Effect of β-1/3,1/6-glucan upon immune responses and bacteria in the gut of healthy common carp (*Cyprinus carpio*)

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ABSTRACT

β-Glucans are frequently included in the diet of healthy common carp *Cyprinus carpio* as a pre-emptive measure for combatting disease. In order to study the effect this has on the relationship between the gut bacteria and host immune response, carp were maintained on either a β-glucan free diet or feed containing 0.1% MacroGard, a β-1/3, 1/6-glucan, for up to 7 weeks and analysis of innate immune gene expression and molecular analysis of the gut bacteria was performed. The data reveals feeding of MacroGard to healthy carp does not induce bactericidal innate immune gene expression in the gut but does appear to alter bacterial species richness that did not have a negative effect on overall health. Analysis of innate immune gene expression within the upper midgut revealed that there were significant changes over time in the expression of Interleukin (*il*)-*1β*, inducible nitric oxide synthase (*inos*), mucin (*muc2*) and C-reactive protein (*crp2*). Diet did not affect the number of copies of the bacterial *16s rDNA* gene in the gut, used as a as a measure of total bacteria population size. However, PCR-denaturing gradient gel electrophoresis (DGGE) analysis revealed a shift in bacterial species richness with MacroGard feeding. Bactericidal immune gene expression of *crp2*, *muc2* and *il-1β* was weakly correlated with gut bacteria population size indicating a potentially limited role of these genes in interacting with the gut bacteria in healthy carp in order to maintain gut homeostatic conditions. These findings highlight the importance of considering both host immunity and the microbiome together in order to fully elucidate the effeect of immunomodulants, such as β-glucans, upon gut health.

KEYWORDS

β-glucan, common carp, *Cyprinus carpio*, gut microbiome, gut health, innate immune gene expression

1 | INTRODUCTION

As the world's population continues to increase, so does the demand upon fisheries and aquaculture to provide food for human consumption (FAO, 2016). The high stocking densities applied in many aquaculture systems risk rapid spread of infections and disease that can result in significant economic losses (Miest *et al*., 2012). Increased concerns about food safety for human consumption (Wang *et al*., 2017) and introduction of legislative policies limit the products available to maintain healthy fish stocks. It is imperative that alternatives to medicines are found to reduce disease outbreaks in aquaculture. The concept of preventing disease outbreaks rather than treating them is well established in both human and veterinary medicine (Falco *et al*., 2012) and there are multiple approaches to the improvement of health that can be easily incorporated into diet. Immunomodulants can be added to the diet in order to modulate the innate immune response and enhance an organism's innate resistance to infections (Dalmo & Bogwald, 2008; Falco *et al*., 2012). Alternatively, the gut microbial population, which aids in the development of the immune system and provides an additional barrier against invading pathogens, can be modulated through the application of biotics (Merrifield *et al*., 2010; Nayak, 2010a).

β-Glucans are immunomodulants that are commonly used in aquaculture to improve health (Selvaraj *et al*., 2005; Dalmo & Bogwald, 2008; Dawood *et al*., 2018, 2019). Increased resistance against infection has been shown following application of β-glucans in common carp *Cyprinus carpio* L. 1758, Nile tilapia *Oreochromis niloticus*(L. 1758), Indian major carp *Labeo catla* (Hamilton 1822), large yellow croaker *Larimichthys crocea* (Richardson 1846), roho labeo *Labeo rohita* (Hamilton 1822) fingerlings and striped catfish *Pangasianodon hypophthalmus* (Sauvage 1878) (Misra *et al*., 2006; Selvaraj *et al*., 2005; Selvaraj *et al*., 2006; Ai *et al*., 2007; Kamilya *et al*., 2008; El-Boshy *et al*., 2010; Pionnier *et al*., 2013; Sirimanapong *et al*., 2015). Indeed, β-glucans have been shown to affect immunity in fish. For example red sea bream *Pagrus major* (Temminck & Schlegel 1843) and carp display altered immune activity in skin mucus after application of β-glucan (Dawood *et al*., 2017a; 2017b, Przybylska-Diaz *et al*., 2013), cytokine gene expression in the spleen of rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) presents dose dependent changes after β-glucan feeding (Douxfils *et al*., 2017) and carp have shown an increase in the number of immune cells in blood (Selvaraj *et al*.,2005).

Feeding carp β-glucan under disease-free conditions has been shown to significantly affect the expression of certain innate immune genes in the gut, including: tumour necrosis factor-α, interleukin 10 (*il*-10; Falco *et al*., 2012), C-reactive protein (*crp*), complement pathway gene *c1rs* (Pionnier *et al*., 2013) and inducible nitric oxide synthase (*inos*; Miest *et al*., 2012). Other immune genes, however, appear to be unaffected by feeding with β-glucan, for example mucin 2 (*muc2* ; van der Marel *et al*., 2012), *il-1β* (Falco *et al*., 2012) and several genes within the complement pathways (Pionnier *et al*., 2013). Although β-glucans are not

classed as prebiotics (Merrifield *et al*., 2010), they have been shown to influence bacterial species richness and diversity within the gut. The bacteria population in the gut of both carp and sea bass *Dicentrarchus labrax* (L. 1758) is altered after feeding diets containing β-glucan at 1% or higher and 0.1% w/w respectively (Kuhlwein *et al*., 2013; Jung-Schroers *et al*., 2016). Similarly, European turbot larvae *Scophthalmus maximus* (L. 1758) and Atlantic cod larvae *Gadhus morhua* L. 1758 (Skjermo *et al*., 2006; Miest *et al*., 2016) have an altered gut microbiome when fed β-glucan enriched rotifers in comparison with control-fed larvae. Increased amounts of β-glucan in the diet has additionally been shown to have a negative effect on the gut bacteria population in some fish species. β-glucan concentration correlates negatively with the quantity of *Vibrio* sp. in pompano fish *Trachinotus ovatus* (L. 1758) (Do Huu *et al*., 2016) and for tinfoil barb *Barbonymus schwanenfeldii* (Bleeker 1854) inclusion of β-glucan in the diet at 1% resulted in reduced bacterial diversity in the gut compared with those fed at 0.1% (Jung-Schroers *et al*., 2019). In contrast, β-glucan did not impact species diversity of either allochthonous or autochthonous microbiota populations in the gut of Nile tilapia (Ran *et al*., 2015). Thus, β-glucans represent a mechanism capable of both directly and indirectly affecting fish health and survival by modulation of the immune response and gut bacterial population.

Whilst there is a wealth of literature that considers the effect of β-glucans upon immune responses and gut bacteria populations, the majority consider these components separately (Dalmo & Bogwald, 2008; Merrifield *et al*., 2010; Rombout *et al*., 2011). Here, we measure the effect of the oral β-glucan supplement MacroGard (Orffa; www.orffa.com) upon both the expression of bactericidal innate immune genes within the gut of carp and the

associated gut bacteria population. We also examine the relationship between the immune response of the fish and the bacteria components of gut health.

2 | MATERIALS AND METHODS

Care and the use of experimental animals were undertaken under UK Home Office licence (PPL: 4003532).

2.1 | Fish

Common carp obtained from Hampton Spring Fisheries (www.fisheries.co.uk/hamptonsprings/index.htm) were maintained in individual recirculation tanks (15 fish per tank) at Keele University under a 12:12 h light/dark cycle and maintained at 15°C. Tanks were 225 l volume with Eheim 2227 filter systems (www.eheim.com) and external water chilling units. Carp were fed a control diet lacking β-glucan produced by Tetra GmbH (www.tetra-fish.com), with details of the formulation being found in Falco *et al*. (2012), at a rate of 1% body mass per day for 6 months. Fish had an initial mean (± SD) mass of 56.5g ±13.5g at the start of the trial.

2.2 | Diet formulation and feeding

Two diets were produced by Tetra as a pellet feed; a control diet without β-glucan and a β-glucan supplemented diet containing 0.1% w/w MacroGard, a β-glucan source, as part of the wheat starch component of the diet (Falco *et al*., 2012). Carp were fed twice daily at 1% body mass per day. Three tanks (15 carp per tank) were maintained on a control diet lacking MacroGard and a further 3 tanks (15 carp per tank) were fed on a diet containing 0.1% MacroGard for up to 7 weeks.

2.3 | Sampling

Three carp per tank at each time point (9 fish per diet) were euthanised in 0.02% 2-phenoxyethanol (Sigma Aldrich; www.sigmaaldrich.com), the intestine removed aseptically and samples of the upper midgut (Jung-Schroers *et al*., 2016), with and without faecal matter, were taken and stored in RNA later at –80°C. Samples were taken from fish fed on either 0% (feed lacking MacroGard) or 0.1% MacroGard before experimentation and then at 7, 21, 35 and 49 days of feeding, whereby day 1 represents commencement of feeding with the MacroGard supplemented diet.

2.4 | Analysis of mRNA gene expression

2.4.1 | RNA isolation and cDNA synthesis

RNA was isolated from gut wall samples using an RNeasy kit (Qiagen; www.qiagen.com) following the protocol optimised for bacterial isolation as per the manufacturer's instructions. RNA samples were suspended in AE buffer (10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0; Qiagen) and stored at –80°C until use. Samples were then treated with DNase (Promega; www.promega.com) before 0.5µg was transcribed to cDNA using Moloney murine leukaemia virus reverse transcriptase and random hexamers (Invitrogen; www.invitrogen), both according to the manufacturer's instructions, using a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems; www.applied-biosystems.com). Complementary (c)DNA was stored in diethylpyrocarbonate (DEPC)-treated water (Sigma Aldrich) at –20°C until further use.

2.4.2 | rt-qPCR analysis

Relative and absolute quantification of gene expression was performed using real time quantitative (rt-q)PCR (Adamek *et al*., 2013; Larionov *et al*. 2005; Livak & Schmittgen, 2001; Weltzien *et al*., 2005). Reactions were performed using SensiFast SYBR HiROX master mix (Bioline; www.bioline.com) in a total volume of 20 µl with primers (Eurofins; www.eurofins.com) used at a final concentration of 0.2µM (Table 1). Two µl of 10x diluted cDNA was utilised as a template in each reaction. Analyses of carp genes were performed using an ABI Prism 9000 sequence detection system (Applied Biosystems) and analysis of bacteria *16s* rDNA expression was carried out using an Mx3000P qPCR System (Agilent; www.agilent.com). The following thermal profile was used for all PCRs: initial denaturation at 95°C for 2 min, 40 cycles of 95°C for 5 s, 62°C for 30 s. The following carp innate

immune genes were analysed: antimicrobial peptides; liver expressed antimicrobial peptide 2 (Leap2), hepcidin antimicrobial peptide 1 (Hamp1), apolipoprotein A-1 (ApoA1); complement proteins including markers for the three different pathways: *c3*, *bf/c2*, *c1rs*, *masp2*; inflammatory cytokine expression: interleukin 1β (*il-1β*), tumor necrosis factor α isoforms 1 and 2 (*tnfα*); c-reactive protein isoform 2 (*crp2*); mucin 2 (*muc2*); inducible nitric oxide synthase (*inos*). Quantification of these genes was performed using a standard (Larionov *et al*. 2005) built from a pool of all cDNA samples (Weltzien *et al*., 2005) subject to a 1:3 serial dilution analysed in tandem with individual samples. For bacterial *16s rDNA* expression (total *16s* rDNA expression and genus specific *16s* rDNA assays for *Aeromonas* sp., *Pseudomonas* sp., *Flavobacterium* sp., *Streptococcus* sp. and *Vibrio* sp.) standards using recombinant plasmids were generated as described in Adamek *et al*. (2013) and subject to a 1:10 serial dilution. Subsequent normalisation of rt-qPCR data for all target genes was performed against the carp *40s* housekeeping gene, as outlined in Adamek *et al*. (2013). Additionally, genus specific *16s* rDNA assays were calculated as percentage of total *16s* rDNA copy number.

2.5 | PCR-DGGE analysis of bacterial species richness

In order to analyse the gut bacteria population, genomic DNA from gut samples (wall plus faecal matter) was isolated using the QIAamp DNA Mini kit following the protocol optimised for bacteria isolation, as per the manufacturer's instructions (Qiagen). PCRs were performed under sterile conditions in a total volume of 25 µl in an Eppendorf Mastercycler

gradient thermocycler (Eppendorf; www.eppendorf.com), as described in Jung-Schroers *et al.* (2016) using 5µl of genDNA. Successful amplification was confirmed by visualising samples on a 1% agarose gel (50 ml 1X TBE buffer, 2.5 µl Roti-Safe gel stain, 105 V for 20 min) before being pooled by treatment–time point. Pooled samples were analysed at 60°C for 820 minutes using a Biostep TV400 denaturing gradient gel electrophoresis (DGGE) vertical electrophoresis system comprising an 8% polyacrylamide gel containing a 40–60% gradient of denaturant (100% denaturant contained 7M urea and 40% w/v formamide). Band patterns were visualised by staining gels with 0.01% w/v SYBRgold (Invitrogen) for 30 min and images obtained using a Nikon D3200 Digital SLR (Nikon; www.nikon.com) with a 55–300mm VR lens (Nikon) and a minimum exposure time of 10 s.

2.6 | Statistical analysis

2.6.1 | Gene expression

Minitab 14 (Minitab; www.minitab.com), SPSS statistics 24 (IBM; www.ibm.com) and Excel (Microsoft; www.products.office.com) were used for statistical analysis. Statistical outliers were identified using Grubbs' outliers test. *Z* scores for each gene analysed were calculated as follows: $Z = Y_1 - \bar{Y})\sigma^{-1}$, where Y_1 is an individual value, $\bar{Y}$ is the mean and σ is the standard deviation. Values > 3.18 were deemed as outliers and removed before further analysis ($n = 90$, $\alpha = 0.05$).

In order to compare gene expression across multiple time points and feed regimes, each gene was analysed using a two-way nested ANOVA with *post hoc* analysis performed using Tukey's test. Normality of the residuals and homoscedasticity were analysed using the Anderson-Darling test and Levene's test respectively. Where data did not meet the assumptions of an ANOVA, it was transformed using a Box-Cox transformation before analysis (Supporting Information Table S1). In cases where data still did not satisfy the assumptions of an ANOVA post transformation, a two-way Scheirer-Ray-Hare test, a non-parametric equivalent, was performed (Dytham, 2003).

Multiple approaches were taken to determine the effect of MacroGard on the relationship between bacterial *16s* expression and carp innate immune gene expression. Pearson's correlation was initially performed and rating as to the strength of this correlation assigned as described by Fowler *et al*. (1998) with a coefficient of 0.00–0.19 being a very weak correlation, 0.20–0.39 being a weak correlation, 0.40–0.69 being a modest correlation, 0.70–0.89 being a strong correlation and 0.90–1.00 being a very strong correlation. Where data distributions permitted, an ANCOVA was used to test for an effect of diet (fixed-level factor) and bacterial *16s* expression (continuous variable) on carp innate immune gene expression. An interaction term to test whether diet affected the relationship between the bacterial *16s* gene and carp innate immune gene expression was also included.

2.6.2 | Multivariate analysis of bacteria species richness

Analysis was performed using Excel and SPSS statistics 24. PCR-DGGE band patterns were analysed in a binary format; *i.e.*, presence–absence of a band was arbitrarily labelled from the top (40% denaturant) to the bottom (60% denaturant) of the gel with all bands being considered irrespective of sample. The distance between different samples was calculated using the Bray-Curtis dissimilarity index (Podani, 2000) and plotted on a non-metric multidimensional scaling (nMDS) ordination (Kuhlwein *et al*., 2013; Attramadal *et al*., 2014).

3 | RESULTS

3.1 | The separate effect of orally applied Macrogard on carp immune parameters and the gut bacteria population

3.1.1 | Changes in innate immune gene expression were seen over time and not due to feeding with MacroGard

Over the course of the 7 week trial, MacroGard feeding did not have any significant effect upon the expression of any of the bactericidal innate immune genes analysed within the gut except *il-1β* (Supporting Information Table S2 for all details and results of statistical analyses). Of the thirteen immune genes analysed, the expression levels of 5 (*tnfα1* and *2*, *c1rs*, *bf/c2* and *masp2*) were not high enough for quantitative analysis. Whilst expression of *inos*, *muc2* and *crp2* were not affected by diet, significant differences over time were

observed (Figure 1). Both *muc2* and *crp2* gene expression was lower 35 days after the start of the trial relative to all other time points ($P < 0.001$ for both genes). Inversely, expression of *inos* varied across the course of the trial and, even with the inclusion of a mathematical outlier (Supporting Information Table S3) at 49 days of feeding, expression at this time point was still significantly lower than expression after 7 and 21 days ($P < 0.01$ and $P < 0.05$, respectively). *il-1β* Expression was highest at the very start of the trial (0 days) and was significantly lower after 7, 21 and 49 days ($P < 0.05$). *il-1β* Was the only immune gene analysed to be significantly affected by feeding with MacroGard ($P < 0.05$) with fish on the β-glucan free diet showing an overall higher level of expression than those fed with MacroGard.

3.1.2 | Overall bacteria numbers within the gut change over time and diet influences species richness

Similar to innate immune gene expression, total bacterial *16s* expression, as a count of bacterial numbers within the gut, revealed no significant differences as a result of diet ($P > 0.05$). There were, however, significant differences in the total *16s* copy number over time. Expression after 21 days was significantly lower ($P < 0.05$) than at the start of the trial and expression at 35 days was significantly higher ($P < 0.05$) than that seen at days 7, 21 and 49 (Figure 2a). Analysis of the *16s* gene from individual genera revealed that neither *Vibrio* sp. and nor *Streptococcus* sp. were detected in any fish analysed. In contrast, *Aeromonas*, *Pseudomonas* and *Flavobacterium* were present at the start and end of the trial (days 0 and

49) with no significant difference in abundance due to diet. There was no difference in the relative abundance of *Aeromonas* ($P > 0.05$) however, for both *Pseudomonas* and *Flavobacterium*, there were significantly more copies of the *16s* gene relative to the total number of *16s rDNA* sequences ($P < 0.05$ and $P < 0.001$, respectively).

In contrast to the quantitative analysis of the gut bacteria population, qualitative analysis of species richness using total number of operational taxonomic units (OTU) separated using PCR-DGGE revealed a significant effect of diet with fewer OTUs in carp fed with the 0.l% MacroGard diet at all time points compared with those on the control diet ($P < 0.01$; Figure 2b). The highest level of species richness was seen 7 days into the trial and the lowest level at 35 days (Figure 2c). This is the inverse of total *16s* expression, however further analysis revealed the absence of any significant correlation between species richness and bacteria population size ($r = 0.285$, $P > 0.05$).

Where analysis of dissimilarity of gut bacteria populations based upon presence/absence of bands (Figure 3) revealed no separation of samples as a result of time or diet overall, comparison within each time point indicated that the inclusion of MacroGard increased dissimilarity between samples after 7, 35 and 49 days of feeding.

3.2 | Correlations between gut immunity and the bacterial population based upon gene expression

During analysis of the relationships between estimations of number of bacteria in the gut and carp bactericidal immune genes, *il-1β*, *crp2* and *muc2* were all shown to be significantly

correlated against total *16s* gene expression using both methods of analysis (*il-1β*: positive modest, $P < 0.001$, *crp2*: negative modest, $P < 0.001$, *muc2* : negative weak, $P < 0.01$ (Figure 4 and Supporting Information Tables S4 and S5). Initial analysis using Pearson's correlation indicated feeding with MacroGard may have reduced the strength–significance of the relationships between the bacteria and *crp2* (0% MacroGard: $r = –0.463$, $P < 0.01$; 0.1% MacroGard: $r = –0.367$, $P < 0.01$) and *muc2* (0% MacroGard: $r = –0.517$, $P < 0.01$; 0.1% MacroGard: $r = –0.104$, $P > 0.05$). Further analysis using ANCOVA, however, did not support this hypothesis ($F_{1,82} = 0.03$ and $F_{1,84} = 0.53$ respectively, $P > 0.05$). All other immune genes analysed did not show a significant relationship when compared with total bacterial *16s* expression (Supporting Information Tables S4 and S5).

Analysis of the relationship between bactericidal innate immune genes and the individual bacteria genera studied revealed no obvious pattern to any of the significant correlations observed. Pearson's correlation analysis identified correlation between *Flavobacterium* and *il-1β* and *muc2* expression in carp fed with the 0% MacroGard diet only ($r = –0.626$, $P < 0.01$; $r = –0.512$, $P < 0.01$, respectively) with no correlation seen in carp fed with the 0.1% MacroGard diet. These differences between diets were not, however, significant ($P > 0.05$). An overall correlation between the relative abundance of *Aeromonas* and *il-1β* was also seen ($P < 0.05$) with, similarly, no effect of diet. No correlations were identified using either method of analysis between immune gene expression and the relative abundance of *Pseudomonas* (Supporting Information Tables S4 and S5).

4 | DISCUSSION

The use of immunomodulants within fish diets to improve health and combat disease is becoming more important as a procedure to reduce the reliance upon treatments such as antibiotics (Dalmo & Bogwald, 2008, Merrifield *et al*., 2010). β-Glucans, which have been studied extensively due to their immunomodulatory properties (Dalmo & Bogwald, 2008), have been shown to reduce mortality in multiple fish species during bacterial infections (Selvaraj *et al*., 2005; Kumari and Sahoo, 2006; Selvaraj *et al*., 2006; Welker *et al*., 2007; Sirimanapong *et al*., 2015) and increase resistance against viral infection (Sealey *et al*., 2008). It is, however, equally important to determine that feeding with β-glucans does not negatively affect health, particularly gut health, under disease free conditions. Whilst the effect of feeding β-glucan upon carp immune status (Miest *et al*., 2012; Pionnier *et al*., 2013; Kuhlwein *et al*., 2014; Pionnier *et al*., 2014*b*) and gut bacteria (Kuhlwein *et al*., 2013; Jung-Schroers *et al*., 2016) under disease-free conditions has been previously considered separately, the relationship and interaction between these two significant parameters in gut health has not been investigated.

Here, the differences in bacterial species richness observed with the inclusion of β-glucan within the diet supports the observations made by Jung-Schroers *et al*. (2016) who fed MacroGard at a higher concentration (1% w/w) within the diet and observed differences in bacteria species richness within the gut of carp. Kuhlwein *et al*. (2013) noted a decrease in culturable aerobic heterotrophic autochthonous bacteria in the gut of carp which suggests that variation in the bacterial population size over time seen in our study could occur naturally irrespective of diet. In contrast, bacteria species diversity is not influenced by β-glucan

enrichment of artemia in turbot larvae (Miest *et al*., 2016) and bacteria population in the gut of cod larvae during colonisation was more stable when algal glucans were included within the diet (Skjermo *et al*. 2006). This suggests the reponse of individual fish species may be an element to consider when studying the effect dietary β-glucan upon the gut bacteria population.

Although there are only limited examples whereby correlation analysis between immune parameters and bacteria numbers have been performed within ichthyology, some similarities can be found. Adamek *et al*. (2013) showed a significant modest positive correlation between total bacteria *16s* expression and *il-1β* expression in the skin of carp during cyprinid herpesvirus 3 (CyHV-3) infection and *il-1β* expression also positively correlates with parasite load in rainbow trout (Gorgoglione *et al*., 2013). Since *il-1β* serves as an inflammatory cytokine, it is possible that it would be upregulated during infection, however, the continued correlation under disease free conditions could indicate a role in maintaining the size of the gut bacteria population. Unlike several previously published *in vitro* trials in carp (Biswas *et al*., 2012; Pietretti *et al*., 2013; Kareem *et al*., 2018), here β-glucan significantly reduced overall expression of *il-1β* in the gut. In contrast to *in vitro* trials, the effect of β-glucan upon inflammatory cytokine gene expression *in vivo* has produced inconsistent results (Djordjevic *et al*., 2009; Falco *et al*., 2012; Medina-Gali *et al*., 2018). In our study however, there was no indication that the downregulation of *il-1β* expression as measured by rt-qPCR had a detrimental effect upon the overall health of the fish. Indeed, *il-1β* expression has been shown to be lower in carp after β-glucan feeding during both *A.*

salmonicida infection (Falco *et al*., 2012) and *A. hydrophila* infection (Syakuri *et al*. 2014) relative to infected carp maintained on a β-glucan free diet.

It should be noted that here, there was a significant negative correlation between the gut bacteria population size and *crp2*, but this may be associated with the maintenance of homeostatic balance. Some of the immune genes studied are also known to play roles within maintaining homeostatic balance including the acute phase protein Crp (Castro-Osses *et al*., 2017). It may be that changes in the homeostatic balance resulted in the significant decrease in *crp2* expression seen at 35 days. An increase in acute phase proteins, including Crp, can be used as a marker of intestinal inflammation in humans (Tibble & Bjarnason, 2001), which can lead to a state of dysbiosis within the gut. Lower *crp2* levels coinciding with high bacterial counts may represent a mechanism by which the gut limits unnecessary immune responses in the absence of infection.

The complement system also plays a role in homeostasis and Kolev and Kemper (2017) suggested different roles for serum and intracellular complement in mammals as immunoprotective and homeostatic respectively. If it is assumed gene expression of *c3* in the gut in healthy carp is indicative of a homeostatic profile, feeding with MacroGard does not appear to alter its role within this system.

Our observations on the expression of *muc2* supports the work of van der Marel *et al*. (2012), which shows that orally applied β-glucan does not affect mucin expression within the gut of carp. Furthermore, secretion of mucus from goblet cells within the gut epithelium have been previously shown to be unaffected by β-glucan feeding in disease-free carp (Kuhlwein *et al*., 2014). However, an upregulation in expression of *muc2* after β-glucan feeding has been

shown in chickens (Cox *et al*., 2010) and pigs (Smith *et al*., 2011) which could indicate differences in mucosal tolerance in the gut environments between aquatic and terrestrial vertebrates.

Many of the data presented here with regard to immune gene expression contradict several previously published studies. The analysed complement genes, inflammatory cytokines, mucin and *crp* all show different expression patterns than have been previously reported in β-glucan fed carp (Falco *et al*., 2012; van der Marel *et al*., 2012; Pionnier *et al.*, 2013, 2014*b*). It has also been shown that different breeding lines of fish species, including carp, have significantly different immune responses under disease free conditions as well as against bacterial and viral pathogens (Adamek *et al*., 2014; Marancik *et al*., 2014; Nath *et al*., 2014; Kobis *et al*., 2015). It is possible that the use of different lines of carp is responsible for the differing expression patterns noted between studies.

In conclusion, this β-glucan feeding trial describes correlations between bactericidal innate immune genes and the gut bacteria of healthy carp. The data presented indicate that feeding of MacroGard to healthy carp does not affect bactericidal innate immune gene expression in the gut, but does alter bacterial species richness. This does not, however, indicate a state of dysbiosis within the gut, but does imply a positive outcome as maintenance of the homeostatic balance within the gut is important for overall health. Indeed, in humans, when the immune response is triggered without the presence of pathogens, it is considered to be a disease state; *e.g*., inflammatory bowel diseases (Blander *et al*., 2017). Although limited, there are significant correlations between the expression of carp immune genes and the overall size of the bacteria population within the gut and there is no effect of feeding with

MacroGard upon the bactericidal genes studied. Overall, these findings highlight that, similar to mammalian models, it is important to consider both host immunity and the associated bacteria and indeed overall microbiome, as a whole rather than as two separate entities in order to fully elucidate the impact of immunomodulants, such as β-glucans, upon gut health.

CONTRIBUTIONS

S.J.H. and D.H. were responsible for experimental design within this manuscript. All data generation and analysis were performed by S.J.H., with M.A. and D.S. hosting S.J.H. in their laboratories to complete analysis of the bacteria populations. D.P.B. and D.R.H. were both involved in the selection of appropriate statistical analyses and interpretation for the data presented. All authors contributed to the production of the manuscript.

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FIGURE 1 Mean (± SE; *n* = 17–18) mRNA expression (the copy number of each gene per copy of the carp housekeeping gene *40s*) of the bactericidal innate immune genes in the midgut of *Cyprinus carpio* during a 7 week feeding trial. (a) Expression of C-reactive protein isoform 2 (*crp2*) and (b) mucin 2 (*muc*2) showed no effect of diet (with or without 0.1% MacroGard w/w). (c) Interleukin 1β (*il-1β*) showed significant differences over time and (d) between groups fed with or without 0.1% MacroGard w/w. (e) Box plot (—, median; □, interquartile range; | range; outliers excluded) showing expression of inducible nitric oxide synthase (*inos*). Time points that share letters are significantly different. *, $P < 0.05$; **, $P < 0.01$

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1 Change A, B etc to (a), (b) etc

2 Delete labelling and numerical values from x-axis of (a).

3 Replace x-axis label of (b), (c) and (e) with Time (days).

4 Change y-axis labels to individual panels to lower case italics (as per legend)

5 Change 40S to *40s*.

FIGURE 2 (a) Mean (± SE; *n* = 17–18) mRNA expression (the copy number of each gene per copy of the carp housekeeping gene *40s*) of the bacterial *16s* rDNA gene in the midgut of *Cyprinus carpio* fed two different diets: with and without 0.1% MacroGard w/w. No significant differences ($P > 0.05$) were seen between the two diet groups so data is shown by time point only. (b) Bacterial species richness within the gut of *C. carpio* over time and (c) with and without 0.1% MacroGard w/w, as measured by average number of bands separated on a 40–60% denaturant gradient gel. A single PCR of the *16s* rDNA gene was performed per fish with 3 fish being pooled by (b) time point, (c) tank before analysis on the denaturing

gradient gel electrophoresis (DGGE) gel was performed. Where a pool was analysed on multiple gels, an average number of bands was taken. $n = 3$ pools per diet per time point. Time points that share letters are significantly different. *, $P < 0.05$; **, $P < 0.01$

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1 Change A, B etc to (a), (b) etc

2 Delete labelling and numerical values from x-axis of (a).

3 Replace x-axis label of (b) with Time (days).

4 Change y-axis labels of (b), (c) to single centred label

5 Change 16S and 40S to *16s* and *40s*.

FIGURE 3 (a) Non metric multidimensional scaling (nMDS) ordination (stress: 0.0902) of Bray-Curtis dissimilarities looking at the differences in bacterial species richness in the gut of *Cyprinus carpio* based on PCR- denaturing gradient gel electrophoresis (DGGE) band patterns of carp that had been fed with either a 0% or 0.1% MacroGard diet.
A single PCR of the *16s* rDNA gene was performed per fish with 3 fish being pooled by time point or feeding regime before analysis on the DGGE gel was performed. Where a comparison between two band patterns was performed on multiple gels, an average dissimilarity was used to generate the nMDS ordination. (b) Ordinations highlighting the location of fish on the two different diets at each time point with arrows indicating the shift in position along Dimension 2. Number of days refers to period of time on 0.1% MacroGard diet with 0 days being the start date of the trial; *i.e.*, all fish had only been fed a non-MacroGard diet: ■, 0% MacroGard diet; ◆, 0.1% MacroGard. Time: ■, 0, 0 days ; ■, 0, 7 days; ■, 0, 21 days; ■, 0, 35 days; ■, 0, 49 days – yellow.

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1 Label single large panel (a) and composite lower panel (b)

FIGURE 4 Correlation analysis between total bacterial *16s* rDNA gene copy numbers as an approximation of total bacteria counts and bactericidal innate immune gene expression within the midgut of *Cyprinus carpio* during a 7 week feeding trial with (□) or without (O) 0.1% MacroGard w/w in the diet (n = 42–45 fish per diet). Expression of each gene is shown as copy number of target gene per copy of the carp housekeeping gene *40s*. Significant correlations were found when comparing total *16s* gene expression with: interleukin 1β (*il-1β*: 0% MacroGard $r = 0.445$, $P < 0.01$; 0.1% MacroGard $r = 0.423$, $P < 0.01$; ANCOVA, effect of diet on correlation $P > 0.05$); C-reactive protein isoform 2 (*crp2*: 0% MacroGard $r = –0.463$, $P < 0.01$; 0.1% MacroGard $r = –0.367$, $P < 0.05$; ANCOVA, effect of diet on correlation $P > 0.05$); mucin 2 (*muc*2: 0% MacroGard $r = –0.517$, $P < 0.001$, 0.1% MacroGard $r = –0.104$ $P > 0.05$; ANCOVA, effect of diet on correlation $P > 0.05$)

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1 Change A, B etc to (a), (b) etc

2 Delete numerical values from x-axis of top three rows

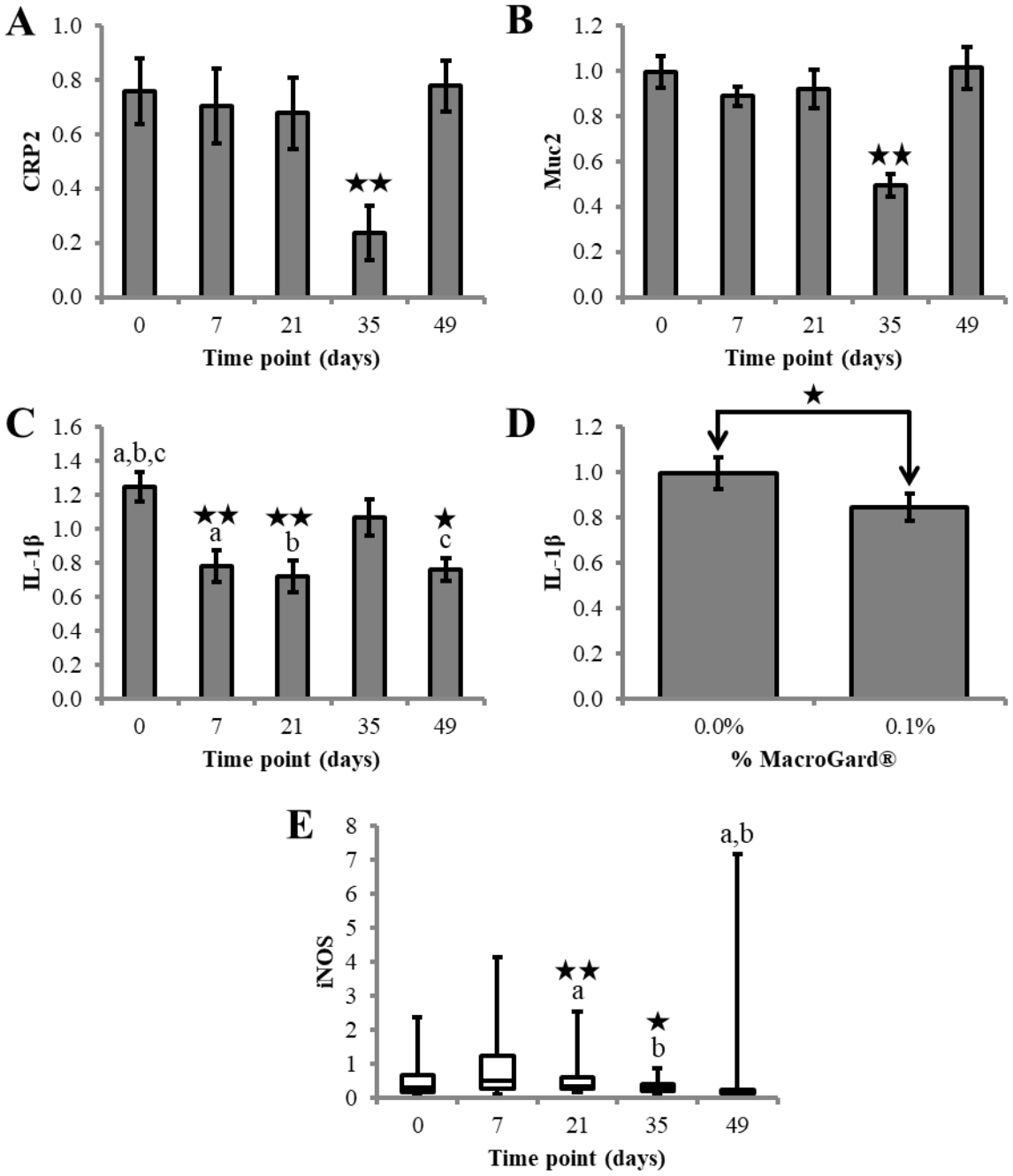
3 Change y-axis labels to individual panels to lower case italics (as per legend)

4 Change 16 S and 40S to *16s* and *40s*.

TABLE 1 A list of PCR primers used in assessing the Effect of β-1/3,1/6-glucan upon immune responses and bacteria in the gut of healthy *Cyprinus carpio*

Target	Sequence 5' → 3' (forward then reverse)	Reference
Total bacteria activity (qPCR) – *16s* rDNA	AGGATTAGATACCCTGGAGTCCA	Adamek *et al.*, 2013
	CATGCTCCACCGCTTGTGC	
Carp – 40S housekeeping gene	CCGTGGGTGACATCGTTACA	Huttenhuis *et al.*, 2006
	TCAGGACATTGAACCTCACTGTCT	
Aeromonas sp. activity – *16s* rDNA	GCGAAGGCGGCCCCCTGGACAAAGA	Adamek *et al.*, 2013
	CCACGTCTCAAGGACACAGCCTCCAAATC	
Carp – Apolipoprotein 1	CCATCTCCGCCTCCTTTC	Dietrich *et al.*, 2014
	ATGTGTTAGTGTGTGTGTGCTTC	
Carp – Complement pathway: *bf/c2*	CGGTCATGGGAAAAAGCATTGAGA	Forlenza *et al.*, 2009
	GATATCTTTAGCATTTGTCGCAG	
Carp – C-reactive protein isoform 2	GATGCTGCAGCATTTTTCAGTC	Falco *et al.*, 2012
	CTCCGCATCAAAGTTGCTCAAAT	
Carp – Complement pathway: C1rs	CAAGCCCATCTTGGCTCCTGG	Forlenza *et al.*, 2009
	GTCCAGATCAAGCGGGGACGT	
Carp – Complement pathway: *c3*	GGTTATCAAGGGGAGTTGAGCTAT	Forlenza *et al.*, 2009
	TGCTGCTTTGGGTGGATGGGT	
Flavobacterium sp. activity – *16s* rDNA	GGGATAGCCCAGAGAAATTTGGAT	Adamek *et al.*, 2013
	AGTCTTGGTAAGCCGTTACCTT	
Carp – Hepcidin antimicrobial peptide 1	TGGAGAGTGAGGCACACCAGGAG	
	TGCCAGGGGATTGGTTTG	
Carp – Interleukin 1β	AAGGAGGCCAGTGGCTCTGT	Huttenhuis *et al.*, 2006
	CCTGAAGAAGAGGAGGCTGTCA	
Carp – Inducible nitric oxidase	AACAGGTCTGAAAGGGAATCCA	Forlenza *et al.*, 2009
	CATTATCTCTCATGTCCAGAGTCTCTTCT	
Carp – Liver expressed antimicrobial peptide 2	GGATCGTGGGCACTAAACCTC	
	GCCTTTCCTGCATATTCCTGTC	
Carp – Complement pathway: *masp2*	CAAGCTGTCCAAGGTGATTG	Forlenza *et al.*, 2009
	AGCAGTGAGGACCCAGTTGT	
Carp – Mucin 2	TGACTGCCAAAGCCTCATTC	van der Marel *et al.*, 2012
	CCATTGACTACGACCTGTTTCTC	
Pseudomonas sp. activity – *16s* rDNA	TGCCTAGGAATCTGCCTGGTAGT	
	AATCCGACCTAGGCTCATCTGATAGCG	
Streptococcus sp. activity – *16s* rDNA	CGGTAACTAACCAGAAAGGGA	
	ATAAATCCGGACAACGCTCGRAGA	
Carp – Tumor necrosis factor α isoform 1	GAGCTTCACGAGGACTAATAGACAGT	Forlenza *et al.*, 2009
	CTGCGGTAAGGGCAGCAATC	
Carp – Tumor necrosis factor α isoform 2	CGGCACGAGGAGAAACCGAGC	Forlenza *et al.*, 2009
	CATCGTTGTGTCTGTTAGTAAGTTC	

Vibrio sp. activity – *16s* rDNA	GTTTGCCAGCGAGTAATGTC	
	TAGCTTGCTGCCCTCTGTATGCG	
PCR- denaturing gradient gel electrophoresis (DGGE)– *16s* rDNA	CGCCCGCCGCGCGCGGCGGCGGGCGGGGCGGGGGCACGGGGGG CCTACGGGAGGCAGCAG	Muyzer *et al.*, 1993
	ATMTCTACGCATTTCACCGCTAC	Steinum *et al.*, 2009



Number of copies of target gene per copy of 40S