Original Research

Pig

Mannan rich fraction from yeast modulates inflammatory responses in intestinal cells (HT-29) exposed to *Escherichia coli*

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Abstract

Mannan from yeast has been demonstrated to limit infection in animals susceptible to gastrointestinal infection, including pigs, poultry and cows, by blocking the mechanism by which gram-negative bacteria adhere to and invade the intestines. Enterotoxigenic *Escherichia coli* (ETEC) cause post weaning diarrhoea (PWD) which results in poor weight gain and potential death at great economic cost to the farmer. A mannan rich fraction (MRF) was assessed *in vitro* for its impact on ETEC infection of HT-29 intestinal cell line. Gene expression markers for inflammation ($TNF\alpha$ and $IL-1\beta$) and TLR4 ($TICAM-1$ and $LY96$) associated recognition of bacteria were significantly elevated following exposure to *E. coli* alone, but not in combination with MRF compared to the control. HT-29 cells exposed to MRF alone demonstrated significantly reduced expression of immune signalling genes $IRAK1$, $IRF7$ and JUN when compared to the control. HT-29 cell protein abundance for $TNF\alpha$ and TLR4 associated proteins were significantly increased in response to *E. coli* exposure alone while no significant change was observed for MRF treatment with *E. coli* infection. *E. coli* adhesion to HT-29 cells was significantly decreased with addition of MRF compared to *E. coli* infection alone. The action of MRF demonstrated its potential capacity to limit infection on an *in vitro* level through blocking bacterial interaction with the intestines that leads to infection as marked by a reduction in proinflammatory responses. MRF on its own demonstrated potential anti-inflammatory effects on intestinal cells with the reduction of proinflammatory responses observed.

Keywords: Enterotoxigenic *Escherichia coli*: HT-29: Post weaning diarrhea: Mannan rich fraction: Inflammatory

(Received 23 January 2019 – Accepted 14 May 2019)

Introduction

The growing issue of microbial resistance and the lack of new antimicrobials entering the market has led to concern regarding the over use and application of antibiotics in the feed industry and subsequent effects on human health. Antibiotics have been given at sub-therapeutic levels as in-feed supplements to a broad spectrum of livestock (Li, 2017), most notably in pigs and poultry. Antibiotics and other antimicrobials are mainly used prophylactically in feed against gastrointestinal diseases such as diarrhoea which reduces mortality (Thacker, 2013). As a result, the food and drug administration

(FDA) in the USA made recommendations to limit medically important antimicrobials to therapeutic use in animals and deemed the use of antimicrobials for in feed purposes to improve animal weight gain and feed efficiency as an inappropriate application (FDA-CMV, 2012). Subsequently the Preservation of Antibiotics for Medical Treatment Act, 2017 phased out the use of antibiotic use in food-producing animals in the USA (Slaughter, 2017). Under the act, medically important antibiotics cannot be administered in feed at sub-therapeutic levels to food-producing animals for disease control. However, if there is a significant risk of disease

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or infection transmission from the farm premises to the animal, it is permissible to use antibiotics. This legislation change followed the prohibition of antibiotic use as a growth promotor in animal feed by the European union (European-Commission, 2005) due to the risk of resistance being passed from livestock through the food chain to human consumers (Phillips *et al.*, 2003).

The World Health Organisation covering South East Asia region (SEAR), is a region that uses intensive farming methods but lack regulations and restrictions on antibiotic use in food producing animals that is seen in the EU and USA. Several challenges and requirements for implementing policy changes need to be addressed to combat antimicrobial resistance in food producing animals in each of the SEAR countries (Goutard *et al.*, 2017). The increased restrictions and regulations on antibiotic usage has exacerbated farmers concerns on issues of controlling livestock infections and poor feed conversions (Rosen, 2004) which have a direct impact on farmers profitability and animal health (Coffman *et al.*, 1999; Cervantes, 2015).

The demand for an alternative to counter infection in livestock and in humans has led to increased research and application of alternative methods to control pathogenic bacteria. This has come in the form of antimicrobials (Guani-Guerra *et al.*, 2010; Leonard *et al.*, 2012), feed enzymes that influence microflora (Halas and Nochta, 2012), probiotics (Anadón *et al.*, 2006) and prebiotics including mannan oligosaccharides (MOS; Spring *et al.*, 2015) and fructooligosaccharides (FOS; Ricke, 2015). Prebiotics, such as MOS products derived from *Saccharomyces cerevisiae*, take advantage of the means by which pathogenic bacteria invade the host *via* the gut through binding to mannan molecules that coat the intestines (Halas and Nochta, 2012).

Certain strains of ETEC are responsible for causing food borne diseases in humans and enteric diseases such as diarrhoea and dysentery in animals. These pathogenic bacteria bind to intestinal epithelial cells through external projections from their cell wall known as pili or fimbriae. *E. coli* expressing type-1 fimbriae have a specific α -D-mannan binding tip which facilitates their adhesion and invasion of epithelial cells, causing disease (Ofek *et al.*, 1977; Hartmann *et al.*, 2012). The mannoproteins isolated from the yeast competitively bind to the type-1 fimbriae thus preventing the ETEC from binding to mannose residues on cells. The *E. coli* strain used in this study possesses H fimbriated mannose binding regions (Fein, 1981; Kennan and Monckton, 1990), that facilitates

binding to mannose residues present on human and pig intestinal cells. Previous findings demonstrated the ability of the *E. coli* strain used here to invade intestinal porcine epithelial cells (IPEC; Horgan *et al.*, 2018).

Humans and pigs are infected in the same manner by similar enteric microbes which makes this study examining the control of infection applicable to both species (Yuan *et al.*, 1996; Steele *et al.*, 2010; Brady *et al.*, 2011) as symptoms for infected pigs are very similar to humans (Meurens *et al.*, 2011). Human and pig intestines are structurally conserved, which enables strong correlations to be derived between pig and human studies *in vivo* (Vitovec and Koudela, 1992; Nedrud, 1999; Zhang *et al.*, 2013) and *in vitro* (Geens and Niewold, 2011; Brosnahan and Brown, 2012). Pigs and humans share similar microflora colonisation of the large and small intestines (Miller and Ullrey, 1987; Pang *et al.*, 2017).

Piglets have a greater susceptibility to severe infections during their weaning phase, most notably in the form of PWD caused by *E. coli* infection of the intestines which features prominently in the first two weeks of weaning (Rhouma *et al.*, 2017). Infection can result in poor growth due to dehydration and a lack of food absorption that ultimately can lead to death, which is costly to pig producers (Amezcuca *et al.*, 2002; Fairbrother *et al.*, 2005). Weaned piglets have long been administered antibiotic-supplemented feed to control harmful intestinal infections during such susceptible periods (Verstegen and Williams, 2002). In-feed antibiotics have an added effect of promoting animal growth (Cromwell, 2002). MOS has demonstrated substantial health benefits and has significantly improved piglet outcomes during their susceptible weaning period in the absence of conventional antibiotics (Smith *et al.*, 2002; White *et al.*, 2002; Burkey *et al.*, 2004; Castillo *et al.*, 2008; Liu *et al.*, 2008; Zhao *et al.*, 2012), without the risk of antimicrobial resistance (AMR) developing.

Feeding MRF derived from MOS has resulted in improved weight gain, feed conversion and intestinal health, while decreasing pathogen load and mortality rates (Hooze, 2004; Spring *et al.*, 2015) in piglets. MRF has similarly shown the ability to bind to and reduce intestinal colonisation by *E. coli* and *Salmonella spp.* in poultry (Biggs *et al.*, 2007; Luquetti *et al.*, 2012). A similar trend in AMR among humans, in the form of gastrointestinal infections, is a growing issue which may benefit from alternative measures such as prebiotic applications of MRF (Al Naiemi *et al.*, 2006; Monira *et al.*, 2017). MRF may offer similar effectiveness in humans against

gastrointestinal infections, due to the similar mode of infection seen in pigs and humans.

The work presented here aimed to determine the impact of MRF at an intestinal cell level through limiting ETEC infection *in vitro* using human colorectal adenocarcinoma cells (HT-29). Assessment of gene pathways associated with bacterial infection and resultant protein abundance changes was carried out following treatment of the cells with MRF and *E. coli* infection. The physical barrier impact MRF had on blocking infection in the intestinal cells was determined.

Materials and Methods

MRF reagent preparation

MRF was obtained from Alltech Inc. (Nicholasville, KY, USA) and an enzymatic digestion was performed on the MRF. Digestion was adapted from a three-step *in vitro* technique by Boisen and Fernandez (1997), including pepsin digestion carried out at pH 2 for 2 h at 39°C followed by a pancreatin digestion at pH 6.8 for 4 h at 39°C, using experimental procedures outlined by Meunier *et al.* (2008). Digests were filtered in 10kDa spin filters (Vivaspin® 20) to remove pepsin and pancreatin following adjustment to a final protein concentration of 16 mg/ml of MRF before application. The same concentration of MRF was applied to all experiments, which was a preoptimised concentration (Horgan *et al.*, 2018).

Mammalian cell culture

Undifferentiated HT-29 cells were grown in sterile Dulbecco's McCoys 5A media that was supplemented with 10% (v/v) foetal bovine serum (FBS) and 1% (v/v) L-glutamine, Gibco® (Grand Island, NY, USA). A stock of HT-29 cells was taken up from frozen in liquid nitrogen at passage 168. Cells were split using Dulbecco's trypsin-EDTA and were maintained at 37°C with a 5% CO₂ atmosphere. HT-29 cells were seeded onto 12 well cell culture plates at a density of 2 x 10⁵ cells per well in McCoys 5A supplemented media. Cells were left to attach to the surface overnight at 37°C in a 5% CO₂ atmosphere prior to use.

Escherichia coli strain: culture conditions

Type 1 fimbriated *E. coli* (O141: K85:H4) NCTC number 10674 (ACDP grade 2), a mannose sensitive strain known to infect swine, was isolated from swine oedema

and used for the duration of these experiments. Cells were grown in liquid nutrient broth (Avantor, Difco™ BD, 90002-660) at 37°C and 150 rpm overnight. Liquid cultures were plated on nutrient broth agar at 37°C overnight and stored at 4°C prior to sub-culturing, plates were stored for a maximum of 14 days. Fresh overnight *E. coli* cultures were sub-cultured and incubated for 2 h at 37°C to enable isolation of *E. coli* in the exponential growth phase. *E. coli* was washed in 10 mM phosphate buffered saline solution (PBS) by centrifugation at 8500 rpm. Optical densities of *E. coli* were measured using a UV spectrophotometer set at 650 nm and adjusted to an optical density (OD) of 0.2 (1 x 10⁸ CFU/ml).

Bacterial adhesion assay

A 12 well cell culture plate was seeded at 2 x 10⁵ HT-29 cells per well and left overnight for cells to adhere to the plate. The HT-29 cells were removed from the incubator the following day and media was replaced with 10% (v/v) FBS and 1% (v/v) L-glutamine supplemented CO₂ independent media containing 1x10⁸ CFU/ml *E. coli* cells with and without MRF or, for controls, MRF on its own in media (treatment control) or media alone (negative control). Following the addition of treatments to the 12 well cell culture plates they were incubated at 37°C for 2 h.

The HT-29 monolayer was washed with CO₂ independent media prior to lysing intestinal cells with the addition of 1 ml of sterile PBS containing 1% (v/v) TritonX-100 for 5 min at room temperature (RT) followed by pipette mixing. The cell suspension (100 µl) was plated on MacConkey agar plates and incubated overnight at 37°C. Colonies were enumerated to determine the number of *E. coli* cells that had adhered to the HT-29 cells. Three biological replicates of adhesion assays were carried out on separate days.

RNA isolation

Cells were washed after exposure to *E. coli* in the presence and absence of MRF, wells were suspended in 350 µl of RLT lysis buffer (Qiagen, Germany) and scraped with a cell scraper. The cell suspension was ruptured for 30 s with a tissue rupture probe on ice to generate a homogenous mixture. The RNA was isolated following the RNeasy Micro Kit (Qiagen, Germany) procedure, as detailed in the user manual, and RNA quality was determined *via* an RNA Nano 6000 LabChip kit using an Agilent 2100 Bioanalyzer (Agilent Technologies,

CA, USA). RNA integrity number (RIN) values of 7.8 and above and RNA concentrations of 0.88 µg and above were obtained.

Quantitative PCR gene array analysis

Reverse transcription was performed on RNA (0.88 µg) acting as a template to synthesise cDNA using SuperScript[®] III First-Strand Synthesis System (Invitrogen, USA) prior to PCR array analysis being performed. PCR array analysis on gene expression changes associated with bacterial infection was analysed using the antibacterial response PCR Array (PAHS-148Z) and RT2 Profiler PCR array system (SABiosciences, Frederick, MD, USA) according to the manufacturer's protocol. Real-time PCR was performed in triplicate using a two-step cycling programme, consisting of a heat activation step (95°C for 10 min) and a cycling step (40 cycles, 95°C for 15 s and 60°C for 1 min; ABI 7500 Fast; Applied Biosystems, USA). Results were analysed using the SABiosciences PCR array data analysis (<https://www.qiagen.com/kr/shop/genes-and-pathways/data-analysis-center-overview-page/>) with cells treated with or without MRF in the presence or absence of *E. coli* compared to untreated HT-29 cells. Criteria for significant changes in gene expression was set at $P \leq 0.05$. RNA used later was supplied by Ambion and SYBR Green supplied by SABioscience, Frederick, MD, USA.

Analysis of the qPCR array results was assessed using pathway analysis software Reactome (<https://reactome.org/>) to determine gene expression changes within pathways. Fold changes from PCR array results were uploaded into Reactome and changes in expression were marked by colour changes, with dark brown /red representing increased fold expression and green representing reduced gene expression seen in the scale bar on the right side of images.

Western blotting

Protein targets LY96 and TICAM-1 ascertained from the gene expression changes of PCR arrays were assessed by Western blotting. Protein was isolated from HT-29 cells following washing the monolayer with cells resuspended in lysis buffer (7M Urea, 2M Thiourea, 4% CHAPS, 30mM Tris) containing a protease inhibitor cocktail (Sigma Aldrich, P2714) and scraped with a cell scraper. Protein was quantified by Bradford (BIORAD, 500-0006) using a bovine serum albumin standard. Protein (48 µg) was loaded in each well of a 12.5% polyacrylamide gel, run at 70–90 V

for 15 min and 110 V until tracking dye reached the end of the gel. Polyacrylamide gels were transferred to a PVDF membrane at 25 V for 45 min on a semi-wet transfer sandwich (Thermo Scientific Pierce G2 Fast Blotter).

Resulting blots were Ponceau stained to check for correct transfer of equal protein prior to blocking in 1% BSA/5% Milk protein resuspended in PBS Tween 20 (0.005%) for 30 min. Blots were probed with target antibody separately for using Rabbit Anti-human LY96 (Sigma, SAB 1410439) or Mouse Anti-human TICAM-1 (Santa Cruz, SC514384) and, for housekeeping, protein actin using Mouse Anti-human Actin (Abcam, AB123034). Each primary antibody was individually incubated for 1 h at RT. Secondary Horseradish Peroxidase (HRP) linked antibody for Anti-mouse-HRP (Sigma Aldrich, A9044) or Anti-rabbit-HRP (Abcam, AB191866) were probed for 1 h at RT to bind to the primary antibody and washed for 10 minutes in triplicate. Resulting Western blotting images were developed by chemiluminescence and analysed using the LICOR scanner and analysis software.

TNFα analysis

TNFα ELISA compatible with cell lysates and cell supernatants was supplied by Sigma Aldrich (RAB1089). All reagents and samples were brought to RT (18–25°C) before use. All standards and secreted supernatant samples were run in duplicate and 100 µl of each standard and sample was added into appropriate wells and incubated for 2.5 h at RT with gentle shaking (150 rpm). Solutions were discarded, and wells washed four times with 1x wash solution between all applications of new reagents. A total of 100 µl of 1x detection antibody was added to each well and incubated for 1 h at RT with gentle shaking. Solutions were removed, and wells washed prior to addition of 100 µl of Streptavidin solution to each well. Wells were covered and incubated for 45 minutes at RT with gentle shaking. Following washing, 100 µl of tetramethylbenzidine (TMB) was added to each well and incubated for 30 minutes at RT in the dark with gentle shaking. Stop solution was added (50 µl) prior to reading at 450 nm absorbance.

Statistics

Statistical analysis of Western blots, ELISA and adhesion experiments were analysed using Students t-test and Tukeys *post-hoc* test with results determined significant at $P \leq 0.05$, MiniTab 18. Bar charts are represented as the mean average and standard error mean (SEM).

Results

qPCR gene array analysis of MRF on bacterial infection

Gene expression was assessed following exposure of intestinal cells to *E. coli* in the presence and absence of MRF to determine any variance in the immune response. Expression changes greater or less than the control was deemed significantly different at $P < 0.05$ (Table 1). *E. coli* treated HT-29 cells had 10 genes expressed at levels significantly higher than the control and one lower than the control. The *E. coli* exposed to HT-29 cells treated with MRF had six genes expressed at levels significantly higher than the control and two significantly lower than the control. MRF treated HT-29 cells alone had three genes with significantly lower expression than the control.

A number of inflammatory genes were significantly upregulated in HT-29 cells in response to *E. coli* exposure (*IL-1 β* , *TNF α* , and *ZBP1*) when compared to the control (Table 1). Similarly, immune cell attraction to the sight of infection in the form of chemo-attractants were significantly elevated for HT-29 cells exposed to *E. coli* alone for both *CXCL2* and *CXCL8*, while *CXCL1* had a 100-fold increase over the control, but was not significantly different. Immune cell signalling for a number of important intracellular signalling molecules demonstrated either a significant increase (*IRAK1*) or higher expression (*IRF7*) in HT-29 cells that were exposed to *E. coli* alone. *BIRC3*, an anti-apoptotic gene, was significantly increased in response to *E. coli*. Genes responsible for upstream pathogen recognition through toll-like receptor 4 (TLR4) were significantly elevated for HT-29 cells following exposure to *E. coli* (*LY96* and *TICAM-1*) when compared to the control.

E. coli exposed to HT-29 cells in the presence of MRF demonstrated significant increases in the inflammatory gene *ZBP1*, chemo-attractants *CXCL1*, *CXCL2* and *CXCL8*, pattern recognition protein *LY96* and anti-apoptotic gene *BIRC3* compared to control cells. The MAP kinase signalling gene *MAP2K3* was significantly decreased in MRF treated HT-29 cells exposed to *E. coli*. MRF treatment alone on HT-29 cells demonstrated significantly decreased expression of immune signalling genes *IRAK1*, *IRF7* and *JUN* when compared to the control. *E. coli* infected HT-29 cells treated with MRF demonstrated greater reductions in gene expression for immune signalling genes *IRAK1* and *IRF7* compared to the HT-29 cells exposed to *E. coli* only. The addition of MRF to *E. coli* infected HT-29 cells reduced

expression of inflammatory genes *IL-1 β* and *TNF α* to non-significant levels when compared to the *E. coli* infected intestinal cells alone. The expression of proinflammatory marker *NLRP3* was significantly reduced in both HT-29 infected groups with and without MRF when compared to the control.

CXCL2 and pathogen recognition gene *CD14*. Proinflammatory gene Caspase-1 (CASP1) expression was reduced by MRF addition to -1.46 compared to the control. Similarly, a number of genes demonstrated decreased expression of equal to or greater than (-1.45) fold compared to the control for inflammatory associated genes *IRF5*, *ZBP1*, *IFNB1*, *TNFRSF1A*, immune signalling genes *NOD1*, *RELA*, *MAPK1*, *MAPK14*, *MAPK3*, *MAPK8*, *TIRAP*, *TICAM-1*, *CARD9*, chemokine gene.

Western blotting of HT-29 cell protein following *E. coli* infection and treatment with MRF

Protein targets LY96 and TICAM-1 were selected due to the significant change in HT-29 gene expression in response to *E. coli* infection. Both TICAM-1 and LY96 proteins have important functions in TLR4 pathogen recognition, with changes in these protein abundances serving as indicators for the level of infection in the presence or absence of MRF. Target antibodies demonstrated binding to both TICAM-1 and LY96 with developed bands corresponding to respective molecular weights 55 kDa and 25 kDa. LY96 protein abundance normalised to the actin control demonstrated a significant rise in abundance of LY96 in the *E. coli* treated HT-29 cells (1.79 ± 0.25) over the control (1.0 ± 0 , $P \leq 0.05$) and the treatment control (1.02 ± 0.09 , $P \leq 0.05$; Figure 1a). *E. coli* exposed to HT-29 cells in the presence of MRF (16 mg/ml) demonstrated a non-significant rise over the control to 1.12 ± 0.09 and the MRF treatment control showed no change to the negative control at 1.02 ± 0.09 . The addition of MRF to *E. coli* infected HT-29 cells (1.12 ± 0.09 , $P \leq 0.05$) significantly reduced LY96 abundance compared to the HT-29 cells infected with *E. coli* alone (1.79 ± 0.25). TICAM-1 demonstrated a similar trend to LY96 abundance, with a significant rise seen only in the *E. coli* (1.40 ± 0.24 , $P \leq 0.05$) treated HT-29 cells over the control (1.0 ± 0.0). HT-29 cells exposed to *E. coli* with MRF (1.064 ± 0.27) demonstrated no change in TICAM-1 abundance to the control (Figure 1b).

Table 1. Table of gene fold changes of HT-29 cells with or without *E. coli* exposure in the presence or absence of MRF. Qiagen array analysis calculates p values based on a Student's t-test of the replicate $2^{(-\Delta\Delta C_T)}$ values for each gene in the control group and treatment groups, and p values less than 0.05 are indicated in bold (n=3).

Symbol	Accession number	Fold Regulation Change				Description
		Control	<i>E. coli</i>	<i>E. coli</i> and MRF	MRF	
AKT1	NM_005163	1	1.60	1.08	-1.46	V-akt murine thymoma viral oncogene homolog 1
BIRC3	NM_001165	1	3.97	4.32	-1.17	Baculoviral IAP repeat containing 3
BPI	NM_001725	1	1.04	1.42	1.08	Bactericidal/permeability-increasing protein
CAMP	NM_004345	1	1.59	1.72	1.36	Cathelicidin antimicrobial peptide
CARD6	NM_032587	1	-1.26	-1.17	-1.17	Caspase recruitment domain family, member 6
CARD9	NM_052813	1	1.58	-1.17	-2.35	Caspase recruitment domain family, member 9
CASP1	NM_033292	1	1.26	1.72	-1.46	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)
CASP8	NM_001228	1	-1.26	-1.47	-1.16	Caspase 8, apoptosis-related cysteine peptidase
CCL3	NM_002983	1	-1.26	-1.14	1.08	Chemokine (C-C motif) ligand 3
CCL5	NM_002985	1	1.26	1.08	-1.17	Chemokine (C-C motif) ligand 5
CD14	NM_000591	1	1.27	1.37	-1.46	CD14 molecule
CHUK	NM_001278	1	-1.59	-1.47	-1.17	Conserved helix-loop-helix ubiquitous kinase
CRP	NM_000567	1	-1.26	-1.14	1.08	C-reactive protein, pentraxin-related
CTSG	NM_001911	1	-1.23	-1.17	1.08	Cathepsin G
CXCL1	NM_001511	1	100.07	108.14	1.08	Chemokine ligand 1, melanoma growth stimulating activity, alpha)
CXCL2	NM_002089	1	101.30	87.29	-1.46	Chemokine ligand 2
FADD	NM_003824	1	1.27	1.09	-1.16	Fas (TNFRSF6)-associated via death domain
HSP90AA1	NM_001017963	1	-1.58	-1.46	1.08	Heat shock protein 90kDa alpha (cytosolic), class A member 1
IFNA1	NM_024013	1	-1.02	-1.17	-1.19	Interferon, alpha 1
IFNB1	NM_002176	1	1.59	1.35	-1.47	Interferon, beta 1, fibroblast
IKBKB	NM_001556	1	1.27	-1.17	-1.47	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
IL12A	NM_000882	1	1.60	2.19	1.37	Natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35
IL12B	NM_002187	1	-1.26	-1.11	1.08	Natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40.
IL18	NM_001562	1	-1.59	-1.47	1.08	Interferon-gamma-inducing factor
IL1 β	NM_000576	1	7.97	8.60	-1.17	Interleukin 1, beta
IL6	NM_000600	1	1.05	-1.11	1.08	Interleukin 6 (interferon, beta 2)
CXCL8	NM_000584	1	25.46	21.71	1.37	Interleukin 8
IRAK1	NM_001569	1	2.00	-1.46	-2.32	Interleukin-1 receptor-associated kinase 1
IRAK3	NM_007199	1	-1.26	-1.17	1.08	Interleukin-1 receptor-associated kinase 3
IRF5	NM_001098629	1	1.26	1.37	-1.84	Interferon regulatory factor 5
IRF7	NM_001572	1	1.57	-1.17	-2.33	Interferon regulatory factor 7
JUN	NM_002228	1	2.01	1.72	-2.94	Jun proto-oncogene
LBP	NM_004139	1	-1.21	-1.12	1.08	Lipopolysaccharide binding protein
LCN2	NM_005564	1	1.27	1.08	-1.16	Lipocalin 2
LTF	NM_002343	1	-1.26	-1.17	1.08	Lactotransferrin
LY96	NM_015364	1	2.00	1.72	1.06	Lymphocyte antigen 96
LYZ	NM_000239	1	1.26	1.08	1.38	Lysozyme
MAP2K3	NM_002756	1	-1.59	-1.86	-1.17	Mitogen-activated protein kinase kinase 3
MAP2K4	NM_003010	1	-1.27	-1.47	1.08	Mitogen-activated protein kinase kinase 4
MAP3K7	NM_003188	1	1.01	1.09	1.08	Mitogen-activated protein kinase kinase kinase 7
MAPK1	NM_002745	1	-1.26	-1.17	-1.47	Mitogen-activated protein kinase 1
MAPK14	NM_001315	1	-1.01	-1.49	-1.87	Mitogen-activated protein kinase 14
MAPK3	NM_002746	1	1.59	-1.17	-2.33	Mitogen-activated protein kinase 3
MAPK8	NM_002750	1	-1.26	-1.48	-1.84	Mitogen-activated protein kinase 8
MPO	NM_000250	1	-4.07	-4.86	1.09	Myeloperoxidase
MYD88	NM_002468	1	1.01	1.09	1.08	Myeloid differentiation primary response gene (88)
NAIP	NM_004536	1	1.26	1.08	1.36	NLR family, apoptosis inhibitory protein
NFKB1	NM_003998	1	-1.27	-1.17	-1.17	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NFKBIA	NM_020529	1	16.06	13.73	-1.16	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
NLRC4	NM_021209	1	-1.26	-1.47	1.36	NLR family, CARD domain containing 4
NLRP1	NM_033004	1	-1.23	-1.11	1.08	NLR family, pyrin domain containing 1
NLRP3	NM_183395	1	-2.01	-1.86	-1.16	NLR family, pyrin domain containing 3
NOD1	NM_006092	1	-1.28	-1.19	-2.36	Nucleotide-binding oligomerization domain containing 1
NOD2	NM_022162	1	-1.26	-1.17	1.08	Nucleotide-binding oligomerization domain containing 2
PIK3CA	NM_006218	1	-1.27	-1.49	-1.16	Phosphoinositide-3-kinase, catalytic, alpha polypeptide
RELA	NM_021975	1	2.01	1.09	-1.46	V-rel reticuloendotheliosis viral oncogene homolog A (avian)
RIPK1	NM_003804	1	-1.26	-1.47	-1.15	Receptor (TNFRSF)-interacting serine-threonine kinase 1
RIPK2	NM_003821	1	-1.27	-1.17	1.08	Receptor-interacting serine-threonine kinase 2
SLC11A1	NM_000578	1	1.59	1.70	1.09	Solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1
SLPI	NM_003064	1	1.26	1.36	1.08	Secretory leukocyte peptidase inhibitor

SUGT1	NM_006704	1	-1.26	-1.85	1.08	SGT1, suppressor of G2 allele of SKP1 (S. cerevisiae)
TICAM-1	NM_182919	1	2.52	2.17	-1.46	Toll-like receptor adaptor molecule 1
TICAM2	NM_021649	1	-4.00	-3.69	1.37	Toll-like receptor adaptor molecule 2
TIRAP	NM_001039661	1	1.00	-1.17	-1.86	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLR1	NM_003263	1	-1.26	-1.17	1.08	Toll-like receptor 1
TLR2	NM_003264	1	-1.23	1.11	1.08	Toll-like receptor 2
TLR4	NM_138554	1	-1.58	-1.47	1.08	Toll-like receptor 4
TLR5	NM_003268	1	1.25	1.39	1.08	Toll-like receptor 5
TLR6	NM_006068	1	1.25	1.72	1.08	Toll-like receptor 6
TLR9	NM_017442	1	-1.23	-1.14	1.08	Toll-like receptor 9
TNF	NM_000594	1	536.42	459.45	-1.18	Tumor necrosis factor
TNFRSF1A	NM_001065	1	-1.26	-1.17	-1.47	Tumor necrosis factor receptor superfamily, member 1A
TOLLIP	NM_019009	1	1.00	-1.47	-1.85	Toll interacting protein
TRAF6	NM_004620	1	-1.59	-1.47	1.09	TNF receptor-associated factor 6
XIAP	NM_001167	1	-1.01	1.70	-1.17	X-linked inhibitor of apoptosis
ZBP1	NM_030776	1	2.00	2.16	-1.47	Z-DNA binding protein 1

Figure 1. Western blotting of HT-29 cell protein following *E. coli* infection and treatment with MRF. LY96 protein abundance graph 1a, TICAM-1 protein abundance graph 1b normalised to Actin housekeeping protein abundance and western blotting representative images for TICAM-1, LY96 and Actin. Data was analysed using a One-way ANOVA with Tukey's multiple comparison test and significance marked as * ($p < 0.05$), ($n = 3$).

TNF α secretion from HT-29 cells exposed to E. coli in the presence and absence of MRF

TNF α , a proinflammatory marker indicative of infection was measured for extracellular secretions using a TNF α ELISA. The HT-29 cells exposed to *E. coli* demonstrated significantly higher TNF α secretion of 161.7 ± 42.0 pg/ml ($P \leq 0.01$), compared to control TNF α levels (74.89 ± 4.25 pg/ml). The same trend was seen when comparing *E. coli* exposed HT-29 cells alone, having significantly higher TNF α levels (161.7 ± 42.0 pg/ml; $P \leq 0.05$) over cells treated with MRF only (85.96 ± 17.7 pg/ml; $P \leq 0.05$) (Figure 2). HT-29 cells exposed to *E. coli* in the presence of MRF (107.23 ± 6.13 pg/ml)

Figure 2. TNF α secretion from HT-29 cells exposed to *E. coli* in the presence and absence of MRF. ELISA measurement of TNF α secretion from HT-29 cells exposed to *E. coli* in the presence or absence of MRF and controls with media (Control) or MRF treatment only (MRF). Data was analysed using a One-way ANOVA with Tukey's multiple comparison test and significance marked as * ($p < 0.05$), ** ($p < 0.01$), ($n = 3$).

had higher than the negative control and the MRF only levels of $\text{TNF}\alpha$, but these were not significantly different (Figure 2).

Bacterial adhesion of HT-29 cells treated with MRF

In a comparison of adherence of *E. coli* to HT-29 cells exposed for 2 h with and without MRF highlighted that treatment of cells with MRF reduced the ability of *E. coli* to bind to the HT-29 cells by 78% at $1.59 \times 10^5 \pm 1.02 \times 10^5$ CFU/ml compared to the control (*E. coli* only) at $6.69 \times 10^5 \pm 7.25 \times 10^4$ CFU/ml with no MRF (Figure 3).

Discussion

Increases in both inflammation and apoptosis are key host responses to *E. coli* intestinal infection (Wong Fok Lung *et al.*, 2014). The expression of genes for the chemo-attractants *CXCL2* and *CXCL8* used by infected host cells to attract immune cells to the sight of infection were significantly higher in HT-29 cells exposed to *E. coli* alone compared to cells exposed to *E. coli* and MRF. Exposure of HT-29 cells to *E. coli* significantly increased *LY96* and *TICAM-1* genes associated with pathogen recognition and intracellular signalling of the TLR4 pathway. Both of these genes are essential components to the TLR4-led recognition of Gram-negative bacteria such as *E. coli* (Rajaiah *et al.*, 2015; Kuzmich *et al.*, 2017). It was evident that MRF in combination with *E. coli* reduced the expression of *TICAM-1* to non-significant

levels and *LY96* to lower levels than were observed in the HT-29 cells exposed to *E. coli* alone. Reduced detection of bacterial infection *via* the TLR4 pathway by HT-29 cells exposed to *E. coli* with MRF may indicate the ability of MRF to impair adhesion of the *E. coli* to the surface of intestinal cells, thus lowering infection rates.

TICAM-1 gene expression appeared to show a similar trend to protein abundance, as HT-29 cells treated with *E. coli* has a significant increase in *TICAM-1* in both gene and protein abundance (Figure 1b) over the negative control. Yet *TICAM-1* protein abundance was not significantly changed for HT-29 cells exposed to *E. coli* with MRF treatment. Similarly, *LY96* protein abundance was significantly elevated in HT-29 cells, responding to the *E. coli* treatment alone (Figure 1a), but not for intestinal cells exposed to *E. coli* with MRF. Interestingly the addition of MRF to *E. coli* infected HT-29 cells reduced *LY96* abundance to non-significant levels when compared to HT-29 cells infected with *E. coli* alone. A comparable study of broilers fed yeast-derived, mannan rich carbohydrates demonstrated lower TLR4 gene expression (Munyaka *et al.*, 2012). Although this result was not duplicated in TLR4 gene expression in the current study, a similar trend was observed with genes and proteins associated to the TLR4 complex when MRF was combined with *E. coli* and for the MRF treatment control group (Table 1, Figure 1a and 1b).

Assessment of the TLR4 associated pathways by Reactome software depicted greater significant changes in gene expression for HT-29 cells exposed to *E. coli* only but not for treatments including *E. coli* combined with MRF or MRF alone when compared to the negative control (Figure 4).

MRF treatment alone demonstrated a much lower activation of genes associated with the TLR4 pathway over the control, which has been previously demonstrated in poultry (Xiao *et al.*, 2010; Brennan *et al.*, 2013), piglets (Che *et al.*, 2012; Samuel and Brennan, 2012), and fish (Zhao *et al.*, 2015). Zhao *et al.*, (2015) suggested that dietary mannose priming of fish switched expression of inflammatory cytokines to mechanisms for resolution and repair, which may explain the impact of MRF on HT-29 cells on its own and when combined with *E. coli* in the current experiment. Although no evidence of priming was evident in this study when MRF was added on its own.

MRF in combination with *E. coli* affected HT-29 gene expression specifically in reducing the expression of genes involved in inflammation, bacterial recognition

E. coli adhesion to intestinal cells

Figure 3. Bacterial adhesion to HT-29 cells treated with MRF. Attachment of *E. coli* to human intestinal HT-29 cells in the presence of MRF (*E. coli* and MRF) or absence of MRF the positive control (*E. coli*). Statistical analysis carried out using a one-tailed students t-test with significance marked as * ($p < 0.05$), ($n = 4$).

Figure 4. representing fold changes in genes for TLR4 Pattern recognition and signalling for (a) Control, (b) *E. coli*, (c) MRF with *E. coli* and (d) MRF treated HT-29 cells respectively. Scale bar of fold expression changes ranges from +2.52-4 fold with red box highlighting change of interest.

Figure 4b. (Continued)

and immune signalling when compared to HT-29 cells exposed to *E. coli* only. Overall gene markers for the signs and severity of *E. coli* infection in the form of

inflammation (*IL-1β* and *TNFα*) and apoptosis (*BIR3*) were decreased with addition of MRF in comparison to the *E. coli* exposed to HT-29 cells alone (Table 1).

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Figure 5. Representing fold changes in genes for TNFR1 signalling pathway for (a) Control, (b) *E. coli*, (c) MRF with *E. coli* and (d) MRF treated HT-29 cells respectively. Scale bar of fold expression changes ranges from +536-1.59 fold. TNF receptor activation through TNFα binding and downstream signalling.

Figure 5b. (Continued)

Figure 5c. (Continued)

Figure 5d. (Continued)

levels of TNF α secretion demonstrated a strong correlation with the gene expression, as *E. coli* exposure to HT-29 cells triggered a significant release of TNF α compared to the control. Interestingly MRF combined with *E. coli* and exposed to HT-29 cells demonstrated a non-significant rise in TNF α over the control in both protein secretion and gene expression. This again highlighted MRFs potential capacity to protect cells from the effects of *E. coli* infection that results in lower TNF α production and release. Previous studies assessing MRF similarly highlighted its ability to lower inflammation while blocking harmful intestinal bacteria (Xiao *et al.*, 2010; Che *et al.*, 2012; Munyaka *et al.*, 2012; Samuel and Brennan, 2012).

The adhesion of *E. coli* to its cellular target has been reported to trigger a signalling pathway response (e.g., MAPK and PI3K) leading to CXCL8 synthesis, which induces the migration of neutrophils to the epithelial site of infection (Cane *et al.*, 2010). This response has been reported to follow the stimulation of epithelial cells to synthesise TNF α and IL-1 β (Betis *et al.*, 2003) which was observed here in both HT-29 cell treatment groups exposed to *E. coli*. The expression of CXCL8, TNF α and IL-1 β , were significantly increased in response to *E. coli* exposure, but not for TNF α and IL-1 β expression in response to *E. coli* treated with MRF. Reduced inflammatory markers from HT-29 cells exposed to *E. coli* and MRF when compared to HT-29 cells exposed to *E. coli* alone demonstrated the potential reduction of infection through MRF addition.

Research has previously shown that yeast mannans have a positive influence on the immune status of sows and their newly weaned piglets (Duan *et al.*, 2016) when sow diets were supplemented. The effect of yeast mannans on the immune response of cells when challenged with ETEC has been examined by Hermes *et al.* (2011). It was subsequently demonstrated that the activation of TLR-4, TLR-5 and the levels of inflammatory markers, such as IL-1 β and TNF α , were reduced similarly to what was observed in the current experiment, although only for the TLR4 pathway.

The anti-inflammatory effects of MRF alone were highlighted by Munyaka *et al.* (2012) in broilers, demonstrating the downregulation of gene expression of the proinflammatory marker interferon. Similarly, it was shown in the current experiment that MRF treatment alone significantly reduced HT-29 cell expression for IRF7 and IRAK1 activators of proinflammatory markers TNF α and IL-1 β respectively, when compared to the control levels (Table 1). Further evidence of anti-inflammatory

effects of MRF was seen by the number of inflammatory genes that were reduced in expression compared to the control. For example, caspase1, an enzyme responsible for cleaving protein precursors to their active inflammatory forms such as IL-1 β (Thornberry *et al.*, 1992), was reduced in expression compared to the control.

Adhesion of *E. coli* to HT-29 intestinal cells facilitates infection, which was demonstrated to be significantly impaired by the presence of MRF (Figure 3). MRF functions as a decoy molecule to *E. coli* blocking the normal interaction by which the bacteria bind to the intestinal cell lining through mannan surface molecules. Interestingly, previous findings demonstrated significantly reduced invasion of IPEC by the same *E. coli* strain used here when MRF was added compared to a control (Horgan *et al.*, 2018). The method of physically blocking bacterial invasion exploits the bacteria's reliance on the mannan-dependent means of invasion. This method of invasion that yeast mannan targets, appears to be evolutionarily difficult for the bacteria to adapt to and generating alternative means against invasion. This is unlike targeting the bacteria *via* antimicrobials, which act as a selective pressure allowing bacteria to evolve to overcome such pressure and attain resistance (Sandegren, 2014). Thus, the method of utilising a mannan decoy *via* MRF to block adhesion may prove more difficult for bacteria such as *E. coli* and *Salmonella spp.* to negate or block.

Previous work on MRF demonstrated beneficial effects in limiting infection by *E. coli* and *Salmonella spp.* in poultry (Biggs *et al.*, 2007; Luquetti *et al.*, 2012), as observed here with HT-29 intestinal cells exposed to pathogenic *E. coli* (Figure 3). Intracellularly, the characteristic markers for infection were elevated in response to *E. coli* infection but were reduced with MRF addition. MRF exposure in the absence of infection demonstrated potential anti-inflammatory effects on cells, as reported in previous research (Munyaka *et al.*, 2012). Yet the MRF didn't demonstrate any priming of intestinal cells to either fungal or bacterial gene pathways (Figure 4d). A balance between pro and anti-inflammatory changes is required, since impairing inflammation can affect the hosts capacity to fight infection, while too strong an inflammatory response results in excessive tissue damage, which could lead to poor nutrient absorption from the gut.

Conclusions

The changes in gene and protein biological markers in response to intestinal infection may be utilised to

determine reoccurrence of pig infections and the effectiveness of in feed prebiotics such as MRF. The incorporation of MRF in feed to improve PWD outcomes in pigs could similarly be assessed by changes in key biomarkers observed here for the severity of infections through measuring inflammatory, apoptotic and immune signalling changes. Feed additives such as MOS and MRF offer the potential to prevent colonisation of the animal's gut by pathogenic bacteria, leading to lower incidence of PWD and ultimately providing a means to improve gut health naturally, without the need for antimicrobial usage on farms.

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