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Probiotic *Pediococcus acidilactici* modulates both localised intestinal- and peripheral-immunity in tilapia (*Oreochromis niloticus*)

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ABSTRACT

The application of probiotics in aquaculture has received concerted research efforts but the localised intestinal immunological response of fish to probiotic bacteria is poorly understood. Therefore, a study was conducted to evaluate the probiotic effect of *Pediococcus acidilactici* on Nile tilapia (*Oreochromis niloticus*) with specific emphasis on intestinal health and probiotic levels as well as system level responses such as growth performance, feed utilization and haemato-immunological parameters under non-challenged conditions. Fish (9.19 ± 0.04 g) were fed either a control diet or a *P. acidilactici* supplemented diet (at 2.81×10^6 CFU g^{-1}) for six weeks. At the end of the study the probiotic was observed to populate the intestine, accounting for ca. 3% (1.59×10^5 CFU g^{-1}) of the cultivable intestinal bacterial load. Real-time PCR indicated that the probiotic treatment may potentiate the immune-responsiveness of the intestine as up-regulation of the gene expression of the pro-inflammatory cytokine TNFα was observed in the probiotic fed fish ($P < 0.05$). Light microscopy observations revealed elevated intra-epithelial leucocyte (IEL) levels in the intestine of *P. acidilactici* fed tilapia after six weeks ($P < 0.05$) of feeding and a trend towards elevated goblet cells was also observed after six weeks feeding ($P = 0.08$). Concomitantly at week six, along with elevated IELs and elevated TNFα mRNA levels in the intestine, an increased abundance of circulating neutrophils and monocytes were observed in fish fed the probiotic supplemented diet ($P < 0.05$). This haemopoietic expansion of innate immune cells could be reflective of an elevated state of immuno-readiness. Together these results suggest that the probiotic has a protective action on the intestinal mucosal cells, stimulating the innate immune response after feeding for a period of six weeks. These immunological modulations did not impair growth performance or the remaining haematological and zootechnical parameters compared to the control group ($P > 0.05$).

1. Introduction

The gut microbiome of vertebrates has been well studied over the past decade and its functions and implications towards host organism health, development, nutrition, disease and recently on reproduction is becoming well documented [1–4]. The application of feed additives, such as probiotics, to fortify these microbial communities and subsequently provide host benefits has therefore received much attention. A number of studies have investigated the efficacy of probiotics in aquafeeds (for review see Refs. [5–7]). The majority of these studies have looked at fish immune responses and disease resistance and, although not in all cases, improved immunological responses and disease resistance have often been reported [5–9]. The application of probiotics can also improve growth, embryo and larval development [10], stress tolerance [11], fecundity [4,12–16], gastrointestinal morphology, and microbial balance [17–24]. These studies provide important information, particularly at the whole organism level, but our understanding of the mechanisms involved in mediating these responses is somewhat limited due to a paucity of information regarding the host–microbe interactions at the intestinal mucosal interface. This is particularly true for tilapia (*Oreochromis niloticus*), which are an important cultured fish species with global production of over 2.5 million metric tonnes in 2010, valued at over $4 billion [25]. Our previous research has demonstrated that dietary application of the probiotic *Pediococcus acidilactici* can modulate the intestinal

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microbiota of tilapia by elevating lactic acid bacterial (LAB) levels, out-competing certain bacterial phylotypes and reducing bacterial diversity [8]. However, the impact of these microbial changes on the host mucosa is poorly understood. In order to utilise probiotics effectively it is vital to gain a clear understanding of the underlying mechanisms at the intestinal interface which initiate and drive host benefits. Pro-inflammatory cytokines, including tumour necrosis factor-α (TNFα), are often used as biomarkers for immune-regulation. This cytokine plays an important role in orchestrating host defence mechanisms in response to bacterial colonization or invasion. The inflammatory process is a vital component of the innate immune response and therefore the first aim of the present study was to assess the effect of dietary *P. acidilactici* on localised intestinal immune status by assessing the expression of TNFα, the intestinal morphology and abundance of immune cells in the tilapia intestine. The secondary aim was to investigate the systemic effects on growth performance and heamato-immunological status.

2. Materials and methods

All experimental work involving fish was conducted under the UK Home Office project licence PPL 30/2644 and was in accordance with the UK Animals (Scientific Procedures) Act 1986 and the Plymouth University Ethical Committee.

2.1. Diet preparation

Two iso-nitrogenous and iso-lipidic diets were formulated (Table 1) according to the known requirements for tilapia [26]. *P. acidilactici* MA 18/5 M (Bactocell®, Lallemand Inc., Canada) was cultured and the diets were prepared as described previously [8] to produce 2 mm pellets. The bacterial cells were diluted to the desired concentration based on a calibration curve (CFU ml^{-1} vs OD_{590}) to achieve the manufacturer's recommended dose in the final pellet (10^6 CFU g^{-1}). Washed cells were then added drop wise into the basal mixture which was thoroughly homogenised, with the addition fish oil, corn oil and warm water until a doughy consistency was achieved. This was the then cold press extruded using a PTM Extruder system (model P6, Plymouth, UK) [8,20]. The same volume of PBS (without *P. acidilactici*) was added to the basal mixture for the control diet. The diets were dried to ca. 5% moisture in an air convection oven set at 50 °C and composition analysed using AOAC [27] protocols (Table 1). Experimental diets were subsequently refrigerated at 4 °C and stored in airtight containers prior to use. The probiotic inclusion levels (2.81×10^6 CFU g^{-1}) were confirmed by spread plating the diets on de Man, Rogosa & Sharpe agar (MRS; Oxoid, Basingstoke, UK) for 24 h at 37 °C. Sequence analysis of 16S rRNA was undertaken to confirm identification of *P. acidilactici* isolates using the primers 27F and 1491R, as described in detail elsewhere [8].

Table 1
Dietary formulations (%) and chemical composition.

	Control	Probiotic
Fishmeal[a]	25.00	25.00
Soyabean meal[b]	16.09	16.09
Corn starch[c]	32.51	32.51
Lysamine pea protein[d]	10.00	10.00
Glutalys[d]	10.00	10.00
Sunflower oil	3.80	3.80
Vitamin–mineral premix[e]	2.00	2.00
CMC-binder[c]	0.50	0.50
Probiotic (CFU g^{-1})[f]	0.00	2.81×10^6
Chemical composition (%)		
Dry matter	95.30	96.55
Crude protein[g]	44.44	43.83
Crude lipid[g]	6.97	7.33
Ash[g]	5.93	6.16
Gross energy (MJ kg^{-1})[h]	19.85	19.64

[a] Herring meal LT92 – United Fish Products Ltd., Aberdeen, UK.
[b] Hamlet HP100 (57% crude protein), Hamlet Protein, Denmark.
[c] Sigma- Aldrich Ltd., UK.
[d] Roquette Frêres, France.
[e] Premier nutrition vitamin/mineral premix contains: 121 g kg^{-1} calcium, Vit A 1.0 μg kg^{-1}, Vit D3 0.1 μg kg^{-1}, Vit E (as alpha tocopherol acetate) 7.0 g kg^{-1}, Copper (as cupric sulphate) 250 mg kg^{-1}, Magnesium 15.6 g kg^{-1}, Phosphorous 5.2 g kg^{-1}.
[f] *P. acidilactici* (CNCM MA 18/5 M), Bactocell® (Lallemand Inc., Canada).
[g] Values given are based on a dry matter basis.
[h] Gross energy (MJ kg^{-1}) calculated according to 23.6 kJ g^{-1} for protein, 39.5 kJ g^{-1} for lipid and 17.0 kJ g^{-1} for NFE.

2.2. Experimental design

Genetically male red Nile tilapia *O. niloticus* (GMT®, Fishgen UK) were obtained from the Institute of Aquaculture, University of Stirling, UK and transported to the Aquaculture and Fish Nutrition Research Aquarium, Plymouth University, UK where they were allowed eight weeks of acclimation. Three hundred and twenty fish were randomly distributed into 8 × 80-l tanks with four replicate tanks per dietary treatment (40 fish per tank; average weight = 9.19 ± 0.04 g) containing aerated recirculated freshwater. Fish were fed experimental diets at a rate of 4% biomass per day provided in equal rations at 09:00, 13:00 and 17:00 for six weeks. Daily feed was adjusted on a weekly basis by batch weighing following a 24-h starvation period. Fish were held at 27 ± 1 °C with a 12: 12 h light: dark photoperiod. Water quality parameters were monitored throughout the trial and were maintained at 6.6 ± 0.4 pH (adjusted with $NaHCO_3$ as necessary) and dissolved oxygen >80% saturation. Ammonium, nitrite and nitrate levels were monitored weekly and water changes (~20% system volume) were conducted every week to minimise the build-up of these compounds as well as to prevent background system build-up of probiotic levels.

2.3. Intestinal probiotic levels

At the end of the trial, six fish per experimental treatment were sampled to enumerate the intestinal microbiota. The gastrointestinal tract was aseptically removed in its entirety and allochthonous microbial populations were isolated from the intestine as described elsewhere [28]. Since inter-fish variation has been reported previously [29,30] the faecal matter from two fish per tank was pooled yielding three samples per treatment. Samples were serially diluted with PBS and 100 μl was spread onto duplicate tryptone soya agar (TSA, Oxoid, Basingstoke, UK) plates to determine total aerobic heterotrophic populations and MRS for LAB using the spread plate method. MRS and TSA plates were incubated at 30 °C (comparable to tilapia culture conditions) for 48 h and colony forming units (CFU) g^{-1} were calculated by counting colonies from statistically viable plates (i.e. plates containing 20–300 colonies). Sequence analysis of 16S rRNA was undertaken on a representative subset of LAB isolates to confirm identification of *P. acidilactici*.

2.4. Intestinal histology

Two fish per tank were sampled for histological appraisal of the mid-intestine at the mid and endpoint of the trial ($n = 8$). For light microscopy, the tissue samples were fixed in 10% formalin, dehydrated in graded ethanol concentrations and embedded in paraffin wax. In each specimen, multiple sets of sections (5 μm thick) were stained with May-Grünwald Giemsa (MGG), haematoxylin and eosin (H&E) and Alcian-Blue-PAS [8,31]. The intestinal perimeter ratio (arbitrary units; AU) was assessed [32] and the numbers of intra epithelial leucocytes (IEL's) and goblet cells in the epithelium,

across a standardized distance of 100 enterocytes (only nucleated cells), was then calculated by averaging the cell numbers from all specimens [8].

Samples for transmission electron microscopy (TEM) were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.2 and 20 °C, for 1 h, and post-fixed in 1.0% osmium tetroxide (OsO_4) in 0.1 M sodium cacodylate buffer at pH 7.2 and 20 °C, for 2 h. Samples were then rinsed in buffer, dehydrated in ethanol, infiltrated in low viscosity resin (Agar Scientific, Essex, UK), and polymerised overnight in coffin moulds at 60 °C. Ultra-thin sections were stained with a saturated uranyl acetate solution, post stained with Reynolds lead citrate and examined with a Jeol JSM 1200 EX II (Tokyo, Japan) transmission electron microscope at 120 kV.

2.5. Intestinal gene expression

2.5.1. RNA extraction, cDNA synthesis and real-time PCR

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, with some modifications as previously indicated [9]. RNA concentration and purity were measured spectrophotometrically (NanoDrop Technologies, Wilmington, USA) and stored at −80 °C until use. Total RNA was treated with DNAse (10 UI at 37 °C for 10 min, MBI Fermentas), and a total amount of 1 μg of RNA was used for cDNA synthesis, employing iScript cDNA Synthesis Kit (Bio-Rad CA, USA). PCRs were performed with the SYBR green method in an iQ5 iCycler thermal cycler (Bio-Rad). Duplicate PCR reactions were carried out for each sample analysed. The reactions were set on a 96-well plate by mixing, for each sample the treatment mix was 1 μL of diluted (1/20) cDNA, 5 μL of 2× concentrated iQ™ SYBR Green Supermix (Bio-Rad), containing SYBR Green as a fluorescent intercalating agent, 0.3 μM of forward primer and 0.3 μM of reverse primer. The thermal profile for all reactions was 3 min at 95 °C and then 45 cycles of 20 s at 95 °C, 20 s at 60 °C and 20 s at 72 °C. Fluorescence monitoring occurred at the end of each cycle. Additional dissociation curve analysis was performed and showed in all cases one single peak. β-actin was used as the reference gene in each sample in order to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. The sequences of specific primers used for β-actin and TNFα are reported in Table 2. No amplification product was observed in negative controls and no primer–dimer formations were observed in the control templates. The data obtained were analysed using the iQ5 optical system software version 2.0 (Bio-Rad).

2.5.2. Haemato-immunological parameters

Blood was taken from two fish per tank at the trial midpoint (i.e. day 22) and four fish per tank at the end of the study (i.e. day 42). Fish were anaesthetized with tricaine methanesulfate (MS222) at 150 mg l^{-1} and samples were taken from the caudal arch using a 25 gauge needle and a 1 ml syringe. Blood smears were prepared for the determination of differential leucocyte counts whilst additional blood was left to clot for a period of 12 h (at 4 °C) to isolate serum. Serum was isolated by centrifugation at 3600 *g* for 5 min and was stored at −80 °C. Haematocrit (measured and read as % packed cell volume; PCV), haemoglobin, erythrocyte counts (RBC), serum lysozyme activity, leucocyte counts (WBC) and differential leucocyte proportions were determined according to standard methods described elsewhere [33].

Table 3
Viable counts (log CFU g^{-1}) of cultivable aerobic bacteria, LAB and % LAB in the digesta of Nile tilapia after six weeks of feeding on the experimental diets ($n = 3$).

	Control	Probiotic	*P*-value
TVC	7.21 ± 0.16	6.62 ± 0.42	0.101
LAB	3.09 ± 0.45[a]	5.20 ± 0.41[b]	0.014
% LAB	<0.01 ± 0.00[a]	3.16 ± 0.12[b]	0.010

Values with different superscripts indicate significant differences ($P < 0.05$).

2.5.3. Growth performance and zootechnical parameters

Growth performance and feed utilisation were assessed by net weight gain (NWG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and condition factor (*K*). Calculations were carried out using the formulae: SGR = 100 ((ln FW − ln IW)/*T*); FCR = FI/WG; PER = WG/PI and $K = (100 \times FW)/(FL^3)$, where FW = final weight (g), IW = initial weight (g), *T* = duration of feeding (days), WG = wet weight gain (g), FI = feed intake (g), PI = protein ingested and FL = final length (cm). At the beginning of the trial, 30 fish were pooled to make three samples to determine initial carcass composition and at the end of the trial four fish per tank were sampled to determine final carcass composition. Proximate composition analysis was conducted according to AOAC protocols [27].

At the end of the trial four fish per tank were euthanized with tricaine methanesulfate (MS222) at 200 mg l^{-1} followed by destruction of the brain. These fish were used to record viscera weight and whole body weight in order to calculate the hepatosomatic index (HSI) and viscerosomatic index (VSI). Calculations were made using the following formulae: HSI = (LW/BW) × 100 and VSI = (VW/BW) × 100. Where LW = liver weight (g), BW = body weight (g) and VW = viscera weight (g).

2.5.4. Statistical analysis

All data are presented as mean ± standard deviation (SD). All data were checked for normality using a Kolmogorov–Smirnov test and analysed using a *t*-test (SPSS Inc., 15.0, Chicago, IL, USA). In all cases significance was accepted at $P < 0.05$.

3. Results

3.1. Intestinal probiotic levels

Bacterial levels within the digesta of fish fed experimental diets are displayed in Table 3. Allochthonous total cultivable levels (TVC) were not affected by the probiotic treatment ($P = 0.101$), however, compared to fish fed the control diet, LAB levels were significantly ($P = 0.014$) higher in the probiotic fed fish. LAB isolated from the probiotic fed fish were identified as *P. acidilactici* by 16S rRNA sequence analysis.

3.2. Intestinal histology

Light microscopy revealed that the intestine of tilapia fed either a control or probiotic supplemented diet showed an intact

Table 2
Primers used for real-time PCR analyses.

Gene	Forward primer	Reverse primer	Amplicon size	Genbank number
TNFα	GGTCATCTGGAGTGGAGGAA	AGCCGTGGTCTGAGAAGCTA	295	AY428948.1
β-Actin	GTGCCCATCTACGAGGGTTA	CTCCTTAATGTCACGAACGA	156	EU887951.1

epithelial barrier and a mucosal arrangement of organised villi-like mucosal folds. The intestinal mucosa consisted of a simple epithelium and a lamina propria (LP) which housed scattered IELs together with acidophilic granulocytes (AGs) (Fig. 1) both in the control and probiotic group. The number of IELs residing between every 100 enterocytes in the intestine of the probiotic fed fish was significantly ($P = 0.039$) elevated at six weeks (140.80 ± 11.94 vs 114.60 ± 16.85 in the control) but did not differ at three weeks (Fig. 2A). The number of goblet cells remained unaffected at week three but a trend towards elevation ($P = 0.080$) was observed in the probiotic fed fish at week six (Fig. 2B). Alcian-Blue PAS stained sections revealed that the goblet cells of the GI tract (both treatments and both timepoints) were filled with abundant acidic mucins which were also present lining the epithelium forming a mucus barrier, in both treatments (Fig. 1E and F). The perimeter ratio (a higher perimeter ratio indicates a higher absorptive intestinal surface area brought about by more numerous and/or longer mucosal folds) of the intestine in the control group (week

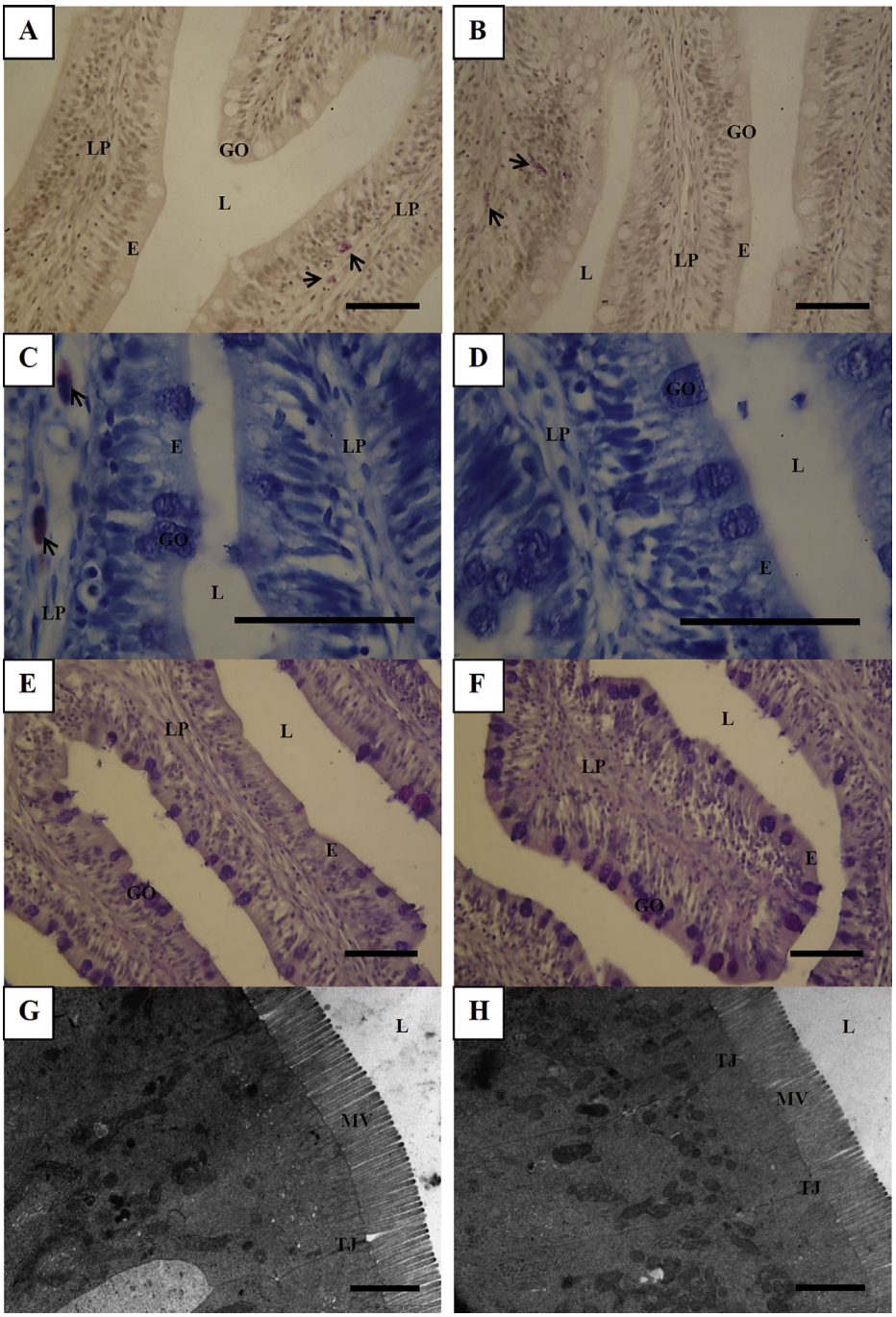

Fig. 1. Light (A–F) and electron (G and H) micrographs of the mid-intestine of Nile tilapia fed either the control (A, C, E and G) or probiotic (B, D, F and H) diet at the end of the experimental period. Note that the goblet cells are filled with abundant acidic mucins in both treatments. Abundant IELs are present in the epithelia and a distinct sub-population of acidophilic granulocytes (stained pink) are scattered amongst the mucosa (arrows). Electron micrographs show the apical brushborder revealing intact epithelia comprised of elongated cuboidal enterocytes and tight junctional complexes. Abbreviations used are E: epithelia; GO: goblet cells; LP: lamina propria, L: lumen, MV: microvilli and TJ = tight junctions. Light microscopy staining: A, B: H&E; C, D: MGG; E, F: Alcian-Blue-PAS. Scale bars = 50 μm (A–F), 2 μm (G and H).

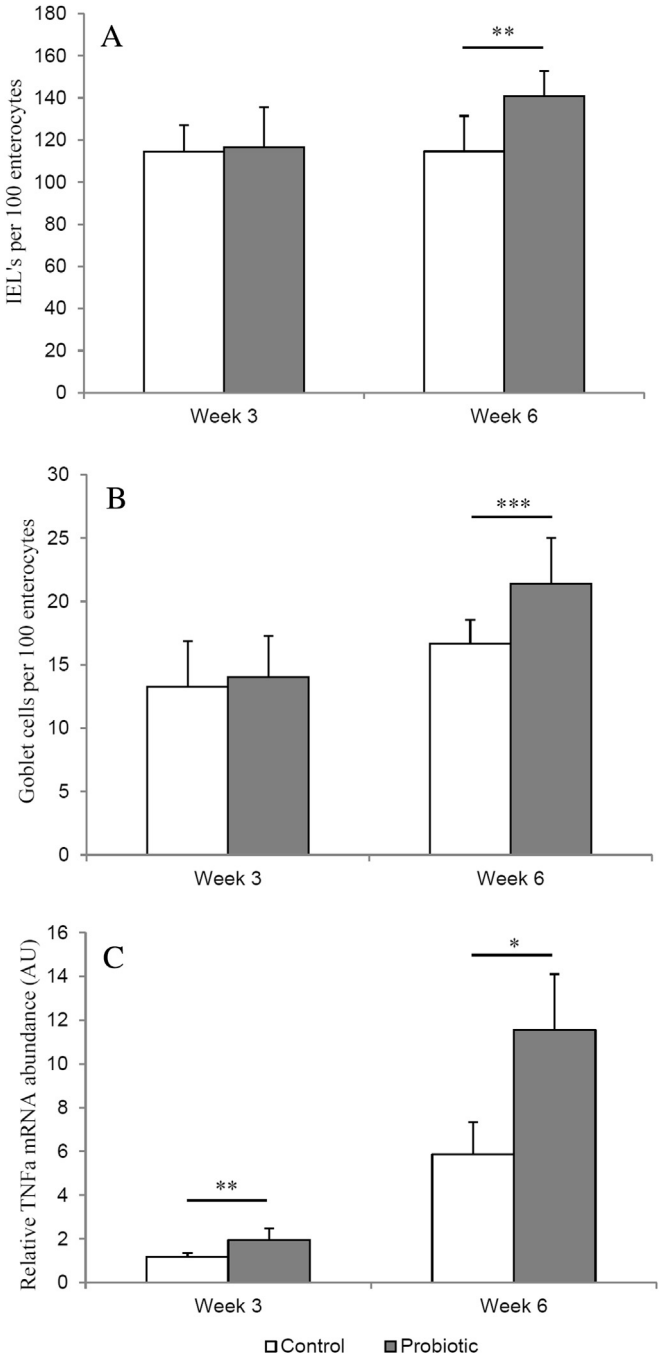

Fig. 2. (A) IEL abundance (per 100 enterocytes), (B) goblet cell abundance (per 100 enterocytes) and (C) TNFα mRNA levels (AU; normalized against β-actin) in the tilapia mid-intestine fed either a control or a probiotic supplemented diet for three and six weeks. Data are given as mean values ± SD ($n = 8$). *, ** and *** denote differences between the treatment groups at the $P < 0.01$, $P < 0.05$ and $P < 0.10$ level, respectively.

three = 4.11 ± 0.71 AU and week six = 3.64 ± 0.43 AU) did not significantly differ to that of the probiotic fed fish at week three (4.66 ± 0.78 AU) or week six (3.99 ± 1.18 AU). Transmission electron microscopy observation of the apical brushborder revealed intact epithelia in both treatments; elongated cuboidal enterocytes were bound together by tight junctional complexes and signs of endocytic activity were observed with pinocytosis occurring between and beneath the microvilli. Below the brush border a high level of mitochondria were present. There were no signs of damage in either dietary treatment (Fig. 1G and H). Densely packed microvilli were present on the enterocyte surfaces and the microvilli height did not differ between treatments (1.61 ± 0.05 μm and 1.62 ± 0.57 μm for the control and probiotic groups, respectively).

Table 4
Haematological and immunological parameters of Nile tilapia after six weeks of feeding on the experimental diets. $n = 16$.

	Control	Probiotic	*P*-value
Haematocrit (%PCV)	30.10 ± 7.74	29.44 ± 3.20	0.899
Haemoglobin (g dl^{-1})	5.66 ± 1.01	7.15 ± 1.67	0.256
RBC (10^6 μl^{-1})	1.26 ± 0.39	1.25 ± 0.25	0.962
WBC (10^3 μl^{-1})	14.33 ± 3.73	16.70 ± 8.38	0.633
MCV (fL)	245.49 ± 42.68	247.88 ± 62.70	0.959
MCH (pg)	47.85 ± 11.57	56.94 ± 3.16	0.281
MCHC (g dl^{-1})	20.16 ± 5.91	24.78 ± 7.23	0.430
Serum lysozyme	178.50 ± 73.05	189.68 ± 69.20	0.774

RBC = red blood cells.
WBC = leucocytes.
MCV = mean corpuscular volume = (haematocrit (%PCV) × 10)/RBC (10^6 μl^{-1}).
MCH = mean corpuscular haemoglobin = (haemoglobin (g dl^{-1}) × 10)/RBC (10^6 μl^{-1}).
MCHC = mean corpuscular haemoglobin concentration = (haemoglobin (g dl^{-1}) × 100)/haematocrit (%PCV).

3.3. Intestinal TNFα gene expression

The expression of the pro-inflammatory cytokine TNFα in the mid-intestine of tilapia is displayed in Fig. 2C. TNFα expression was significantly up-regulated in the probiotic group at week three ($P = 0.042$) and week six ($P = 0.005$) compared to the control fish (Fig. 2C).

3.4. Haemato-immunological parameters

Haematological and immunological parameters are displayed in Tables 4 and 5. Haematocrit levels remained unaffected by the probiotic treatment (PCV = ~30% in both groups), as did haemoglobin levels (~6 g dl^{-1}). There were no significant differences between total numbers of erythrocytes (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) between dietary groups ($P > 0.05$). Additionally, there were no significant differences of serum lysozyme activity between the two dietary treatments. Although there were no significant differences between total numbers of circulatory leucocytes (WBC), the proportions of leucocyte types was significantly altered in the probiotic fed fish at week six (Table 5). At week six fish fed *P. acidilactici* displayed

Table 5
Circulatory leucocyte proportions of Nile tilapia at the midpoint (week three) and end of the trial (week six).

Leucocyte type (%)	Control	Probiotic	*P*-value
Week three[a]			
Lymphocytes	68.25 ± 5.50	66.00 ± 13.00	0.662
Neutrophils	19.25 ± 9.88	24.10 ± 12.30	0.397
Monocytes	nd	nd	
Thrombocytes	12.40 ± 12.20	9.90 ± 11.30	0.678
Week six[b]			
Lymphocytes	83.44 ± 9.38	81.19 ± 8.20	0.476
Neutrophils	8.25 ± 5.60^A	12.94 ± 6.95^B	0.045
Monocytes	0.13 ± 0.34^A	1.69 ± 2.06^B	0.009
Thrombocytes	8.19 ± 7.84	4.19 ± 4.61	0.091

^{AB} Values with different superscripts indicate significant differences ($P < 0.05$).
nd = not detected (below detection threshold).
[a] $n = 8$.
[b] $n = 16$.

elevated proportions of neutrophils ($P < 0.05$) and monocytes ($P < 0.01$) and a trend towards a significantly lower proportion of thrombocytes ($P = 0.091$). These differences were not observed at week three.

3.5. Growth performance and zootechnical parameters

Growth performance, feed utilisation and somatic indices are displayed in Table 6. Both dietary groups grew very well with >250% increase in body weight after six weeks feeding. There were no significant differences ($P > 0.05$) between the groups with respect to growth, feed utilisation, hepatosomatic index (HSI), viscerosomatic index (VSI), condition factor or survival. Additionally, body composition was also unaffected (data not shown).

4. Discussion

In the present study dietary *P. acidilactici* provision led to high populations of *P. acidilactici* (1.59×10^5 CFU g^{-1}) in the intestine which is in the same range reported in previous studies using this probiotic in tilapia [8], rainbow trout [28,34] and shrimp [35]. Our previous studies, using both culture-dependent and culture-independent methodology, indicate that this probiotic is able to survive gastric passage, populate the intestinal tract, out-compete some endogenous phylotypes and form associations with the tilapia intestinal mucosa [8]. These are important criteria for a probiotic candidate and it is essential that probiotic candidates survive gastric passage in high numbers in order to exert their effect at the site of action, the intestine.

The present study shows that the functionality of the tilapia immune system, at the local level, was influenced by signals from the *de novo* probiotic colonizers, or transient probiotic bacteria, administered. This study revealed the effect of feeding probiotic supplemented diets on the expression of the pro-inflammatory cytokine TNFα, which is commonly used as a biomarker for immune-regulation. TNFα contributes to the defence mechanisms of the host in response to bacterial colonization, or invasion, by inducing an inflammatory response which acts to recruit more lymphoid cells to the site of colonisation/infection. The cellular response in the tilapia intestine in the present study, increased TNFα production, implies that probiotic fed fish are in an immunologically elevated state which may aid in resisting pathogenic insults by improving the immune-readiness. These findings are in agreement with previous studies that have shown that the supplementation of probiotic bacteria increases the expression of pro-inflammatory cytokines, including IL-1β and TNFα in fish [9,36,37]. In mammals, previous studies have evidenced that cell surveillance of gut microbiota is routinely exerted by the gastrointestinal immune system [38] and regulatory signals generated by probiotics may be able to stimulate host immunity to afford a degree of enhanced protection against pathogens [39].

TNFα is an acute phase protein which initiates a cascade of cytokines which subsequently recruits macrophages and neutrophils to the site of inflammation. It would be tempting to suppose therefore that the elevated IELs observed in the intestine of the probiotic fed fish at week six were likely to be macrophages and neutrophils but from the methods used in the present investigation it is not possible to accurately quantify the leucocyte sub-populations in the epithelium. The increase in monocytes and neutrophil proportions in the blood, combined with an increased expression of TNFα supports the stimulation of the innate immune response in fish fed the probiotic. MGG staining revealed that acidic granulocytes were present in the epithelium. These findings are consistent with previous studies which revealed elevated IELs (specifically acidic granulocytes, T cells and Ig+ cells) in the intestine of gilthead sea bream and European sea bass fed dietary *Lactobacillus* spp. [40,41]. In the present study elevated goblet cell levels were also present at week six in the probiotic group. Alcian-Blue PAS stained micrographs showed that goblet cells residing in the GI tract of fish fed either dietary treatment were filled with abundant acidic mucins. This was also evident in the mucus layer lining the epithelia. This viscous barrier helps prevent pathogenic invasion by trapping and enabling the removal of potentially harmful antigenic material as well as containing many antibacterial substances providing protection [42]. The gastrointestinal tract of fish is a portal of entry for a number of important fish pathogens [43] and elevated IELs and goblet cell levels may therefore provide an elevated resistance to intestinal pathogenesis. Our previous studies using this probiotic in rainbow trout experiments have also revealed elevated goblet cells and IELs in the intestine and this appeared to lead to a qualitative reduction in the extent of epithelial damage induced by *V. anguillarum* in *ex vivo* intestinal sac experiments [44]. In the present study there was no significant difference in perimeter ratio between dietary treatments after three weeks ($P = 0.603$) or six weeks ($P = 0.726$). Transmission electron microscopy of the intestine of fish fed *P. acidilactici* revealed no signs of histological damage and the epithelium conformed to healthy morphology as observed in the control group; the apical brushborder exhibited a typical structure composed of uniformly formed microvilli structures with epithelial integrity maintained by tight junctions between enterocytes.

Despite changes in the localised immune status there was little effect on the systemic immune parameters investigated. *P. acidilactici* has previously been reported to positively influence tilapia immunity by enhancing circulatory leucocyte numbers and increasing serum lysozyme activity [8]. However, contradictory results are present in the literature regarding the response of serum lysozyme activity and circulatory leucocyte levels in probiotic fed fish [22,28,45]. These differences may be attributed to the different fish species, stage of development, strain of probiotic, dosage, duration of feeding, feed composition, mode of supplementation and/or environmental/rearing conditions. In the present study changes in the proportions of leucocytes were observed as monocytes and neutrophils proportions were significantly elevated at week six in the probiotic fed fish. Monocytes and neutrophils are key components of the innate immune response; neutrophils are one of the first cells to respond to inflammation and monocytes also react quickly to replenish macrophage and dendritic populations at inflammatory sites [46]. Their elevation in the bloodstream is indicative of a potentiated systemic response, which may provide a more rapid non-specific immune response upon pathogenic infection.

Table 6
Growth performance and zootechnical parameters of Nile tilapia after six weeks of feeding on the experimental diets. $n = 4$.

Parameters	Control	Probiotic	*P*-value
Initial body weight (g $fish^{-1}$)	9.19 ± 0.05	9.19 ± 0.03	1.000
Final body weight (g $fish^{-1}$)	33.77 ± 1.04	34.50 ± 1.51	0.459
Weight gain (g $fish^{-1}$)	24.57 ± 1.06	25.31 ± 1.48	0.456
Specific growth rate (% day^{-1})	3.55 ± 0.07	3.59 ± 0.12	0.655
Feed conversion ratio (g g^{-1})	0.99 ± 0.03	0.98 ± 0.04	0.857
Mean daily feed intake (g $fish^{-1}$ day^{-1})	0.97 ± 0.03	0.96 ± 0.03	0.620
Condition factor (*K*)[a]	1.74 ± 0.07	1.71 ± 0.12	0.642
Apparent protein utilisation (%)	27.53 ± 6.35	33.97 ± 3.06	0.142
Protein efficiency ratio (PER)	1.66 ± 0.06	1.71 ± 0.10	0.470
VSI (%)[a]	10.48 ± 0.53	10.27 ± 0.30	0.537
HSI (%)[a]	1.53 ± 0.18	1.84 ± 0.17	0.056
Survival (%)	96.05 ± 3.40	98.68 ± 1.52	0.230

[a] $n = 16$.

Fish grew well with >250% increase in biomass and >95% survival in all treatments. The growth performance, feed utilisation, body composition, somatic indices and haematological parameters were not affected by dietary treatment. This is perhaps not surprising given that the fish were subjected to good environmental conditions (e.g. water quality, temperature dissolved oxygen etc.), high husbandry care and were provided with a highly nutritive value diet meeting, or exceeding, the known requirements of tilapia [19]. However, it is important to note that despite the elevated immunological responses observed, the probiotic fed fish maintained high growth performance in line with that of the control group.

The present study suggests therefore that *P. acidilactici* modulates both localised intestinal- and peripheral- innate immunity in tilapia without detrimental effects on the intestinal morphology, haematological parameters or growth performance. Future studies should assess disease resistance, against enteropathogens in particular, to determine the degree of benefit afforded by these elevated immunological responses.

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Author contributions

Conceived and designed the experiments: DLM SJD MC. Performed the experiments: BS MDR GG. Analyzed and interpreted the data: BS MDR GG OC DLM AF. Contributed reagents/materials/analysis tools: SJD DLM OC MC. Wrote the paper: BS MC AF OC DLM.

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