnostic laboratory to confirm the fish died of ESC.

Three additional tanks of fish (25 fish $tank^{-1}$) of similar weight (~55 g $fish^{-1}$) served as non-challenged controls. The fish originated from the same group of fish used in the feeding study, were mock-challenged (same volume of sterile brain heart infusion [BHI] broth; Sigma-Aldrich) at the same time, and were fed the control diet. No mortalities from the non-challenged controls were recorded during the 21 d challenge.

Studies were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, US Department of Agriculture/Agriculture Research Service Warmwater Aquaculture Research Unit.

Sampling and RNA isolation

Tissue distribution of RBL1a, -1b, -1c, -3a, -3b, and -5a mRNA has been previously published (Small et al. 2008) and was not repeated in the current study. Two fish from each tank were randomly chosen to extract samples on Days 1 and 2 post-challenge. Two fish were also taken at each time point for the non-challenged controls. Fish were euthanized with an overdose (0.3 g l^{-1}) of MS-222 and small samples (approximately 100 mg) of skin, proximal small intestine, and gill were harvested from each fish. These tissues were chosen as possible sites for pathogen adhesion as reported previously (Thongda et al. 2014). Tissue samples were placed in 1 ml TRI-Reagent (Molecular Research Center), snap-frozen in liquid nitrogen, and stored at –80°C until RNA isolation.

For RNA isolation, samples were allowed to thaw and then homogenized in TRI-Reagent® using a TissueLyser (Qiagen). After homogenization, the Direct-zol™ RNA MiniPrep kit (Zymo Research) was

used according to manufacturer's recommendations. To prevent degradation, 20 samples were run at a time and kept on ice until transfer to a Zymo-Spin IIC column and collection tube (Zymo Research). After isolation, RNA was treated with a commercially available DNase I (Zymo Research) according to the manufacturer's instructions. Total RNA was quantified by measuring the absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The integrity of the RNA was verified by visualization of the 18S and 28S ribosomal bands stained with ethidium bromide after electrophoresis on 2.0% agarose gels.

Real-time polymerase chain reaction

Expression of RBL1a, -1b, -1c, -3a, -3b, and -5a mRNA was measured by real-time PCR using SYBR Green technology. Two housekeeping genes (β-2-microglobulin and α-tubulin) were chosen as internal controls based on the results of a previous study (Small et al. 2008). β-2-microglobulin and α-tubulin were shown to be stable during the disease challenge study in all tissues that were sampled. Fig. 1 shows the cycle threshold (C_T) values of the housekeeping genes as well as the genes of interest on Days 1 and 2 post-challenge. These 2 reference genes have also been shown to be suitable reference genes in previous catfish challenge studies (Peterson et al. 2015). The primers for these genes were designed based on the sequences deposited in GenBank (Table 1). For each gene that was measured, one primer was designed to overlap an exon/intron junction. The reaction mixture consisted of 10 µM of forward and reverse primers for β-2-microglobulin, α-tubulin, 20 µM of forward and reverse primers for all 6 RBL genes, 5 µl SSofast™ EvaGreen® supermix (Bio-Rad) and 2.9 µl diethyl pyrocarbonate (DEPC)-treated water (Thermo Fisher Scientific). In addition , 200 ng of cDNA (iScript™ cDNA synthesis kit; Bio-Rad) was added to each well. In non-template control wells (reactions without cDNA template), DEPC-treated water (2 µl) was added to the reaction in place of cDNA. The final volume of reaction mixture was 10.0 µl well^{-1}. Triplet technical reactions were carried out in Bio-Rad CFX96™ real-time detection system (Bio-Rad). The thermal cycling profile con-

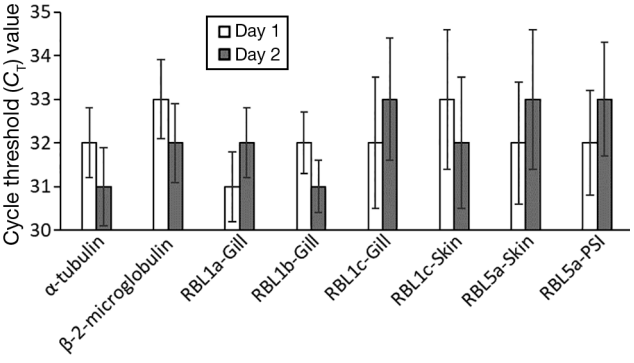

Fig. 1. Cycle threshold (C_T) values of housekeeping genes α-tubulin and β-2-microglobulin; and genes of interest rhamnose-binding lectin (RBL) 1a, -3a, and -3b in the gill; RBL1c and -5a in the skin; and RBL5a in the proximal small intestine (PSI), in channel catfish on Days 1 and 2 post-challenge. Results are mean ± SD of all samples collected

Table 1. Sequence of oligoprimers used in real-time PCR assay for catfish rhamnose-binding lectin (RBL) 1a, -1b, -1c, -3a, -3b, -5a, α-tubulin, and β-2-microglobulin

Primer	GenBank accession no.	Product length (bp)	Sequence (5′–3′)
RBL1a forward	KF725628	167	GTC ATG TCC AAA GAC TCA CTT G
RBL1a reverse	KF725628		GGT CAG GGT TGC CAA GTA ATT C
RBL1b forward	KF725629	210	GTC ATG TCC AAA GAC TCA CTT G
RBL1b reverse	KF725629		GGT CAG GGT TGC CAA GTA ATT C
RBL1c forward	KF725630	210	TAT TGC AGC TCA GGG CTT GT
RBL1c reverse	KF725630		TGA CAA CCT CAG ATG GCG AC
RBL3a forward	KF725631	150	AGA CGG ATT TAC TTG GCA ACC C
RBL3a reverse	KF725631		CAG CAC GTC CGT AGT TCG CA
RBL3b forward	KF725632	171	TGC TAC GAT GCC GAA ACA AC
RBL3b reverse	KF725632		CTT GGT CAA ACC ACT GGG GA
RBL5a forward	KF725633	140	AAT TTG CCC TGC TCT GGT GA
RBL5a reverse	KF725633		GCA CAC GTT CGC GAA TCA AT
α-tubulin forward	CB938582		Small et al. (2008)
α-tubulin reverse	CB938582		Small et al. (2008)
β-2-microglobulin forward	AF016042		Small et al. (2008)
β-2-microglobulin reverse	AF016042		Small et al. (2008)

sisted of an initial denaturation at 95°C (for 30 s), followed by 40 cycles of denaturation at 94°C (5 s), and an appropriate annealing/extension temperature (58°C, 5 s). An additional temperature ramping step was utilized to produce melting curves of the reaction from 65 to 95°C.

Statistical analysis

The C_T values were compared and converted to fold differences by the relative quantification method using Relative Expression Software Tool 384 v.1 (REST; Pfaffl et al. 2002). Comparisons were made in different tissues using analysis of variance (ANOVA) procedure in SAS v.9.2 software. Performance data were also subjected to ANOVA. Relative expression was calculated using pooled group data from each tank. Tank served as the experimental unit for each variable measured. When appropriate, data were also subjected to Duncan's multiple range test for means separation. Differences were considered significant at $p < 0.05$.

RESULTS

Growth performance and survival

There was no difference in weight gain, specific growth rate (SGR), or FCR between the control and EO fish (Table 2). However, survival during the 21 d challenge was higher (64.4 ± 3.3 vs. 48.0 ± 1.5%) for the EO fish compared to controls ($p < 0.05$). No fish died in the non-challenged control group.

RBL mRNA

All 6 RBL genes were examined in gill, proximal small intestine, and skin of channel catfish. After challenge with pathogenic *Edwardsiella ictaluri*, levels of RBL mRNA were examined on Days 1 and 2 post-challenge; non-challenged controls (those that received only BHI) were also sampled on Days 1 and 2 post-challenge. The limit of detection for the assays was calculated to be 6 target molecules using the methods described by Forootan et al. (2017). Depending on the tissue, many of the RBL genes were not detected in the non-challenged fish so a relative comparison could not be made.

We found that, relative to non-challenged controls, gill RBL1a mRNA was higher in fish fed the EO diet on Day 1 ($p < 0.05$) (Fig. 2a). Gill RBL3a mRNA was similar in EO and control fish on both days post-challenge (Fig. 2b). In contrast, gill RBL3b mRNA was higher on Days 1 and 2, respectively, in fish fed only EO diet ($p < 0.01$) (Fig. 2c). Skin RBL1c and -5a mRNA did not change significantly relative to non-challenged fish on Days 1 and 2 of the disease challenge (Fig. 2d,e). RBL5a mRNA was present in the proximal small intestine but was not significantly different between EO and control treatments relative to non-challenged fish on either day (Fig. 2f).

Table 2. Weight gain (mean initial weight was 13.4 ± 0.1 g), specific growth rate (SGR), feed conversion ratio (FCR), and survival of channel catfish (after challenge with *Edwardsiella ictaluri*) fed control or Digestarom® (EO) diets. $SGR = 100 \times [\ln(BW_2) - \ln(BW_1)] / t$, where BW_1 and BW_2 are initial and final weights, respectively, and t is feeding period (days). FCR = ingested food (g) / weight gain (g). All indices are mean ± SD. Values with different superscript letters in a column show significant differences ($p < 0.05$)

Treatment	Weight gain (g fish^{-1})	SGR	FCR	Survival (%)
Control	43.4 ± 3.8	2.3 ± 0.1	1.30 ± 0.1	48.0 ± 19.6^a
EO	38.8 ± 3.4	2.1 ± 0.1	1.39 ± 0.1	64.4 ± 12.5^b

DISCUSSION

Phytogenic feed additives like essential oils have been tested in cattle, poultry, and swine (Botsoglou et al. 2002, Christaki et al. 2004, Mao et al. 2005, Peeters et al. 2006, Vieira et al. 2008, Bartos et al. 2016) and many of the results showed improvements in growth performance and innate immunity to disease. The impetus for this research is that essential oils will likely provide alternatives to synthetic growth promoters and antibiotics. Likewise, developing feed supplements to improve the growth and health status of aquaculture species has become a major trend in the last decade (Huynh et al. 2017). Phytogenic feed additives have been examined in channel catfish (Zheng et al. 2009, Peterson et al. 2014, 2015) and rainbow trout (Ahmadifar et al. 2011, Giannenas et al. 2012) and a few of these studies have also found improvements in growth performance and innate immunity to disease. However, little information can be gleaned from these studies with respect to mechanisms of action.

This study examined the effects of a phytogenic feed additive, Digestarom® P.E.P. MGE, on growth performance, disease susceptibility, and roles that

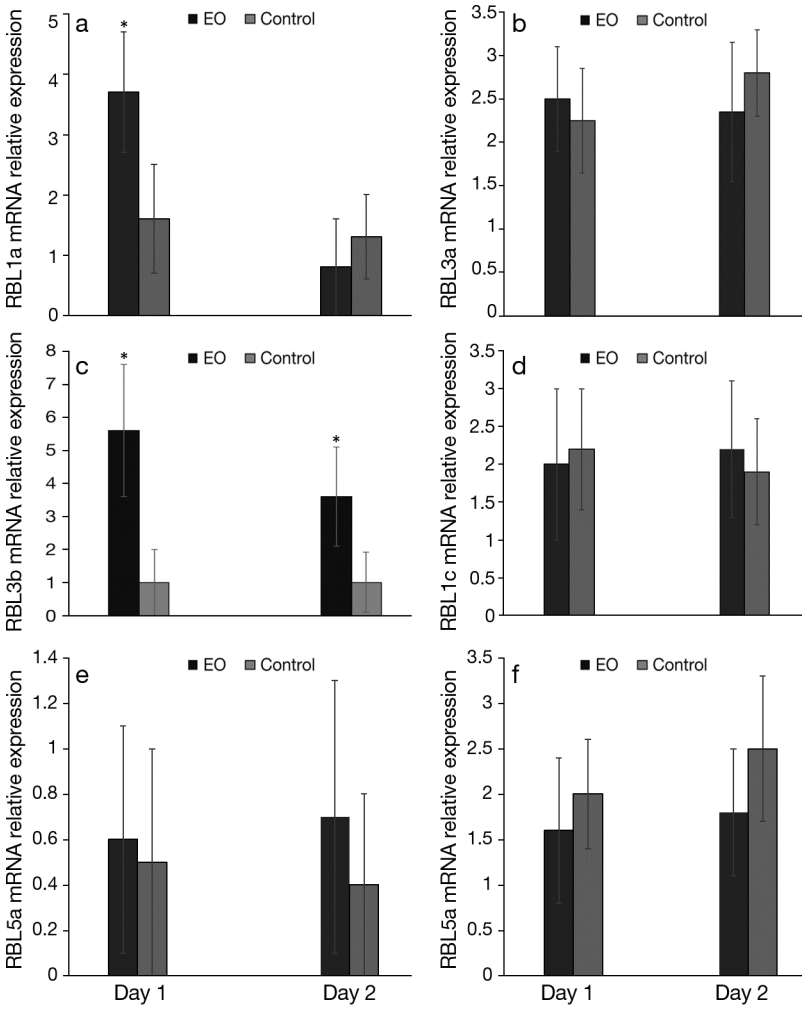

Fig. 2. Real-time PCR analysis for (a) rhamnose-binding lectin (RBL) 1a, (b) RBL3a, and (c) RBL3b mRNA in the gill; (d) RBL1c and (e) RBL5a mRNA in the skin; and (f) RBL5a mRNA in the proximal small intestine of channel catfish following *Edwardsiella ictaluri* infection. Expression was measured on Days 1 and 2 post-infection in fish fed control and essential oil (EO) diets. Fold-change was calculated by the change in expression at a given time point relative to the non-challenged fish and normalized by changes in α-tubulin and β-2-microglobulin housekeeping genes. Relative expression was calculated using pooled group data from each tank. Results are mean ± SD of fold-changes; asterisk indicates significant difference between treatments ($p < 0.05$)

RBL may play on innate immunity of channel catfish challenged with *Edwardsiella ictaluri*. The current study, as well as other catfish studies from our lab, showed no improvement in weight gain or FCR when fed essential oils (Peterson et al. 2014, 2015). It is clear from these 3 studies that the essentials oils carvacrol, thymol, anethol, and limonene (Digestarom® P.E.P. MGE) are not effective at improving growth performance in catfish grown in a tank environment.

The current study showed that survival was about 34% higher when fish were fed essential oils and challenged with *E. ictaluri*, while a previous study showed an increase in survival of 43% in fish fed essential oils and challenged with *E. ictaluri* (Peterson et al. 2015). Our previous tank study also showed that MBL might be involved in protecting catfish against pathogenic *E. ictaluri* (Peterson et al. 2015). In the current study, all 6 RBL genes were examined in skin, gill, and intestine after challenge to *E. ictaluri*. However, many of the genes were detected at very low levels or not at all in the non-challenged fish, so relative comparisons could not be made. It is possible that many of the RBL genes are either downregulated or expressed only upon challenge to infection. We found that relative to non-challenged controls, gill RBL1a mRNA was higher in fish fed EO diet 1 d post-challenge. However, gill RBL1a mRNA returned to non-challenged levels 2 d post challenge. Beck et al. (2012) also showed that RBL1a mRNA in the gill was higher in channel catfish exposed to *Flavobacterium columnare* and in fish that were fasted. Thongda et al. (2014) found that gill RBL1a mRNA in channel catfish was upregulated after exposure to *E. ictaluri*. Those authors suggest that perhaps RBL1a plays a role in blocking *E. ictaluri* adhesion to the gill surface, thus preventing bacteria from gaining entry into the fish.

We found that gill RBL3a mRNA was upregulated in EO and control fish 1 and 2 d post-infection, respectively. Thongda et al. (2014) also found that gill RBL3a mRNA was upregulated after exposure to *E. ictaluri*. We also observed that gill RBL3b mRNA was higher on Days 1 and 2 in fish fed the EO diet. Thongda et al. (2014) showed that gill RBL1b and -3b were increased 4 h after infection with pathogenic *E. ictaluri*. Similar to gill RBL1a, -3a and -3b may play roles in blocking bacterial adhesion to the gill surface.

We also examined the roles of RBLs in the skin and proximal intestine of channel catfish exposed to disease. Skin RBL1c and -5a mRNA did not change significantly relative to non-challenged fish on Days 1 and 2 of the disease challenge. Similarly, RBL5a mRNA was present in the proximal small intestine but was not significantly different between EO and

control treatments relative to non-challenged fish on either day. Thongda et al. (2014) found that most of the genes in the skin and intestine showed reduced expression following infection, with a significant down-regulation of RBL1c (400-fold) at Day 3 post-infection. It is not clear whether suppression of RBL genes in skin and intestine aid in pathogen adhesion or not; additional studies will be needed to further elucidate the role of RBLs with respect to those mucosal tissues.

Our results do not provide evidence that Digestarom® P.E.P. MGE improves weight gain or FCR in channel catfish. Results showed that Digestarom® P.E.P. MGE increased survival of catfish challenged with *E. ictaluri*. The mechanisms through which survival was increased may include enhanced mucosal gill expression of RBL1a and -3b. Further elucidation of the mechanisms through which essential oils affect bacterial pathogenicity will be critical in determining whether these and other feed additives are useful for aquaculture.

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