

ENHANCING CHICKEN INNATE IMMUNITY AND  
DISEASE RESISTANCE BY BOOSTING HOST  
DEFENSE PEPTIDE SYNTHESIS

By

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## **CHAPTER I**

### **INTRODUCTION**



The nonspecific innate immune system fights off infections quickly and further controls the development of the highly specialized adaptive immune system for sustained protection. Weapons of the innate immune system mainly include pattern recognition receptors, leukocytes, complement system, cytokines/chemokines, and antimicrobial peptides/proteins including HDPs. Host defense peptides (HDPs) are comprised of a large diverse group of small cationic antimicrobial peptides of less than 100 amino acid residues adopting an amphipathic conformation. They are widespread in nature and have been found in virtually all forms of life.

Based on the structure, HDPs are broadly classified into four major classes: including (1)  $\alpha$ -helical peptides, (2)  $\beta$ -sheet peptides, (3) peptides with flexible extended structures rich in certain amino acids, and (4) peptides with a loop structure. Most HDPs are produced by mucosal epithelial and myeloid cells, and processed post-translationally to give rise to mature, biologically active peptides. Mature HDPs are capable of killing a broad spectrum of pathogens directly by physical disruption of their membranes or by interacting with intracellular anionic molecules like DNA, RNA, and proteins.

Apart from antimicrobial action, HDPs also modulate innate and adaptive immunity. Many HDPs have strong capacities to chemoattract different types of immune cells, stimulate production of chemokines and cytokines, and promote differentiation and maturation of antigen-presenting cells like dendritic cells. HDPs also bind to bacterial products thereby neutralizing their ability to stimulate the production of pro-inflammatory cytokines. Additionally, HDPs limit inflammation by inducing the synthesis of anti-inflammatory cytokines such as IL-10 and promoting apoptosis of

activated immune cells. With such an array of desirable properties, HDPs have potential in the control of infection and inflammation. Because of the cost associated with use of synthetic peptides, strategies to stimulate the production of endogenous HDPs may be an attractive approach to boost host immunity and enhance disease resistance without relying on traditional antibiotics.

In this dissertation, we have summarized the latest progresses on HDPs regarding their classification, structure, and expression as well as their antimicrobial, anti-inflammatory, and immunomodulatory properties (Chapter II). In addition, we have presented a novel finding on the modulation of chicken HDPs by butyrate, a major short chain fatty acid produced by bacterial fermentation of undigested fiber in the intestinal tract (Chapter III). We revealed that butyrate is a potent inducer of many, but not all, chicken HDPs both in vitro and in vivo. Remarkably, oral supplementation of butyrate to chickens significantly reduced colonization of *Salmonella enteritidis* in the cecum following an experimental infection. We further screened a series of short-, medium-, and long-chain fatty acids for their ability to induce chicken HDP gene expression (Chapter IV). We found that the aliphatic carbon chain length is largely in an inverse correlation with the HDP-inducing activity of fatty acids. Among all fatty acids, short-chain fatty acids are the most potent inducers, while medium-chain fatty acids have a moderate effect, and long-chain fatty acids are largely ineffective. Importantly, a combination of three short-chain fatty acids namely acetate, propionate and butyrate synergistically induced HDPs synthesis, resulting in a more pronounced reduction of the *S. enteritidis* load in the chicken cecum.

We have further investigated the molecular mechanisms involved in the butyrate-mediated induction of the avian  $\beta$ -defensin 9 (AvBD9) gene in the chicken (Chapter V). We discovered that histone deacetylation is highly beneficial for HDP gene expression, as histone deacetylase inhibitors increased AvBD9 synthesis while histone acetyltransferase inhibitors reduced butyrate-mediated AvBD9 induction. Notably, JNK and p38, but not ERK1/2, MAP kinase pathways are also involved in butyrate-triggered AvBD9 expression. Furthermore, activation of cAMP signaling results in an enhanced AvBD9 gene expression. Strikingly, a combination of histone deacetylase inhibitor (butyrate) and cAMP signaling agonists synergistically augmented AvBD9 induction. The results suggested that chicken HDPs synthesis is regulated by a complex mechanism involving histone deacetylation and cAMP and MAP kinase signaling pathways.

Given an urgent need for antibiotic-alternative approaches in disease control and prevention, discovery of the molecular mechanisms of HDP gene regulation and an array of HDP-inducing agents provide an important first step toward development of novel antimicrobial strategies for improvement of animal and human health.

## **CHAPTER II**

### **REVIEW OF LITERATURE**

## **1. INTRODUCTION**

Host defense peptides (HDPs) are critical effectors of the innate immune system that protects the host from harmful pathogens. Being short cationic amphipathic antimicrobial peptides, HDPs have been discovered in nearly all forms of life from prokaryotes to eukaryotes and from invertebrate to mammalian species. HDPs are widely expressed in leukocytes as well as mucosal epithelial cells lining the respiratory, gastrointestinal and urogenital systems of the host. They are synthesized as prepropeptides and processed by different serine proteases to release mature peptides possessing biological functions. They directly kill a myriad of microbes ranging from Gram-positive and Gram-negative bacteria to fungi, protozoa, parasites, and enveloped virus. In addition to their direct antimicrobial activity, they act as antiinflammatory, wound healing and immunomodulatory agents. Because of these unique features, they are being actively explored as novel antimicrobials for disease control and prevention. This review summarizes structural features, expression patterns, and biological properties of HDPs as well as the mechanism of action and gene regulation. Their potential applications in animal agriculture and public health were also discussed.

## **2. CLASSIFICATION AND EXPRESSION PATTERN OF HDPs**

Innate immunity is an important first line of host defense [1-4]. Invertebrates have only innate immune mechanisms, while vertebrates possess both innate and adaptive

immunity [1]. Effector components of the innate immune system include natural physical barriers like skin and mucosal surfaces, natural microflora, complement system, pattern recognition receptors, cytokines, leukocytes, and antimicrobial peptides/proteins [2, 5-8]. Antimicrobial peptides/proteins are comprised of peptidoglycan-recognition proteins, iron metabolism proteins (lipocalin and lactoferrin), and more importantly, host defense peptides (HDPs).

HDPs are present in virtually all forms of life [1, 9-14]. To date, more than 1,200 such HDPs have been discovered, and approximately 1,000 are present in eukaryotic organisms [15-18]. These peptides generally contain less than 100 amino acid residues with an overall net positive charge [19]. Based on their structures, HDPs are broadly classified into four major classes including peptides with  $\alpha$ -helices, peptides with  $\beta$ -sheets, peptides adopting flexible structures enriched for certain amino acids like arginine, histidine, proline, and tryptophan, and peptides with a loop structure due to the presence of a disulfide bond [4, 9, 20-23].

Cathelicidins and defensins represent two major families of HDPs found in vertebrates [24-30]. Cathelicidins were first isolated from bovine neutrophil lysates as cyclic dodecapeptides [31]. Since then, cathelicidins have been found in many mammalian species as well as in fish, snakes, and birds [32-39]. The name cathelicidin was coined from the presence of a highly conserved cathelin domain in the N-terminal prosequence of cathelicidins. However, the C-terminal domains of cathelicidins are highly variable among species and possess different biological functions (**Fig. 1**) [5, 34, 39]. A large group of cathelicidin genes are encoded in the porcine, ovine, and bovine

genomes; however, only a single cathelicidin gene exists in dogs, primates, and humans [3, 30]. Four cathelicidin genes were found recently in chickens [32, 40].

Defensins, present in plants, invertebrates, and vertebrates, are rich in cysteines, and comprised of 3-4 disulfide bonds forming 3-4 anti-parallel  $\beta$ -sheet structures [41-45]. Each vertebrate defensin consists of a signal peptide, proregion and cationic mature peptide with six conserved cysteine residues forming three intramolecular disulfide bridges creating a “defensin-like” fold with an amphipathic feature [46, 47] (**Fig.2**). Based on the spacing pattern and pairing of cysteine residues, defensins are classified into three major subfamilies namely  $\alpha$ -,  $\beta$ -, and  $\theta$ - defensins [45, 47]. The disulfide bridges are formed between C1-C6, C2-C4, and C3-C5 for  $\alpha$ -defensins, whereas C1-C5, C2-C4, and C3-C6 are pairing for  $\beta$ -defensins, and C1-C6, C2-C5 and C3-C4 pairing for  $\theta$ -defensins.  $\alpha$ - and  $\beta$ -defensins consist of flat triple-stranded  $\beta$ -sheets, while  $\theta$ -defensins are composed of circular double-stranded  $\beta$ -sheets (**Fig. 2**) [3, 26, 42, 48-50].

Cathelicidins and defensins are expressed strategically in leukocytes, skin keratinocytes, and mucosal epithelial cells of respiratory, gastrointestinal, and urogenital tracts [10, 51]. For example, human cathelicidin LL-37 is mainly found in both leukocytes and epithelial cells. While  $\alpha$ -defensins and  $\theta$ -defensins are commonly expressed in neutrophils and Paneth cells of the small intestine, the primary source of  $\beta$ -defensins are mucosal epithelia and skin [24, 52]. HDPs are synthesized as prepropeptides, processed posttranslationally by different proteolytic enzymes and stored either as propeptides (cathelicidins) or mature peptides (defensins) [53, 54]. Cathelicidins are further processed by serine proteases like proteinase 3 in neutrophils and kallikrein 5 and 7 in the skin in humans [55, 56] and elastase in cattle and pigs [57-59].  $\alpha$ -defensins

from intestinal Paneth cells including HD5 and HD6 are processed by metalloproteinase 7 [2].

### **3. ANTIMICROBIAL PROPERTIES OF HDPs**

HDPs are broad-spectrum natural antibiotics that kill or suppress the growth of a wide-range of microbes from Gram-positive and Gram-negative bacteria to virus, fungi, and parasites [60, 61]. They kill microbes by formation of pores and physical disruption of membranes or by inhibition of cellular transcription, translation and/or posttranslational machineries [14]. Cationic HDPs initially accumulate onto and electrostatically interact with anionic membrane components such as lipopolysaccharide of Gram-negative bacteria and lipoteichoic acid of Gram-positive bacteria cell. Penetration into negatively charged phospholipids of microbial membranes then takes place, resulting in membrane perturbation and leakage of intracellular contents and ultimately cell death [13, 62-65]. Because, it is very difficult for microbes to change the overall negative charge of their membrane phospholipids, development of resistance against HDPs is extremely difficult [65]. Targeted disruption of microbial but not host membranes is believed to be due to the difference between prokaryotic and eukaryotic cell membranes. While the former is heavily negatively charged with high membrane potential (-140 mV), the latter is largely uncharged with a high cholesterol content and low membrane potential of approximately -15 mV [22, 66].



The mechanism of pore formation on microbial membranes varies among individual HDPs. Depending upon the net charge and spatial structure, HDPs permeate membranes via “barrel-stave”, “toroidal-pore”, “molecular electroporation”, “sinking raft”, or “carpet-wormhole” mechanism [42, 67-69]. In addition to direct disruption of membranes, several HDPs, particularly  $\alpha$ - and  $\theta$ -defensins, suppress viral proliferation by acting as collectins. For example, retrocyclins (primate  $\theta$ -defensins) bind to glycoproteins gp41 and gp120 of HIV as well as host CD4 (Cluster of differentiation 4) and prevent viral entry by blocking the conformational change of gp41, which is required for attachment and fusion of viruses with host cells [70]. Similarly, human neutrophil peptides 1, 2, and 3 bind to envelop glycoprotein B (gB) of herpes simplex virus to minimize viral entry into the host cells [71].

#### **4. ANTI-INFLAMMATORY EFFECTS OF HDPs**

Besides antimicrobial and antiviral properties, HDPs suppress inflammation and protect the host from excessive production of proinflammatory mediators triggered by microbial products. HDPs are capable of neutralizing bacterial endotoxins, inhibiting proinflammatory cytokine production, and inducing antiinflammatory cytokines and preventing classical and lectin complement cascades [52, 72]. For example, human cathelicidin LL-37 binds and neutralizes LPS and LTA, thereby abolishing the production of proinflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6. It also stimulates antiinflammatory cytokine, IL-10 expression [73]. In a murine infection model, LL-37

protects mice from septic shock induced by *Pseudomonas aeruginosa* [74]. It also promotes secondary necrosis of apoptotic neutrophils without causing loss of membrane integrity and provoking proinflammatory response of macrophages [75]. Likewise, a chicken fowlicidin-1 analog prevents LPS-induced production of nitric oxide and TNF- $\alpha$  [76]. Porcine cathelicidin PR-39 inhibits the production of reactive oxygen species while bovine myeloid antimicrobial peptide-28 induces apoptosis of activated lymphocytes [10].

Similar to cathelicidins, defensins also inhibit production of proinflammatory cytokines by binding to microbial membranes, surface adhesins, and bacterial toxins and neutralizing the ability of their attachment to host cells [77]. For example, human neutrophil peptide (HNP) 1 attenuates LPS-mediated production of proinflammatory cytokines including IL-1 $\beta$  from monocytes [78]. Human neutrophil peptides 2-3 reduce production of several proinflammatory cytokines including IL-1  $\beta$ , IL-6, IL-8 and TNF-  $\alpha$  from LPS-stimulated human monocyte-derived macrophages [79]. Human  $\beta$  defensins-3 also abrogates the induction of IL-6, and TNF- $\alpha$  from human myeloid dendritic cells stimulated with *Porphyromonas gingivalis* [80]. Moreover, there is evidence that expression of human  $\alpha$ -and  $\beta$ -defensins is reduced in inflammatory diseases like Crohn's disease, emphasizing the role of defensins in regulation of inflammation [56, 81].

## 5. IMMUNOMODULATION OF HOST IMMUNITY BY HDPs

HDPs have capacity to directly kill pathogens, but their antimicrobial activity is often diminished by the monovalent and divalent cations, serum, and polyanionic molecules like glycosaminoglycans present in the biological fluids [82]. In fact, HDPs may not always have a direct antimicrobial action under physiological conditions. However, they still protect the host from infections [10, 14, 83]. For example, cathelicidin LL-37 protects mice from Gram-positive bacteria when administered exogenously but cannot inhibit bacterial growth in tissue culture medium containing physiologically similar salt concentrations [82], implying its role in immunomodulation.

HDPs promote diverse immunomodulatory functions by acting as chemoattractants, by stimulating the production of chemokines and cytokines, and by regulating complement activation and promoting wound healing (**Fig. 3**) [30, 49, 84]. For example, Human  $\beta$  defensin (HBD) 1 and HBD3 chemoattract immature dendritic cells and memory T-cells while human  $\alpha$ -defensins are chemotactic to naïve T cells [53]. Similarly, HNP1-3 and HBD3-4 stimulate migration of neutrophils and monocytes, whereas LL-7 and HNP1-3 are chemotactic to mast cells and induce degranulation to release histamine and prostaglandin 2 respectively [53]. HDPs induce production of various pro-inflammatory cytokines such as IL-1 $\beta$ , TNF- $\alpha$  and IL-6 as well as chemokines such as IL-8 and monocyte chemoattractant protein-1 from mononuclear phagocytes and epithelial cells [53, 85].

In addition to modulation of host immunity, HDPs appear to be promising wound healing agents. HDPs promote re-epithelialization, angiogenesis and vascularization by inducing proliferation of epithelial cells and vascular endothelial cells and chemoattracting fibroblasts and macrophages [3]. HDPs enhance synthesis of growth factors and cytokines in keratinocytes and epithelial cells that are essential for wound repair [14]. For example, human LL-37 and HBDs enhances IL-18 secretion from keratinocytes [86] that is involved in angiogenesis process [87]. Porcine PR-39 is involved in wound healing by increasing the expression of extracellular matrix proteoglycans syndecan-1 and 4, which are important for activation of many growth factors [88]. HBD-2 and 3 are also shown active involvement in re-epithelialization of damaged skin [89]. Given such an array of immunomodulatory properties of HDPs, it is highly desirable to harness these properties for antimicrobial therapies to boost host immunity without directly acting on microbes, thereby minimizing the risk of developing resistance [90].

## **6. TRANSCRIPTIONAL MODULATION OF HDP EXPRESSION**

Many HDPs expression is inducible in response to infection. Human cathelicidin LL-37 expression is induced in response to gram negative bacteria such as *Salmonella enterica serovar Dublin*, and enteroinvasive *Escherichia coli* in human colonic epithelium [91], *Helicobacter pylori* in human gastric epithelial cells [92] and *Pseudomonas aeruginosa* in corneal epithelium [93] and Gram-positive bacteria

including *S. aureus* in keratinocytes [94], *Mycobacterium* species in human alveolar macrophages, monocytes, neutrophils and epithelial cells [95, 96], bacterial products (LPS and LTA) in sinus epithelial cells [97], flagellin in corneal epithelial cells [93]. On the other hand, *Shigella dysenteriae*, *Vibrio cholera* [98] and *Nisseria gonorrhoeae* [99] downregulate LL-37 expression in intestinal epithelial cells.

In addition, stressors like injury [100], endoplasmic reticulum stress [101], and inflammatory disorders [102] also enhance LL-37 expression in keratinocytes. Moreover, various proinflammatory cytokines (IL-1 $\alpha$ , IL-6, and IL-17) [103-105] and growth factors [insulin like growth factor-1, transforming growth factor (TGF)- $\alpha$  and TGF- $\beta$ 1] [106] promote LL-37 expression in skin epithelial cells while proinflammatory cytokines display no effect on colonic epithelium [91]. IL-10 and IL-13 also mitigate LL-37 expression in the skin [5], and IL-18 stimulates LL-37 expression in colonic epithelial cells [107].

Apart from infection and stress, human LL-37 expression is also induced by several dietary factors including short-chain fatty acids, flavones, zinc, and vitamin D3. For example, short-chain fatty acids including butyrate and propionate induce LL-37 expression in intestinal and hepatic cells as well as lung epithelial cells by acting as histone deacetylase (HDAC) inhibitors [108-110]. Other HDAC inhibitors including phenylbutyrate and trichostatin (TSA) are also able to augment LL-37 expression in epithelial and monocytic cells [108, 110, 111]. Furthermore, oral supplementation of rabbits with butyrate or phenylbutyrate has shown reduced dysentery symptoms in Shigellosis infections through upregulation of rabbit cathelicidins in colon and lung epithelia [112, 113]. In animal agriculture, particularly in poultry, organic acids including

butyrate and propionate have been used for decades and shown an overall improved resistance to *S. enteritidis* [114] and *Clostridium perfringens* [115]. Many antibacterial mechanisms of organic acids have been proposed, including a reduction of intestinal pH, direct antibacterial activities, and suppression of bacterial attachment to host intestinal cells [114, 116-118]. It is also possible that organic acids, particularly short-chain fatty acids, enhance disease resistance by inducing HDP gene expression.

Besides fatty acids, vitamin D3 stimulates LL-37 synthesis in lung epithelial cells, keratinocytes, and monocytes, but not in colon epithelial cells [119-122]. LL-37 expression is also augmented by various cAMP analogs and agonists in mucosal epithelial cells [123]. In addition, zinc has the capacity to enhance LL-37 expression in human Caco-2 intestinal epithelial cells [124]. In humans, probiotic *E. coli* enhances human beta defensin synthesis [125]. Likewise, several probiotics and prebiotics have been used to control infections and inflammatory disorders like inflammatory bowel disease and irritable bowel syndrome [126]. It is likely that probiotics and prebiotics stimulate bacterial fermentation of short-chain fatty acids, which in turn promote HDP synthesis, host immunity, and disease resistance.

## **7. MOLECULAR MECHANISMS OF HDP MODULATION**

The regulatory mechanisms involved in cathelicidin gene expression are very complex. Histone acetylation and the signaling pathways mediated by mitogen-activated protein (MAP) kinases, cAMP, vitamin D receptor (VDR), and NF- $\kappa$ B are all capable of

transactivating HDP gene expression. In some cases, the pathways cross-talk with each other, leading to a synergistic induction of HDP synthesis. Short-chain fatty acids and HDAC inhibitors induce human LL-37 gene expression primarily through histone hyperacetylation of the gene promoter and also global core histone acetylation [108, 109, 121]. Three classic MAP kinase (p38, JNK, and ERK1/2) pathways, but not NF- $\kappa$ B, are involved in most epithelial cells in HDAC inhibitor-mediated induction of LL-37 expression [109]. Vitamin D3 enhances LL-37 expression in keratinocytes through activation of VDR, which in turn binds to vitamin D response element (VDRE) on the promoter [60, 119, 127, 128]. Additionally, vitamin D3 induces LL-37 expression through activation of PPAR $\gamma$ , resulting in activation of p38 MAP kinase pathway and binding of transcription factor AP-1 to the gene promoter [129]. cAMP signaling agonists were recently found to turn on LL-37 gene expression by activation of the protein kinase A (PKA) pathway, which ultimately leads to phosphorylation of cAMP response element binding protein (CREB) and AP-1 and gene transactivation [123].

In psoriatic skin, overexpressed LL-37 binds to self-DNA to turn on TLR9-dependent signaling pathway, leading to excessive inflammation and psoriasis [5, 103, 105]. TLR2 agonists also increase the expression of the genes for CYP27B1 and VDR. CYP27B1 further convert inactive vitamin D3 to 1, 25-vitamin D3, which leads to LL-37 upregulation via VDR activation [127, 128]. Bacteria, bacterial products, and proinflammatory cytokines activate HDP gene expression primarily through Toll-like receptor-mediated NF- $\kappa$ B activation, although MAP kinase pathways are also activated in most cases [105,130-132,133,134,135]. Zinc regulates LL-37 expression in epithelial cells through p38 and ERK1/2 MAP kinase pathways [124].

A synergist effect on HDP gene induction has been demonstrated with HDAC inhibitors, VDR and cAMP signaling. For example, butyrate and vitamin D3 synergize with each other in inducing LL-37 expression in keratinocytes through activation of SRC3, which has inherent histone acetyltransferase activity [121]. Butyrate also synergizes with forskolin, a cAMP agonist, in inducing LL-37 expression through prolonged activation of CREB [123].

In summary, HDPs possess a myriad of beneficial functions with potent antimicrobial, anti-inflammatory, and immunomodulatory activities. A growing body of evidence suggests that dietary factors including vitamin D3, short-chain fatty acids, zinc, and certain amino acids are capable of inducing HDP synthesis in humans. Convenient dietary modulation of the endogenous HDP synthesis may have potential to be explored as a novel antibiotic-free strategy to disease prevention and control for both animal and human health. We have explored the potential and found that short-chain fatty acids and their structural and functional analogs are strong inducers of HDP production, with the capacity to enhance innate immunity and disease resistance in chickens.

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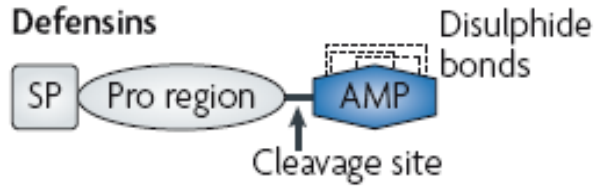
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## FIGURE AND FIGURE LEGENDS

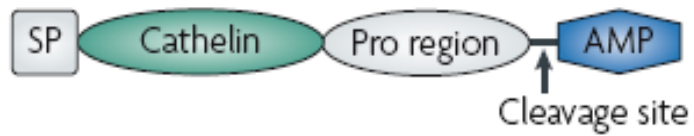
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#### Defensins

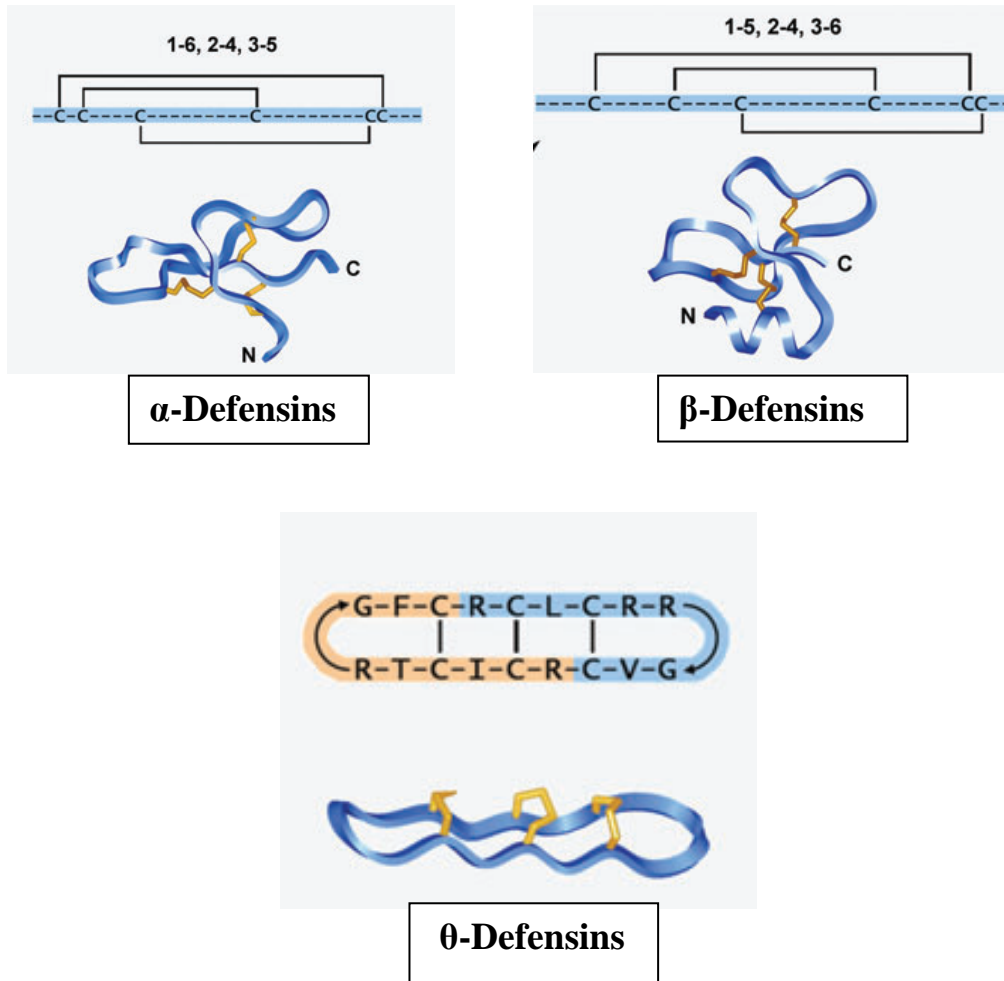


Disulphide bonds confer macrocyclic structure

#### Cathelicidin (LL37)

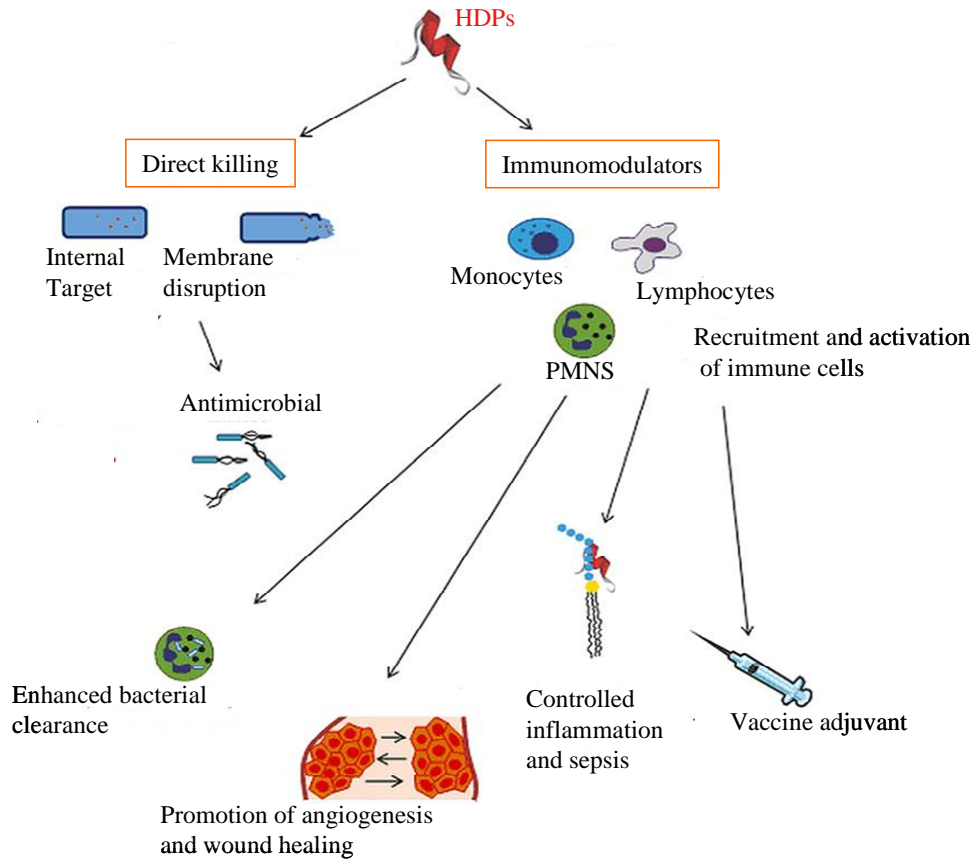


**Fig. 1. Structure and functional domains of defensins and cathelicidins.** SP: Signal peptide, AMP: mature antimicrobial peptide. Figure was adopted from reference [5].



**Fig. 2. Linkage of disulfide bonds and spatial structures of mammalian defensins.**

Figure was adapted from reference [26].



**Fig. 3. Biological functions of host defense peptides (HDPs).** HDPs simultaneously possess direct antimicrobial, immunomodulatory, anti-inflammatory, and wound healing activities. Figure was adopted from reference [14].

## CHAPTER III

### BUTYRATE ENHANCES DISEASE RESISTANCE OF CHICKENS BY INDUCING ANTIMICROBIAL HOST DEFENSE PEPTIDE GENE EXPRESSION<sup>†</sup>

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## ABSTRACT

Host defense peptides (HDPs) constitute a large group of natural broad-spectrum antimicrobials and an important first line of immunity in virtually all forms of life. Specific augmentation of synthesis of endogenous HDPs may represent a promising antibiotic-alternative approach to disease control. In this study, we tested the hypothesis that exogenous administration of butyrate, a major type of short-chain fatty acids derived from bacterial fermentation of undigested dietary fiber, is capable of inducing HDPs and enhancing disease resistance in chickens. We have found that butyrate is a potent inducer of several, but not all, chicken HDPs in HD11 macrophages as well as in primary monocytes, bone marrow cells, and jejunal and cecal explants. In addition, butyrate treatment enhanced the antibacterial activity of chicken monocytes against *Salmonella enteritidis*, with a minimum impact on inflammatory cytokine production, phagocytosis, and oxidative burst capacities of the cells. Furthermore, feed supplementation with 0.1% butyrate led to a significant increase in HDP gene expression in the intestinal tract of chickens. More importantly, such a feeding strategy resulted in a nearly 10-fold reduction in the bacterial titer in the cecum following experimental infections with *S. enteritidis*. Collectively, the results indicated that butyrate-induced synthesis of endogenous HDPs is a phylogenetically conserved mechanism of innate host defense shared by mammals and aves and that dietary supplementation of butyrate has potential for further development as a convenient antibiotic-alternative strategy to enhance host innate immunity and disease resistance.

## 1. INTRODUCTION

Host defense peptides (HDPs), also known as antimicrobial peptides, are present in virtually all species of life and constitute a critical component of the innate immunity [2-6]. Defensins and cathelicidins represent two major families of HDPs in vertebrates [7-12]. While defensins are categorized by the presence of six conserved cysteine residues in the C-terminal mature sequence [7-9, 12], all cathelicidins consist of a conserved cathelin domain in the pro-sequence with a highly diversified C-terminal mature sequence [10, 11]. The chicken genome was recently found to encode a total of 14  $\beta$ -defensins known as AvBD1-14 [13-15] and four cathelicidins, namely fowlicidins 1-3 [13, 16, 17] and cathelicidin-B1 [18]. All AvBDs are densely clustered on chicken chromosome 3q [14, 15], whereas cathelicidin genes are located on chromosome 2p [17, 18]. Both chicken AvBDs and cathelicidins are expressed in a wide range of tissues, with cathelicidins expressed most abundantly in the bone marrow or bursa of Fabricius [16-18] and  $\beta$ -defensins in the liver and throughout the digestive, respiratory, and reproductive tracts [13, 15]. HDPs possess broad-spectrum antimicrobial activities against bacteria, protozoa, enveloped virus, and fungi mainly through direct binding and lysis of microbial membranes [6, 19].

Because of such physical interactions, it is extremely difficult for pathogens to develop resistance to HDPs. Many chicken HDPs such as AvBD9 (formally known as gallinacin-6) and cathelicidin B1 have been found to possess potent antibacterial



activities against a broad range of bacteria including *Salmonella* [17, 20-26]. Besides direct microbicidal activities, HDPs have a strong capacity to modulate the innate immune response by inducing chemotaxis and activation of various types of leukocytes [3, 5]. Because of these pleiotropic effects, HDPs have been actively explored as a new class of therapeutic agents against antibiotic-resistant microbes and other inflammatory diseases [3, 6].

Butyrate, a major species of short-chain fatty acids produced by bacterial fermentation of undigested carbohydrates in the intestine [27, 28], was recently found to be capable of inducing HDP expression in humans and rabbits [29-31]. To test whether butyrate can augment HDP gene expression in a non-mammalian species, we studied the effect of butyrate on HDP gene expression and the antibacterial activity of monocytes in the chicken. Furthermore, we examined the effect of supplementing butyrate in the feed on the titer of *Salmonella enteritidis* in the cecum following experimental infections. We concluded that butyrate-mediated induction of HDP synthesis is phylogenetically conserved in both mammals and aves. Additionally, butyrate may be further exploited as a cost-effective feed or food additive in enhancing host immunity and disease resistance.

## **2. MATERIALS AND METHODS**

### **2.1. Isolation, culture, and stimulation of chicken cells and intestinal tissue explants**

Chicken HD11 macrophage cells [32] were cultured in complete RPMI 1640 containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin, and

seeded at  $2 \times 10^6$  cells/well in 6-well cell culture plates overnight, prior to stimulation with different concentrations of sodium butyrate (Sigma) in duplicate and incubated at 37°C and 5% CO<sub>2</sub> for indicated times. Chicken peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-anticoagulated venous blood of adult layers through gradient centrifugation using Histopaque 1077 (Sigma). Monocytes were obtained by seeding PBMCs at  $3 \times 10^7$  cells/well in 6-well plates overnight and washing off non-adherent cells twice with calcium- and magnesium-free Hank's balanced salt solution (HBSS). Monocytes were replenished with fresh complete RPMI 1640 prior to stimulation with sodium butyrate. Bone marrow cells were collected from femur bones of 1- to 2-week-old broiler chickens, lysed of erythrocytes, and cultured at  $1 \times 10^7$  cells in 60-mm tissue culture dishes in RPMI 1640 containing 20 mM HEPES, 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin, followed by butyrate stimulation.

Jejunal and cecal explants were obtained by washing thoroughly a segment of the jejunum and cecum of 1- to 2- week-old broiler chickens with cold HBSS containing 50 µg/ml of gentamicin, followed by slicing in a series of 0.5-cm long segments and placing individually in 6-well tissue culture plates in RPMI 1640 containing 20 mM HEPES, 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µg/ml gentamicin. Jejunal and cecal explants were cultured at 37°C and 5% CO<sub>2</sub> in the presence of different concentrations of sodium butyrate in duplicate for 24 h.

## **2.2. Real-time RT-PCR analysis of chicken HDP gene expression**

Following treatment with sodium butyrate, chicken cells and tissue explants were lysed in Tri Reagent (Sigma) for extraction of total RNA. The first-strand cDNA was synthesized

from 300 ng of total RNA using QuantiTect Reverse Transcription Kit (Qiagen) in a total volume of 4  $\mu$ l. Real-time PCR was then performed using QuantiTect SYBR Green PCR kit (Qiagen) and MyiQ Real-Time PCR Detection System (Bio-Rad) in 10  $\mu$ l reactions containing 1/40 or 1/20 of the first-strand cDNA and gene-specific primers for 14 AvBDs (Table 1), 4 chicken cathelicidins, multiple cytokines and GAPDH (Table 2) as described [17, 26, 33]. PCR cycling conditions were 95°C for 10 min, followed by 45 cycles of 94°C for 15 sec, 55°C for 20 sec, and 72°C for 30 sec. The specificity of PCR reaction was confirmed by the melt curve analysis. The gene expression levels were quantified using the comparative  $\Delta\Delta$ Ct method with the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene as a reference for normalization.

### **2.3. Cell cytotoxicity of butyrate in HD11 cells**

The cytotoxicity assay was performed as described previously [26, 33, 34]. Briefly, HD11 cells ( $1 \times 10^5$ ) were seeded overnight in 96-well tissue culture plates. Butyrate was added in duplicate from 0 to 16 mM for 18 h, following by addition of 10% of alamarBlue (Invitrogen) for another 6 h. The fluorescence was read at 545 nm excitation and 590 nm emission. Cell death (%) was calculated as  $[1 - (F_{\text{butyrate}} - F_{\text{background}})/(F_{\text{control}} - F_{\text{background}})] \times 100$ , where  $F_{\text{butyrate}}$  is the fluorescence of cells exposed to different concentrations of butyrate,  $F_{\text{control}}$  is the fluorescence of cells only, and  $F_{\text{background}}$  is the background fluorescence of 10% alamarBlue in cell culture medium without cells.

### **2.4. Antibacterial activity of monocytes treated with butyrate**

Following overnight adherence of PBMCs to cell culture dishes, chicken monocytes were replenished with fresh antibiotic-free RPMI 1640 and incubated with 0, 0.5, 1, 2, and 4

mM of sodium butyrate for 24 h. Cells were then scraped, stored at  $-80^{\circ}\text{C}$  overnight, lysed with 1% Triton X-100, and centrifuged at  $12,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . Serial 2-fold dilutions were then prepared from the cell supernatants and incubated with  $2 \times 10^4$  CFU of *Salmonella enteritidis* (ATCC 13076) in 20% Trypticase Soy Broth containing 1 mM  $\text{NaH}_2\text{PO}_4$  and 25 mM  $\text{NaHCO}_3$  for 9 h in a 96-well plate at  $37^{\circ}\text{C}$  as described [35]. Bacterial turbidity was measured at  $\text{OD}_{590 \text{ nm}}$  using an ELISA plate reader. Different concentrations of sodium butyrate were also directly added to *S. enteritidis* in the same growth medium to measure turbidity after 9 h incubation.

## **2.5. Phagocytosis assay of HD11 cells**

Phagocytosis of *S. enteritidis* phage type 13a by HD11 cells was measured as described with slight modifications [36]. After seeding  $6 \times 10^6$  cells in complete RPMI 1640 overnight in 60- mm tissue culture plates, HD11 cells were stimulated with and without 0.5, 1 or 2 mM sodium butyrate for 24 h. Cells ( $2.5 \times 10^6$ ) were then incubated with  $2.5 \times 10^7$  CFU of *S. enteritidis* phage type 13a in 1 ml RPMI 1640 containing 5% chicken serum for 30 min at  $37^{\circ}\text{C}$ . To kill extracellular bacteria, cells were washed twice with ice-cold HBSS, re-suspended with 1ml RPMI 1640 containing 100  $\mu\text{g}/\text{ml}$  gentamicin for 1 h at  $37^{\circ}\text{C}$ . Cells were then lysed by incubating with 1% Triton X-100 for 15 min, serially diluted, and spread on Brilliant Green agar plates (Becton Dickinson) containing 20  $\mu\text{g}/\text{ml}$  of nalidixic acid and incubated overnight at  $37^{\circ}\text{C}$  for enumeration.

## **2.6. Oxidative burst assay of HD11 cells**

The assay of oxidative burst activity was performed as previously described with slight modifications [37]. Briefly, HD11 cells were seeded at  $1 \times 10^5$  cells in a 96-well plate in

complete RPMI 1640 and cultured overnight. After addition of 0, 0.5, 1, and 2 mM of sodium butyrate for 24 h, cells were washed with HBSS to remove antibiotics, replenished with fresh RPMI 1640 free of Phenol Red and antibiotics, and rested for 30 min. Phorbol 12-myristate 13-acetate (PMA, Sigma) and 2',7'-dichlorodihydrofluorescein diacetate (DCFDA, Sigma) were added to cells to final concentrations of 0.5 µg/ml and 10 µM, respectively. The fluorescence was monitored at 485 nm excitation and 528 nm emission using FLx800 Multi-Detection Microplate Reader (Bio-Tek Instruments) 1 h after incubation at 37°C. The results were normalized against protein concentrations, which were measured using the Bradford assay (Bio-Rad) as per manufacturer's instructions.

## **2.7. Flow cytometric analysis of MHC class I and II surface markers**

Following stimulation with 4 mM butyrate, 1 µg/ml LPS from *E. coli* O111:B4 (Sigma) or left untreated for 24 h, HD11 cells were scraped, washed, and adjusted to  $1 \times 10^6$ /ml with the FACS buffer (0.1% BSA + 0.02% sodium azide in phosphate buffered saline). Cells were preincubated in the FACS buffer containing 1% chicken serum and 1% of rat FCγ III/II receptor blocker (clone 2.4G2, eBioscience) for 15 min, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated mouse anti-chicken MHC class I (clone F21-2, SouthernBiotech) and R-phycoerythrin (R-PE)-conjugated mouse anti-chicken MHC class II (clone 2G11, SouthernBiotech) monoclonal antibodies for another 30 min. Flow cytometry was performed on a FACSCalibur Flow Cytometer (Becton-Dickinson) and analyzed with BD CellQuest Pro-software.

## **2.8. Butyrate feeding and *S. enteritidis* infection of chickens**

Two chicken trials were conducted to test the in vivo effect of butyrate on HDP gene expression and disease resistance. In trial 1, a total of 20, five-day-old male Cornish Rock broiler chickens (Ideal Poultry, Cameron, TX) were equally divided and fed with a standard antibiotic-free ration mixed with or without 0.2% sodium butyrate for 48 h prior to intraesophageal infections with 0.5 ml of Lysogeny broth (LB) containing  $1 \times 10^6$  CFU of *S. enteritidis* phage type 13a [38]. After continuous feeding with butyrate-supplemented feed for another 4 days, the birds were euthanized and cecal contents were aseptically collected from each animal, serially diluted in PBS, and plated on Brilliant Green agar plates (Becton Dickinson) containing 20 µg/ml of nalidixic acid for bacterial enumeration. Trial 2 was conducted similarly with a total of 30, five-day-old male broilers fed with or without 0.1% or 0.2% sodium butyrate supplementation in the feed for two days, with 10 chickens per treatment. An intraesophageal infection with  $1 \times 10^6$  CFU of *S. enteritidis* phage type 13a was conducted 2 days later and butyrate supplementation was continued for another 4 days. Cecal contents were then collected from each chicken for bacterial counting. All animal procedures were approved by the Institutional Care and Use Committee of Oklahoma State University. Unpaired Student's *t*-test was performed among groups, and  $p < 0.05$  was considered statistically significant.

### 3. RESULTS

#### **3.1. Butyrate induces HDP gene expression in chicken HD11 macrophage cells, primary monocytes, bone marrow cells, and jejunal and cecal explants**

To elucidate the effect of butyrate on HDP gene expression in the chicken, we first stimulated HD11 macrophage cells and primary chicken monocytes with different concentrations of sodium butyrate for various times, followed by real-time RT-PCR analysis of the expressions of the genes for all 14 AvBDs and 4 cathelicidins. Butyrate enhanced HDP gene expression significantly in all chicken cell types tested (Fig. 1). The avian  $\beta$ -defensin 9 (*AvBD9*) gene was dramatically induced in HD11 cells in a time-dependent manner peaking at 24-48 h following stimulation with 4 mM butyrate (Fig. 1A). A dose-dependent induction was also evident in HD11 cells, with 4 mM butyrate giving nearly 5400-fold induction of *AvBD9* after treatment for 24 h (Fig. 1B). Similarly, the *AvBD9* gene expression was dose-dependently augmented in primary monocytes, resulting in a 200- and 650-fold increase following 24 h stimulation with 4 and 8 mM butyrate, respectively (Fig. 1C). A 700-fold augmentation of the *AvBD9* gene was also observed in chicken bone marrow cells treated with 4 mM butyrate for 24 h (Fig. 1D). It is noteworthy that the kinetics of butyrate-mediated HDP gene expression is similar in humans, where a peak response occurred in intestinal cell lines 1-2 days following treatment with 4 mM butyrate [30, 31]. However, it is not clear why the sensitivity of the two chicken cell types to butyrate differs. Butyrate at 4 mM gave an optimal induction of the *AvBD9* gene in HD11 and bone marrow cells, whereas a peak response occurred at 8 mM in primary monocytes, although no appreciable impact on the viability of the cells was observed in any cell type in response to up to 8 mM butyrate (data not shown).

Besides *AvBD9*, several other chicken HDP genes including *cathelicidin B1*, *AvBD3*, *AvBD4*, *AvBD8*, *AvBD10*, and *AvBD14*, also showed largely dose-dependent inductions in response to butyrate treatment in HD11 cells, albeit at a lesser magnitude

than *AvBD9* (Fig. 2). A similar trend also occurred in chicken primary monocytes, where butyrate triggered dose- dependent up-regulation of *cathelicidin B1*, *AvBD3*, *AvBD5*, and *AvBD14* (Fig. 2). Notably, a subset of HDP genes including *AvBD1*, *AvBD6*, and *fowlicidins 1-3* were essentially not modulated by butyrate in either cell type (Fig. 2). Furthermore, *AvBD2* and *AvBD7* were even slightly down-regulated in primary monocytes and HD11 cells, respectively (Fig. 2), suggesting differential regulation of HDPs by butyrate. To further examine whether butyrate is capable of augmenting HDP gene expression in intestinal cells, chicken jejunal and cecal explants were prepared and stimulated with butyrate for 24 h. Three representative HDPs, namely *AvBD9*, *AvBD14*, and *cathelicidin B1*, were induced significantly in a dose-dependent manner in both the jejunum (Fig. 3A) and cecum (Fig. 3B), although the magnitude of induction was generally less pronounced in the cecum than in the jejunum.

To confirm the HDP-inducing activity of butyrate *in vivo*, we fed 2-day-old broiler chickens with and without 0.1% and 0.2% butyrate in standard ration for 2 days and harvested the crop, cecal tonsil, and cecum for real-time RT-PCR analysis of the *AvBD9* gene expression. As shown in Fig. 4, significantly induced *AvBD9* expression was observed in the crop, with 0.1% and 0.2% butyrate leading to 22- and 7.5-fold increase, respectively. A similar, but less dramatic trend also occurred in the cecal tonsil and cecum (Fig. 4). It is not known why a reduced response was seen with 0.2% butyrate supplementation compared to 0.1% butyrate. Perhaps higher concentrations of butyrate are more potent in inducing growth arrest and apoptosis [27, 28]. The finding that *AvBD9* induction is more pronounced in the crop than in the lower digestive tract is perhaps related to tissue specificity. However, it is more likely because local



concentrations of supplemented butyrate are much higher in the crop than in other parts of the intestinal tract, similar to earlier findings that the majority of butyrate is absorbed in the crop before reaching the lower digestive tract [39, 40]. Collectively, these results strongly suggest that butyrate is a potent inducer of the chicken HDP expression in multiple cell types both in vivo and in vitro, although cell- and tissue-specific induction patterns are also evident.

### **3.2. Butyrate triggers no or minimum inflammatory response**

Butyrate generally exerts anti-inflammatory effects and has been used to treat inflammatory bowel diseases [27, 28]. To confirm butyrate-mediated specific augmentation of HDP gene expression without triggering a proinflammatory response, we treated HD11 cells with and without butyrate for 3 and 24 h and analyzed the expressions of three representative cytokines, namely *IL-1 $\beta$* , *IL-8*, and *IL-12p40*. Butyrate had essentially no effect on either *IL-1 $\beta$*  (Fig. 5A) or *IL-12p40* expression (Fig. 5B) at both time points. No influence on *IL-8* expression was observed after 3 h stimulation with a moderate induction only after 24 h (Fig. 5C). In contrast, *IL-1 $\beta$* , *IL-8*, and *IL-12p40* were induced markedly in response to 1  $\mu$ g/ml LPS (Fig. 5). These results demonstrated that butyrate selectively induces HDPs with a minimum impact on proinflammatory cytokine expression, consistent with earlier transcriptional profiling results that butyrate is generally anti-inflammatory, suppressing expression of certain cytokines with no effect on the majority of them [41, 42].

### **3.3. Butyrate augments the antibacterial activity of chicken monocytes through induction of HDPs**

To investigate the functional consequence of butyrate-induced HDP expression, we stimulated chicken primary monocytes with and without different concentrations of butyrate for 24 h, lysed cells, incubated cell lysates with *S. enteritidis*, and measured bacterial turbidity after 9 h. As shown in Fig. 6, a dose-dependent, statistically significant suppression of bacterial growth in butyrate-treated monocyte lysates was observed, with 4 mM butyrate giving greater than 3-fold reduction in turbidity. It is worth noting that incubation of bacteria with butyrate alone had no impact on bacterial growth at up to 4 mM (Fig. 6), implying that butyrate is incapable of killing bacteria directly at the HDP-inducing concentrations. Furthermore, given that butyrate in the cell culture medium was completely washed off prior to cell lysis and the antibacterial assay, an enhancement in the antibacterial activity of the cell lysates is unlikely due to the direct bacterial killing activity of butyrate.

To further rule out the possibility that butyrate-induced augmentation of the antibacterial activity was not attributed to a change in phagocytosis of chicken macrophages by butyrate, we first incubated HD11 cells with different concentrations of butyrate for 24 h and then measured the phagocytic capacity of the cells to *S. enteritidis*. In comparison with non-treated cells, essentially no difference in phagocytosis was observed with any concentration of butyrate (Fig. 7A). We further examined the influence of butyrate on the oxidative burst activity of chicken macrophages. As seen in Fig. 7B, PMA triggered a significant oxidative burst in HD11 cells; however, butyrate had a minimum impact on the cells treated with and without PMA.

To test whether butyrate is capable of activating chicken macrophages, we quantified a surface marker of cell activation, i.e., MHC class II, on HD11 cells by flow

cytometry following stimulation with 4 mM butyrate for 24 h, using MHC class I as a house-keeping control. As expected, LPS stimulation induced surface expression of MHC class II in nearly 50% cells; however, essentially no change in MHC class II expression was observed in butyrate-treated HD11 cells (Fig. 8). These results collectively indicated that butyrate is incapable of modulating phagocytosis, oxidative burst or activation status of macrophage cells. Augmentation of the antibacterial activity in response to butyrate treatment, therefore, is likely due to specific induction of endogenous synthesis of HDPs.

### **3.4. Oral supplementation of butyrate reduces *S. enteritidis* colonization in the cecum of infected chickens**

Because enhanced HDP gene expression and antibacterial activities were observed in cells in response to butyrate treatment, we evaluated whether supplementation of feed with butyrate can reduce the survival of pathogenic bacteria in the intestinal tract of 5-day-old broilers in two separate trials. Chickens were fed with and without 0.1% and/or 2% butyrate for 2 days prior to intraesophageal inoculation of *S. enteritidis* phage type 13a for another 4 days. The cecal contents, where *S. enteritidis* most heavily colonizes, were aseptically harvested and subjected to serial plating on Brilliant Green agar plates containing 20 µg/ml of nalidixic acid for specific enumeration of *S. enteritidis* 13a. In trial 1, oral supplementation of 0.2 % butyrate resulted in 1-log reduction in the median counts of inoculated bacteria in the cecal content, relative to the control group (Fig. 9A). In trial 2, 0.1% butyrate significantly reduced bacterial load ( $P = 0.03$ ) in the cecal content of the chickens, whereas 0.2% butyrate led to a less reduction of bacterial counts (Fig. 9B). This is perhaps not surprising, given the earlier findings

that, as compared to 0.1% butyrate, 0.2% butyrate supplementation caused less induction of the HDP genes in the intestinal tract (Fig. 4).

#### 4. DISCUSSION

As a major species of short-chain fatty acids produced from fermentation of undigested dietary fiber by intestinal microflora, butyrate exerts a plethora of effects on intestinal health and disease [27, 28, 40]. In addition to being a primary energy source for colonocytes in mammals, butyrate has been found to play an important role in the digestive tract by stimulating mucin synthesis and intestinal motility, cell proliferation and differentiation, while suppressing inflammatory diseases [27, 28, 40]. In the present study, we have revealed a novel role for butyrate in host defense and extended earlier findings that butyrate-induced synthesis of HDPs not only occurs in humans and rabbits [29-31], but is also conserved in chickens. We have presented both *in vitro* and *in vivo* evidence showing that butyrate strongly induces the expressions of multiple HDPs in different cell and tissue types including HD11 macrophages, primary monocytes, bone marrow cells, jejunum and cecal explants as well as in crop, cecum, and cecal tonsils of chickens. The results clearly suggest that transcriptional regulatory mechanisms of many HDPs are phylogenetically conserved across mammals and aves.

It is important to note that only a subset of chicken HDPs are regulated by butyrate (Fig. 2), implying that HDPs are differentially regulated even within the same family. Consistently, only LL-37 and human  $\beta$ -defensin-2 were reported to be regulated

by butyrate in humans [30, 31, 43]. For those chicken HDP genes that are modulated by butyrate, we observed a clear cell-specific regulation pattern as evidenced by marked differences in the magnitude of induction among different cell types. For example, treatment with 4 mM butyrate for 24 h induced the *AvBD9* gene approximately 3,000- to 5,000-fold in HD11 macrophage cells, but only 200-fold in primary monocytes, 700-fold in bone marrow cells, 140-fold in jejunal explants, and 5-fold in cecal explants (Figs. 1 and 3). Several other HDPs, e.g., *AvBD14* and *cathelicidins B1* were also regulated differently among individual cell types (Fig. 3 and data not shown).

Although we could not detect the synthesis of chicken HDPs at the protein level in response to butyrate treatment due to a lack of specific antibodies, we observed an increased HDP gene synthesis leading to an enhanced antibacterial activity in monocytes in vitro and augmented intestinal bacterial clearance in vivo following butyrate treatment. A nearly 10-fold reduction in the bacterial titer was achieved in the cecal contents of the chickens fed 0.1% or 0.2% butyrate (Fig. 9). Given the rapid rate of absorption and metabolism, the majority of supplemented butyrate is known to be taken up by the upper digestive tract, with very small quantities reaching the lower intestinal tract or general circulation [39, 40]. A more pronounced reduction in the cecal bacterial titer may be achieved if supplemented butyrate can be protected when passing through the upper digestive tract or if more butyrate can be produced in the cecum by manipulating the conditions of local bacterial fermentation [39, 40].

It is noteworthy that 0.1% butyrate gave a better bacterial reduction than 0.2% butyrate in our feeding trial (Fig. 9B), in agreement with the finding that 0.1% butyrate supplementation led to a higher level of the *AvBD9* gene transcription in the crop, cecum,

and cecal tonsil of chickens than 0.2% butyrate (Fig. 4). Consistently, 8 mM butyrate failed to stimulate the synthesis of a higher amount of the *AvBD9* transcripts in HD11 cells than 4 mM butyrate (Fig. 1B). In fact, higher concentrations of butyrate often lead to cytotoxicity, growth arrest, and apoptosis [27, 28, 40]. The optimal dose of butyrate for in vivo applications, therefore, needs to be investigated carefully for each animal species.

It was reported earlier that oral supplementation of 0.63 g/kg or 0.92 g/kg of butyrate reduces colonization and shedding of *S. enteritidis* in the cecum of chickens [44, 45]. However, the mechanism by which butyrate suppresses bacterial growth remain elusive, although it was proposed to be a result of the direct antibacterial activity of butyrate [46, 47] or a decrease in the invasiveness of *Salmonella* through intestinal epithelial cells following exposure to butyrate [36, 47]. However, because especially high concentrations of butyrate (25, 50, and 100 mM) were needed to kill bacteria or negatively impact on bacterial invasiveness [36, 46, 47], it is uncertain whether these proposed mechanisms may occur in vivo, given that most butyrate is absorbed in the upper digestive tract if supplemented orally [39, 40] and that cecal concentrations of butyrate are only < 6 mM in 18-day-old healthy broiler chickens and < 1 mM in 4-day-old chickens [47]. More importantly, an increased invasion to intestinal epithelial cells was observed in the same study when *S. enteritidis* was pre-incubated with a mixture of short-chain fatty acids mimicking the in vivo cecal concentrations [47]. Here, we uncovered a novel mechanism that we believe accounts primarily for butyrate-mediated suppression of intestinal bacterial colonization. We found that at physiological concentrations butyrate fails to inhibit bacteria directly, but increase the antibacterial activity of host innate immune cells by inducing the synthesis of an array of HDPs with a

minimum impact on the phagocytic and oxidative killing capacity as well as activation status of host cells. Therefore, it is the production of HDPs that is mainly responsible for a reduction of bacterial colonization in the intestinal tract of chickens following oral supplementation of butyrate.

Our *in vitro* and *in vivo* studies have firmly established that butyrate has a strong capacity to induce HDP synthesis and that supplementation of butyrate can augment disease resistance and reduce bacterial colonization in chickens. Therefore, the strategies for efficient delivery of butyrate to the lower intestinal tract will have important implications in animal health and food safety. Indeed, the microencapsulated form of butyrate proves to be more efficient in suppressing bacterial growth in the ceca of chickens than the free unprotected form [44, 45]. Alternatively, identification and application of less labile forms of butyrate analogs in the feed may also prove to be more desirable. In fact, several butyrate analogs have been shown to be capable of inducing HDP gene expression in humans [48] and such analogs await further testing for their antibacterial efficacy in other animal species such as chickens. Besides direct administration of butyrate and its analogs, the dietary approaches that promote the proliferation of butyrate-producing bacteria and stimulate the fermentation of butyrate through the use of prebiotics may also have good prospect to augment HDP synthesis and host defense.

In summary, we have revealed that butyrate-induced synthesis of endogenous HDPs is a phylogenetically conserved mechanism of innate host defense shared by both mammals and chickens. Moreover, we propose that butyrate-induced HDP synthesis represents a newly discovered mechanism that mainly accounts for the suppression of

bacterial colonization and shedding in farm animals by butyrate. Coupled with anti-inflammatory effects and other beneficial properties, butyrate, butyrate analogs, and perhaps other short-chain fatty acids may have potential for further development as antibiotic-alternative food or feed additives to boost innate immunity and disease resistance of humans and animals without provoking a harmful proinflammatory response.

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**Table 1. Primer sequences of chicken Avian  $\beta$ -defensins (AvBDs) for real time PCR<sup>a</sup>**

Gene	Forward primer	Reverse primer	Product size (bp)	
			cDNA	Gene
AvBD1	ATGCGGATCGTGTACCTGCTC	CTGCTTGGGATGTCTGGCTCT	219	1197
AvBD2	CTCTCTCCTCTTCCTGGCAC	GAGGGGTCTTCTTGCTGCTG	265	1122
AvBD3	ATGCGGATCGTGTACCTGCTC	CAGAATTCAGGGCATCAACCTC	196	2379
AvBD4	CATCTCAGTGTGTTTCTCTGC	ACAATGGTTCCTCAAATCCAAC	321	899
AvBD5	CTGCCAGCAAGAAAGGAACCTG	TGAACGTGAAGGGACATCAGAG	300	1100
AvBD6	AGGATTTACATCCCAGCCGTG	CAGGAGAAGCCAGTGAGTCATC	249	1203
AvBD7	CTGCTGTCTGTCCTCTTTGTGG	CATTTGGTAGATGCAGGAAGGA	230	665
AvBD8	TTCTCCTCACTGTGCTCCAA	AAGGCTCTGGTATGGAGGTG	124	383
AvBD9	GCAAAGGCTATTCCACAGCAG	AGCATTTCAGCTTCCCACCAC	211	1802
AvBD10	TGGGGCACGCAGTCCACAAC	ATCAGCTCCTCAAGGCAGTG	298	2285
AvBD11	ACTGCATCCGTTCCAAAGTCTG	TCGGGCAGCTTCTCTACAAC	301	1299
AvBD12	CCCAGCAGGACCAAAGCAATG	GTGAATCCACAGCCAATGAGAG	335	731
AvBD13	CATCGTTGTCATTCTCCTCCTC	ACTTGCAGCGTGTGGGAGTTG	175	4514
AvBD14	CTCCTGTTTCTTGTTCCTCTG	CACTTTGCCAGTCCATTGTAG	149	501

<sup>a</sup> Primers for AvBD4-13 are adopted from reference 15.

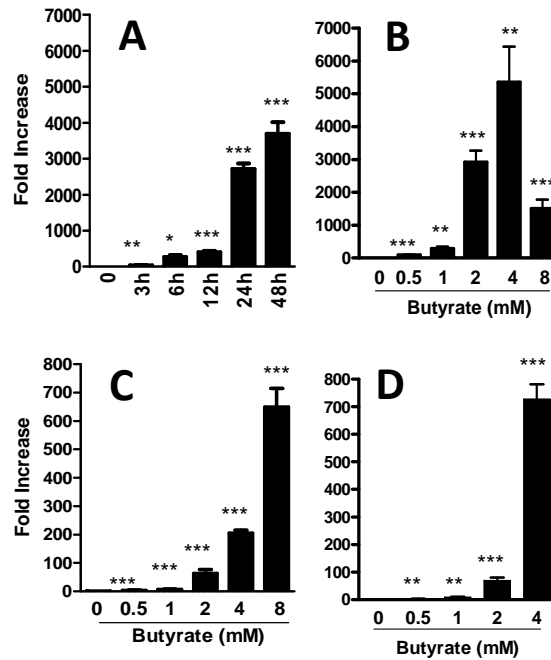


**Table 2. Primer sequences of GAPDH, chicken cathelicidin/fowlicidin family and cytokines for real time PCR<sup>a</sup>**

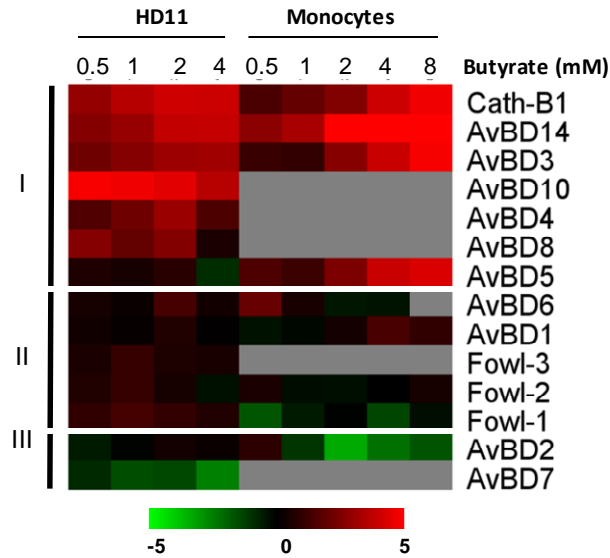
Gene	Forward primer	Reverse primer	Product size (bp)	
			cDNA	Gene
Cath-B1	CCGTGTCCATAGAGCAGCAG	AGTGCTGGTGACGTTTCAGATG	170	251
Fowlicidin-1	GCTGTGGACTCCTACAACCAAC	GGAGTCCACGCAGGTGACATC	261	882
Fowlicidin-2	CAAGGAGAATGGGGTCATCAG	CGTGGCCCCATTTATTCATTCA	221	584
Fowlicidin-3	GCTGTGGACTCCTACAACCAAC	TGGCTTTGTAGAGGTTGATGC	352	1095
IL-1 $\beta$	GACATCTTCGACATCAACCAG	CCGCTCATCACACACGACAT	215	384
IL-8	GCTGATCGTAAAGGCACTTATG	GTGAAAGGTGGAAGATGGAATG	159	727
IL-12p40	GACCCACCTCAATGTCAGTATG	GCCCAGTCTTTGGAATCTGAAT	184	1456
GAPDH	GCACGCCATCACTATCTTCC	CATCCACCGTCTTCTGTGTG	356	876

<sup>a</sup> Primers for GAPDH are adopted from reference 15.

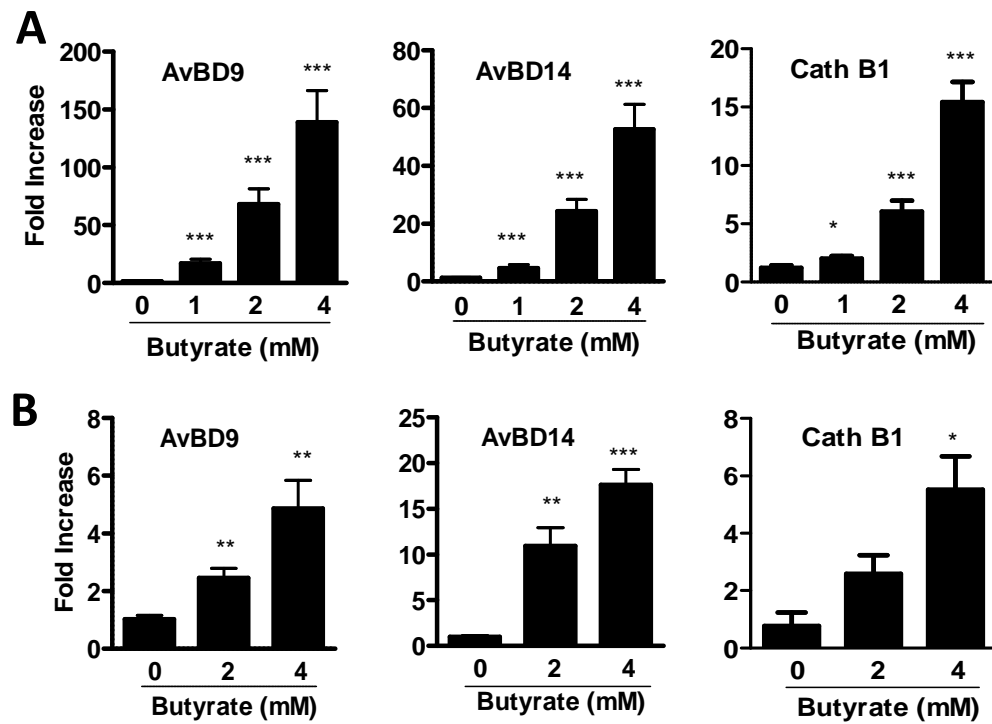
## FIGURE AND FIGURE LEGENDS



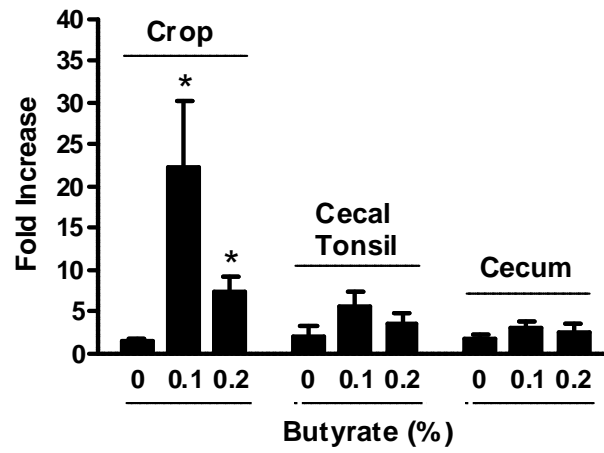
**Fig. 1. Butyrate-induced expression of the *AvBD9* gene in different chicken cell types.** HD11 macrophage cells were incubated in duplicate with 4 mM sodium butyrate for indicated time points (A) or indicated concentrations of butyrate for 24 h (B). Chicken primary monocytes (C) or bone marrow cells (D) were exposed to different concentrations of butyrate in duplicate for 24 h prior to isolation of total RNA. The *AvBD9* gene expression was analyzed by real-time RT-PCR, and the relative fold increase over the control group was calculated using the comparative  $\Delta\Delta C_t$  method and the *GAPDH* gene for normalization. The bars represent means  $\pm$  standard error of the data from 2-3 independent experiments. \* $P < 0.05$ , \*\*  $P < 0.001$ , and \*\*\*  $P < 0.0001$  by unpaired Student's *t*-test as compared to the untreated control.



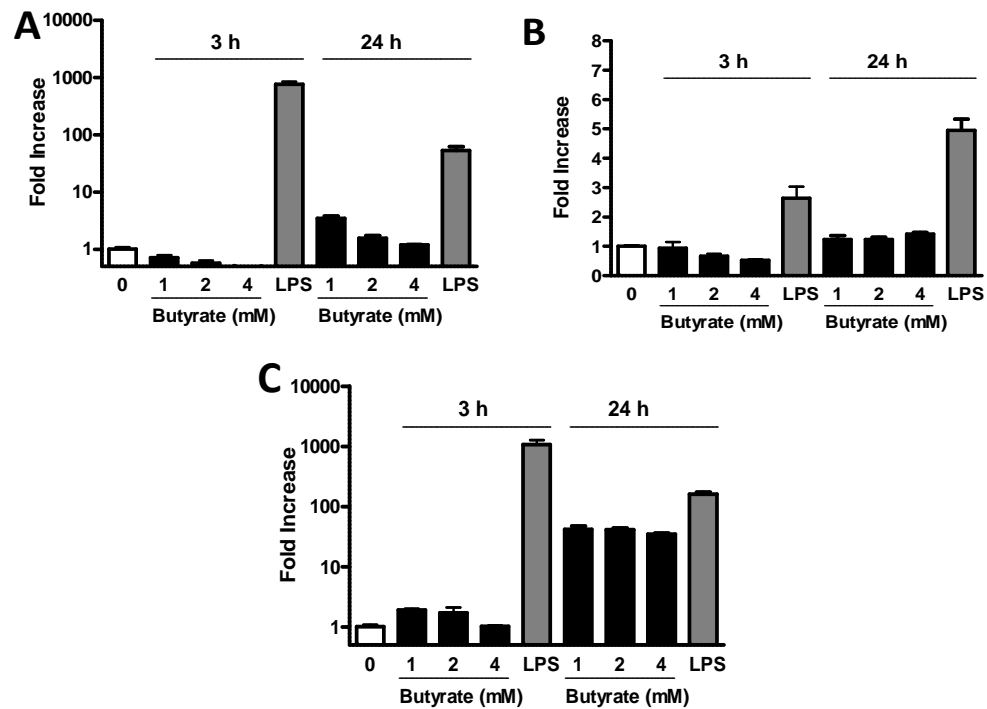
**Fig. 2. Induction of HDP gene expression in chicken HD11 macrophages and primary monocytes.** Chicken HD11 macrophage cells and primary monocytes were incubated in duplicate with and without different concentrations of butyrate for 24 h, followed by RNA isolation and real-time RT-PCR analysis of all 14 chicken  $\beta$ -defensins (*AvBDs*) and 4 cathelicidins (*fowlicidins 1-3* and *cathelicidin B1*). The color elements represent average  $\log_2$  ratios of fold change from 2-3 independent experiments. Red indicates up-regulation, whereas black means no induction and green down-regulation. Gray areas are an indication of no data due to extremely low expression levels of certain HDPs in primary monocytes. Three groups of chicken HDPs, namely generally induced (I), non-regulatable (II), and generally down-regulated (III), can be classified according to their mode of modulation by butyrate. *AvBD11*, *AvBD12*, and *AvBD13* could not be reliably detected in either cell type, and therefore, were not shown. The heat map was generated by using MultiExperiment Viewer [1].



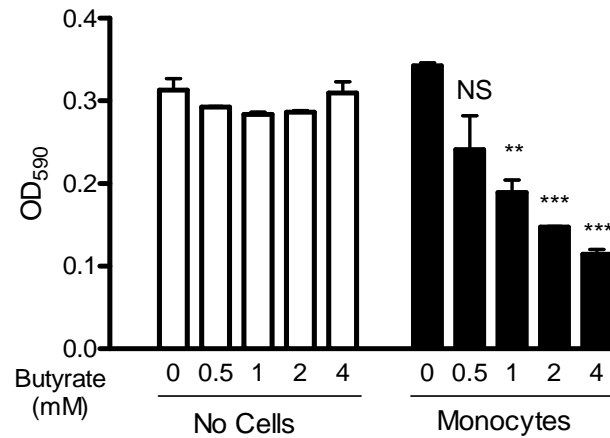
**Fig. 3. Up-regulation of three representative HDPs in chicken jejunal (A) and cecal explants (B) by butyrate.** Chicken jejunum and cecal explants were obtained by culturing slices of 0.5 cm long segments, followed by incubation with indicated concentrations of butyrate in duplicate for 24 h. Real time RT-PCR was performed and the relative fold increase over the control group was calculated using the comparative  $\Delta\Delta C_t$  method and the *GAPDH* gene for normalization. The bars represent means  $\pm$  standard error of the data from two independent experiments. \* $P < 0.05$ , \*\*  $P < 0.001$ , and \*\*\*  $P < 0.0001$  by unpaired Student's *t*-test as compared to the untreated control.



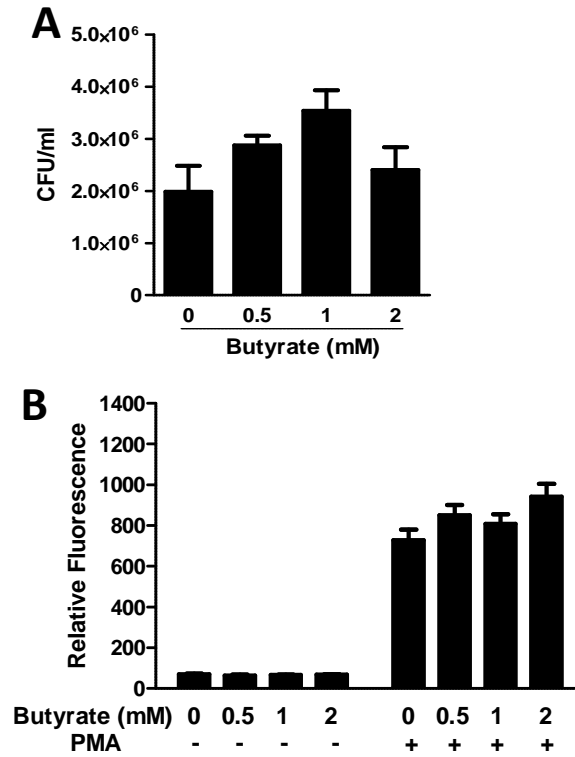
**Fig. 4. In vivo induction of the *AvBD9* gene expression in the intestinal tract of chickens by butyrate.** Two-day-old male Cornish Rock broilers were fed with standard ration with or without supplementation of 0.1% and 0.2% butyrate for 2 days. The crop, cecal tonsil, and cecum were collected from each chicken and the *AvBD9* gene expression was evaluated by real-time PCR. Each bar represents means  $\pm$  standard error of the data from 6 different chickens. \*  $P < 0.05$  by unpaired Student's *t*-test.



**Fig. 5. Minimum triggering of proinflammatory cytokine synthesis in HD11 cells by butyrate.** Chicken HD11 macrophage cells were incubated with indicated concentrations of butyrate or 1  $\mu$ g/ml LPS in duplicate for 3 and 24 h, followed by real-time PCR analysis of the gene expressions of *IL-1 $\beta$*  (A), *IL-12p40* (B), and *IL-8* (C). The bars represent means  $\pm$  standard error of the data from two independent experiments. Essentially no induction of *IL-1* and *IL-12p40* was observed at both 3 and 24 h after butyrate stimulation, with moderate induction of *IL-8* occurring only following butyrate treatment for 24 h.

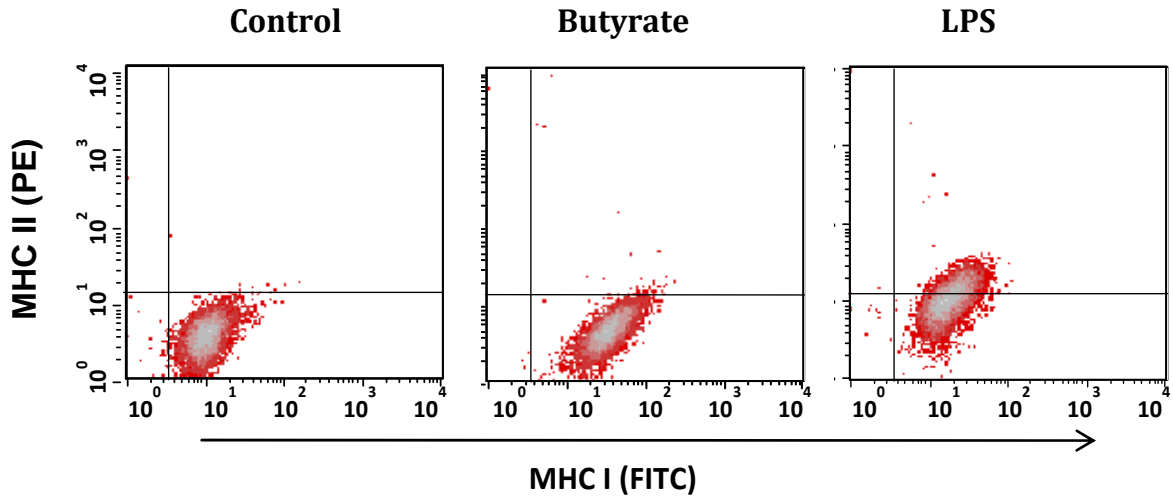


**Fig. 6. Augmentation of the antibacterial activity of monocytes following stimulation with butyrate.** Chicken monocytes were treated with or without different concentrations of butyrate for 24 h. Cell lysates were then prepared and incubated with *S. enteritidis* (ATCC 13076) for 9 h at 37°C. Bacterial turbidity at OD<sub>590nm</sub> was measured as an indication of the bacterial density. *S. enteritidis* was also directly incubated with different concentrations of butyrate in cell culture medium alone without monocytes as controls (white bars). The bars represent means ± standard error of the data from two independent experiments. \*\*  $P < 0.001$ , and \*\*\*  $P < 0.0001$  by unpaired Student's *t*-test as compared to the untreated control.

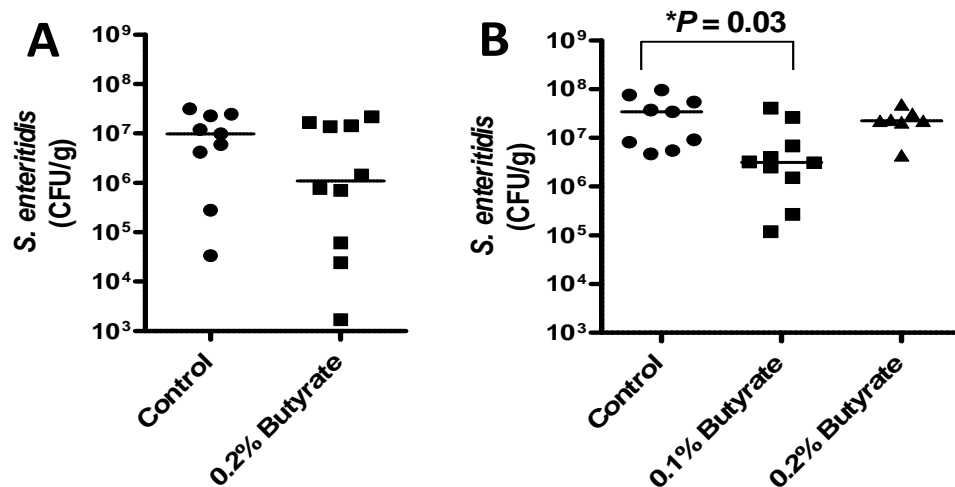


**Fig. 7. No impact of butyrate on phagocytic (A) or oxidative burst activities (B) of HD11 cells.** For both assays, chicken HD11 macrophage cells were incubated with different concentrations of butyrate in duplicate for 24 h, followed by exposure to *S. enteritidis* phage type 13a for 30 min at 37°C in the presence of 5% chicken serum for phagocytosis assay. Extracellular bacteria were then killed by gentamicin, and internalized bacteria were enumerated from lysed HD11 cells by serial plating on Brilliant Green agar plates containing 20 µg/ml nalidixic acid overnight at 37°C. In the oxidative burst assay, the fluorescence was monitored following 1 h incubation with DCFA in the presence or absence of phorbol 12-myristate 13-acetate (PMA). The results were normalized against protein concentrations of each sample. The bars represent means ± standard error of the data from two independent experiments.





**Fig. 8. No influence on the activation status of HD11 cells by butyrate.** HD11 cells were incubated with 4 mM butyrate, 1  $\mu$ g/ml LPS or left untreated for 24 h, followed by flow cytometric analysis of surface expression of MHC class I and II using fluorescein isothiocyanate (FITC)-conjugated anti-chicken MHC class I and R-phycoerythrin (R-PE)-conjugated anti-chicken MHC class II monoclonal antibodies. The data shown are representative of two independent experiments.



**Fig. 9. Reduction of the *S. enteritidis* titer in the cecal contents of chickens following oral supplementation of butyrate.** In trial 1 (A), 5-day old male broilers were equally divided into two groups of 10 and fed with a standard antibiotic-free diet mixed with and without 0.2% sodium butyrate for 2 days. Birds were then inoculated with  $1 \times 10^6$  CFU of *S. enteritidis* phage type 13a and continued with butyrate feeding for another 4 days. The *S. enteritidis* titer in the cecal content was quantitated from each animal by serial plating on Brilliant Green agar plates containing 20  $\mu\text{g/ml}$  nalidixic acid. Trial 2 (B) was similarly conducted with an additional group of 10 broilers fed with 0.1% butyrate. Each dot represents the bacterial titer from a bird and the solid line represents the median value of each treatment. Brackets indicate the statistical significance of differences ( $*P = 0.03$ , unpaired Student's *t*-test).

## CHAPTER IV

### MODULATION OF THE CHICKEN $\beta$ -DEFENSIN 9 GENE EXPRESSION BY FREE FATTY ACIDS

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## ABSTRACT

Widespread use of antibiotics as growth promoters in food animal production has been criticized to be a major driving force for emergence of antimicrobial resistant pathogens, which has become a serious public health concern worldwide. Development of antibiotic-alternative approaches to disease control and prevention is imperatively needed. Previously, we showed that butyrate, a major species of short-chain fatty acids (SCFAs) fermented from undigested fiber by intestinal microflora, is a potent inducer of endogenous antimicrobial host defense peptide (HDP) genes in the chicken. In the present study, we further revealed that, in chicken HD11 macrophage cells and primary monocytes, expression of HDPs is largely in an inverse correlation with the aliphatic carbon chain length of free fatty acids, with SCFAs being the most potent, medium-chain fatty acids moderate and long-chain fatty acids essentially ineffective. Additionally, three SCFAs, namely acetate, propionate, and butyrate, exerted a strong synergy in augmenting HDP synthesis in chicken cells. Consistently, supplementation of chickens with a combination of the three SCFAs in water resulted in a further reduction of *S. enteritidis* in the cecum as compared to feeding of individual SCFAs. More importantly, free fatty acids enhanced HDP gene expression without triggering proinflammatory interleukin-1 $\beta$  production. Taken together, oral supplementation of SCFAs is capable of boosting host immunity and disease resistance, with potential in disease control and prevention in animal agriculture without relying on antibiotics.

## 1. INTRODUCTION

Use of antibiotics as growth promoters is suspected to be a major source for the development of antibiotic-resistant pathogens, which have become a major public health concern worldwide. Enhancing host immunity and disease resistance by specifically boosting the synthesis of endogenous host defense peptides (HDPs) may represent a promising antibiotic-alternative strategy. HDPs have been found in nearly all forms of life and play an important role in the first line of defense [1-3]. HDPs kill a broad range of microbes including bacteria, fungi, parasites, and enveloped viruses mainly through physical interaction and disruption of the membranes [1-3]. It is, therefore, extremely difficult for pathogens to develop resistance [1-3]. In addition to their direct antimicrobial activities, HDPs play a profound role in potentiating the immune response to infections by recruiting and activating immune cells, binding and neutralizing bacterial endotoxins, and promoting wound healing [1-4]. Because of these pleiotropic effects, it is beneficial to specifically enhance the synthesis of endogenous HDPs for disease control and prevention.

As an important source of energy, fatty acids are represented by a large group of carboxylic acids with an aliphatic hydrocarbon chain that are either saturated or unsaturated. Based on the number of carbon atoms in the aliphatic chain, fatty acids are broadly classified into three groups, namely SCFAs ( $\leq C5$ ), medium-chain fatty acids (MCFAs) ( $C6$  to  $C11$ ), and long-chain fatty acids (LCFAs) ( $\geq C12$ ) [5]. Butyrate, acetate and propionate are the major species of SCFAs produced by bacterial fermentation of

resistant starch, cellulose, and sugar in the intestine [6-8]. The concentrations of acetate, propionate, butyrate vary in molar ratios from 48:29:23 to 70:15:15 in human feces [7] and 33:12:6 in chicken cecal contents [9]. Besides being a major source of energy and constituents of cellular membranes, fatty acids also play an important role in maintaining homeostasis of intestinal physiology by regulating fluid absorption, gut motility, gut microbiota, and mucosal inflammation as well as proliferation, differentiation, and apoptosis of intestinal epithelial cells [10-15].

Earlier studies reported that SCFAs including butyrate and propionate are capable of inducing the synthesis of LL-37, a HDP in humans [12], which is largely due to their histone deacetylase inhibitory activity [14]. Inhibition of histone deacetylase is known to promote hyper-acetylation of the lysine residues in nucleosome core histones leading to a less compact chromatin and transcriptional activation of a subset of genes [16, 17]. Indeed, several other histone deacetylase inhibitors were also found to be capable of inducing HDP gene expression in humans, albeit with a varying potency [14, 18].

We recently reported that butyrate enhances HDP expression in several different cell types including macrophages, monocytes, and intestinal epithelial cells [19]. In the present study, we further compared the relative potency in HDP induction by free fatty acids of various aliphatic chain lengths (C1 to C18). There was an inverse correlation between the expression of HDPs and the length of aliphatic chain of fatty acids, with SCFAs being the strongest inducers. Saturation or unsaturation of the aliphatic tails of fatty acids appeared to play a minimum role of HDP induction. We further revealed a strong synergy among three SCFAs including acetate, propionate, and butyrate in enhancing AvBD9 expression and reducing bacterial colonization in the chicken,

suggesting the potential for dietary supplementation of SCFAs in disease control and prevention.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals

Formate, acetate, propionate, butyrate, hexanoate, n-octanoate, decanoate, linoleic acid ( $\omega$ -6),  $\alpha$ -linolenic acid ( $\omega$ -3), conjugated linoleic acid (CLA), and trichostatin A (TSA) were purchased from Sigma-Aldrich (St. Louis, MO), whereas heptanoate, nonanoate, dodecanoate, tetradecanoate, octadecanoate were from TCI America (Portland, OR). All free fatty acids were purchased in the sodium salt form, except for linoleic acid, linolenic acid, and CLA, which were in the free acid form. SCFAs (formate, acetate, propionate, and butyrate), MCFAs (hexanoate, heptanoate, n-octanoate, nonanoate, and decanoate) were dissolved in RPMI 1640 cell culture medium, while LCFAs (dodecanoate, tetradecanoate, and octadecanoate) were dissolved in methanol and free linoleic acid, linolenic acid and CLA were dissolved in ethanol. Bacterial lipopolysaccharide (LPS) from *E. coli* O111:B4 was purchased from Sigma-Aldrich and dissolved in RPMI 160 medium.

### 2.2. Isolation, culture, and stimulation of chicken cells

Chicken HD11 macrophage cells (kindly provided by Dr. Hyun S. Lillehoj from USDA, ARS) were cultured in 6-well plates in RPMI 1640 containing 10% FBS and 1%

streptomycin/ penicillin at  $2 \times 10^6$  cells/well. After overnight growth, HD11 cells were incubated with various fatty acids. Chicken peripheral blood mononuclear cells (PBMCs) were isolated from EDTA-anticoagulated venous blood by gradient centrifugation using Histopaque 1077 (Sigma). Cells in the interphase were then collected, washed in Hank's balanced salt solution (HBSS), and then resuspended in RPMI 1640 containing 10% FBS, 1% streptomycin/pencillin, and 20 mM HEPES in 60-mm tissue culture dishes at  $6 \times 10^7$  cells/dish. After overnight incubation at 37°C and 5% CO<sub>2</sub>, non-adherent cells were washed off with HBSS, and adherent monocytes were used subsequently for stimulation with fatty acids. Each treatment was performed in duplicate or triplicate and repeated at least 2-3 times. For all experiments, equal amounts of solvents were added to cells as negative controls. All chemicals were tested for their toxicity to chicken cells, and the subtoxic concentration ranges that gave the maximal induction of HDP expression were presented.

### **2.3. Analysis of chicken gene expression by real time RT-PCR**

Following stimulation, cells were harvested with RNAzol (Molecular Research), and total RNA was extracted according to the manufacturer's instructions. The first-strand cDNA was synthesized from 300 ng of total RNA with QuantiTect Reverse Transcription Kit (Qiagen), and real time PCR was performed with QuantiTect SYBR Green PCR Kit (Qiagen) using 1/40 (for GAPDH) or 1/10 (for HDP genes) of the first-strand cDNA and gene-specific primers in a total volume of 10 µl as previously described [20-22]. The PCR was set for initial denaturation at 95°C for 10 min, followed by 45 cycles of 94°C for 15 sec, 55°C for 20 sec, and 72°C for 30 sec. A melt curve analysis step was also included to ensure the specificity of PCR amplification. Chicken glyceraldehyde-3-



phosphate dehydrogenase (GAPDH) was used as a house keeping gene for data normalization. The forward and reverse primers for chicken GAPDH, HDPs (AvBD9 and cathelicidin B1), and proinflammatory cytokines (IL-1 $\beta$ , IL-8, and IL-12p40) were previously described [20]. Relative changes in the gene expression level were quantified with the  $\Delta\Delta C_t$  method as described [20-22].

#### **2.4. Histone deacetylase (HDAC) assay**

HDAC assay was performed using the Fluor-de-Lys<sup>®</sup> HDAC Fluorimetric Cellular Activity Assay Kit (Enzo Life Sciences, PA) according to the manufacturer's instructions. Chicken HD11 cells ( $1 \times 10^5$ ) were cultured in phenol red-free RPMI 1640 containing 10% FBS in a 96-well tissue culture plate overnight. Cells were treated in duplicate with or without SCFAs in the presence of 100  $\mu$ M of Fluor-de-Lys<sup>®</sup>, a fluorogenic, cell-permeable HDAC substrate for 4 h. The deacetylation reaction was then stopped by addition of TSA, a strong HDAC inhibitor, in a cell lysis buffer containing 1% NP-40. The fluorescent signal was generated by addition of a developer solution to a final concentration of 1  $\mu$ M, and the fluorescence was recorded at 360 nm excitation and 460 nm emission using FLx800 Multi-Detection Microplate Reader (Bio-Tek Instruments). The HDAC inhibitory activity (%) was calculated as  $[1 - (F_{\text{treatment}} - F_{\text{background}})/(F_{\text{max}} - F_{\text{background}})] \times 100$ , where  $F_{\text{treatment}}$  is the fluorescence of cells exposed to SCFAs,  $F_{\text{max}}$  is the maximum fluorescence of cells without being exposed to SCFAs, and  $F_{\text{background}}$  is the fluorescence of cell culture medium without cells.

#### **2.5. Oral supplementation of SCFAs and experimental infection of chickens with *Salmonella enteritidis***

A total of 20, day-of-hatch male Cornish Rock broiler chickens were purchased from a commercial hatchery (Ideal Poultry, Cameron, TX) and were equally divided into four groups with 5 birds/group and fed with a standard antibiotic-free ration and deionized water ad libitum for 4 days. Water containing 0.5% sodium acetate, 0.2% propionate and/or 0.1% butyrate was provided ad libitum for each group for 2 days, prior to an intraesophageal infection with 0.5 ml of Lysogeny broth (LB) containing  $1 \times 10^7$  CFU of *S. enteritidis* phage type 13a (a kind gift from Dr. Susan Lamont at Iowa State University) [23]. After administration of SCFAs in water for another 4 days, the birds were euthanized and cecal contents were aseptically collected from each animal, serially diluted in PBS, and plated on Brilliant Green agar plates (Becton Dickinson) containing 20 µg/ml of nalidixic acid for overnight growth and bacterial enumeration. All animal procedures were approved by the Institutional Animal Care and Use Committee of Oklahoma State University.

## **2.6. Statistical analysis**

Unpaired Student's two-tailed *t*-test was used to evaluate the statistical significance using GraphPad Prism 5 (GraphPad Software, La Jolla, CA).  $P < 0.05$  was considered statistically significant.

## **3. RESULTS AND DISCUSSION**

### **3.1. Inverse correlation between the HDP-modulating ability and aliphatic chain length of free fatty acids**

To examine the effect of free fatty acids of various aliphatic carbon chain lengths on the expression of a representative chicken  $\beta$ -defensin (AvBD9), we incubated chicken macrophage HD11 cells and primary monocytes with different concentrations of fatty acids for 24 h and then examined AvBD9 gene expression by real-time RT-PCR. As shown in Fig. 1A, we observed a clear dose-dependent induction of AvBD9 in HD11 cells in response to SCFAs and MCFAs, with LCFAs being largely inactive. A peak response occurred with SCFAs, with greater than 1000-fold induction of AvBD9 gene expression in HD11 cells when exposed to 80, 64, and 4 mM of acetate, propionate, and butyrate, respectively. The magnitude of AvBD9 induction was dramatically reduced with MCFAs (Fig. 1A) when compare to SCFAs. A similar trend was also observed in primary chicken monocytes, with SCFAs being the most potent inducers (Fig. 1B). However, a notable difference is that LCFAs including dodecanoate (C12), tetradecanoate (C14), and octadecanoate (C18) maintained a comparable, if not slightly better, AvBD9-inducing activity than MCFAs in primary monocytes (Fig. 1B).

Besides AvBD9, we also examined another representative chicken HDP, namely cathelicidin B1, in response to free fatty acids in chicken primary monocytes. Similar to AvBD9, cathelicidin B1 was most readily induced by SCFAs including acetate, propionate, and butyrate (Fig. 2). The maximum induction of cathelicidin B1 expression was 17-fold for acetate, 37-fold for propionate, and 29-fold for butyrate, respectively. However, MCFAs and LCFAs had little or no impact on cathelicidin B1 synthesis.

To further examine the effect of the saturation status of the aliphatic chain on HDP expression, different concentrations of oleate [C18:(n-9)], linoleic acid [C18:2(n-6)],  $\alpha$ -linolenic acid [C18:3(n-3)], and CLA were used to stimulate HD11 cells for 24 h. Real-time RT-PCR revealed that, in contrast to saturated LCFAs, unsaturated long chain fatty acids including oleate, linoleic acid,  $\alpha$ -linolenic acid, and CLA were incapable of inducing AvBD9 gene expression in HD11 cells (Fig. 3A), but clearly showed a statistically significant, dose-dependent induction of the AvBD9 expression in primary chicken monocytes (Fig. 3B), showing a seemingly more potent HDP-modulating activity than saturated, unbranched LCFAs. Over all, these findings surprisingly suggested the significance of double bonds in the regulation of HDP expression. It appears that presence of double bonds in LCFAs tends to increase their ability to modulate AvBD9 gene expression, with an opposite effect seen with saturated LCFAs. However, additional unsaturated fatty acids need to be tested in order to strengthen the conclusion, and the underlying mechanisms warrant further investigations.

### **3.2. Impact of free fatty acids on the inflammatory response in HD11 cells**

SCFAs, particularly Butyrate, generally exert anti-inflammatory effects and have been used to treat inflammatory bowel diseases [7, 8]. To confirm augmentation of HDP gene expression by free fatty acids without triggering a proinflammatory response, we treated HD11 cells with or without different fatty acids at optimal HDP-inducing concentrations for 3 and 24 h and analyzed the expressions of three representative cytokines including IL-1 $\beta$ , IL-8, and IL-12p40. Bacterial lipopolysaccharide (LPS) from *E. coli* O111:B4 at 1  $\mu$ g/ml was used as a positive control. All representative fatty acids, including acetate, propionate, butyrate, hexanoate, and octanoate, had essentially no

effect on *IL-1 $\beta$*  at both time points (Fig. 4). No influence on *IL-12p40* expression was observed following fatty acid stimulation for 3 h; however, a 3- to 10-fold induction was seen with all fatty acids except for butyrate. As compared with LPS that caused >1000-fold induction, a minimum influence (~10-fold increase) on *IL-8* expression was observed after 3 h stimulation; however, all fatty acids showed a *IL-8*-inducing activity comparable to LPS after 24 h (Fig. 4). Taken together, these results demonstrated that fatty acids generally have no or a mild influence on triggering the inflammatory response while promoting the production of HDPs.

### **3.3. Synergistic induction of AvBD9 expression and reduction of bacterial colonization by SCFAs**

Because acetate, propionate, and butyrate are among the most potent fatty acids in inducing AvBD9 gene expression and they also represent the major species of SCFAs being produced simultaneously by intestinal microflora, we sought to determine the synergistic effect of these three SCFAs on HDP synthesis. Chicken HD11 cells and primary monocytes were treated with acetate, propionate, and butyrate individually or in combinations for 24 h and followed by real-time RT-PCR analysis of AvBD9 gene expression. Individual SCFAs at low concentrations gave a minimum induction of AvBD9 gene in both HD11 cells and primary monocytes (Figs. 5A and 5B). However, a combination of propionate and acetate showed an obvious synergism (Fig. 5). More strikingly, an addition of all three SCFAs resulted in a significant induction of the AvBD9 gene in both cell types when compared to individual fatty acids (Fig. 5). A combination of all three free fatty acids enhanced AvBD9 gene expression with a maximum increase of 4,000-fold in HD11 cells and 25- to 50-fold in primary monocytes.

SCFAs and butyrate in particular are well-known histone deacetylase inhibitors [16, 17]. To study the impact of histone deacetylation on the AvBD9-inducing activity in chickens by SCFAs, we treated HD11 cells with or without acetate, propionate, and butyrate individually or in combination for 4 h and then performed HDAC assays using Fluor-de-Lys<sup>®</sup> HDAC Fluorimetric Cellular Activity Assay Kit (Enzo Life Sciences). As shown in Fig. 6, low concentrations of butyrate (0.5 mM) and acetate (40 mM) showed a similar HDAC inhibitory activity of approximately 50%, while propionate (4mM) suppressed the HDAC activity by 67% (Fig. 6). Moreover, a combination of any two SCFAs showed comparable or higher HDAC inhibitory activity than any individual SCFAs. More importantly, simultaneous treatment of HD11 cells with all three SCFAs resulted in the greatest inhibition of the HDAC activity (83%) (Fig. 6). These results are precisely correlated with the relative capacity of SCFAs to stimulate AvBD9 gene expression, where individual SCFAs gave marginal induction, combination of two caused a marked increase in AvBD9 expression, and the most dramatic augmentation occurred with three SCFAs (Fig. 5). Our data are also consistent with earlier findings that SCFAs induced HDP synthesis mainly through inhibition of HDACs in humans [14].

To further confirm whether SCFA-mediated synergistic induction of HDP could confer animals an enhanced resistance to bacterial infection, we supplemented 4-day-old male broiler chickens with 0.5% acetate, 0.2% propionate, and 0.1% butyrate individually or in combination in water for 2 days, followed by an inoculation with  $1 \times 10^7$  CFU of *S. enteritidis* for another 4 days. The bacterial titer in the cecal content was examined. As seen in Fig. 7, a significant reduction of the *S. enteritidis* load was observed with supplementation of acetate, propionate, and butyrate individually. Importantly, the most

dramatic reduction in bacterial colonization was seen in the chickens receiving a combination of three SCFAs, consistent with their ability to induce AvBD9 gene expression in vitro (Fig. 5). It is likely that newly synthesized HDPs are released into extracellular compartments, killing microbes on mucosal surfaces [24].

In the present study, we have shown among all free fatty acids, SCFAs are the most potent inducers of HDP gene expression in the chicken without provoking excessive proinflammatory response. Furthermore, the HDP-inducing activity of SCFAs is strongly correlated with their ability to inhibit the HDAC activity. It is worth noting that, in addition to the capacity to promote HDP synthesis, SCFAs and MCFAs were also found to possess direct antibacterial activities, albeit at high concentrations [9, 25]. Additionally, MCFAs and SCFAs, except for acetic acid, reduce the ability of *Salmonella* to invade intestinal epithelial cells [9, 25]. Given such a plethora of antibacterial properties, free fatty acids, particularly SCFAs, have potential for disease control and prevention and may represent promising alternatives to antibiotics.

## **ACKNOWLEDGEMENTS**

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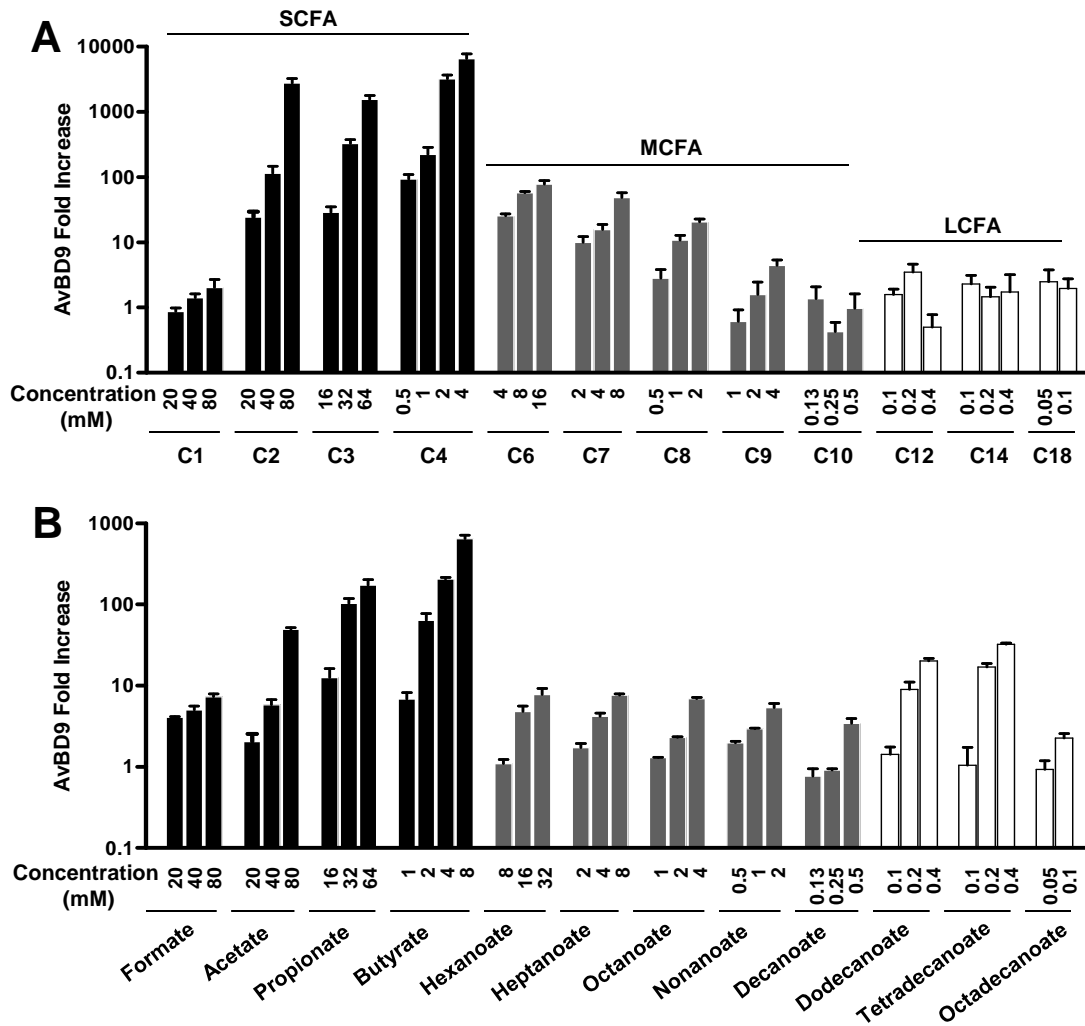


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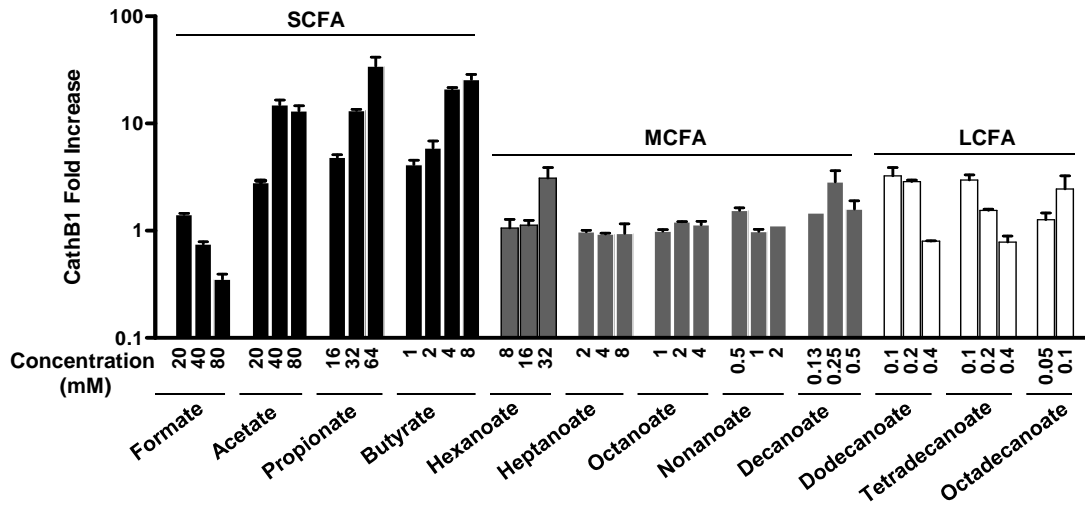
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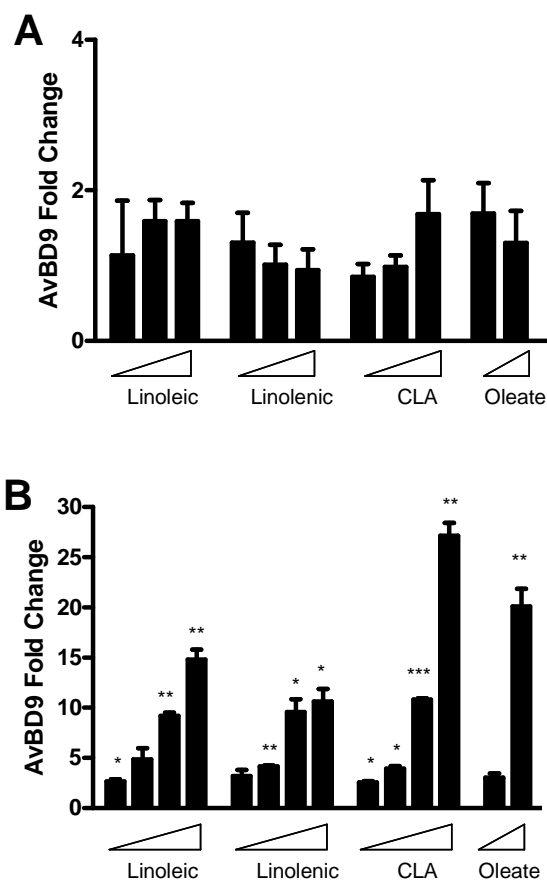
## FIGURE AND FIGURE LEGENDS



**Fig. 1. Regulation of AvBD9 gene expression by free fatty acids.** Chicken macrophage HD11 cells (A) and primary monocytes (B) were treated in duplicate with or without indicated concentrations of fatty acids (mM) for 24h, followed by real-time RT-PCR analysis of AvBD9 gene expression. Data was normalized with GAPDH, and relative fold change of each treatment versus solvent control was calculated using  $\Delta\Delta C_t$  method. Each bar indicates mean  $\pm$  standard error of the data from 2-3 experiments.

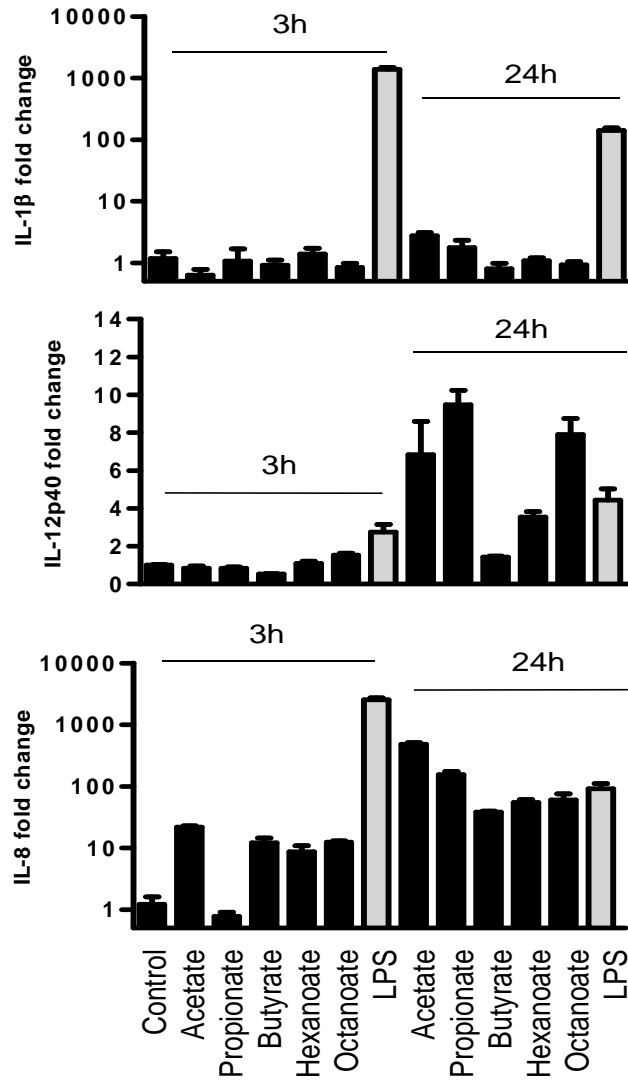


**Fig. 2. Modulation of cathelicidin B1 gene expression by free fatty acids.** Primary chicken monocytes were treated in duplicate with or without indicated concentrations of free fatty acids (mM) for 24h, followed by real-time RT-PCR analysis of cathelicidin B1 gene expression. Data was normalized with GAPDH, and relative fold change of each treatment versus solvent control was calculated using  $\Delta\Delta C_t$  method. Each bar indicates mean  $\pm$  standard error of the data from 2-3 experiments.

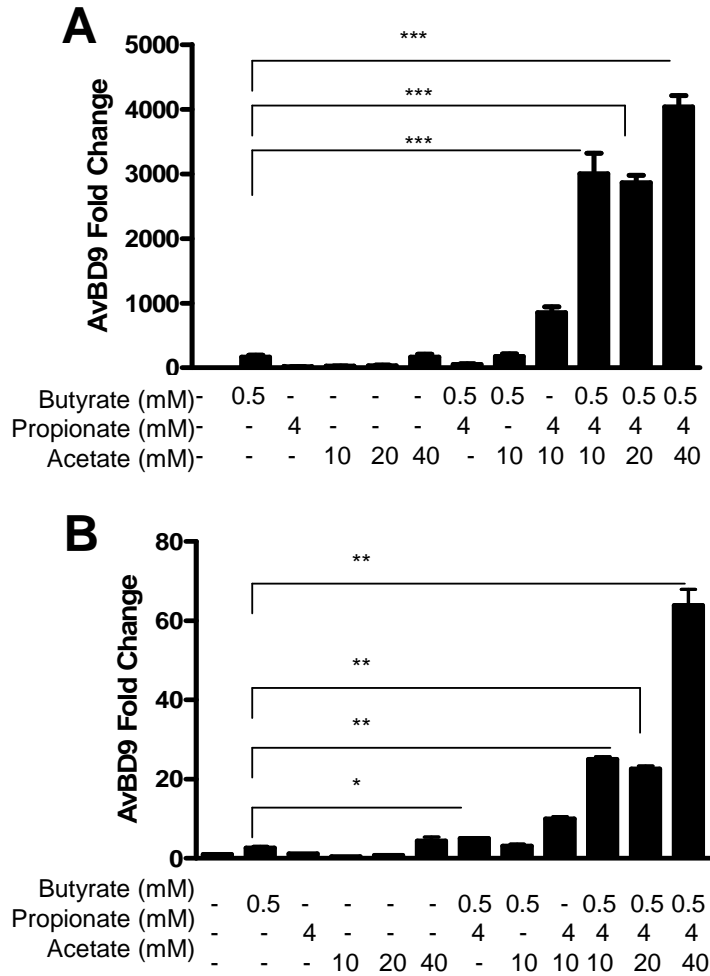


**Fig. 3. Differential expression of AvBD9 in response to unsaturated fatty acids.**

Chicken HD11 macrophage cells (A) and primary monocytes (B) were treated in duplicate with different concentrations of sodium oleate (0.1 and 0.2 mM) and linoleic,  $\alpha$ -linolenic, and conjugated linolenic acids (0.05, 0.1, 0.2, and 0.4 mM for all three) for 24 h, followed by real-time RT-PCR analysis of AvBD9 gene expression. Each bar indicates mean  $\pm$  standard error of the data from two independent experiments. \* $P < 0.05$ , \*\*  $P < 0.001$ , and \*\*\*  $P < 0.0001$  by unpaired Student's  $t$ -test as compared to the untreated control.

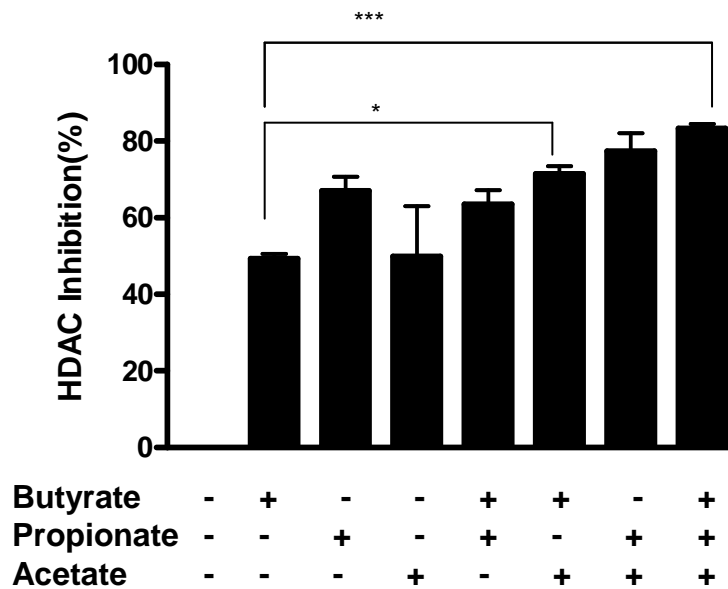


**Fig. 4. A minimum impact of free fatty acids on the expression of proinflammatory cytokines.** Chicken HD11 cells were stimulated with different fatty acids at optimal HDP-inducing concentrations (80 mM acetate, 32 mM propionate, 4 mM butyrate, 16 mM hexanoate, and 2 mM octanoate) or LPS (1  $\mu\text{g}/\mu\text{l}$ ) as a positive control for 3 and 24 h, followed by real-time RT-PCR analysis of the expression of IL-1 $\beta$  (A), IL-12p40 (B), and IL-8 (C). The bars represent means  $\pm$  standard errors from 2-3 experiments.



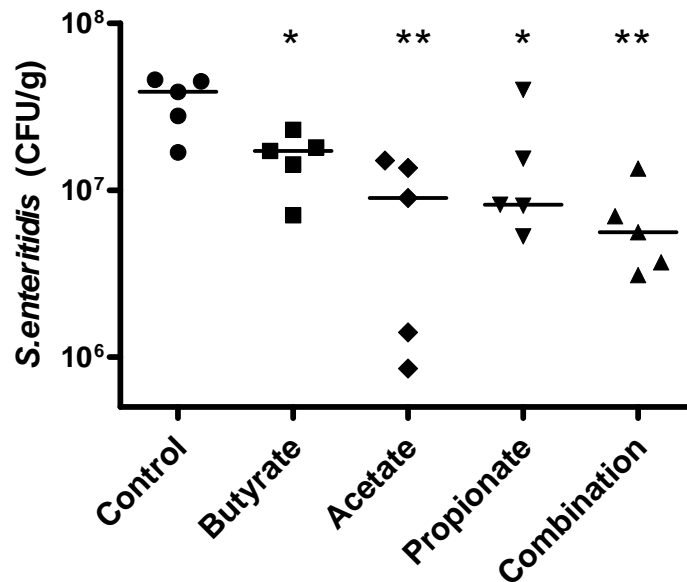
**Fig. 5. Synergistic induction of AvBD9 with acetate, propionate and butyrate in chicken HD11 cells (A) and primary monocytes (B).** Cells were incubated with acetate, propionate and butyrate alone or in combinations for 24h, followed by real-time RT-PCR analysis of AvBD9 expression. Each bar represents mean  $\pm$  standard error of the data from 3 independent experiments. \* $P < 0.05$ , \*\*  $P < 0.001$ , and \*\*\*  $P < 0.0001$  by unpaired Student's *t*-test as compared to the cells treated with butyrate alone.





**Fig. 6. Inhibition of the HDAC activity by acetate, propionate, and butyrate.**

Chicken HD11 cells were incubated in duplicate with or without three SCFAs in the presence of Fluor-de-Lys<sup>®</sup>, a fluorogenic, cell-permeable HDAC substrate for 4 h. The deacetylation reaction was stopped and the fluorescent signal was generated by addition of a developer solution containing trichostatin A and NP-40. Fluorescence was monitored at 360 nm excitation and 460 nm emission. HDAC inhibition by SCFAs was calculated relative to the cells without being exposed to any HDAC inhibitor. Each bar represents mean  $\pm$  standard error of the data. \* $P < 0.05$ , \*\*  $P < 0.001$ , and \*\*\*  $P < 0.0001$  by unpaired Student's *t*-test as compared to the cells treated with butyrate alone.



**Fig. 7. Synergistic reduction of the *S. enteritidis* load in the cecum of chickens by a combination of acetate, propionate and butyrate.** Four day-old male broiler chicks were supplemented with or without 0.5% acetate, 0.2% propionate, and 0.1% butyrate alone or in combinations in water for 2 days with 5 birds per group, followed by an inoculation with *S. enteritidis* phage type 13a ( $1 \times 10^7$ ). SCFA supplementation was continued for another 4 days before the cecal content was collected and bacterial number enumerated. Each dot indicates the bacterial titer in a bird and the solid line represents the median value of each treatment. \* $P < 0.05$  and \*\* $P < 0.01$  (by unpaired Student's *t*-test).

## CHAPTER V

### UPREGULATION OF CHICKEN HOST DEFENSE PEPTIDE EXPRESSION BY BUTYRATE: ROLE OF HISTONE ACETYLATION, cAMP SIGNALING, AND MITOGEN-ACTIVATED PROTEIN KINASES

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## ABSTRACT

As an important component of innate immunity, host defense peptides (HDPs) protect the host from invading pathogens by acting as direct antimicrobials and immunomodulators. Butyrate, a histone deacetylase (HDAC) inhibitor, was shown to induce the HDP expression and reduce the *Salmonella enteritidis* colonization in chickens. However, the molecular mechanism by which butyrate induces chicken HDP expression remains elusive. Here we studied the involvement of histone acetylation and cAMP and MAP kinase signaling pathways in butyrate-mediated regulation of AvBD9, a chicken  $\beta$ -defensin in chicken HD11 macrophage cells. We showed that, similar to butyrate, most HDAC inhibitors are capable of inducing AvBD9 gene expression, although varying in the efficacy. On the other hand, histone acetyltransferase (HAT) inhibitors reversed butyrate-induced AvBD9 gene expression. Inhibition of p38 MAP kinase or c-Jun N-terminal kinase (JNK), but not extracellular signal-regulated kinase (ERK) pathway, obliterated butyrate-triggered AvBD9 synthesis. In addition, cAMP analogs and adenylate cyclase agonists upregulated AvBD9 gene expression. More importantly, butyrate and adenylate cyclase agonists acted synergistically in enhancing AvBD9 gene expression. Taken together, our studies revealed a critical involvement of histone acetylation, cAMP signaling, and p38 and JNK pathways in the regulation of AvBD9 gene transcription mediated by butyrate. A detailed understanding of the underlying mechanisms of the HDP gene regulation will pave the way for development of novel antibiotic-free strategies in diseases control and prevention in both animal agriculture and public health.

## 1. INTRODUCTION

Host defense peptides (HDPs) are a critical, evolutionarily conserved component of the innate immune system. HDPs are represented by a large group of small cationic peptides with generally less than 100 amino acid residues [1-4]. HDPs are expressed strategically in leukocytes, skin keratinocytes, and mucosal epithelial cells lining the digestive, respiratory, and urogenital tracts, providing an important first line of host defense. They are either constitutively expressed or differentially regulated in response to infection or injury. HDPs kill bacteria, enveloped virus, protozoa, and fungi mainly by physically disrupting their membranes [2, 5-8]. In addition to their direct bacterial killing activity, HDPs also modulate innate and adaptive immunity [2, 9]. Two major families of HDPs, namely cathelicidins and defensins, exist in vertebrates [4, 10, 11]. Most defensins are composed of six conserved cysteine residues in the C-terminal region [10-13], whereas cathelicidins consists of a conserved cathelin domain in the N-terminal region and a highly variable C-terminal sequence [4, 14]. The chicken genome encodes a total of 14 avian  $\beta$ -defensins also known as AvBDs and 4 cathelicidins known as fowlicidins 1-3 [15-17] and cathelicidin B1 [18].

Butyrate and a group of histone deacetylase (HDAC) inhibitors were recently found to specifically augment LL-37 cathelicidin gene expression in human HT29 colonic epithelial cells [19-21]. As an important epigenetic mechanism for remodeling of the chromatin structure and controlling of gene expression, histone acetylation is achieved by a balanced act of histone acetyltransferases (HATs) and HDACs. HATs

acetylate the lysine residues of nucleosomal core histones leading to a relaxed and transcriptionally active chromatin. Conversely, HDACs remove the acetyl groups from the lysine residues resulting in a condensed and transcriptionally silenced chromatin. HDAC inhibitors block the action of HDACs, leading to hyper-acetylation of histones, thereby affecting gene expression [22-25]. It will be important to reveal the significance of histone acetylation in regulating HDP expression in a non-mammalian species. Besides epigenetic control, binding of cAMP-response element-binding protein (CREB) and activator protein 1 (AP-1) to the promoter region were shown to play a major role in butyrate-mediated induction of human LL-37 expression in intestinal epithelial cells [26]. Consistently, blockage of cAMP and MAP (mitogen-activated protein) kinase signaling essentially abrogated the transcriptional activation of the LL-37 gene by butyrate [26].

We found previously that butyrate upregulates the expression of several HDPs and reduces the *Salmonella enteritidis* colonization in the chicken [27]. In the present study, we extended our work to have further revealed a critical role of histone acetylation and cAMP and MAPK signaling pathways in butyrate-mediated regulation of AvBD9, a chicken  $\beta$ -defensin. We also discovered a synergistic induction of AvBD9 gene expression by a combination of butyrate and adenylate cyclase agonists. These results will have important implications in devising novel immune boosting strategies in disease control and prevention without the use of antibiotics.

## **2. MATERIALS AND METHODS**

### **2.1. Chemicals and cells**

Sodium valproate, Sodium butyrate, pertussis toxin (PT) and cholera toxin (CT) were purchased from Sigma-Aldrich (St. Louis, MO). Forskolin (FSK), 8-Bromo-cAMP, dibutyryl-cAMP (DB-cAMP), SB203580 (p38 inhibitor), PD98059 (MEK inhibitor), and SP600125 (JNK inhibitor) were from Santa Cruz Biotechnology (Santa Cruz, CA). HDAC inhibitors including trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA), CAY10433/BML-210, and CAY10398 were obtained from Cayman Chemicals (Ann Arbor, MI). Epigallocatechin gallate (EGCG), garcinol, and anacardic acid (HAT inhibitors) were acquired from Cayman Chemicals (Ann Arbor, MI), Enzo Life Sciences (Farmingdale, NY), and Santa Cruz, respectively. Sodium valproate, sodium butyrate, 8-Bromo-cAMP, DB-cAMP, and PD98059 were dissolved in RPMI 1640 medium. TSA, SAHA, CAY10433, and CAY10398, FSK, SB203580, and SP600125 were dissolved in dimethyl sulfoxide (DMSO), whereas CT and PT were dissolved in sterile water. Chicken HD11 macrophage cell line [28] was a generous gift from Dr. Hyun S. Lillehoj at the USDA, ARS.

### **2.2. Isolation of chicken primary monocytes**

Chicken blood was collected intravenously using EDTA as anticoagulant (Sigma), and peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation with Histopaque 1077 (Sigma). Chicken blood was mixed 1:1 with 1% methyl cellulose (Sigma), and centrifuged at  $25 \times g$  for 20 min. Cells remaining in suspension were

collected, mix 1:1 with prewarmed Hanks balanced salt solution (HBSS), and centrifuged for 15 min at  $600 \times g$ . Cells were resuspended with warm HBSS and overlaid onto Histopaque 1077 for centrifugation for 30 min at  $400 \times g$ . Interphase containing PBMC was collected into a fresh tube and washed with HBSS. Cell pellet was then resuspended in RPMI 1640 (Hyclone) supplemented with 10% fetal bovine serum (FBS), 100  $\mu\text{g/ml}$  streptomycin, 100 U/ml penicillin, and 20 mM HEPES. PBMCs ( $6 \times 10^7/\text{well}$ ) were dispensed in 60 mm tissue culture dishes and let adhere overnight at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ . Non-adherent cells were then removed, and adherent monocytes were washed once with prewarmed HBSS. Monocytes were replenished with fresh complete RPMI 1640 medium and incubated for another 2 h prior to be exposed to different agents.

### **2.3. Culture and stimulation of cells**

Chicken HD11 macrophages ( $2 \times 10^6/\text{well}$ ) were grown in 2 ml RPMI 1640 supplemented with 10% FBS and 1% antibiotics in 6-well tissue culture plates. After overnight incubation at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ , cells were treated with different agents. To study the signaling mechanisms in butyrate-mediated HDP induction, cells were incubated with cAMP agonists, MAPK kinase inhibitors for 1h and with HAT inhibitors for 2 h, followed by butyrate treatment for up to another 24 h. All experiments were performed 2-3 times independently, with 2-3 biological replicates for each treatment.

### **2.4. Total RNA extraction and cDNA synthesis**

Following treatment with different agents, cells were harvested with RNazol (Molecular Research) for isolation of total RNA. The quantity and quality of RNA were measured by Nanodrop (NanoDrop Products, Wilmington, DE), and QuantiTect Reverse Transcription



Kit (Qiagen, Valencia, CA) was used to synthesize the first-strand cDNA from total RNA following the manufacturer's recommendations. Briefly, 0.3  $\mu$ g of total RNA was first eliminated of genomic DNA contamination in a genomic DNA wipeout buffer for 5 min at 42°C. Reverse transcription was then performed in a total volume of 4  $\mu$ l using Quantiscript reverse transcriptase and a mixture of random hexamers and oligo(dT) primers for 30 min at 42°C, followed by 3 min at 95°C to inactivate reverse transcriptase. The cDNA was then diluted 10-fold with RNase-free water prior to use in real-time PCR.

### **2.5. Real-time PCR analysis of gene expression**

QuantiTect SYBR Green PCR Kit (Qiagen) was used for real-time amplification of the first-strand cDNA using MyiQ Real Time PCR Detection System (Bio-Rad, Hercules, CA, USA) as previously described [17]. Briefly, each PCR reaction was set up in a 96-well PCR plate in a total volume of 10  $\mu$ l using 0.1  $\mu$ g of the first-strand cDNA and gene-specific primers (Table 1). Real-time PCR was programmed as follows: initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 20 s, and extension and data collection at 72°C for 30 s. The forward and reverse primers for chicken GAPDH and AvBD9 and proinflammatory cytokines were previously described [29]. Melting curve analysis was conducted to confirm the specificity of PCR amplifications. Comparative  $\Delta\Delta C_t$  method was used for quantification of gene expression using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as the reference for data normalization [17].

### **2.6. Statistical analysis**

All data were subjected to statistical analysis using Student's *t* test and GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Each data point represented mean  $\pm$  standard error from 2-3 independent experiments. The results were considered statistically, if  $P < 0.05$ .

### 3. RESULTS

#### 3.1. Induction of AvBD9 gene expression by HDAC inhibitors

We showed recently that butyrate upregulates several chicken HDPs and enhances resistance of chicken against *S. enteritidis* [27]. Since butyrate is a well-known HDAC inhibitor, we evaluated the ability of several HDAC inhibitors (Fig. 1A) to stimulate AvBD9 synthesis. We treated chicken HD11 macrophages and primary monocytes with different concentrations of a few selected HDAC inhibitors for 24 h, followed by RNA isolation and real-time RT-PCR analysis of the AvBD9 gene expression. As expected, TSA, SAHA, sodium valproate, CAY10433, and CAY10398, all stimulated AvBD9 gene expression significantly in a dose-dependent manner both in HD11 cells (Fig. 1B) and primary monocytes (Fig. 1C), albeit at lower magnitudes than butyrate, which peaked with an approximately 2,000-fold AvBD9 induction in HD11 cells and greater than 250-fold induction in primary monocytes. On the other hand, all other HDAC inhibitors showed a similar efficacy in inducing AvBD9 expression in HD11 cells with an approximately 100-fold maximum induction (Fig. 1B). In primary monocytes, valproate and SAHA led to 100-fold increase in AvBD9 expression, but TSA, CAY10433, and CAY10398 showed a reduced efficiency, with approximately 10-, 30-, and 6-fold

maximum increase, respectively (Fig. 1C). The results collectively are suggestive of a beneficial role of histone hyperacetylation in AvBD9 gene induction. The variation in the magnitude of AvBD9 gene regulation among different HDAC inhibitors could be due to their relative potency in HDAC inhibition in different cell types.

### **3.2. Suppression of butyrate-induced AvBD9 gene expression by HAT inhibitors**

Acetylation of nucleosomal core histones is achieved by either activation of HATs or inhibition of HDACs [22, 23, 25]. If hyperacetylation of histones through HDAC inhibition promotes the AvBD9 gene expression, inhibition of the HAT activity will have an opposite effect. To confirm the effect of HAT inhibition on butyrate-induced AvBD9 expression, we pretreated HD11 cells with different concentrations of HAT inhibitors for 2 h prior to stimulation with 1 mM butyrate for another 24 h. Cells were then harvested and subjected to total RNA extraction and real time RT-PCR. As expected, EGCG dose-dependently reversed the induction of the AvBD9 gene by butyrate (Fig. 2A). EGCG at 200  $\mu$ M suppressed butyrate-induced AvBD9 expression by 15-fold. Anacardic acid (Fig. 2B) or garcinol (Fig. 2C) also similarly inhibited AvBD9 gene induction caused by butyrate, although with a less efficacy. The stronger inhibitory effect of EGCG than that of anacardic acid or garcinol on the HAT activity is likely to be attributed to the fact that EGCG inhibits a broader spectrum of HATs than other two HAT inhibitors [30-33]. It is noted that, due to a low expression level of AvBD9 under the basal condition, a further decrease by HAT inhibitors could not be reliably detected in HD11 cells. Nevertheless, these studies reinforced a critical role of histone acetylation in regulation of the AvBD9 gene.

### **3.3. Involvement of p38 MAPK and JNK pathways in AvBD9 gene expression**

MAPK signaling was shown to be involved in butyrate-mediated LL-37 induction in human intestinal epithelial cells and lung epithelial cells [19, 34, 35]. To determine the effect of three classical MAPK pathways on AvBD9 gene expression, we pretreated HD11 cells with or without p38 MAPK, ERK1/2, and JNK inhibitors for 1 h, followed by incubation with butyrate for another 24 h. Real-time RT-PCR analysis of AvBD9 expression revealed that SB203580 and SP600125, p38 MAPK and JNK inhibitors, respectively, significantly attenuated butyrate-mediated AvBD9 induction (Fig. 3). On the other hand, PD, a specific ERK1/2 inhibitor, failed to suppress AvBD9 expression induced by butyrate. These results suggested that p38 MAPK and JNK, but not ERK1/2, pathways are involved in butyrate-triggered AvBD9 expression.

### **3.4. Impact of cAMP signaling on AvBD9 synthesis**

In addition to histone acetylation and MAPK signaling, cAMP analogs and adenylate cyclase agonists were shown to induce LL37 expression in human intestinal epithelial cells [26]. To study whether cAMP signaling is also involved in the regulation of HDP synthesis in chickens, we first treated HD11 cells with different concentrations of two cAMP analogs, 8-bromo-cAMP and DB-cAMP for up to 48 h. As shown in Fig. 4, the two analogs triggered both a time- and dose-dependent induction of AvBD9 gene expression. Treatment with 0.5 mM 8-bromo-cAMP led to an approximately 5-fold increase in AvBD9 expression at 24 h and 15-fold AvBD9 induction at 48 h (Fig. 4A). More strikingly, DB-cAMP caused a much more pronounced augmentation of AvBD9 gene expression, with approximately 200-, 500-, and 1,000-fold induction following

stimulation with 2 mM of DB-cAMP for 6, 12, and 24 h, respectively (Fig. 4B). The results clearly confirmed the role of cAMP in the HDP induction in the chicken. The marked difference in AvBD9 regulation between two cAMP analogs are likely due to the release of two butyrate molecules from the cAMP motif after DB-cAMP is taken up into the cells. Therefore, unlike 8-bromo-cAMP, the effect seen with DB-cAMP is likely due to the combined actions of both butyrate and cAMP. In fact, a growing body of evidence suggested a consideration of the biological effect of butyrate when DB-cAMP is used as a cAMP analog [36-39]. Nevertheless, it is beneficial to use DB-cAMP to enhance host immunity and disease resistance by taking advantage of the HDP-inducing activity of both butyrate and cAMP motifs existing in DB-cAMP.

In addition to cAMP analogs, we further examined the AvBD9-inducing efficacy of adenylate cyclase agonists, which promote the endogenous synthesis of cAMP. As shown in Fig. 5, 10  $\mu$ M forskolin stimulated AvBD9 gene expression in a time-dependent fashion peaking significantly with nearly a 9-fold induction at 24 h, consistent with the potency of a cAMP analog, 8-bromo-cAMP. Similarly, CT at 0.5  $\mu$ g/ml also exerted a statistically significant 6-fold increase in AvBD9 gene expression following 24 h stimulation, whereas PT caused a marginal 2-fold enhancement at 12 or 24 h (Fig. 5) demonstrated negligible induction of AvBD9 at and 24h time period. Overall, these data indicated that, in addition to cAMP itself, any agent that stimulates the synthesis of cAMP is also capable of promoting AvBD9 gene expression in the chicken.

### **3.5. Synergistic induction of AvBD9 gene expression by HDAC inhibitors and adenylate cyclase agonists**

Since both HDAC inhibitors and cAMP signaling activators induce AvBD9 gene expression, we sought to test whether there is a synergistic interaction between these two groups of agents. To our surprise, we observed a clear, statistically significant synergy between butyrate and three different adenylate cyclase agonists. Stimulation of HD11 cells with 1 or 2 mM butyrate for 24 h led to 200- to 800-fold increase in AvBD9 gene expression, whereas forskolin gave a maximum, less than 10-fold induction (Fig. 6A). However, a nearly 3,000-fold increase in AvBD9 expression was observed in HD11 cells in response to a combination of both 2 mM butyrate and 5  $\mu$ M forskolin, which reflected an additional 3-fold increase over butyrate alone (Fig. 6A). Similarly, CT or PT led to a marginal increase in AvBD9 gene expression in HD11 cells; however, simultaneous treatment with butyrate and CT or PT resulted in an additional 3- to 4-fold increase over butyrate alone (Fig. 6B and 6C). The results revealed a clear synergistic interaction between histone deacetylation and cAMP signaling.

It is worth noting that forskolin regulated butyrate-mediated AvBD9 expression in a biphasic manner, with higher concentrations from 10 to 200  $\mu$ M suppressing AvBD9 induction (Fig. 6A). The same is true with forskolin alone, with low concentrations inducing gene expression and high concentrations causing a dose-dependent abrogation of AvBD9 induction (Fig. 6A, insert). These results perhaps are not surprising, given the existence of negative feedback mechanisms in cAMP signaling. In fact, prolonged production of cAMP negatively regulates the expression of LL-37 in human intestinal cells, due to the presence of an inducible cAMP early repressor in the LL-37 gene promoter [26]. It is likely that such a similar cAMP repressor is also present in the AvBD9 gene promoter; however, it needs to be experimentally confirmed.

#### 4. DISCUSSION

HDAC inhibitors including butyrate, sulforaphane, phenylbutyrate, and TSA were found to induce the HDP expression in humans [[19-21, 34, 35]]. We also revealed the role of butyrate on regulation of several HDP gene expression in chickens [27]. Here, we revealed for the first time that several additional HDAC inhibitors such as sodium valproate, SAHA, CAY10433, and CAY10398 are all capable of stimulating AvBD9 gene expression in chicken HD11 macrophage cells and primary monocytes, albeit with different efficacies. Furthermore, we showed that HAT inhibitors suppressed the HDP gene expression. The results made it evident that HDP regulation by histone deacetylation is conserved in both mammals and aves. However, it is likely that differences exist among species and/or cell types. For example, phenylbutyrate was shown to be more potent than butyrate in inducing LL-37 expression in human HT29 intestinal cells [21]. Sulforaphane also exhibited higher efficiency than butyrate in triggering human  $\beta$ -defensin 2 (HBD-2) expression in the same cell line [20]. However, when compared to butyrate, phenylbutyrate had a less stimulating effect on AvBD9 expression in chicken HD11 cells while sulforaphane had no effect (data shown). Therefore, it is prudent to confirm the HDP-inducing efficacy of individual HDAC inhibitors in each species.

cAMP activates gene expression through protein kinase A (PKA)-mediated phosphorylation of intracellular transcription factors such as cAMP response element-binding protein (CREB), which in turn promotes recruitment of several HATs including

CREB binding protein (CBP) and p300 to the target gene promoter, leading to chromatin remodeling and gene transactivation [40]. HDAC inhibitors act to prolong CREB phosphorylation, thereby potentiating CBP/p300 recruitment and cAMP-dependent gene transcription [41]. Therefore, it is not surprising to see a clear synergy between adenylate cyclase agonists and HDAC inhibitors in triggering AvBD9 gene expression. It will be important to explore such a synergistic interaction between cAMP signaling and histone deacetylation in boosting HDP synthesis, host immunity, and disease resistance.

We have also shown JNK and p38 MAPK signaling pathways are critically important in regulating butyrate-mediated AvBD9 gene induction, which is consistent with an earlier report on the existence on the AvBD9 (also known as gallinacin-6) promoter region of several binding sites for activator protein 1 (AP-1) [42], which is a common target transcription factor activated by MAP kinases [43]. It is intriguing to note that ERK1/2 MAPK pathway appears not to be involved in regulating AvBD9 expression in HD11 cells. However, both ERK1/2 and JNK, but not p38 MAP kinase pathways are implicated in LL-37 induction in human lung and intestinal epithelial cells stimulated with butyrate [19, 35] or phenylbutyrate [21]. The reason for such a discrepancy between humans and chickens remains unknown. It is plausible that species- or gene-specific regulatory pattern of HDP expression may exist.

Taken together, our results clearly showed that histone deacetylation, cAMP signaling, and MAP kinase pathways are involved in AvBD9 gene regulation. All these three events are likely to cooperate with each other in providing a fine tuning of the AvBD9 expression. HDAC inhibitors enhance histone acetylation and relax the AvBD9 gene promoter, achieving two benefits simultaneously. First, it prolongs cAMP signaling



resulting in enhanced recruitment of CREB to the AvBD9 promoter. Secondly, it facilitates binding of AP-1 activated by MAP kinases to the gene promoter. It is also known that cAMP and MAPK signaling pathways cross-talks in that cAMP-dependent activation of PKA ultimately activates all three classical MAP kinase pathways [26].

Because of many desirable host defense roles of HDPs, further exploration of the regulatory mechanisms of HDPs will facilitate development of strategies for optimal production of HDPs, which will have enormous implications in boosting host immunity and disease resistance without resorting to conventional antibiotics.

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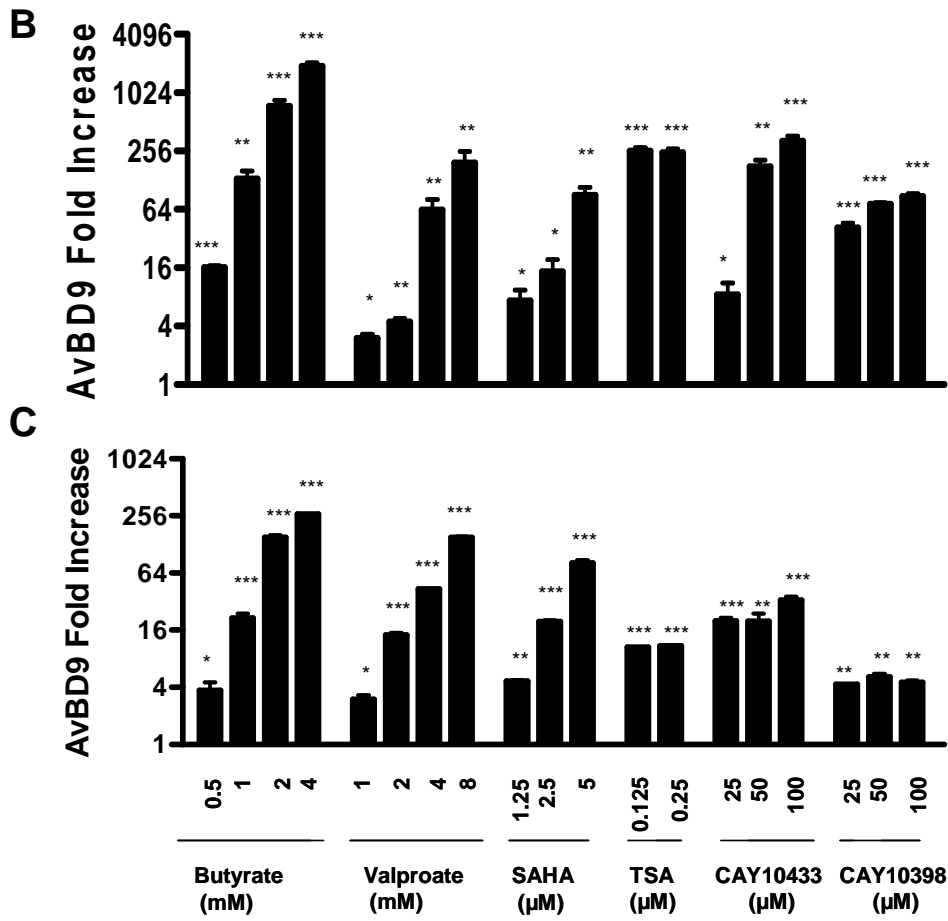
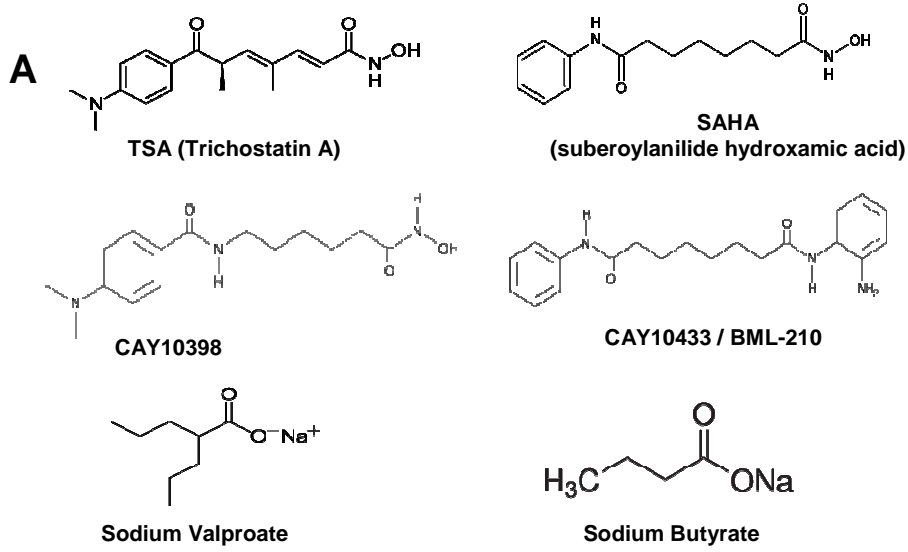
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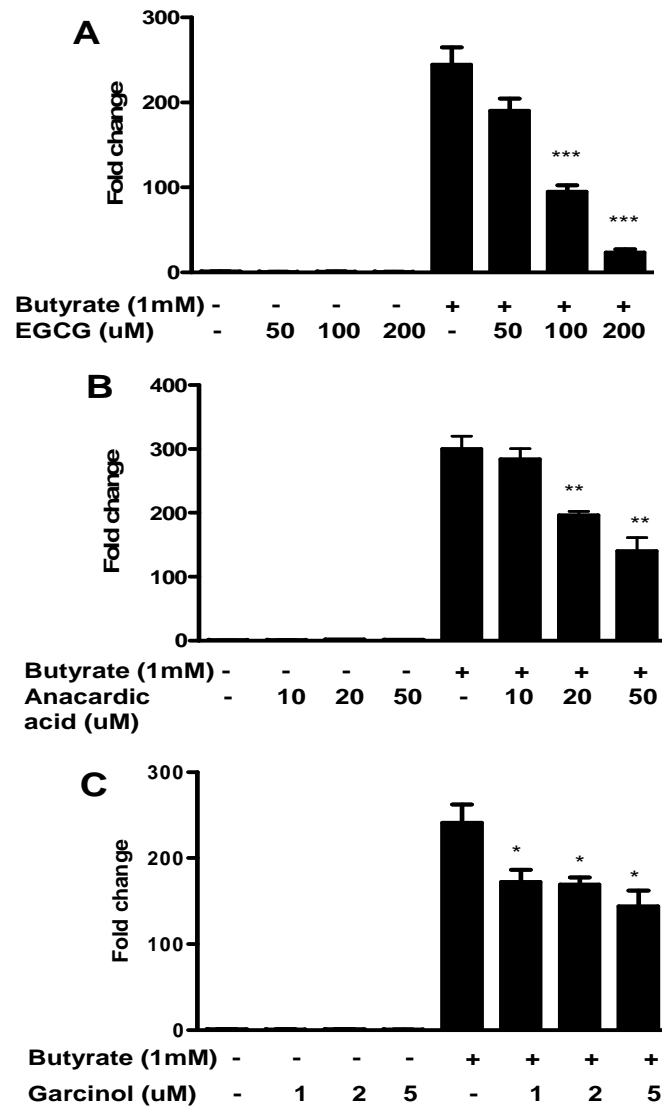
**FIGURE AND FIGURE LEGENDS**



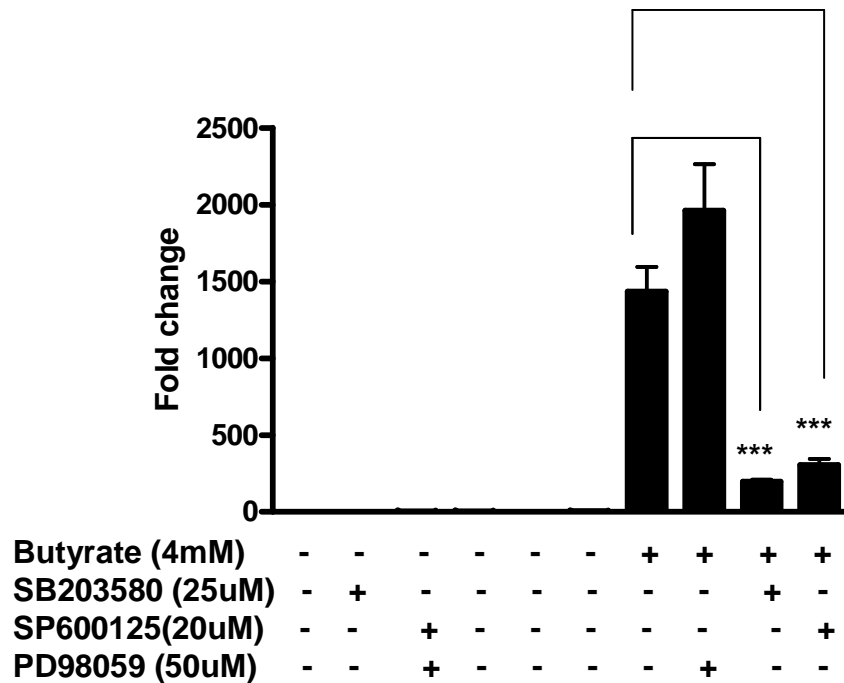
**Fig. 1. Induction of AvBD9 gene expression by histone deacetylase inhibitors.**

Different concentrations of histone deacetylase inhibitors (A) were incubated in duplicate with chicken HD11 macrophages (B) or primary monocytes (C) with for 24 h, followed by real-time RT-PCR analysis of AvBD9 expression. Each bar represents mean  $\pm$  standard error of the data from 2-4 independent experiments. \* $P < 0.05$ , \*\*  $P < 0.001$ , and \*\*\*  $P < 0.0001$  by unpaired Student's  $t$ -test as compared to the untreated control.

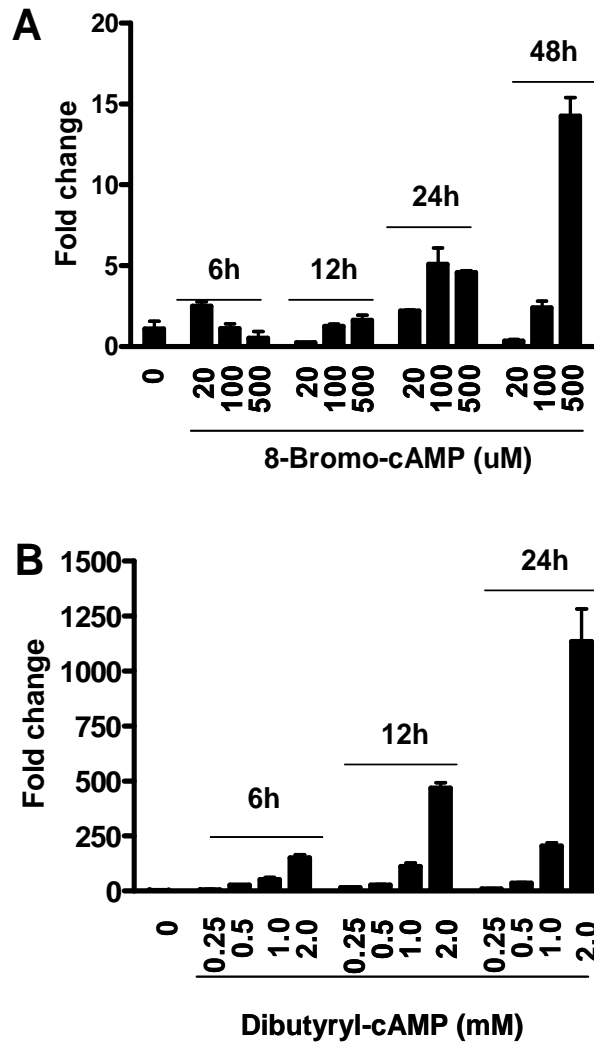




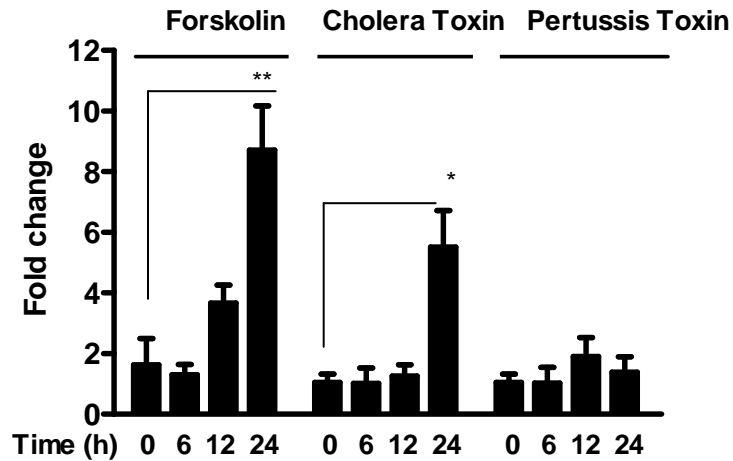
**Fig. 2. Suppression of butyrate-mediated AvBD9 gene induction by histone acetyltransferase inhibitors.** Chicken HD11 cells were treated in duplicate with indicated concentrations of epigallocatechin gallate (EGCG) (A), anacardic acid (B) and garcinol (C) for 2 h before treatment with 1 mM butyrate for another 24 h. Real-time RT-PCR analysis was carried out to evaluate AvBD9 gene expression. Each bar shows mean  $\pm$  standard error of the data from 3-4 experiments. \* $P < 0.05$ , \*\*  $P < 0.001$ , and \*\*\*  $P < 0.0001$  by unpaired Student's  $t$ -test.



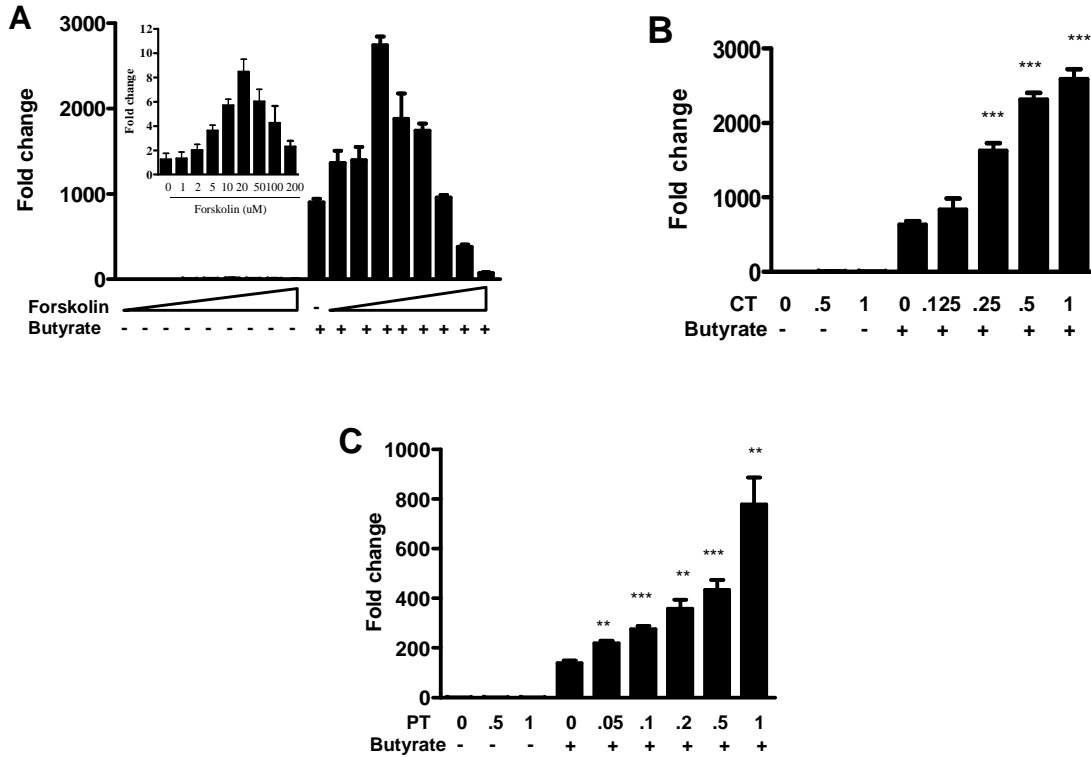
**Fig. 3. Role of p38 and JNK mitogen-activated protein kinase pathways on AvBD9 gene induction.** Chicken HD11 cells were incubated in duplicate with 25  $\mu$ M p38 inhibitor (SB203580), 20  $\mu$ M JNK inhibitor (SP600125) or 50  $\mu$ M MEK inhibitor (PD98059) for 1 h, followed by stimulation with 4 mM butyrate for another 24 h. AvBD9 expression was evaluated with real-time RT-PCR. Data from 2-4 experiments are presented in bars showing means  $\pm$  standard error. \*\*\* $P \leq 0.0001$  by unpaired Student's *t*-test.



**Fig. 4. Upregulation of AvBD9 gene expression by cAMP analogs.** Chicken HD11 cells were treated in duplicate with different concentrations of 8-bromo-cAMP (A) and dibutyryl-cAMP (B) for 6, 12 or 24 h. Real-time RT-PCR analysis was performed to evaluate AvBD9 gene expression and the results were normalized against GAPDH. Each bar shows mean  $\pm$  standard error of the data from 2-3 experiments.



**Fig. 5. Induction of AvBD9 gene expression by adenylyate cyclase agonists.** Chicken HD11 cells were stimulated with 10  $\mu$ M forskolin, 0.5  $\mu$ g/ml cholera toxin, or 0.5  $\mu$ g/ml pertussis toxin for 6, 12 or 24 h. AvBD9 gene expression was analyzed by real-time RT-PCR, and relative fold change was calculated as compared to the negative control. Values represent means  $\pm$  standard error of the data from 2 to 3 experiments. \* $P$  < 0.05, and \*\*  $P$  < 0.001 by unpaired Student's  $t$ -test.



**Fig. 6. Synergistic increase in AvBD9 gene expression by butyrate and adenylate cyclase agonists.** HD11 cells were pretreated in duplicate for 1 h with different concentration of forskolin (FSK ( $\mu\text{M}$ ): ( ): 0, 1, 2, 5, 10, 20, 50, 100, and 200) (A) or cholera toxin (CT;  $\mu\text{g/ml}$ ) (B), followed by 2 mM butyrate for another 24 h. Similarly, different concentrations of pertussis toxin (PT;  $\mu\text{g/ml}$ ) (C) were added to HD 11 cells for 1 h prior to 1 mM butyrate incubation for another 24 h. Real-time RT-PCR analysis was used to evaluate AvBD9 gene expression, and the relative fold change was quantitated using  $2^{-\Delta\Delta\text{Ct}}$  method. Each bar demonstrates means  $\pm$  standard error of the data from 2 to 3 experiments. The effect of FSK alone on AvBD9 induction was shown in the insert, panel A. \* $P < 0.05$ , \*\*  $P < 0.001$ , and \*\*\*  $P < 0.0001$  by unpaired Student's  $t$ -test as compared to the cells treated with butyrate alone.

VITA

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Candidate for the Degree of

Doctor of Philosophy

Thesis: ENHANCING CHICKEN INNATE IMMUNITY AND DISEASE RESISTANCE BY BOOSTING HOST DEFENSE PEPTIDE SYNTHESIS

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Pages in Study: 126

Candidate for the Degree of Doctor of Philosophy

Major Field: Animal Science (Molecular Immunology)

Scope and Method of Study: Routine use of antibiotics for growth promotion and disease prevention in animal agriculture has caused the concern for rapid emergence of antimicrobial resistance in public health worldwide. In this study, we attempted an antibiotic-alternative approach to disease control and prevention by enhancing host defense peptide (HDP) synthesis and animal immunity. We evaluated a diverse group of dietary supplements for their capacity to stimulate chicken HDP gene expression *in vitro* and *in vivo* by real-time RT-PCR. Chicken infection studies were further conducted to confirm an enhanced resistance to *Salmonella enteritidis* following oral supplementation of selected dietary factors. We also evaluated the role of histone acetylation as well as cAMP and MAP kinase signaling in the transcriptional regulation of HDP synthesis in chicken HD11 macrophage cells.

Findings and Conclusions: Butyrate, a short-chain fatty acid and a well-known histone deacetylase inhibitor, enhances a large set of chicken HDPs and confers resistance to *S. enteritidis*. In addition, the induction of chicken HDP synthesis is largely inversely correlated with the aliphatic carbon chain length of free fatty acids, with short-chain fatty acids being the most potent, medium-chain fatty acids moderate, and long-chain fatty acids mostly ineffective. Desirably, free fatty acids enhance HDP expression with a minimum impact on proinflammatory response. Additionally, a combination of three short-chain fatty acids, namely acetate, propionate, and butyrate, induced HDP expression in a synergistic manner, leading to more significant reduction of the *S. enteritidis* load in the chicken than individual fatty acids. Moreover, cAMP signaling agonists stimulated chicken HDP gene expression and synergized with butyrate in HDP induction. We confirmed that p38 and JNK, but not ERK1/2, MAP kinase signaling pathways are involved in butyrate-mediated chicken HDP induction. Identification of potent HDP-inducing dietary supplements and a better understanding of transcriptional regulatory mechanisms of HDP gene expression will undoubtedly facilitate development of antibiotic-free approaches to disease control and prevention with applications in both animal and public health.

ADVISER'S APPROVAL: Dr. Guolong Zhang

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