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EFEITO DA MISTURA DO LÍQUIDO DA CASCA DA CASTANHA DE CAJU E DO ÓLEO DE MAMONA NO DESEMPENHO, NA IMUNIDADE E NA MICROBIOTA DE FRANGOS DE CORTE DESAFIADOS POR COCCIDIOSE

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Tese apresentada como um dos requisitos para a obtenção do grau de Doutor em Zootecnia Área de Concentração em Produção Animal

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TESE

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para obtenção do Grau de
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Á Andréa Machado Leal Ribeiro,
com todo meu respeito, graditão e admiração por seu trabalho.

Aos meus pais, Jurema e Daniel,
por abrir mão de seus sonhos para que eu pudesse realizar os meus.

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“Se vi mais longe foi por estar de pé sobre ombros de gigantes”
(Isaac Newton)

EFEITO DA MISTURA DO LÍQUIDO DA CASCA DA CASTANHA DE CAJU E DO ÓLEO DE MAMONA NO DESEMPENHO, NA IMUNIDADE E NA MICROBIOTA DE FRANGOS DE CORTE DESAFIADOS POR COCCIDIOSE[1]

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RESUMO

O objetivo deste estudo foi avaliar o efeito da mistura comercial do líquido da casca de castanha de caju e do óleo de mamona (Essential, Oligo Basics Agroind. Ltda., Cascavel, Brasil) no desempenho, na microbiota e no sistema imune de frangos de corte desafiados ou não por coccidiose. Ao total 864 pintos machos (Cobb) de um dia de idade foram distribuídos aleatoriamente em 6 tratamentos (8 boxes/tratamento e 18 pintos/box) em um desenho fatorial 3 x 2 com 3 aditivos: controle (sem aditivo), 100 ppm de monensina ou 0,15% de Essential e 2 níveis de desafio aos 14 dias de idade: não desafiados ou inoculados por gavagem com 1mL de solução contendo oocistos esporulados de *E. tenella*, *E. acervulina* e *E. máxima*. Os resultados foram divididos em dois artigos. Artigo 1: Na primeira semana após desafio, as aves desafiadas suplementadas com monensina apresentaram maior ganho de peso (GP), consumo de ração (CR) e melhor conversão alimentar (CA) (P <0,05), porém na segunda semana o Essential apresentou maior GP e melhor CA (P <0,05), aos 42 dias de idade, ambos os grupos não se diferiram em GP, CR, PV e foram maiores do que o controle (P <0,05). A utilização de monensina em aves desafiadas reduziu o número cópias do domínio bactéria e de *E.coli* (P<0,05), por sua vez, a suplementação com Essential reduziu *Clostridium Cluster* XIV, *Clostridium perfringens* e *Staphylococcus aureus* em relação aos demais tratamentos (P<0,05). As aves não desafiadas que receberam Essential ou monensina apresentaram menor população de *C.perfringens e S. aureus* (P<0,05). Artigo 2: O grupo que recebeu Essential aumentou a expressão gênica de IFN-y, IL-6 e TNF-α (P<0,05) e o grupo controle aumentou a expressão gênica de COX-2 e IL-1 em relação aos demais tratamentos (P<0,05). As aves não desafiadas que receberam monensina apresentaram maior expressão gênica de IFN-y, COX-2 e IL-1 comparadas aos demais tratamentos (P<0,05), ao contrário do grupo com Essential que reduziu a expressão gênica com exceção do TNF-α. Aos 7 e 14 dias após o desafio houve maior excreção de oocistos para o grupo controle, Essential e monensina não diferiram-se (P>0,05). Assim, o Essential melhorou o desempenho de frangos de corte infectados por coccidiose após a segunda semana do desafio e atuou como um modulador da microbiota intestinal e do sistema imune, direcionando a resposta inflamatória contra o parasita.

Palavras-chave: Citocinas. Eimeria. Imunidade. Microbiota. Saúde intestinal.

[1]Tese de Doutorado em Zootecnia - Produção Animal, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (129 p.). Setembro, 2017.

EFFECT OF BLEND OF CASHEW NUT SHELL LIQUID AND CASTOR OIL ON GROWTH PERFORMANCE, IMMUNITY AND MICROBIOTA IN BROILERS CHALLENGED WITH COCCIDIOSIS[1]

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ABSTRACT

The objective of this study was to evaluate the effects of a commercial mixture of cashew shell liquid and castor oil (Essential, Oligo Basics Agroind. Ltda., Cascavel, Brazil) on growth performance, immunity and microbiota in broilers challenged with coccidiosis. A total of 864 one-day-old male chicks (Cobb) were randomly assigned to 6 treatments (8 pens/treatment and 18 birds/pen) in a 3 x 2 factorial design with 3 additives: control (no additive), 100 ppm of monensin, and 0.15% of Essential; and 2 challenge levels at 14 days of age: no challenge and inoculation by gavage of 1 ml of a solution containing sporulated oocysts of E*. tenella, E. acervulina*, and *E. maxima.* The results were divided into two articles. Article 1: In the first week after challenge, challenged birds supplemented with monensin showed higher LW, WG, FI and better FCR ($P < 0.05$), but in the second week Essential presented higher WG and better FCR ($P < 0.05$), at 42 days of age, both groups did not differ in WG, FI, and LW and were higher than the control ($P < 0.05$). The use of monensin in challenged birds reduced the number of copies of the bacteria domain and of E.coli ($P<0,05$). In turn, Essential supplementation reduced Clostridium Cluster XIV, Clostridium perfringens and Staphylococcus aureus in relation to the other treatments ($P<0,05$). The unchallenged birds that received Essential or monensin presented a lower population of C.perfringens and S. aureus ($P<0,05$). In addition, Essential presented higher number of copies of *Lactobacillus* spp., followed by monensin and control ($P < 0.05$). Article 2: The group that received Essential increased the gene expression of IFN-y, IL-6 e TNF-α ($P<0,05$) and the control group increased the gene expression of COX-2 and IL-1 in relation to the other treatments ($P < 0.05$). The unchallenged birds that received monensin presented upregulated expression of IFN-y, COX-2 and IL-1 compared to the other treatments ($P < 0.05$), unlike the Essential group, which reduced gene expression with the exception of TNF-α. At 7 and 14 days after the challenge there was a higher excretion of oocysts for the control group, Essential and monensin did not differ ($P>0,05$). Thus, Essential improved the performance of coccidiosis-infected broiler chickens after the second week of challenge, as well as acts as a modulator of intestinal flora and immune system, directing the inflammatory response against the parasite.

Keywords: Cytokines. Eimeria. Immunity. Gut health. Microbiota.

[1]Doctoral thesis in Animal Science, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. (129 p.) September, 2017

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Lista de Abreviaturas e Símbolos

ACTH: hormônio adenocorticotrófico
ChTL: receptores *Toll-like* de frangos
COX-2: ciclooxigenase-2
CR: consumo de ração
CT: cycle threshold
DAMP: padrões moleculares associados a danos
DNA: ácido desoxirribonucleico
GHRH: hormônio liberador do hormônio do crescimento
GP: ganho de peso
FI: Feed intake
IFN-γ: interferon-gama
IL: interleucina
IEL: linfócitos intraepiteliais
IRF3: Interferon regulatory factor 3
LCCC: liquido da casca de castanha do caju
LPS: lipopolissacarídeos
MAPK: mitogen-activated protein kinase
MHC: complexo de histocompatibilidade
mRNA: RNA mensageiro; RNA: ácido ribonucleico
MyD88: Myeloid differentiation primary response gene 88
NF-κB: fator nuclear Kb
NK: células natural killers
RT-qPCR: real time quantitative polymerase chain reaction
PBS phosphate-buffered saline
PAMP: padrões moleculares associados a patógenos
RNA: ácido ribonucleico
RRP: receptores de reconhecimento de padrões
Th: linfócito T *helper*
TLR: receptores *toll-like*
TNF: Fator de necrose tumoral
TRH: hormônio de liberação de tireotropina
ΔBW: variação no ganho de peso entre o tratamento indicado e o controle
ΔFI: variação no consumo de ração entre tratamento indicado e o controle

SUMÁRIO

CAPÍTULO I

1. INTRODUÇÃO

A interação entre a nutrição, a imunidade e a microbiota intestinal está diretamente envolvida no crescimento e na produtividade animal. Tanto a nutrição quanto a microbiota pode modular a suscetibilidade da ave contra desafios infecciosos; a suscetibilidade pode estar relacionada com a resistência ou resiliência. A resistência é a capacidade de exclusão do patógeno; por outro lado, a resiliência é a capacidade do frango de manter sua produtividade durante o desafio infeccioso. O intuito da nutrição e aumentar a resiliência das aves (Machado e Fontes, 2005).

A coccidiose aviária é uma doença intestinal causada por protozoários do gênero Eimeria spp. que se multiplicam no intestino causando destruição tecidual e prejudicando a digestão e a absorção de nutrientes, resultando em diarreia aquosa ou hemorrágica (Berchieri Jr. et al., 2009). A infecção por *Eimeria* spp. também permite a proliferação de microrganismos patogênicos como as bactérias do gênero *Clostridium spp*. Estudos têm demonstrado que a coccidiose altera drasticamente a microbiota intestinal (Hume et al., 2006; Oviedo-Rondón et al., 2006). Em conjunto com o desequilíbrio da microbiota intestinal, a coccidiose desencadeia no hospedeiro uma resposta imune complexa, porque o parasita exibe um ciclo de vida extracelular e intracelular. No entanto, a alta mortalidade não é comum. Na maioria dos casos ocorre a infecção subclínica, o que dificulta o diagnóstico da doença em tempo hábil para começar um tratamento antes que ocorra a perda de desempenho. Durante a infecção subclínica ocorre uma diminuição da digestão e absorção de nutrientes em virtude das lesões no trato gastrointestinal e consequentemente queda no desempenho (Cornelissen et al., 2009).

Alguns fitogênicos podem atuar como agentes antimicrobianos e anti-inflamatórios com efeitos similares aos fármacos utilizados na produção animal, pois são compostos formados por uma mistura complexa de substâncias voláteis como os hidrocarbonetos terpênicos, os álcoois simples, os aldeídos, entre outros que são farmacologicamentes ativos (Applegate et al.,

2010). A vantagem é que essa mistura de compostos ativos pode diminuir o desenvolvimento de resistência microbiana e reduzir o potencial de resíduos tóxicos nos produtos de origem animal.

Dentro da categoria dos fitogênicos os óleos funcionais são definidos como aqueles que têm uma ação além do valor nutricional (Murakami et al., 2014). Um desses produtos trata de uma mistura do líquido da casca de castanha de caju e do óleo de mamona cujo produto comercial é chamado de Essential® (Essential, US Patent N°. 8,377,485 B2: Oligo Basics Agroind. Ltda., Rua Sérgio Gasparetto 503, Cascavel, PR-CEP, Brazil). O Essential tem como compostos ativos o cardanol (200g/kg), o acido ricinoleico (90g/kg) e o cardol (40g/kg). Avaliando essa mistura na dieta de frangos de corte desafiados por coccidiose, Murakami et al (2014) observaram um aumento no ganho de peso e uma melhoria na conversão alimentar, além de um ganho de 100 kcal de energia metabolizável na dieta quando comparada com a controle (Bess et al., 2012; Murakami et al., 2014). Segundo Bess et al (2012), esse aumento na disponibilidade de energia pode estar relacionado com os efeitos antimicrobianos e anti-inflamatórios dos óleos funcionais . No entanto, não há na literatura trabalhos que comprovem a ação antimicrobiana deste produto in vivo.

Entender os mecanismos fisiológicos e metabólicos pelos quais os aditivos utilizados na dieta afetam o desempenho, a imunidade e o ecossistema da microbiota permite a formulação de dietas práticas de produção que otimizem a resistência a doenças e melhorem o desempenho animal. Assim, o presente trabalho foi desenhado com o objetivo de avaliar os efeitos da suplementação da mistura comercial do líquido da casca de castanha de caju e do óleo de mamona na dieta de frangos de corte desafiados por coccidiose.

2. REVISÃO BIBLIOGRÁFICA

2.2 Sistema imunológico

O sistema imune é dividido didaticamente em três linhas de defesa. A primeira linha de defesa é constituída pelas barreiras físicas que evitam a penetração do agente; a segunda é a imunidade inata, composta por células e moléculas responsáveis pela resposta imediata, conferindo uma proteção inicial; e a terceira é a imunidade adquirida (Tizard, 2009). A imunidade inata é linha de defesa do hospedeiro capaz de induzir uma resposta rápida e menos específica. O reconhecimento dos patógenos na imunidade inata é mediado pelos Receptores de Reconhecimento de Padrões (RRPs), que reconhecem padrões moleculares associados aos patógenos, os denominados de PAMPs e padrões moleculares associados aos danos, conhecidos como DAMPs. Os PAMPs são as estruturas microbianas comuns e quimicamente diferentes dos componentes normais do organismo. Uma das principais famílias dos PAMPs é a dos receptores Toll-like (TLRs). Os DAMPs são ligantes endógenos liberados durante o dano tecidual ocorrido em sítios infecciosos e de necrose celular (Abbas et al., 2011).

A ativação dos TLR, conduz à ativação do fator neural kappa B (NF-kB) via de transdução do sinal de indução de uma grande variedade de genes do hospedeiro envolvidos na imunidade inata, tais como citocinas, quimiocinas e de síntese de óxido nítrico. Além disso, fazem parte da imunidade inata as células dendríticas, células natural killer e as células fagocíticas como os macrófagos e os neutrófilos Nas aves os neutrófilos são denominados heterofilos. A defesa do organismo é centralizada no local da infecção, estabelecendo uma resposta inflamatória através das citocinas pró-inflamatórias (TNFα, lL-1, IL-6, IL-12), que juntas às células defensoras, evitam que os patógenos se desloquem para áreas não infectadas. Se o sistema imune inato não for capaz de prevenir o acesso ou destruir o microrganismo invasor, a imunidade adquirida é desencadeada (Scroferneker, 1996; Tizard, 2009; Abbas, et al., 2012).

O sistema imune adquirido está dividido em dois tipos de resposta e é dependente do microrganismo invasor. A primeira é a humoral, que é mediada

por linfócitos B e é desencadeada por microrganismos que permanecem nos fluidos corporais, ou seja, extracelularmente. A segunda é a resposta imune celular, mediada por linfócitos T e ocorre quando o patógeno encontra-se intracelularmente (Tizard, 2009; Abbas et al., 2012).

A imunidade humoral é mediada por anticorpos produzidos pelos linfócitos B com função de neutralizar o desenvolvimento do agente infeccioso e sinalizar para eliminação dos mesmos. A resposta imune celular é composta pelos linfócitos T que têm uma especificidade restrita, reconhecem somente antígenos peptídicos ligados a moléculas do hospedeiro que são as proteínas do complexo de histocompatibilidade (MHC). A resposta imune celular conta com a ativação de linfócitos T citotóxicos, células natural killers (NK) e macrófagos. Os linfócitos T citotóxicos agem liberando produtos citolíticos na área de contato com a célula infectada induzindo-a a apoptose. A resposta mediada pelas células T é extremamente efetiva no mecanismo de defesa contra agentes intracelulares, como vírus, protozoários, fungos e bactérias intracelulares. As células T podem exercer sua função através da citotoxicidade mediada por células CD8+ ou através da secreção de citocinas que vão ativar macrófagos para destruir os agentes intracelulares (Abbas et al., 2015).

2.1.1 Custo do sistema imune

A interação entre a nutrição e a imunidade é particularmente importante para o crescimento e produtividade animal. A composição da dieta pode modular a suscetibilidade da ave contra desafios infecciosos. A suscetibilidade pode estar relacionada com a resistência ou resiliência, sendo a resistência a capacidade de exclusão do patógeno e a resiliência a capacidade do animal em manter a produtividade durante o desafio infeccioso. O intuito da nutrição e aumentar a resiliência dos animais (Machado e Fontes, 2005; Yang et al., 2003).

Durante o desafio imunológico o custo energético é maior que o proteico ou de outros substratos, devido a alta taxa de *turnover* celular com eficiente reutilização de nutrientes. Quando há um sistema imune ativo há uma hipertrofia do fígado para a produção de proteínas da fase aguda de resposta.

Também o sistema imune aumenta sua exigência de minerais traço que atuam como cofatores para as proteínas da fase aguda de resposta (Klasing, 2007).A fase aguda de resposta é caracterizada pela diminuição do apetite, aumento da taxa metabólica basal, aumento da degradação do músculo esquelético e aumento da síntese hepática de proteínas da fase aguda. Essas mudanças ocorrem mediadas pelas citocinas pró-inflamatórias (IL-1, IL-6 e TNF-α), que por sua vez desencadeiam a liberação de hormônios que reduzem a taxa de crescimento. Como exemplo, o estímulo do hormônio adenocorticotrófico (ACTH) pelos linfócitos T e macrófagos vai aumentar a síntese de glicocorticóide que vai atuar na musculatura esquelética causando liberação de aminoácidos para glicogeneogênese ou síntese de proteínas da fase aguda de resposta (Klasing; Korver, 1997). Além disso, quando receptores específicos para TNF-α do cérebro de aves são ativados há uma redução significativa de hormônios como o GHRH (hormônio de liberação do hormônio de crescimento) e TRH (hormônio de liberação de tireotropina), reduzindo assim a taxa metabólica e o crescimento (Elsasser et al., 1997).

Essas respostas do sistema imune têm efeito sistêmico alterando o partilhamento de nutrientes (Klasing et al., 1997). Há uma relação antagônica entre os processos fisiológicos da imunidade e do crescimento. Durante a resposta inflamatória cerca de 70% da queda de desempenho é devido à diminuição do consumo de alimentos, porém o restante fica por conta da redução do metabolismo e da menor absorção de nutrientes (Klasing et al., 1987). Jiang et al. (2010), observaram queda de 22,5% na taxa de ganho de peso de frangos de corte desafiados com lipopolissacarídeos bacterianos (LPS) comparado ao grupo não desafiado, alimentado ad libitum. O grupo de aves que não foi desafiado, mas que teve o fornecimento de ração pareado com os desafiados com LPS também reduziu o crescimento, porém não tão intensamente. Conclui-se que apenas 59% da queda de desempenho foi em função da redução no consumo de ração, enquanto 41% foi devido a outros fatores, provavelmente associados a resposta imune. Comparando a exigência de lisina do sistema imune em mantença e ativado, o requerimento de lisina passou de 1-2% para 6-7%, respectivamente. Isso porque houve um aumento

na produção de anticorpos, linfócitos antígeno-específicos, fagócitos e, principalmente, pela produção das proteínas de fase aguda no fígado (Klasing, 2007).

2.2 Microbiota intestinal de frangos de corte

A saúde intestinal está diretamente relacionada com o perfil da microbiota que interage com o hospedeiro regulando a eficiência absortiva, apresentando mecanismos antagônicos a bactérias patogênicas, reforçando a integridade intestinal e modulando a imunidade (Oviedo-Rondón et al., 2009; Pan e Yu 2014). Ao contrário do genoma do hospedeiro, que raramente é manipulado por intervenção xenobiótica, o microbioma é facilmente alterável, principalmente pela dieta, ingestão de antibióticos e infecção por agentes patogênicos (Day et al., 2015).

Os microrganismos que compõe a microbiota intestinal estabelecem relações de cooperação e competição por nutrientes e locais de aderência no lúmen, estabelecendo equilíbrio da comunidade microbiana. No intestino delgado dos frangos predominam bactérias do gênero *Lactobacillus, Clostridiaceae, Streptococcus* e *Enterococcus*, ao contrário do que ocorre no ceco, onde predomina a família *Clostridiaceae*, seguida dos gêneros *Fusobacterium, Lactobacillus* e *Bacteroides* (Lu et al., 2003).

Por outro lado, o aumento no número de Lactobacillus, geralmente, está associado com uma melhor saúde intestinal. No entanto, Torok et al. (2011), observaram que o pior desempenho está relacionado com o aumento *L. salivarius, L. aviarius* e *L. crispatus* no íleo de frangos. Segundo Guban et al. (2006), a espécie *L. salivarius* pode estar associada ao pior desempenho em frangos por desconjugar os sais biliares prejudicando a emulsificação de gordura (Guban et al., 2006).

Dentre os membros da família *Clostridiaceae* a espécie *Clostridium perfringens* é conhecida por ser um patógeno causador da enterite necrótica. Esta bactéria é um habitante normal do ceco, no entanto, quando o ambiente torna-se favorável há uma rápida proliferação, podendo estender-se para o intestino delgado, onde geralmente está em baixa concentração, levando à

enterite necrótica (Shojadoost et al., 2012). A população de C. perfringens no intestino delgado de aves saudáveis é normalmente 102 a 104 UFC/g de conteúdo intestinal; quando instaurada a enterite necrótica, a população fica em tono de 10^7 a 10^9 UFC / g de conteúdo intestinal (Shojadoost et al., 2012). Sabe-se que um dos fatores que favorece a proliferação desse patógeno é a coccidiose, por causar imunossupressão, danos na barreira da mucosa, aumento da produção de muco e aumento de proteínas plasmáticas, disponibilizando substrato para o crescimento do *Clostridium perfringens* (Kitessa et al., 2014). Entretanto, é importante destacar que nem todas as espécies do gênero *Clostridium* são consideradas patogênicas A espécie *Clostridium butyricum* é utilizada como probiótico melhorando o desempenho e a eficiência alimentar de frangos (Yang et al., 2012). Zhang et al. (2016), observaram que em frangos desafiados por *Escherichia coli*, a utilização de *C. butyricum* melhorou o ganho de peso e reduziu a população de *E.coli*. Além disso, o C*lostridium* coccoide produz acido butírico e o *C. cellulosi* degrada a celulose melhorando a saúde intestinal, demonstrando que a presença do gênero *Clostridium* pode trazer benefícios ao desempenho animal (Rubio et al., 2014; Stanley, 2012) .

As bactérias comensais são importantes para o desenvolvimento e maturação da resposta imune inata (Muir et al., 2000; Brisbin et al., 2011). Estudos com mamíferos mostraram que bactérias comensais específicas têm um papel vital na indução de células imunes (Kogut, 2013). As bactérias pertencentes ao filo Bacteroidetes apresentam associação com o desenvolvimento de células T-helper produtoras de IL-17 (Mazmanian et al., 2005). Por sua vez, o grupo Lactobacilos possui capacidade de ativar o sistema imunológico e aumentar a resistência a doenças, em parte através da liberação de peptídeos de baixo peso molecular que induzem ativação imune (Muir et al., 2000) .

A disbiose pode afetar a morfologia da parede intestinal e induzir reações imunes, ou ainda aumentar a população de bactérias patogênicas, induzindo a resposta imune e desviando a energia e os nutrientes do crescimento para a resposta inflamatória, aumentando assim a demanda para

a mantença (DiAngelo et al., 2009; Humphrey e Klasing, 2003). A resposta inflamatória é de extrema importância para a eliminação do patógeno (Kogut, 2013). No entanto, se for descontrolada, essa ativação imune representa um risco de inflamação excessiva e danos intestinais, que por sua vez, podem prejudicar as funções digestivas do intestino (Brisbin et al., 2011). Além disso, a inflamação excessiva também pode causar distúrbios no metabolismo do hospedeiro (Kogut, 2013). Foi relatado que a microbiota comensal desempenha papéis importantes na manutenção da homeostase imune intestinal e na prevenção da inflamação intestinal (Lan et al., 2005).

2.3 A coccidiose aviária

A coccidiose aviária é uma doença intestinal causada por protozoários do gênero *Eimeria* que se multiplicam no intestino causando destruição tecidual e prejudicando a digestão e a absorção de nutrientes, resultando em diarreia aquosa ou hemorrágica (Berchieri Jr. et al., 2009). A Eimeria afeta espécies de animais de maneira específica, sendo que a *E. acervulina*, *E. brunetti*, *E. máxima, E. mitis, E. praecox, E. necatrix* e *E. tenella* possuem especificidade para aves doméstica. O sistema de produção intensiva com elevada densidade no interior dos galpões, propicia um ambiente favorável para o acúmulo de uma elevada quantidade de parasitas, bem como aumenta a facilidade de transmissão do parasita entre as aves (Shirley et al., 2007).

Cada espécie de *Eimeria* possui características próprias quanto à prevalência, local de infecção, patogenicidade e imunogenicidade (Berchieri Jr. et al., 2009). Comparando a *E. tenella* com a *E. máxima* , a primeira é uma das mais patogênicas entre as sete espécies que infectam os frangos. Ela invade a mucosa cecal, causando inflamação e danos aos enterócitos. Possui curta duração, mas com sinais clínicos severos e mortalidade elevada, que ocorrem, em geral após o quinto dia de infecção. (Lillehoj et al., 2004). Kipper et al. (2013), realizaram uma meta-análise para verificar as variações no desempenho produtivo que ocorreram em frangos desafiados com diferentes

espécies de Eimeira e observaram que o desafio não afetou o consumo de ração para aves infectadas com *E. acervulina* e *E. tenella*. No entanto, na infecção com ambas as espécies de Eimeria houve um menor ganho de peso, e uma pior conversão: para *E. tenella* esses decréscimos fora na ordem de 10% e 49%, respectivamente.

Alta mortalidade com coccidiose clínica com a coccidiose não é comum. Na maioria dos casos ocorre a infecção subclínica, o que dificulta o diagnostico da doença em um tempo hábil para começar um tratamento antes que ocorra a perda de desempenho. Durante a infecção subclínica, ocorre a piora na digestão e absorção de nutrientes, em virtude das lesões no trato gastrointestinal, e queda no desempenho (Cornelissen et al., 2009). Além disso, o desafio por coccidiose pode mudar drasticamente a comunidade bacteriana no intestino, diminuindo a diversidade microbiana e criando um ambiente favorável para disseminação de outros patógenos como a bactéria gram-positiva *C. perfringens* (Kley et al., 2012). O trabalho de Oviedo-Rondon et al (2010), mostrou que após o desafio por *Eimeria spp*. o perfil microbiano no íleo e no ceco foi alterado em 45 e 64%, respectivamente. No entanto, com a utilização de dietas suplementadas com uma mistura de fitogênicos, essa alteração foi de apenas 19 e 32%.

2.3.1 Resposta imunológica contra coccidiose

A infecção por *Eimeria* spp. induz uma resposta imune complexa ao hospedeiro, porque o parasita exibe um ciclo de vida extracelular e intracelular Durante a fase inicial de infecção, o sistema imune inato do hospedeiro pode detectar e responder rapidamente à infecção do protozoário através dos receptores de reconhecimento padrão (Gazzinelli e Denkers, 2006). No entanto, a imunidade mediada por células, principalmente por linfócitos intra-epiteliais (IELs) e linfócitos da lâmina própria, representam o principal componente da imunidade protetora contra a coccidiose aviária. Estudos com as principais espécies de *Eimeria* revelaram um papel essencial para o sistema imune inato e o mediado por células, tanto pela produção de citocinas como

por ataque citotóxico direto nas células afetadas (Yun et al., 2000; Laurent et al., 2001)

Zhou et al. (2014), identificaram durante a infecção por *E. tenella* um aumento na expressão gênica de Toll-like receptor tipo ChTLR3, ChTLR15 e MyD88 no ceco. A maioria dos TRLs utilizam a proteína adaptadora denominada MyD88, responsável pela ativação dos três principais fatores de transcrição o MAPK, NF-kB e IRF3 que ativam os genes de quatro principais proteínas pro-inflamatórias IL-1, IL-6, IL-12, TNF-α e IFN- γ (Tizard, 2009). Já o IFN-γ desempenha um papel crítico na mediação de imunidade protetora contra parasitas Eimeria, estimulando macrófagos a produzir óxido nítrico, o que inibe a replicação de *E. tenella* no interior das células hospedeiras (Lillehoj e Choi, 1998). A expressão do gene de IFN-γ como resposta à infecção por *Eimeria* em frangos parece restrita ao local do intestino parasitado (Laurent et al., 2001). Assim, em aves inoculadas com *E. tenella*, a expressão de IFN-γ detectada por RT-qPCR aos 7 dias após inoculação esteve restrita ao ceco, enquanto que em aves inoculadas com *E. maxima,* foi detectada no jejuno e no íleo (Laurent et al., 2001). Por último, o TNF-α é estimulado pelos macrófagos e NF-ƙB. Frangos infectados com coccídias aumentam a expressão de TNF-α e quando tratados com anticorpos policlonais para TNF-α revertem a perda de peso causada pela coccidiose. Tal fato sugere que essa citocina esteja envolvida na fisiopatologia da coccidiose (Allen e Fetterer, 2002).

2.4 Aditivos para a saúde intestinal

Existe um grande interesse na produção animal em desenvolver aditivos alimentares com a capacidade de melhorar o desempenho, controlar os patógenos e modular a microbiota intestinal. Neste sentido, os antibióticos são utilizados como promotores de crescimento por inibir o crescimento bacteriano, a infecção subclínica endêmica, reduzindo assim os gastos metabólicos do sistema imunitário (inato) também; melhoram a absorção e utilização de nutrientes, pois a parede intestinal dos animais alimentados com antibióticos promotores de crescimento torna-se mais fina (Niewold, 2007).

No entanto, os resíduos encontrados na carne e a resistência aos antibióticos podem ter consequências críticas para o meio ambiente, segurança alimentar, bem-estar dos animais e aceitabilidade por parte do consumidor. O aumento do interesse dos consumidores nessas questões e das regulamentações governamentais relativas a práticas prudentes de uso de antibióticos estimularam pesquisas com o intuito de encontrar substâncias alternativas (Roberts et al., 2015). Para buscar substâncias alternativas é preciso ir além dos resultados de desempenho animal e entender o impacto do produto no ecossistema do intestino. Para isso é necessário avaliar a interação entre os fatores que o compõem o sistema microbiota x nutrição x sistema imune, possibilitando o desenvolvimento de ferramentas para modular-loe melhorar o desempenho animal. Algumas substâncias já foram estudadas e incluem probióticos, prebióticos, simbióticos, enzimas, fitogênicos e ácidos graxos voláteis (Sugiharto, 2016; Roberts et al., 2015).

2.4.1 Líquido da casca da castanha e óleo de mamona

O líquido da casca da castanha do caju (LCCC) é um subproduto que representa aproximadamente 25% do peso da castanha. Diferentes processos podem ser empregados para a obtenção do LCCC: extração a frio (prensas), extração por solvente e processo térmico-mecânico. O LCC proveniente da extração com solventes é constituído principalmente de ácido anacárdico (60-65%), cardol (15-20%), cardanol (10%) e traços de 2-metil cardanol (KUMAR et al., 2002). Quando proveniente do processo térmico-mecânico o LCC é constituído por cardanol (60-65%), cardol (15 -20%), material polimérico (10%) e traços do 2-metil cardol. Isso acontece porque o ácido anacárdico é termicamente instável e é facilmente descarboxilado durante o processo de extração por aquecimento, quando é transformado em cardanol (Costa et al., 2004).

Lopez et al. (2012), estudando níveis crescentes de LCC extraído com o processo térmico-mecânico na dieta de frangos (0,1; 0,2; 0,3; e 0,4 ml de LCC.kg-1de ração) e comparando com um promotor de crescimento (virginiamicina) observaram que o LCC apresentou desempenho e rendimento

de abate semelhantes ao promotor de crescimento, além de reduzir a concentração de *E. coli* no conteúdo intestinal em aves não desafiadas.

Segundo Abbas et al. (2012), os componentes do líquido da casca de castanha de caju têm ação semelhante a um ionóforo monovalente, causando danos à membrana celular da bactéria. A atividade antimicrobiana do líquido está associada ao número de terpenóides e de compostos fenólicos presente na sua estrutura, com ação contra bactérias gram-positivas (Kanehashi et al., 2015). Além disso, o LCCC possui atividade anfipática, principalmente o cardanol e tende a se distribuir na interface dos fluidos e assim reduzir a tensão interfacial das moléculas, atuando assim como um agente surfactante (Nitschke e Pastore, 2002).

O óleo de mamona é um óleo funcional, composto majoritariamente (90%) de ácido ricinoleico conhecido por sua ação laxativa (Vieira et al., 2001). O ácido ricinoleico exerce efeito antimicrobiano desnaturando e coagulando as proteínas da parede celular bacteriana. O grupamento éster que compõe a molécula de ácido ricinoleico favorece a hidrólise por esterases plasmáticas que formam álcool e inibem a enzima transpeptidadase responsável pela síntese de peptídeoglicanos, presentes principalmente na parede das bactérias gram-positivas (Guimarães et al., 2010). Assim o óleo de mamona pode atuar como um inibidor da síntese da membrana celular.

Em função das propriedades químicas descritas acima, foi desenvolvida uma mistura comercial do líquido da casca de castanha de caju e do óleo de mamona comercialmente chamada de Essential® (Essential, US Patent N°. 8,377,485 B2: Oligo Basics Agroind. Ltda., Rua Sérgio Gasparetto 503, Cascavel, PR-CEP, Brazil). Os compostos ativos do Essential são o cardanol, acido ricinoleico e o cardol, respectivamente nas concentrações de 200g/kg, 90 g/kg e 40g/kg. Avaliando essa mistura comercial na dieta de frangos de corte desafiados por coccidiose, observou-se um aumento no ganho de peso e uma melhoria na conversão alimentar (Murakami et al., 2014), além de uma melhora de 100 kcal de energia metabolizável quando comparada com a dieta controle (Bess et al., 2012; Murakami et al., 2014). Esse aumento na disponibilidade de

energia pode estar relacionado com os efeitos antimicrobianos dos óleos funcionais (Bess et al., 2012).

3. HIPÓTESES E OBJETIVOS

3.1 Hipóteses

- A mistura do líquido da casca da castanha de caju e do óleo de mamona aumenta o desempenho de frangos de corte desafiados por algum agente inflamatório, nesse experimento representado pela coccidiose.
- A mistura do líquido da casca da castanha de caju e do óleo de mamona tem efeito anti-inflamatório, minimizando a perda de desempenho causada por uma reação inflamatória.
- A mistura do líquido da casca da castanha de caju e do óleo de mamona atua como um modulador da microbiota intestinal em frangos desafiados por coccidiose.

3.2 Objetivos

3.2.1 Geral

Avaliar a mistura comercial do líquido da casca de castanha de caju e do óleo de mamona (Essential, Oligo Basics Agroind. Ltda., Cascavel, Brasil) no desempenho, na microbiota e no sistema imune de frangos de corte desafiados ou não por coccidiose.

3.2.2 Específicos

- Avaliar a mistura do óleo da casca da castanha de caju e do óleo de mamona como promotor de crescimento em frangos de corte.
- Avaliar a atividade anti-inflamatória e antimicrobiana da mistura de óleos.

- Analisar a microbiota intestinal de frangos de corte suplementados com a mistura de óleos após o desafio por coccidiose.

CAPÍTULO II

Functional oils on performance and microbiota of broilers challenged with coccidiosis:

COMPARISON BETWEEN A COMMERCIAL BLEND OF FUNCTIONAL OILS AND MONESIN ON PERFORMANCE AND MICROBIOTA OF BROILERS CHALLENGED WITH COCCIDIOSIS[1]

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ABSTRACT

The aim of the study was to evaluate a cashew nut shell oil and castor oil blend commercial (CNSL - Castor oil) effect on performance and microbiota of broiler chickens challenged or not with coccidiosis. A total of 864 one - day - old male chicks (Cobb) were randomly distributed in 6 treatments (8 pen / treatment and 18 chicks / pen) in a 3 x 2 factorial with 3 additives: control (non- additive), 100 ppm of sodium monensin or 0.15% Essential, and 2 challenge levels at 14 days of age: unchallenged or inoculated by gavage with 1mL of solution containing oocysts sporulated with *Eimeria tenella, Eimeria acervulina* and *Eimeria maxima*. In the period before the challenge and for birds that were not challenged, no differences in the productive performance among treatments were observed (P > 0.05). After seven days of challenge, birds receiving monensin presented better performance compared to the positive control group (non-additive and challenge) or CNSL- Castor oil (P > 0.05). However, 14 days after the challenge, birds supplemented with CNSL- Castor oil presented higher weight gain and better feed conversion (P > 0.05), without altering feed intake (P > 0.05). In the accumulated period (1-42 days of age) the live weight, weight gain and feed intake did not differ between the CNSL- Castor oiland monensin groups, and these were larger than the positive control. The challenged birds increased the number of *Lactobacillus* spp*.* and *Clostridium perfringens* copies (P < 0.05). CNSL- Castor oil supplementation reduced *Clostridium* cluster XIV, *Clostridium perfringens* and *S. aureus*, compared with monensin and control groups (P > 0.05). In addition, Essential presented higher number of Lactobacillus spp. copies, followed by monensin and positive control groups (P > 0.05). Thus, monensin and CNSL - Castor oil were effective in minimizing the impact of coccidiosis at

different times. While monensin acts as an antimicrobial, CNSL- Castor oil acts as an intestinal microbiota modulator with antimicrobial action against gram-positive bacteria, mainly *C. perfringens* and *S. aureus*.

Key Words*:* Coccidiosis, Functional oils, Gut health, Microbiota, Monensin

INTRODUCTION

The microbiota evolved with the host as a mutualistic partner, and its balance is linked to the abundance and diversity of species. However, dysbiosis can cause a number of disorders affecting the intestinal wall morphology, reducing diversity by the increase of pathogenic bacteria population, inducing the immune response, diverting energy and nutrients from the growth to the inflammatory response and, consequently, reducing performance (Kogut, et al., 2013, DiAngelo et al., 2009). In this context, there is great interest in developing food additives with the ability to improve performance, control pathogens and modulate intestinal microbiota. The phytogenetics have presented interesting results improving intestinal health and modulating the microbiota as additives for animal production (Kim et al., 2013; Abdel-Wareth et al., 2012; Kley et al., 2012; Oviedo-Rondón et al., 2010; Hume et al., 2006; Oviedo-Rondón et al., 2006).

Some factors like age, diet, food additives and the presence of pathogens are known to alter the intestinal microbiota. Coccidiosis challenge can drastically change the bacterial community in the gut, reducing microbial diversity (Kley et al., 2012) and creating a favorable environment for pathogens dissemination such as gram-positive bacteria *Clostridium perfringens* (Baba et al., 1997). Oviedo-Rondón et al. (2010) observed that the microbial profile in the ileum and cecum was altered in 45 and

64%, after *Eimeria* spp. challenge. However, using diets supplemented with phytogenic blend, this change was only 19% and 32%.

Within the phytogenic category, the functional oils are defined as oils that have an action beyond nutritional value (Murakami et al., 2014). Castor oil is a functional oil, composed of 90% ricinoleic acid (Dabdoub and Bronzel, 2007), known for its laxative action (Vieira et al., 2001). Also has an antimicrobial action: the ester derivatives break the glycosidic bonds of the peptideoglycans present, mainly, in the wall of the gram-positive bacteria (Oliveira et al., 2005; Guimarães et al., 2010).

The cashew nuts liquid is mainly composed of cardanol, cardol and anacardic acid (Mazzetto et al., 2009). The antimicrobial activity of the liquid is associated with the number of terpenoids and phenolic compounds present in its structure (Kanehashi et al., 2015), acting against gram-positive bacteria (Himejina and Kubo 1991, Parasa et al., 2011). *In vitro* studies observed that both functional oils had mechanisms of action of an ionophore (Maenz and Forsyth, 1982; Vieira et al., 2001; Toyomizu et al., 2003).

Due to its chemical properties, a blend of cashew shell liquid and castor oil was developed and commercially called Essential® (Essential, US Patent N°. 8,377,485 B2: Oligo Basics Agroind. Ltda., Rua Sérgio Gasparetto 503, Cascavel, PR-CEP, Brazil). Evaluating this commercial blend in the diet of broilers challenged with coccidiosis, increasing in weight gain and an improvement in feed conversion were observed (Murakami et al., 2014), as well as an improvement in a 100 kcal of ME (Bess et al., 2012 and Murakami et al., 2014). This increase in energy availability may be associated to the functional oils antimicrobial effects (Bess et al., 2012). However, there is no literature demonstrating its antimicrobial action *in vivo.*

The aim of the present study was to evaluate the effect of cashew shell liquid and castor oil blend on performance and microbiota of broilers challenged with coccidiosis, comparing to monensin ionophore.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee at Federal University of Rio Grande do Sul reviewed and approved the protocol number 29814 used in the present study.

Animals and diets

A total of 864 one-day-old male chicks (Cobb 500) were obtained in a commercial hatchery and housed in two identical experimental rooms, for challenged and unchallenged birds, thus avoiding cross-contamination. The rooms were composed of 48 pens with an initial density of 18 birds per pen. The nutritional program consisted of three diets: pre-starter (1 to 7 days), starter (8 to 21 days) and grower (22 to 28 days) based on the nutritional requirements recommended by the Brazilian Tables of Pigs and Swine (Rostagno et al., 2011). The nutritional composition was the same for all treatments, varying only the additive used.

Every week broilers were weighed, feed intake was measured and in the calculation of feed conversion, the weight of dead bird was considered (Sakomura and Rostagno 2016).

Experimental design

The experimental design was completely randomized in a 3x2 factorial arrangement: food additives (basal diet, 100 ppm sodium monensin or 0.15% Castor oil-CNLS) and sanitary challenge (challenged or unchallenged with coccidiosis). Both

food additives, Essential and monensin sodium, were introduced by replacing inert (kaolin) in the basal diet at all phases.

Challenge and sample collection

At 14 days of age, 1mL of sporulated oocysts of *E. tenella* ($10x10^3$), *E. acervulina* ($200x10^3$) and *E. maxima* ($80x10^3$) was inoculated by gavage. The oocyts were acquired at the *Laboratório de Biologia Molecular de Coccídias* (University of São Paulo/ Brazil). Unchallenged chickens received 1mL of saline, providing the same management stress.

After 7 and 14 days of oocysts inoculation (21 and 28 days of age), three average weight birds from each replicate were euthanized by cervical dislocation and evaluated for lesion score by *Eimeria* spp. Lesions were ranked from 0 (absence of macroscopic lesions) to 4 (presence of severe macroscopic lesions), according to the method described by Johnson and Reid (1970).

At 28 days of age, the same birds euthanized for the lesion score, were used to collect the intestinal contents. A portion of 10 cm of the duodenum segments (from pylorus exit to the end of the descending duodenal loop), jejunum (descending duodenal loop to the Meckel's diverticulum) and ileum (diverticulum to ileocecal insertion) were removed and immediately stored at -20 ° C .

DNA extraction

The intestinal contents were separated and the concentrated bacterial fraction was obtained according to the procedure proposed by Apajalahti et al. (1998). The DNA was extracted with the PowerFecal ™ DNA Isolation Kit (MoBio, UK), following the manufacturers recommendations. After extraction, its quality was verified using a NanoDrop 2000 (Invitrogem) and quantified using Qubit 3.0 (Invitrogen). The DNA obtained was diluted to a concentration of 2 ng / ul.

q-PCR absolute curve

The sequence of primers selected, the size and annealing temperature are shown in Table 2. The reactions were conducted on the StepOnePlus ™ Real-Time PCR System (Applied Biosystems), in a final volume of 15 µL, containing 2,0 µL of PCR buffer 10x; 1,6 µL of MgCl2 [50mM] 0,5 µL of each primer [10 µM]; 0,2 µL of dNTP [(5 mM); 2,0 µL Syber green (1x), 0,05 µL Platinum® Taq DNA Polymerase [5U/ µL] 5 µL of DNA. and ultra pure water to complete the volume. The conditions for q-PCR were 94 ° C for 5min, 35 cycles at 94 ° C for 30s, annealing temperature specific for each oligonucleotide pair (Table 2) for 30s and 72 ° C for 30s. After the amplification cycles, the dissociation curve of the amplification products was performed by raising the temperature from 60 to 95 °C to obtain the dissociation curve of the reaction products.

An ATCC bacterium according to the primer (Table 1) was used to construct the standard curve. The bacteria were cultured in specific media without antibiotics. Bacterial genomic DNA was extracted using PureLink® Genomic DNA Kits (Invitrogen). Serial dilutions of gene copies were from $3x10^9$ to $3x10^2$ on each plate. The threshold was adjusted for each standard curve to achieve an amplification efficiency close to 100%. The CT (cycle threshold) was determined for each sample and compared to the standard curve to determine the number of copies in 2 ng of genomic DNA. The number of copies per gram of intestinal contents was calculated taking into account the initial mass of the starting material, extraction yield and the DNA dilution.

Statistical analysis

The number of gene copies was transformed into log10 to obtain a normal distribution. An ANOVA of the factorial arrangement was performed, including the

challenge effects, additives and their interactions for all variables of performance, lesion score and microbiota. Means were compared by LSmeans when significant differences were found. The GLM procedure of the statistical package SAS, version 9.0 (SAS Institute 2002) was used.

RESULTS

Growth performance

In the period before the challenge and for the unchallenged birds, there was no statistical difference in performance among treatments for all periods evaluated. Mortality was less than 1% after challenge (data not shown / supplementary material).

Animal performance was negatively affected by coccidiosis in the week after the challenge (14 to 21 days of age), decreasing BWG by 49% and worsening FCR by 58% ($P < 0.0001$). In the second week after the challenge (21 to 28 days of age) the negative challenge effect was less intense, 26% lower BWG and 20% worse FCR (Table 2). The lowest performance of the challenged birds was also observed in the total rearing period (1 to 42 days of age-Table 3), with lower weight gain, feed intake and worse feed conversion. The challenged birds showed 17% live weight reduction at 42 days of age.

In the week following the challenge (14 to 21 days of age) there was an interaction between additives and challenge for all variables analyzed (Table 3). In the challenged birds, weight gain and feed intake were higher and feed conversion was better in the monensin group, and no differences were seen between the other groups ($P < 0.05$). Two weeks after the challenge (21 to 28 days of age), birds

supplemented with Castor oil-CNLS presented greater weight gain ($P < 0.05$) and better feed conversion ($P < 0.05$) than the other treatments. In the accumulated period (1 to 42 days of age), positive control group presented lower live weight than the other groups ($P < 0.01$), which showed no differences between them, demonstrating that monensin and CNSL- Castor oil compensated the coccidiosis negative effect. No interaction for feed conversion was observed.

Analyzing the factors individually, challenge decreased feed conversion and, regardless of the challenge, monensin presented better feed conversion when compared to control; Castor oil-CNLS was intermediate, but not differ for control an monensin.

Lesion score

In the first week after infection, broilers receiving monensin had a lower lesion score for *E. acervulina* compared to the other groups ($P < 0.0001$). In the following week, the broilers with CNSL- Castor oil had a lower lesion score for *E. tenella* ($P < 0.0480$). There was no difference ($P > 0.05$) among groups for lesion score of *E. maximus* in any week evaluated (Figure 1).

Microbiota modulation using monensin or CNSL- Castor oil

There was interaction among the factors for bacterial domain (total bacteria number), *Lactobacillus* spp., *Clostridium* cluster XIV, *C. perfringens, E. coli* and *S. aureus* ($P < 0.05$) (Tables 4 and 5). In the challenged birds, monensin reduced bacterial domain and *E. coli* compared to the other groups. CNSL- Castor oil reduced copy number of *Clostridium* cluster XIV, *C. perfringens* and *S. aureus*, monensin and positive control did not differ. In the unchallenged birds bacterial domain, Clostridium cluster XIV and S. aureus did not presented statistical difference among groups. *Lactobacillus* spp. copy number was lower for the positive control, followed by

monensin and CNSL- Castor oil. Both monensin and CNSL- Castor oil reduced *C. perfringens* and *E.coli*. copy number.

Regardless of the challenge, the positive control group presented more *Bifidobacterium* spp ($P < 0.05$) copies than CNSL- Castor oil, and monensin group was intermediate. The copy number of *Enterococcus* spp. genus was higher for positive control group and lower for monensin and CNSL- Castor oil that did not differ.

DISCUSSION

The present study was conducted based on recent studies using coccidiosis challenge (Bortoluzzi et al., 2015; Cox et al., 2010; Kim et al., 2011; Orengo et al., 2012; Scheurer et al., 2013). In this study, the challenge presented irrelevant mortality (<1%). However, it was enough to decrease animal performance throughout the evaluation period.

Anti-coccidial drugs have high efficiency in reducing losses caused by coccidiosis infection. On the other hand, intensive use may stimulate parasite resistance (Chapman et al., 2010). Some phytogenetics studied have achieved a recovery in performance similar to the drugs (Bess et al., 2012; Kley et al., 2012; Mohiti-Asli and Ghanaatparast-Rashti, 2015). In this study, the use of monensin improved performance in the week immediately post challenge. However, CNSL-Castor oil provided late but consistent compensatory gains, noting that at 42 days of age, both groups did not differ in weight gain, feed intake and live weight.

The worst performance during coccidiosis challenge is associated with the reduction in the intestinal absorption area, nutrient absorption deficit and

inflammation caused in the first week after the challenge (Giannenas et al., 2012, Laurent et al., 2001, Cornelissen et al., 2009, Cox et al., 2010). It is possible to speculate that CNSL- Castor oil has lower anticoccidial action than monensin, but its mechanism of action may be associated with recovery of intestinal health after the inflammation peak, allowing a similar growth performance to the ionophore in the accumulated period (1 -42 days).

Intestinal health is directly related to the profile of the microbiota that interacts with the host. The microbiota regulates the absorptive efficiency, presents antagonistic mechanisms to pathogenic bacteria, enhances intestinal integrity and modulates the immunity (oviedo-Rondón, 2009; Pan & Yu 2013). The results show that coccidiosis challenge did not significantly affect the total number of bacteria, but change the microbiota profile, increasing the population of *Lactobacillus* spp., *Clostridium* cluster XIV, *C. perfringens* and *S. aureus*. Some studies show that after coccidiosis challenge there is an increase of *Clostridium* bacteria and lactic acid fermenters such as *Lactobacillus* (Klim et al. 2015; Stanley et al. 2014; Enberg et al. 2012; Kley et al. 2012; M'sadeq et al. 2012). This is due to the increase in the amount of mucus and the presence of proteins from the cells damaged by the coccidia (Collier et al., 2008), serving as a substrate for beneficial and pathogenic bacteria (Deplancke and Gaskins, 2001).

Broilers that received monensin or CNSL- Castor oil and were unchallenged showed *Lactobacillus* increase, and *C.perfringens* and *E. coli* decrease, when compared to the control group. *Lactobacillus* have beneficial characteristics for the host, such as modulating the immune system and antagonizing pathogenic bacteria (Yousaf et al., 2010; Klose et al., 2010; Servin et al., 2004). They are usually considered as a beneficial group, however the presence of three species of

Lactobacillus, L. salivarius, L. aviarius and *L. crispatus*, may be associated with the worst performance in broilers, because they deplete the bile salts and impair fat emulsification (Guban et al., 2006).

Monensin is an ionophore known for its coccidiostatic, antimicrobial and growth promoting action (Huyben et al., 2001). Challenged birds, compared with the unchallenged receiving monensin, showed total number of bacteria reduction and increase *Clostridium* cluster XVI, *C. perfringens* and *S. aureus* increase. This change was similar to the positive control, showing that monensin, even causing a reduction in the total number of bacteria, provided a profile similar to the challenged control group. A quantitative and qualitative change in the microbiota profile is characteristic of dysbiosis process, in this case caused by coccidiosis. Dysbiosis is defined as an undesirable microbiota alteration, resulting in an imbalance between beneficial and pathogenic bacteria, and it may negatively affect animal performance (Ducatelle et al., 2014).

Studies suggest that phytogenic additives use has shown positive results in intestinal microbiota modulation even in the presence of a coccidiosis challenge (Abdel-Wareth et al., 2012; Hume et al., 2006; Kim et al., 2013; Kley et al., 2012; Oviedo-Rondón et al., 2006; Oviedo-Rondón et al., 2010). In this study, CNSL-Castor oil appears as beneficial modulator of the intestinal microbiota, because it did not caused differences for *Lactobacillus* spp., *Clostridium* ssp., *Clostridium perfringens* and *S. aureus* population in challenged broilers, although it did not reduce the total bacteria domain. This balance in the microbiota may have aided in the performance recovery after challenge. The microbiota composition may be directly associated to the best animal performance, but it is not entirely clear how this relationship works (Stanley et al., 2013). In this study, *Enterococcus* genus was

reduced in challenged animals receiving the functional oil blend. Some species of this genus are pathogenic; for example, *Enterococcus cecorum* is related to bone diseases, such as osteomyelitis (Kense and Landman, 2011). In addition, *Enterococcus* spp. are opportunistic and can spread rapidly when dysbiosis occurs (Cao et al., 2013). Lunedo et al. (2014) associated a worse feed conversion in chickens receiving low tannin sorghum with the increase of *Enterococcus* genus and *Enterobacteriaceae* family in the ileum.

In this study, it was possible to observe an increase in *S. aureus* species copy number in the challenged broilers, except for the group that received the functional oil blend. In general, the genus *Staphylococcus* spp. is a normal habitant of the skin and mucous membranes, also considered opportunistic (Jonsson and Wadstrom 1993). In poultry, *S.aureus* is the most common species that cause disease. Its rapid spread in the intestine occurs when immune resistance is low due to infection by other pathogens, immunosuppression and skin or mucosal lesions, causing diseases such as salpingitis, folliculitis, bursitis, gangrenous dermatitis and cellulitis (Ferreira and Ferreira, 2009).

The results of this study show that the functional oil blend has an action against gram-positive bacteria acting as a modulator of the intestinal microbiota. According to Abbas et al. (2002), the liquid cashew nutshell components, cardoles and anacardic acid have a similar action than a monovalent ionophore, causing damage to the bacterial cell membrane. Moreover, ricinoleic acid has antimicrobial effect denaturing and coagulating proteins of the bacterial cell wall. The ester group that composes the ricinoleic acid molecule favors hydrolysis by plasma esterase that form alcohol and inhibit the transpeptidase enzyme, responsible for the peptide

glycols synthesis (Guimarães et al., 2010). Thus, castor oil may act as an inhibitor of cell membrane synthesis.

The functional oils blend improved the performance of coccidiosis-challenged broilers in the second week, resulting in similar performance to those receiving the ionophore monensin. The blend showed to be a good option in a coccidiosis challenge, acting as a modulator of the intestinal microbiota, with antimicrobial action against gram-positive bacteria, mainly *C. perfringens* and *S. aureus*.

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Table 1. Ingredient formulas and chemical composition of experimental diets according to the rearing period

Ingredients (%)	Pre Starter (1-7 d)	Starter (8- 21d)	Grower (22- 42d)
Corn	538.55	571.05	597.75
Soybean Meal	384.60	354.60	321.10
Vegetal Oil	31.80	33.30	43.00
Dicalcium Phosphate	19.00	16.60	16.30
Limestone	10.10	10.10	7.80
Salt	5.10	4.80	4.60
L-Lys HCl	3.00	2.50	2.70
DL-Met	3.70	3.20	3.00
L-Tre	1.20	0.80	0.70
Vit-min Premix [1]	1.05	1.05	1.05
Choline Chloride	0.40	0.50	0.50
Inert/Monensin/CNSL-Castor oil [2]	1.50	1.50	1.50
Total (kg)	1000.00	1000.00	1000.00
Calculated composition			
Metabolizable Energy (Kcal/kg)	3000	3050	3150
Crude Protein (g/kg)	222.0	210.0	196.9
Calcium (g/kg)	9.2	8.6	7.6
AvailableP (g/kg)	4.7	4.2	4.1
Digestible P (g/kg)	3.9	3.6	3.5
Potassium (g/kg)	8.6	8.1	7.6
Sodium (g/kg)	2.2	2.1	2.0
Chlorine (g/kg)	3.5	3.4	3.2
Dig. Lysine (g/kg)	13.2	2.2	1.5
Dig. Methionine (g/kg)	6.5	5.9	5.6
Dig. Met+Cys (g/kg)	9.5	8.8	8.3
Dig. Threonine (g/kg)	8.6	7.9	7.3
Dig. Tryptophan (g/kg)	2.5	2.3	2.2
Choline (mg/kg)	1550	1550	1450
(Na+K)-Cl (mEq/kg)[3]	216.91	202.59	191.1

[1] Composition (per kg): 150000 mg of Mn, 100000 mg of Zn, 80000 mg of Fe, 15000 mg of Cu, 1200 mg of I, 700 mg of Se, 23200000 UI of vitamin A, 5600000 UI of vitamin D, 52000 mg of vitamin K, 6000 mg of vitamin B1, 18000 mg of vitamin B2, 9000 mg of vitamin B6, 132000 mg of niacin, 44000 mg of pantothenic acic, 2400 mg of folic acid, 200000 µg of biotin, 40000 µg of vitamin B12.

[2] At all phases addition varied according to the treatment (1.50 g / kg of kaolin or CNSL- Castor oil or 0.250g / kg monensin + 1.25 g / kg of kaolin). [3] Electrolytic balance.

Table 2. Target gene, annealing temperature (TA °C), base pairs and the ATCC bacterium used for the standard curve, primer sequence, and the reference of the groups and bacterial species studied

Microorganisms	Target Gene	TAºC	Amp (pb)	ATCC control	Sequence (5' 3')	References
Bacteria domain	16S	60	200	*E. coli* (10536)	F: CGGYCCAGACTCCTACGGG R: TTACCGCGGCTGCTGGCAC	Wise et al. (2007)
Escherichia coli	16S	56	475	*E. coli* (10536)	F: CCTACGGGAGGCAGCAGT R: CGTTTACGGCGTGGACTAC	Chiang et al. (2006)
Lactobacillus grup	16S	58	341	*L. plantarum* (8014)	F: CACCGCTACACATGGAG R: AGCAGTAGGGAATCTTCCA	Wise et al. (2007)
Staphylococcus aureus	nuc	60	279	*S. aureus* (4163)	F: GCGATTGATGGTGATACGGTT R: AGCCAAGCCTTGACGAACTAAAGC	Rinttila et al. (2004a)
Salmonella enteric	invA	58	195	*S. choleraesuis* (10708)	F: ATTTCAATGGGAACTCTGCC R: ATCGAGATCGCCAATCAGTC	Zhang et al. (2009)
Clostridium cluster XIV	16S	60	116	*C. perfringes* (13124)	F:ACTCCTACGGGAGGCAGC R:GCTTCTTAGTCARGTACCG	Louie et al. (2012)
Clostridium perfringens	16S	56	120	*C. perfringes* (13124)	F: ATGCAAGTCGAGCGA(G/T)G R: TATGCGGTATTAATCT(C/T)CCTTT	Rinttila et al. (2004)
Bifidobacterium spp.	16S	58	437	*B. animalis* (27672)	F: GGGTGGTAATGCCGGATG R TAAGCCATGGACTTTCACACC	Bartosh et al. (2005)
Enterococcus spp.	16S	50	124	*E. faecalis* (29212)	F: GAGAATGATGGAGGTAGAGC R: GACTACGGATCTTATCACTC	Lehner et al. (2005)

Table 3. Feed intake (FI), weight gain (WG) and feed conversion ratio (FCR) of unchallenged (UD) and challenged (CD) broilers in the period of 14 – 21days and 21 - 28 days of age

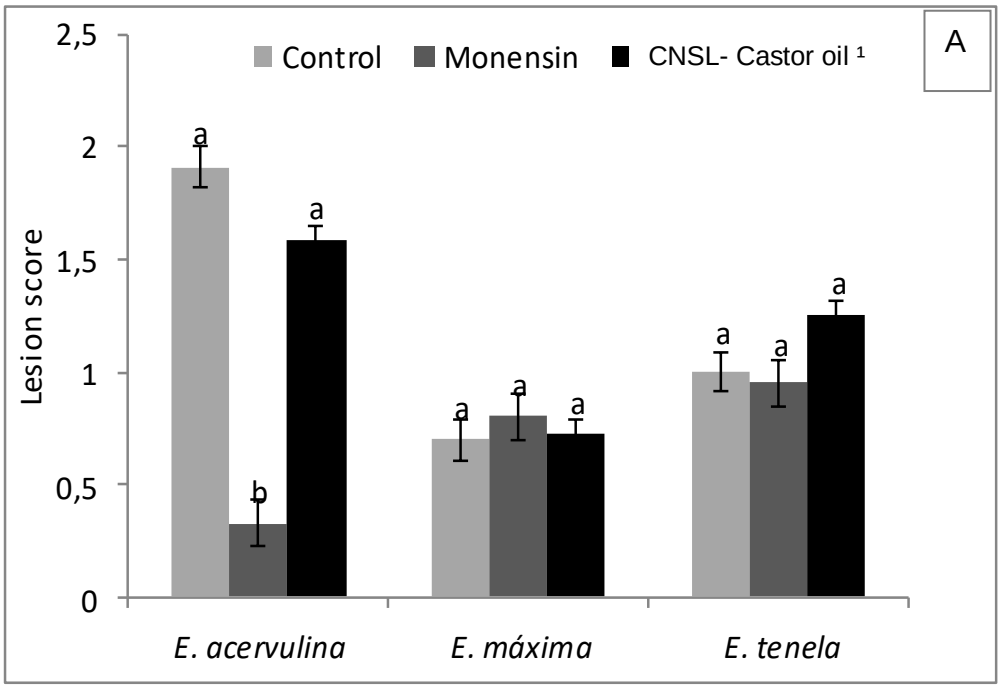
Treatments	14 - 21 d						21-28 d					
	FI		WG		FCR		FI		WG		FCR	
	UD	CD	UD	CD	UD	CD	UD	CD	UD	CD	UD	CD
					Challenge							
	643 A	486 B	419 A	215 B	1.54 A	2.43 B	870 A	754 B	536 A	396 B	1,63 B	1,95 A
					Interaction							
Control	644 Aa	445 Bb	422 Aa	175 Bb	1.52 Aa	2.59 Bb	847 A	741 B	518 Aa	357 Bb	1,63 Ba	2,12 Aa
Monensin	621 Aa	531 Ba	415 Aa	296 Ba	1.52 Aa	1.82 Ba	887 A	741 B	551 Aa	374 Bb	1,61 Ba	2,01 Aa
CNSL- Castor oil [1]	653 Aa	481 Bb	422 Aa	174 Bb	1.55 Aa	2.86 Bb	878 A	780 B	540 Aa	457 Ba	1,64 Ba	1,71 Ab
					Additive							
Control	545		298.22 b		2.06 a		794		437 b		1.88 a	
Monensin	581		355.25 a		1.67 b		814		462 b		1.81 a	
CNSL- Castor oil	567		297.62 b		2.21 a		829		499 a		1.68 b	
					Probability[2]							
Challenge	***		***		***		***		***		***	
Additive * Challenge	**		***		***		ns		**		***	
Additive	ns		***		***		ns		**		***	
SEM	12.61		13.32		0.1		15.29		19.92		0.04	

[1]Essential (US Patent N°. 8,377,485; Oligo Basics Ind. Ltda., Cascavel, Paraná, Brazil). [2] Probabilities: *** P <0.001, ** P <0.05 and ns: not significant; Means with different letters differ statically by LSMEANS, lower case in the column and uppercase in the row within the same variable. [2] SEM: standard error of the mean

Table 4. Body weight (BW), feed intake (FI), weight gain (WG) and feed convertion ratio (FCR) of unchallenged (UD) and challenged (CD) broiles in the period of 1- 42 days of age

Treatments	BW (g)		FI (g)		WG (g)		FCR	
	UD	CD	UD	ND	UD	ND	UD	ND
				Challenge				
	2860 A	2373 B	4539 A	3964 B	2816 A	2330 B	1,61 B	1,73 A
				Interaction				
Control	2868 Aa	2267 Bb	4597 Aa	3848 Bb	2824 Aa	2225 Ba	1,63	1,73
Monensin	2873 Aa	2416 Ba	4497 Aa	4038 Ba	2829 Aa	2393 Bb	1,59	1,69
CNSL- Castor oil[1]	2879 Aa	2435 Ba	4576 Aa	4088 Ba	2836 Aa	2372 Bb	1.62	1,72
				Additive				
Control	2568 b		4223		2525		1.69 a	
Monensin	2654 a		4267		2611		1.62 b	
CNSL- Castor oil [1]	2628 a		4332		2584		1.67 ab	
				Probability				
Challenge	***		***		***		**	
Additive * Challenge	**		**		**		ns	
Additive	**		ns		ns		**	
SEM[2]	44.40		42.28		445.578		0.036	

[1]Essential (US Patent N°. 8,377,485; Oligo Basics Ind. Ltda., Cascavel, Paraná, Brazil). [2] Probabilities: *** P <0.001, ** P <0.05 and ns: not significant; [2]SEM: standard error of the mean. Means with different letters differ statically by LSMEANS, lowercase in the column and uppercase in the row within the same variable



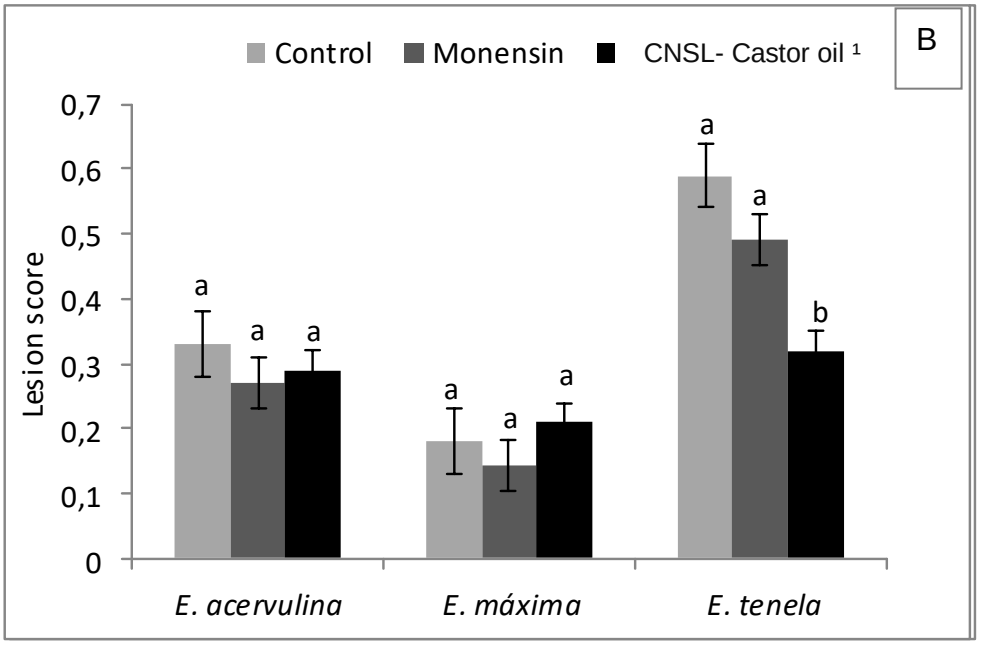


Figure 1. Lesion score in coccidiosis challenged broilers at 21 (A) and 28 (B) days of age - 7 and 14 days after challenge. ¹ Essential (US Patent N°. 8,377,485; Oligo Basics Ind. Ltda., Cascavel, Paraná, Brazil). Means with different letters differ statically by *LSMeans*

Table 5. Total bacteria copy number[1] (Bacteria Domain), *Lactobacillus* group, *Bifidobacterium* and *Enterococcus* genus and *Clostridium* group in the intestinal contents of broilers 14 days after challenge with coccidiosis

Treatments	*Bacteria domain*		*Lactobacillus spp.*		*Bifidobacterium spp.*		*Enterococcus spp.*		*Clostridium* cluster XIV	
	UD	CD	UD	CD	UD	CD	UD	CD	UD	CD
					Interaction					
Control	10.59 Aa	10.57 Aa	6.22 Bc	9.03 Aa	4.79	5.32	6.13	6.37	9.94 Ba	10.48 Aa
Monensin	10.08 Aa	9.71 Bb	8.21 Bb	9.53 Aa	4.96	4.97	6.12	6.21	9.85 Ba	10.17 Aa
CNSL- Castor oil[1]	10.54 Aa	10.68 Aa	8.88 Aa	8.88 Aa	4.45	4.52	6.07	5.63	9.80 Aa	9.51 Ab
					Challenge					
Challenge	10.4	10.32	7.78 B	9.15 A	4.91	4.76	6.11	6.04	9.81	9.84
					Additives					
Control	10.58 a		7.63 b		5.05 a		6.25 a		10.06 a	
Monensin	9.89 b		8.88 a		4.97 ab		6.12 b		9.91 a	
CNSL- Castor oil	10.38 a		8.89 a		4.49 b		5.86 b		9.66 b	
					Probability					
Additive*challenge	<0.0001		<0.0001		0.8323		0.0863		0.0085	
Challenge	0.5094		<0.0001		0.3515		0.5452		0.4291	
Additive	0.0166		<0.0001		<0.0127		0.0499		0.0013	
SEM	0.24		0.21		0.138		0.11		0.074	

[1] log10 copy number of 16S RNA gene in 1 gram of intestinal contents. UD: unchallenged broilers, CD: broilers challenged with coccidiosis. [1] Essential (US Patent N°. 8,377,485; Oligo Basics Ind. Ltda., Cascavel, Paraná, Brazil).SEM: Standard Error of the Mean. Means with different letters differ statically by LSMEANS, lowercase in the column and uppercase in the row within the same variable. Values represent a pool of intestinal contents of 3 birds per box, totaling an average of 24 birds per treatment.

Table 6. Copy number of *Salmonella enterica, Clostridium perfringens*, *Escherichia coli, Staphylococcus aureus* in the intestinal content of broiler 14 days after challenge with coccidiose

Treatment	*S. enterica*		*C. perfringens*		*E.coli*		*S.aureus*	
	UD	CD	UD	CD	UD	CD	UD	CD
				Interaction				
Control	4.17	4.18	6.60 Ba	7.14 Aa	6.63 Aa	6.53 Aa	6.97 Ba	7.39 Aa
Monensin	4.12	4.24	5.28 Bb	6.80 Aa	5.89 Ab	6.00 Ab	6.83 Ba	7.42 Aa
CNSL- Castor oil[2]	4.23	4.31	5.18 Ab	5.27 Ab	5.60 Bb	6.66 Aa	6.77 Aa	6.62 Ab
				Challenge				
Challenge	4.18	4.23	5.50 B	6.81 A	6.00 A	6.10 A	7.05	6.95
				Additives				
Control	4.27		6.62 a		6.07		7.18 a	
Monensin	4.18		5.23 b		5.95		7.13 a	
CNSL- Castor oil [2]	4.25		5.87 b		6.15		6.70 b	
				Probability				
Additive*challenge	0.924		0.0129		0.0308		0.0018	
Challenge	0.6566		0.0115		0.7449		0.3359	
Additive	0.7855		<0.0001		0.8647		0.0009	
SEM	0.145		0.154		0.252		0.0952	

[1] log10 copy number of 16S RNA gene in 1 gram of intestinal contents. UD: unchallenged broilers, CD: broilers challenged with coccidiosis. . [2] Essential (US Patent N°. 8,377,485; Oligo Basics Ind. Ltda., Cascavel, Paraná, Brazil). SEM: Standard Error of the Mean. Means with different letters differ statically by LSMEANS, lowercase in the column and uppercase in the row within the same variable. Values represent a pool of intestinal contents of 3 birds per box, totaling an average of 24 birds per treatment

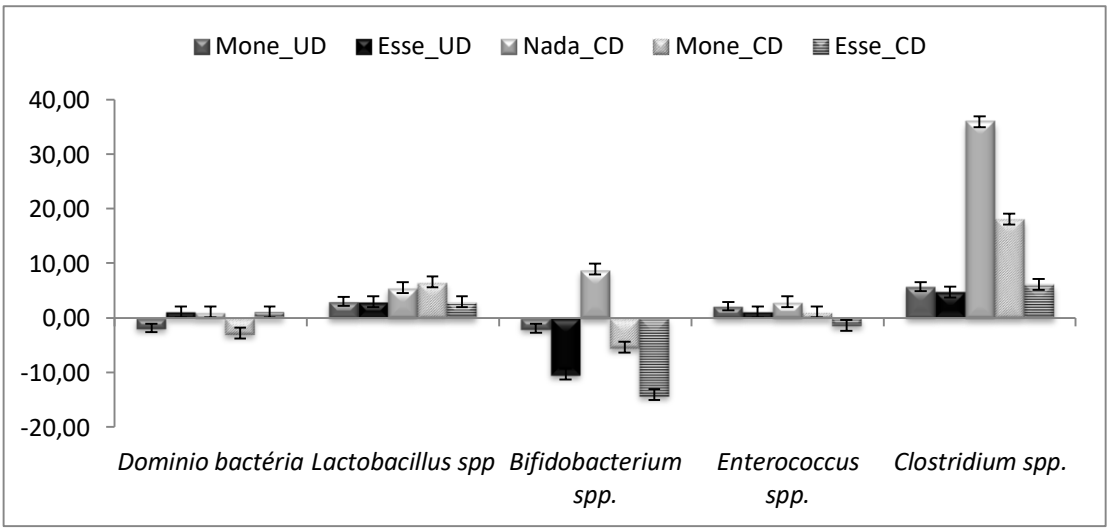


Figure 2. Fold-change of genus 16S rRNA copy number of bacterial group of broilers challenged or not with coccidiosis and receiving different additives compared to the unchallenged group with no additive. Means ± standard.

CAPÍTULO III

Effect of functional oils on the immune response of broilers challenged with Eimeria spp. [2]

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[2] Artigo científico nas normas da revista A*nimal*

Abstract

The aim of this study was to evaluate the effect of a blend commercial of liquid cashew nut shell liquid and castor oil (CNSL - Castor oil) on the immune response of broilers challenged or not with coccidiosis. A total of 864 one - day - old male chicks (Cobb) were randomly distributed in 6 treatments (8 pen / treatment and 18 chicks / pen) in a 3 x 2 factorial design with 3 additives: control (non- additive), 100 ppm of Monensin or 0.15% CNSL - Castor oil; and 2 challenge status at 14 days of age: unchallenged birds or inoculated by gavage with 1 mL of solution containing oocysts sporulated with amount of *Eimeria tenella, Eimeria* acervulina and *Eimeria maxima*. Although the positive control treatment (non-additive and challenged) and CNSL - Castor oil had similar variation in weight gain (ΔBWG = -132% and -133%, respectively) compared to unchallenged birds fed without additives, in birds fed diets containing CNSL - Castor oil most of the variation presented is due to the higher maintenance requirement, not associated with food efficiency. In the second week after the challenge, the treatment with CNSL - Castor oil presented a ΔBWG less expressive than the other treatments, respectively -52%; -43% and -15% for the positive control, monensin and CNSL - Castor oil treatments. At 7 and 14 days post challenge, there was a higher excretion of oocysts in the control group, and CNSL - Castor oil and monensin did not differ ($P>0.05$). The CNSL - Castor oil group increased the gene expression of IFN, IL-6 and TNF ($P<0.05$) and the control group increased COX and IL-1 ($P>0.05$). The heterophils / lymphocyte ratio was lower for monensin treatment ($P>0.05$). The unchallenged birds that received monensin presented higher gene expression of IFN, COX and IL-1 compared to the other treatments ($P<0.01$), and the CNSL - Castor oil group

reduced gene expression, with the exception of TNF. The commercial blend of cashew nut liquid and castor oil modulated the inflammatory response against *Eimeria* spp .. In the absence of the parasite, there was no stimulation of the genes involved inflammatory response, demonstrating that the blend is an effective tool in modulating the imune system.

Keywords: cashew nut, castor oil, challenge, coccidiosis, interleukins

Implications

Coccidiosis reduces broiler performance, increases the physiological cost of the immune system, both by the presence of the parasite and its effect on intestinal health. The blend of functional oils potentiated the host immunity against Eimeria, with a reduced weight gain, but reducing the excretion of oocysts similar to the ionophore. Inflammatory response increase was essential for the birds protection, with a smaller variation in the weight gain in the two week after challenge. These results demonstrate that the functional oil blend use provided a slower recovery of the animals, but as effective as those with ionophore.

Introduction

Coccidiosis usually occurs subclinically in birds, which makes it difficult to diagnose the disease in a timely manner to begin treatment before loss of performance occurs (Cornelissen *et al.*, 2009). Studies with the major species of Eimeria have revealed that innate and cell-mediated immunity have a fundamental action against the pathogen, both by the production of cytokines and by the direct cytotoxic attack on the affected cells. (Lillehoj e Choi, 1998; Laurent *et al.*, 2001).

The major proinflammatory cytokines, such as IL-1, IL-6 and TNF-α, are responsible for the acute-phase results that is related to systemic and metabolic changes, characterized not only by appetite decrease, but also by increased basal metabolic rate, skeletal muscle degradation, and acute-phase hepatic protein synthesis (Kogut e Klasing, 2009). Jiang *et al.* (2010) using lipopolysaccharide (LPS) challenge, concluded that only 59% of the performance loss is due to the reduction in feed intake, while 41% is due to other factors, probably associated with the immune response. Anticoccidial drugs are given preventively and continuously in the diet to minimize problems with coccidiosis. The monensin is an ionophore widely used in poultry production, however *Eimeria* spp. strains resistant to ionophores were already identified (Chapman *et al.*, 2010). In addition, the constant discussion about reducing the use of antibiotics as growth promoters has stimulated the search for alternative methods that can reduce the impact of this parasite and act as growth promoters at the same time.

Phytogenic may act as antimicrobial and anti-inflammatory agents with similar effects of some drugs used in animal production, because, in the general, they are composed of a complex blend of volatile substances such as terpene hydrocarbons, simple alcohols, aldehydes, among others that are pharmacologically active (Applegate *et al.*, 2010). Within the category of phylogenetic, the functional oils are defined as those that have an action beyond the nutritional value (Murakami *et al.*, 2014). One such product is a blend of cashew nut liquid and castor oil. Evaluating this blend in the diet of broilers challenged with coccidiosis, Murakami *et al.* (2014) observed weight gain increase and feed conversion improvement, as well as the gain of 100 kcal of metabolizable energy in the diet when compared to the control treatment (Bess *et al.*, 2012;

Murakami *et al.*, 2014). According to Bess *et al.* (2012), this increase in energy availability may be associated to antimicrobial and anti-inflammatory effects of functional oils. However, there is no literature demonstrating the anti-inflammatory action of this product in vivo.

Thus, the aim of this study was to evaluate the effect of commercial blend of cashew shell liquid and castor oil on the immune response of broilers challenged or not with coccidiosis.

Material and methods

The Institutional Animal Care and Use Committee at Federal University of Rio Grande do Sul reviewed and approved the protocol used in the present study (register number 29814).

Animals, diets and experimental design

A total of 864 one-day-old male chicks (Cobb 500) were obtained in a commercial hatchery and housed in two identical experimental rooms, composed of 48 pens (8 pens / treatment and 18 birds / pens). The nutritional program consisted of three diets: pre-initial (1 to 7 days), initial (8 to 21 days) and growth (22 to 28 days) based on the levels of nutritional requirements recommended by the Brazilian Tables of Poultry and Swine (Rostagno *et al.*, 2011). The nutritional composition was the same for all treatments, varying only the additive used in the diet. Metabolizable energy and crude protein were, respectively, in the three phases 3000 kcal / kg and 22.2%; 3050 kcal / kg and 21%; 3150 kcal / kg and 19.69%. The experimental design was completely randomized in a 3x2 factorial design with 3 additives: control (non- additive), 100 ppm of Monensin or 0.15% CNSL- Castor

oil; challenged or not with coccidiosis. Both additives, CNSL- Castor oiland sodium monensin, were introduced by replacing the inert (kaolin) in the basal diet. The treatment unchallenged without additives was denominated negative control and the challenged treatment was positive control.

Health challenge

At 14 days of age, 1mL of sporulated oocysts of E. tenella ($10x10^3$), E. acervulina ($200x10^3$) and E. maxima ($80x10^3$) were inoculated by gavage. The oocysts were obtained from the *Laboratório de Biologia Molecular de Coccídias* (University of São Paulo, Brazil). Unchallenged chickens received 1 mL of saline.

Sampling and data collection

Weight gain and feed intake were measured at 7 and 14 days after the health challenge, respectively, 21 and 28 days of age. At 21 days of age (7 days after the challenge), three birds per replicate were euthanized by cervical dislocation and 1 cm of the duodenum (in the final portion), the jejunum (before the Meckel's diverticulum) and the cecal tonsils were aseptically collected. The samples were washed in cold PBS (phosphate buffered saline), minced, immediately frozen in liquid nitrogen and stored at -80° C until analysis of gene expression.

From these three birds, one was randomly chosen to collect 5 cm of fragment of duodenum (final portion), jejunum (before Meckel's diverticulum) and ileum (before cecum entry) to evaluate intestinal health. In the period before euthanasia, blood from the ulnar vein was collected to perform leucogram of one bird per repetition.

Oocysts count

After 7 and 14 days of challenge the litter as collected in five different points, forming a pool of samples per pen. The number of oocysts was determined as described by Costa and Paiva (2009), using a McMaster chamber and according to the formula: total oocysts / pen = (oocysts counted × dilution factor × [sample volume / counting chamber volume) / 2.

Intestinal health index (ISI)

The ISI – ““I See Inside” (INPIBR1020150036019) is an intestinal health index generated from the histological changes that are submitted to the following formula ISI = Σ (EL * FI). Where: Σ sum, EL is the lesion score (0-3 being 3 the most severe) attributed to the observed histological changes, and FI is the pre-established Impact Factor (ranges from 1-3 being 3 the most severe) that is attributed to the change according to how much it affects the evaluated organ function. Details of the method are presented in Table 1 (Kraieski *et al.*, 2017). The fragments collected in the experiment were fixed in buffered 10% formalin solution, later prepared in paraffin blocks and stained with hematoxylin and eosin with Alcian Blue. For the evaluation of intestine was carried out the reading in 10x objective in 5 villi per bird selected from the count performed on the 4X objective. Leica DM1000 LED Optical Microscope was used for microscopic analysis.

Gene expression

Total RNA was extracted from the pool of samples obtained separately in the duodenum, jejunum and cecal tonsils of 3 birds per pen using the protocol of the Invitrap® Spin Tissue RNA kit (Stratec). An aliquot of 20-20 mg of each sample

was used. The RNA was eluted by washing the column membrane twice with 25 µl of RNase-free water. The total concentration of RNA was determined by Qubit (Qubit 3.0 Fluorometer) and RNA purity by optical density (OD) (NanoDrop-1000, Thermo Fisher Scientific, Waltham, MA) checking the ratio of OD 260/280. Reverse transcription was performed using the high throughput cDNA transcription kit of QuantiTec Kit (Qiagen), following the manufacturer's protocol and the cDNA was stored at -20 ° C. A Step One Plus (Applied Biosystems) was used for quantitative PCR (rt-qPCR).

The cDNA was diluted 1: 100 in nuclease-free water and 10 µL were added to each well of the plate. Subsequently, 10 µL of a mix containing 2.0 µL of 10x PCR buffer was added; 1.6 µl of MgCl2 [50 mM] 0.5 µl of each primer [10 µM]; 0.2 µl dNTP [5 mM]; 2.0 µL Syber green [1x], 0.05 µL Platinum® Taq DNA Polymerase [5U / µL] 5 µL of DNA, and the remainder of ultra pure water to complete the volume. The conditions for qPCR were 94 °C for 5min, 35 cycles at 94 °C for 30 s, annealing temperature specific for each oligonucleotide pair (Table 2) for 30s and 72 °C for 30 s. The specificity of the PCR products was checked at the end of the reaction by analysing the dissociation curve. In addition, the size of the amplicons was verified by electrophoresis. The values obtained for each gene were normalized and the gene expression was calculated in relation to the negative control group (without challenge and without additive), as described by Livak e Schmittgen (2001).

Calculations and statistical analysis

The Pearson correlation coefficient was determined as a measure of the linear correlation between the weight gain variation (ΔBWG) and the feed consumption

variation (ΔFI) between the negative control and the indicated treatment. The relationship between ΔFI and ΔBWG for the birds challenged the negative control, receiving monensin or CNSL- Castor oil, exposure was analyzed using linear regression: $\Delta BWG = \alpha + \beta \times \Delta FI$ as described by Pastrorelli et al. (2012). The analysis was carried out using the REG procedure in SAS. The intercept (α) represents reduction in BWG related to changes in maintenance (i.e., not associated with changes in FI). The slope (β) represents the extent of BWG change associated with the reduction in feed efficiency in challenged birds.
The factorial was analysed by ANOVA and included the effects of the additives, challenge and their interactions for all the studied variables. The LSmeans compared the means when significant differences were found. The GLM procedure of the SAS statistical package, version 9.2 (SAS Institute 2002) was used.

Results

Growth Performance

The ΔBWG showed a linear fit to the ΔFI in the first and second weeks evaluated (Fig 1 and 2). The ΔBWG was lower for all treatments in the second week, demonstrating that the greatest impact resulting from coccidiosis occurred in the first week after infection. In addition, the intercepts of all equations were negative in both weeks, suggesting that changes in maintenance requirements contributed to a negative ΔBWG even when feed intake was not affected.

Most of the ΔBWG observed in the challenged groups in relation to the control birds was due to worsening of feed efficiency, except for the group that received

CNSL- Castor oil in the first week. The maintenance-related fraction was twice large (2-fold) in CNSL- Castor oil group than in the positive control, and 24 times (24-fold) higher than Monensin treatmet, which had the lowest ΔBWG. Groups fed diets containing CNSL- Castor oil presented lower ΔBWG, both due to maintenance (4%) and feed efficiency (11%) in the second week.

Oocysts count

The total oocysts count for birds fed diets with monensin or CNSL- Castor oil was reduced when compared to the positive control ($P<0.05$) (Fig. 3). As expected, in unchallenged chickens no oocysts were detected into excreta.

Blood analysis and ISI score

The percentage of heterophiles and the heterophiles / lymphocyte ratio presented interaction ($P<0.05$).The non-challenged birds had no difference between the treatments ($P> 0.05$), but the group challenged of the positive control and the CNSL- Castor oil had a higher percentage of heterophiles and a higher heterophiles / lymphocyte ratio than those of Monensin (Fig.4). In addition, birds receiving Monensin showed similar between non-challenged and challenged. The number of total leukocytes and the percentage of eosinophils did not present a difference between the treatments, and the percentage of lymphocytes and monocytes differed only for the challenge factor ($P<0.05$), in which unchallenged birds had a higher percentage of lymphocytes and lower monocytes when compared to challenged birds (data not shown).

Figure 5 shows the average of the maximum scores obtained by the ISI morphometric index, described by Kraieski et al. (2016). There was no interaction

between the factors, therefore, regardless of the challenge, CNSL- Castor oil presented higher ISI in all evaluated segments, and monensin and control did not differ. The detailed ISI results are presented in annex 1.

Gene expression

The mRNA expression of the genes evaluated in the duodenum and jejunum at 7 days after the challenge (21 days of age) is shown in Figure 6. In these two segments, there was interaction between the additives and challenge for all genes evaluated. Contrary to the cecal tonsils where there was no interaction between the factors, however, all the genes evaluated presented higher expression for the challenged birds ($P<0.05$). In this segment, there was an effect of the additive only for Inos expression, which presented higher expression in the control group, followed by monensin and CNSL- Castor oil ($P<0.05$). (Data not shown)

The challenged birds that received CNSL- Castor oil presented lower Inos gene expression and higher NF-kB and IL-6 than monensin in the duodenum and jejunum ($P<0.05$). There was upregulated expression of IFN and TNF in relation to the positive control and monensin in the jejunum ($P<0.05$). In both segments of the birds of the positive control, there was a upregulated expression of IL-1 and in the jejunum greater COX expression in relation to the monensin and CNSL- Castor oil birds; between these treatments, there were no differences for both genes ($P>0.05$). The non-CNSL- Castor oil-challenged birds presented lower gene expression in relation to the other treatments in at least one of the segments ($P<0.05$), except for the TNF gene, which showed no difference among treatments in the jejunum ($P>0.05$) and was lower for the positive control treatment in the duodenum ($P<0.05$). The treatment with monensin presented higher gene

expression of IFN, COX and IL-1 than the other treatments in both segments ($P < 0.01$).

Discussion

The CNSL- Castor oil additive stimulated the inflammatory response of birds by increasing the immune response against the parasite in the week after the challenge. This is seen in the increase of some proinflammatory interleukins (IFN, TNF and IL-6), the heterophiles / lymphocyte ratio in the blood, the ISI and the higher maintenance expenditure. However, birds excreted a quantity of oocysts similar to birds with monensin. The opposite occurred with the positive control, which presented lower inflammatory response and greater oocysts excretion, demonstrating that the parasite continues to proliferate.

Regardless of the additives used, as expected, the challenged birds presented worse ISI, higher heterophiles / lymphocyte ratio and higher expression of Inos, TNF, IFN, NF-kB, Cox, IL-1 and IL-6 in the week following the challenge in all analyzed intestinal segments, characterizing an ongoing inflammatory process against the pathogen (Laurent *et al.*, 2001; Cox *et al.*, 2010; Zhou *et al.*, 2014; Chen *et al.*, 2016; Zhang *et al.*, 2016). According to Kogut e Klasing (2009) and Klasing (2004), the acute phase of the immune response is the first defence mechanism and is related to systemic and metabolic alterations.

The immune response against the parasite is complex and there are mechanisms that are not clear yet. It is known that the innate immune system and cell mediated has a fundamental action in response to the pathogen. The immune system does not prevent sporozoite invasion into enterocytes, but avoids their development (Allen e Fetterer, 2002). Innate immunity recognizes the pathogen through toll-like

receptors (TLRs) that use the MyD88 adapter protein to activate the genes of four major pro-inflammatory proteins IL-1, IL-6, IL-12, TNF-α and IFN-γ (Tizard 2009). These cytokines play important regulatory roles modulating the course of the immune response during infection (Allen & Fetterer, 2002). It was observed that the reaction developed in the treatment that received CNSL- Castor oil modulates the immune system to increase gene expression of TNF-α, IL-6 and IFN-y and reduce IL-1 expression and COX-2, contrary to the control Positive that increased Cox-2 and IL-1, being the first one more effective than the second, which can be observed by the reduction of oocysts.

The efficacy of CNSL- Castor oil treatment in reducing oocysts can be explained by the increase in IFN-γ. Because IFN-γ expression stimulates cell-mediated immunity (Th1) that is crucial in the immune response against Eimeria (Lillehoj e Choi, 1998). IFN-γ produced mainly by CD4 + cells can directly inhibit sporozoite development by increasing the cytotoxic activity of CD8 + cells and activating macrophages (Allen e Fetterer, 2002). Lee *et al.* (2008) observed that the use of a phytogenic composed of *Prunus salicin* in the feed increased the expression of IFN-γ, promoting the protective immunity against coccidiosis, with reduction in oocyst excretion and weight loss 10 days after infection, in agreement with the results obtained in this study.

The TNF-α and interleukin IL-6 are inflammatory markers stimulated by macrophages and NF-kB (Kim *et al.*, 2008), and in the present study, the heterophiles / lymphocyte ratio increased in the blood, reflecting the ISI worsening. According to Kraieski et al., (2016, the increase in ISI is related to the increased infiltration of lymphocytes in the lamina propria, with epithelial thickness,

goblet cells, congestion and enterocytes proliferation or infiltration of inflammatory cells.

When applying the Pastorelli et al. (2012) aproach to evaluate the link between feed intake and weight gain, although the positive control treatment and CNSL-Castor oil presented a similar ΔBWG (-132%), the partition of this variation was different. While the positive control presented a more significant change due to the fraction associated with the change in feed efficiency, that may be associated with a reduction in feed consumption (-86%), the variation in the CNSL- Castor oil group was due to changes associated with maintenance, not associated with ΔFI (-94%). Zhang *et al.* (2016) observed that ΔBWG in broilers after coccidiosis challenge was attributed to change in maintenance. The nutrients requirements for maintenance are higher during the challenge with coccidiosis, particularly, in the first week after challenge (Laurent *et al.*, 2001; Cornelissen *et al.*, 2009; Cox *et al.*, 2010). The increased requirement probably includes metabolic costs for repairing damaged tissues, immune system stimuli, and reduced ability of animals to utilize nutrients (Chen *et al.*, 2016; Grenier *et al.*, 2016). The innate immune system may causes considerable collateral damage, such as fever and inflammatory reactions that consume resources, reducing nutrient reserves and increasing catabolism (Klasing e Iseri, 2013; Iseri e Klasing, 2014). These consequences make inflammation a highly undesirable phenomenon in animal production. One week after the challenge, the greater inflammatory response in CNSL- Castor oil treatment was important to transform the immune system in a more effective mechanism, not only against coccidia, but also against pathogenic bacteria, preventing intestinal dysbiosis.

This fact was proven the following week, in which CNSL- Castor oil reduced the population of *C. perfringens* and *S. aureu*, considered opportunistic bacteria (Moraes et al. / Article in process). In this sense, it is possible to say that the mode of action of CNSL- Castor oil is similar to a vaccine, potentiating the host immune system against the pathogens and acting as a modulator at the same time. The monensin group presented a lower inflammatory process, characterized by the downregulate of TNF, IFN, NF-kB, Cox, IL-1 and IL-6 in at least one of the evaluated segments, greater anticoccidial activity acts on the parasite when it is outside the host cell and can kill the coccidia on the third day after the challenge, in the form of merozoites, before the inflammatory process peak, which occurs at the end of the first week (Laurent *et al.*, 2001; Chapman *et al.*, 2010; Cox *et al.*, 2010).

This modulation was also observed in unchallenged birds, which presented lower gene expression of most of the interleukins evaluated when supplemented with CNSL- Castor oil, unlike the monensin treatment that increased IFN-γ, COX -2 and IL-1. This may be a better microbiota balance reflection, confirmed by the analysis of the microbiota at 28 days of age, when a larger population of *Lactobacillus* spp was observed for the CNSL- Castor oil group (Moraes et al. / Article in process). These bacteria have the ability to modulate the gene expression of cytokines, toll-like receptors and T cells (Brisbin *et al.*, 2011).

The commercial blend of cashew nut liquid and castor oil modulated the inflammatory response against *Eimeria* spp .. In the absence of the parasite, there was no stimulation of the genes involved inflammatory response, demonstrating that the blend is an effective tool in modulating the imune system.

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Table 1. Intestinal helth index classification table

Alteration	Impact Factor
Self-blade thickness	2
Epithelial thickness	1
Proliferation of enterocytes	1
Epithelial plasma infiltration	1
Mixed inflammatory infiltration of lamina propria	3
Goblets cells	2
Congestion	2
Necrosis (apical karyolysis)	3
Presence of oocysts	3
Maximum score [1]	54

[1] Maximum score represents the results considering an observation of score 3 for each alteration, multiplied by the impact factor (fixed value for each alteration) and summed at the final. (adapted from Kraiski et al., 2016)

Table 2. Target gene and primers used for the analysis of gene expression

Target	Sequence	ID	Reference
*B- actin_F	5' ACCTGAGCGCAAGTACTCTGTCT 3'		
*B- actin_R	5' CATCGTACTCCTGCTTGCTGAT 3'	NM205518.1	Xie et al. (2014)
*GAPDH_F[1]	5' CCTAGGATACACAGAGGACCAGGTT 3'		
*GAPDH_R	5' GGTGGAGGAATGGCTGTCA 3'	NM_204305	Tan et al. (2014)
IL-1B_ F	5' ACT GGG CAT CAA GGG CTA 3'		
IL-1B_R	5' GGT AGA AGA TGA AGC GGG TC 3'	NM_204524	Tan et al. (2014)
IL-6_ F	5' TTTATGGAGAAGACCGTGAGG 3'		
IL-6_R	5' TGTGGCAGATTGGTAACAGAG 3'	NM_204628	Long et al.(2011)
TNF-α_F	5' TGCTGTTCTATGACCGCC 3'		
TNF-α_R	5' CTTTCAGAGCATCAACGCA 3'	AY765397	Hu et al. (2015)
IFN-y_F	5' AGCTGACGGTGGACCTATTATT 3'		
IFN-y_R	5' GGCTTTGCGCTGGATTC 3'	Y07922	Lee et al. (2012)
Cox-2_F	5' GGTGAGACTCTGGAGAGGCAAC 3'		
Cox-2_R	5' GTTGAACAGAAGCTCAGGGTCA 3'	M64990	Laurent et al. (2001)
iNOS_F[2]	5' CCTGTACTGAAGGTGGCTATTGG 3'		
iNOS_R	5 AGGCCTGTGAGAGTGTGCAA 3'	D85422	Cox et al. (2010)
NF-kB_F[3]	5' GTGTGAAGAAACGGGAACTG 3'		
NF-kB_R	5' GGCACGGTTGTCATAGATGG 3'	NM_205129	Tan et al. (2014)

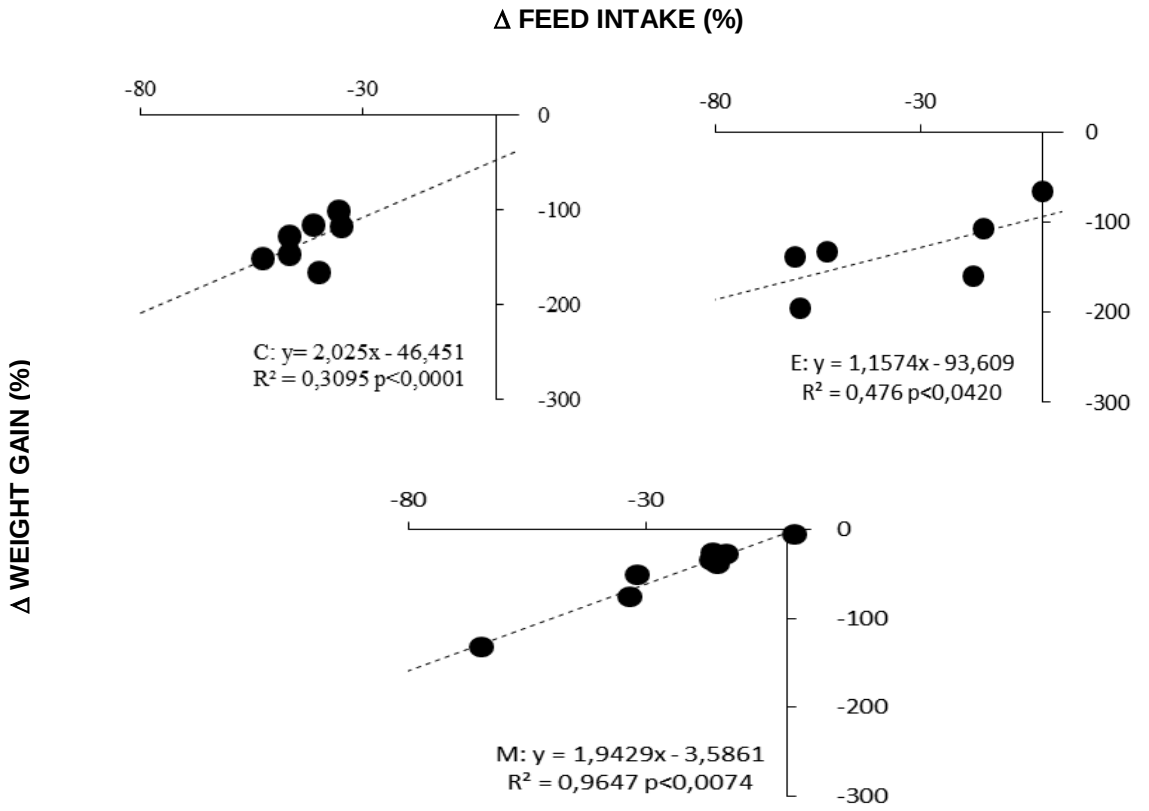
F: forward; R reverse; ID: GenBank access number ; *Housekeeping genes

[1] Glyceraldehyde-3-phosphate dehydrogenase

[2] Inducible nitric oxide synthase

[3] Nuclear factor kappa B

(a)



(b)

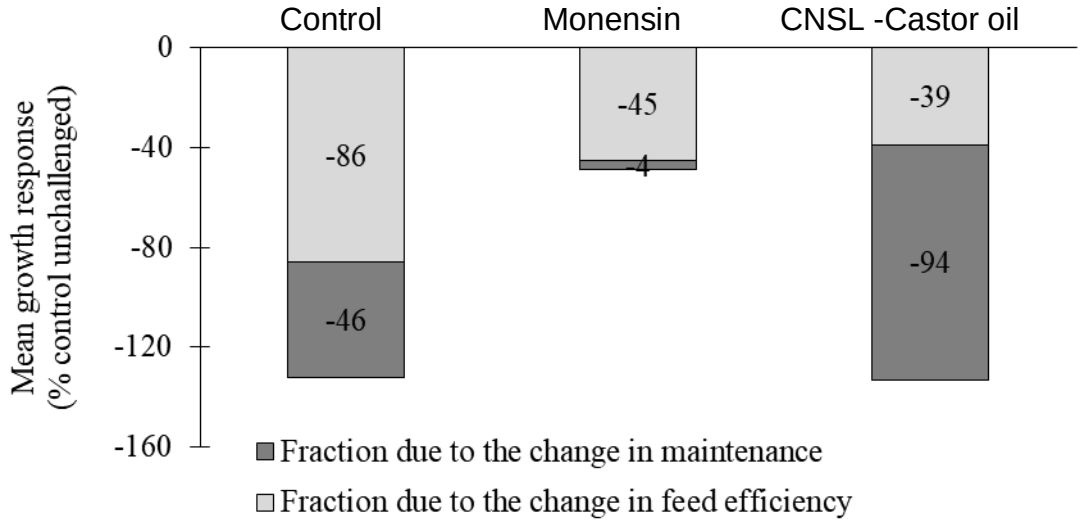


Figure 1. *(a) . Relationship between the change in BW gain (ΔBWG) and feed intake (ΔFI) of broiler chicks at 14–21 d of age challenged with Eimeria spp. supplemented with inert (C) CNSL - Castor oil (*Essential (US Patent N°. 8,377,485; Oligo Basics Ind. Ltda., Cascavel, Paraná, Brazil) *(E) monensin (M) in the diet. Responses are expressed as results of the challenged birds relative to negative control. (b) Partitioning of the reduction in average BW gain between the fraction due to the change in maintenance (■, not associated with ΔFI) or change in feed intake (□, associated with ΔFI). ΔFI, Change in feed intake.*

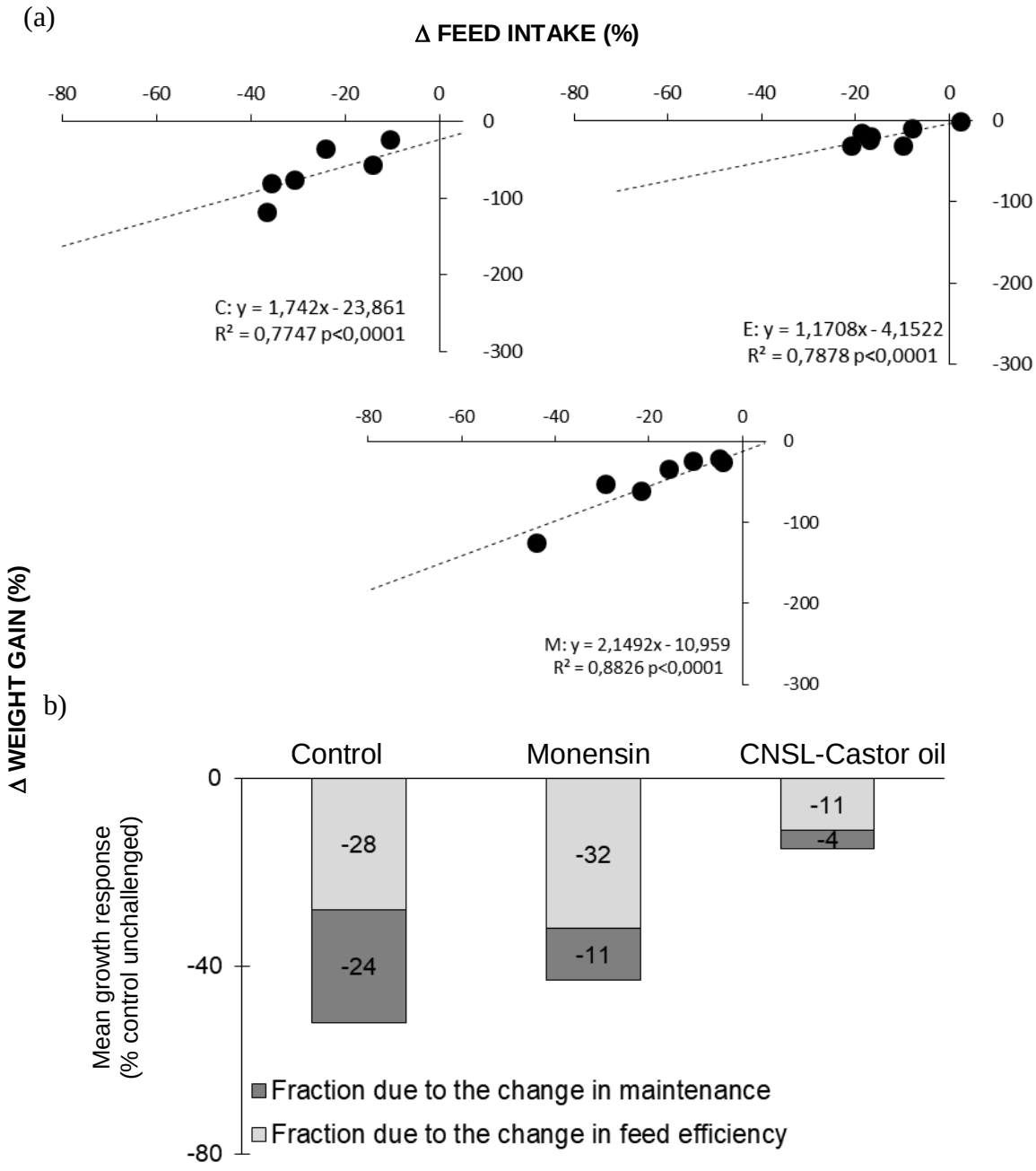


Figure 2. *Relationship between the change in BW gain (ΔBWG) and feed intake (ΔFI) of broiler chicks at 21–28 d of age challenged with Eimeria spp. supplemented with inert (C) CNSL- Castor oil (*Essential (US Patent N°. 8,377,485; Oligo Basics Ind. Ltda., Cascavel, Paraná, Brazil) *(E) monensin (M) in the diet. Responses are expressed as results of the challenged birds relative to negative control. (b) Partitioning of the reduction in average BW gain between the fraction due to the change in maintenance (■, not associated with ΔFI) or change in feed intake (□, associated with ΔFI). ΔFI, Change in feed intake.*

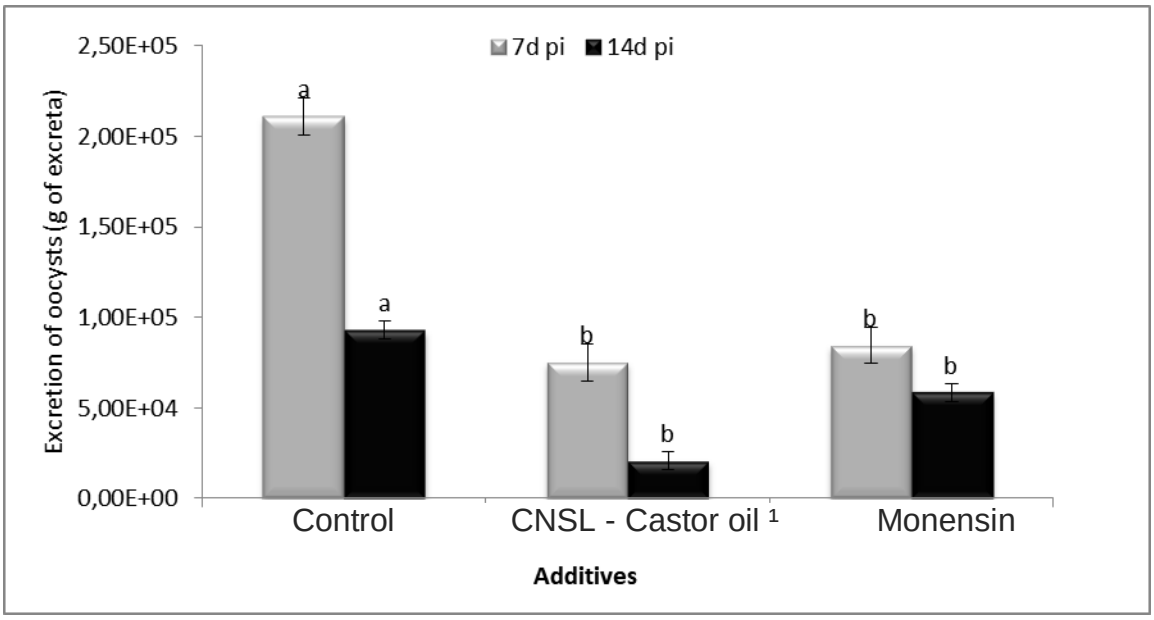


Figure 3. Excretion of oocysts in chickens challenged with coccidiosis at 7 and 14 days post infection. ¹ Essential (US Patent N°. 8,377,485; Oligo Basics Ind. Ltda., Cascavel, Paraná, Brazil Averages with different lowercase letters differ statically by LSMEANS in the week evaluated. Values represent the box, totaling 8 boxes per treatment

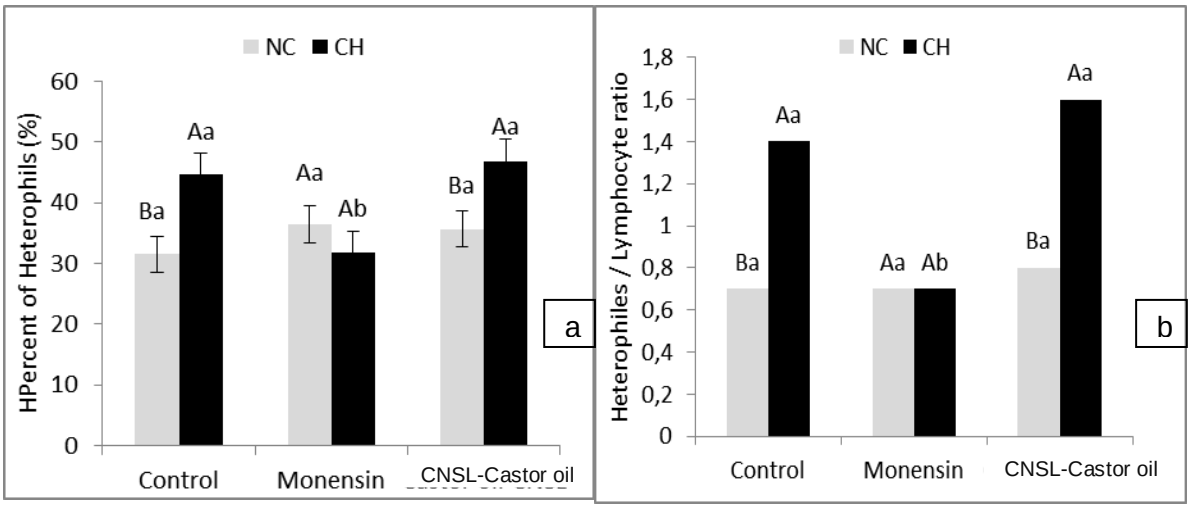


Figure 4. Percent of Heterophils (a) and Heterophiles / Lymphocyte ratio (b) in challenged broilers (CH) or non (NC) with coccidiosis.[1] Essential (US Patent N°. 8,377,485; Oligo Basics Ind. Ltda., Cascavel, Paraná, Brazil. Means with different letters differ statically by LSMEANS, lowercase for additive and uppercase for challenged. Values represent 1 birds by box, totaling an average of 8 birds per treatment

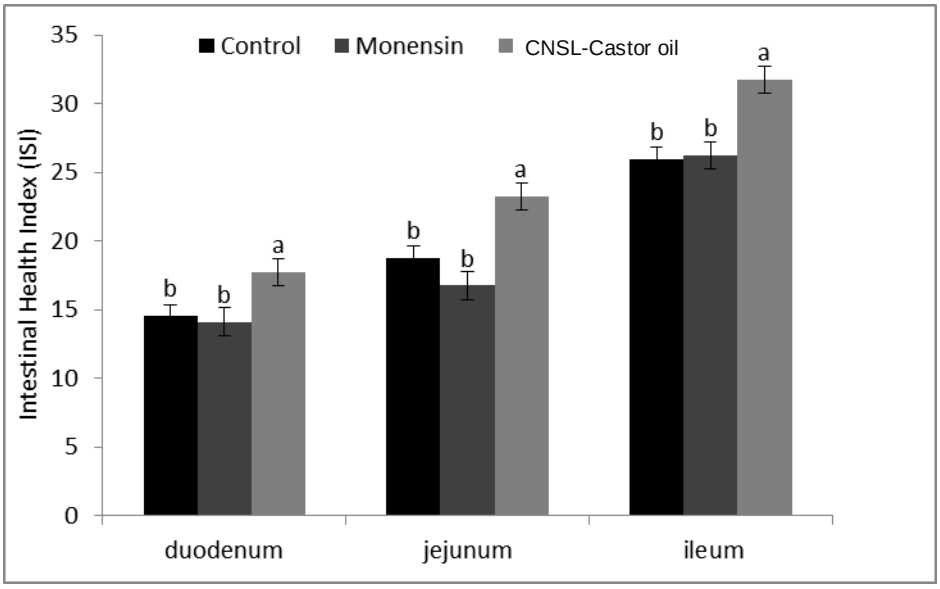
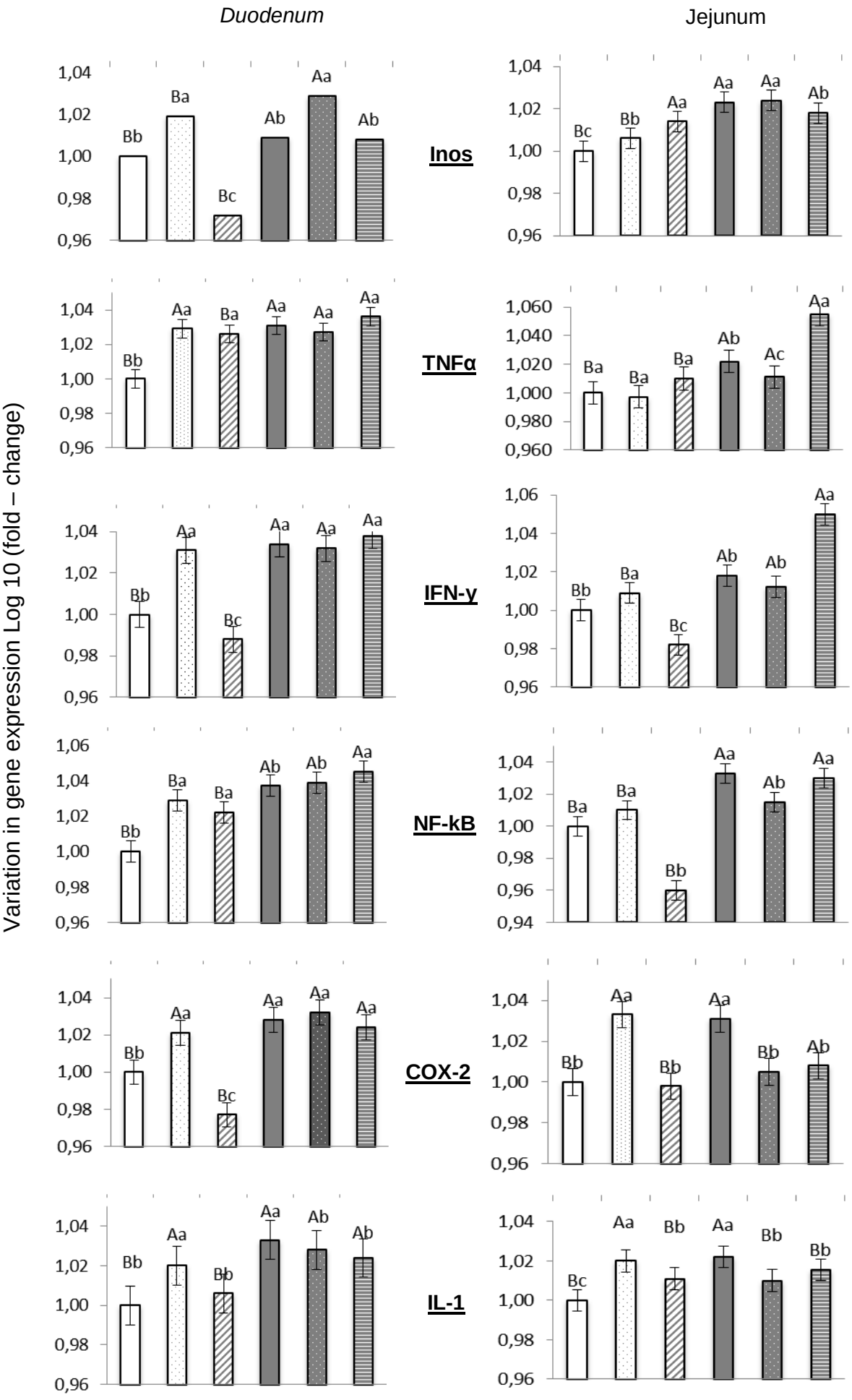


Figure 5. Intestinal Health Index (ISI) of broilers challenged or not at 21 days of age receiving different additives.[1] Essential (US Patent N°. 8,377,485; Oligo Basics Ind. Ltda., Cascavel, Paraná, Brazil. Averages with different lowercase letters differ statically by LSMEANS in the week evaluated. Values represent the 1 bird by box, totaling 8 boxes per treatment


Duodenum
Jejunum
Variation in gene expression Log 10 (fold – change)
Inos
TNFα
IFN-y
NF-kB
COX-2
IL-1

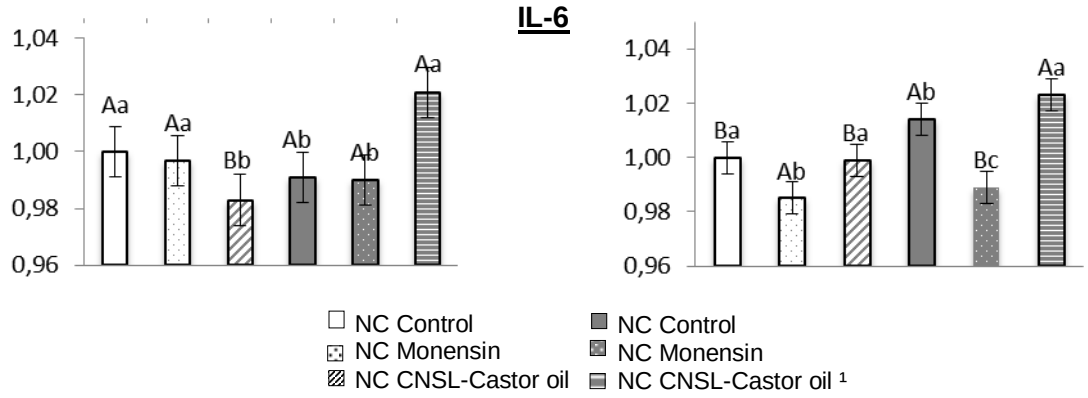


Figure 6. *Interaction between coccidiosis challenge and different additives in the gene expression of interleukins in the duodenum and jejunum. Gene expression was calculated in relation to treatment that did not receive challenge or additive.*[1] Essential (US Patent N°. 8,377,485; Oligo Basics Ind. Ltda., Cascavel, Paraná, Brazil. *Capitalized averages differ statistically for the challenge, with lowercase letters differing for the additive within the challenge by LSMEANS using the data transformed into Log10*

Annex

Anexx 1 - ISI histological analysis scores mean and standard error different treatments in samples of duodenum of broilers with 21 days of age

Treatmens	Self-blade thickness	Epithelial thickness	Proliferation of enterocytes	Epithelial plasma infiltration	Mixed inflammatory infiltration of lamina propria	Goblets cells	Congestion	Necrosis (apical karyolysis)	Presence of oocysts	Score
					Challenge					
	1,76±0,14 b	2,67±0,10 b	2,47±0,10 b	0,77±0,03 a	1,63±0,18	1,91±0,15	0,90±0,12 b	0,05±0,03	0,13±0,06	12,33±0,46 b
	3,65±0,15 a	3,22±0,09 a	3,11±0,10 a	0,61±0,04 b	1,55±0,17	1,75±0,16	1,46±0,17 a	0,05±0,039	3,20±0,33	18,62±0,57 a
					Additives					
Control	2,37±0,19 b	2,76±0,12 b	2,51±0,13 b	0,61±0,04	1,29±0,20	1,37±0,18	1,00±0,17	0,08±0,05	2,53±0,40	14,55±0,70 b
Monensin	2,37±0,19 b	2,81±0,13 b	2,65±0,13 b	0,75±0,04	1,83±0,22	1,52±0,19	0,97±0,17	0±0	1,20±0,25	14,12±0,69 b
CNSL - CO [1]	3,37±0,20 a	3,26±0,11 a	3,21±0,11 a	0,70±0,05	1,66±0,22	2,60±0,191	1,57±0,20	0,08±0,05	1,26±0,28	17,75±0,64 a
					Interaction					
Control_Un	1,60±0,23	2,56±0,17	2,23±0,17	0,68±0,06	1,08±0,26	2,10±0,29 ab	0,70±0,20	0,08±0,083	0,00±0,00 c	11,05±0,66
Monensin_Un	1,50±0,24	2,53±0,19	2,30±0,17	0,80±0,057	0,58±0,07	1,20±0,24 bc	0,90±0,21	0±0	0,00±0,00 c	11,48±0,81
Control _CH	3,15±0,28	2,96±0,18	2,80±0,18	0,55±0,073	1,50±0,29	0,65±0,18 c	1,30±0,27	0,083±0,08	5,06±0,66 a	18,06±1,08
Monensin_CH	3,25±0,26	3,10±0,16	3,00±0,18	0,70±0,064	1,41±0,29	1,85±0,28 ab	1,05±0,28	0±0	2,40±0,45 b	16,76±1,02
CNSL - CO _UN	2,20±0,28	2,93±0,16	2,90±0,16	0,83±0,072	1,58±0,32	2,45±0,24 a	1,10±0,21	0,08±0,08	0,40±0,20 c	14,48±0,85
CNSL - CO _CH	4,55±0,20	3,60±0,14	3,53±0,14	0,58±0,07	1,75±0,31	2,75±0,29 a	2,05±0,33	0,08±0,08	2,13±0,50 b	21,03±0,77
					Probability					
Challenge	0,000	0,000	0,000	0,004	0,740	0,437	0,080	1,000	0,000	0,000
Additives	0,000	0,006	0,000	0,132	0,198	0,000	0,340	0,368	0,000	0,000
Additive*Challenge	0,257	0,735	0,927	0,507	0,101	0,001	0,303	1,000	0,000	0,598

[1] CNSL- Castor oil_ Essential (US Patent N°. 8,377,485; Oligo Basics Ind. Ltda., Cascavel, Paraná, Brazil. [a,b,c,d] Different letters in the same column indicate a significant difference of P ≤ 0.0.5 by Tukey's test.

Anexx 2 - ISI histological analysis scores mean and standard error different treatments in samples of jejunum of broilers with 21 days of age

Treatmens	Self-blade thickness	Epithelial thickness	Proliferation of enterocytes	Epithelial plasma infiltration	Mixed inflammatory infiltration of lamina propria	Goblets cells	Congestion	Necrosis (apical karyolysis)	Presence of oocysts	Score
					Challenge					
Unchallenged	1,83±0,13 b	2,50±0,07 a	1,03±0,08 b	0,38±0,03 b	3,75±0,18	3,78±0,11 a	1,31±0,13	1,47±0,19 b	0,04±0,03	16,11±0,60 b
Challenge	3,13±0,13 a	2,16±0,08 b	1,55±0,10 a	0,61±0,03 a	4,86±0,15	3,13±0,12 b	1,15±0,13	3,41±0,25 a	3,02±0,23	23,05±0,68 a
					Additives					
Control	2,65±0,17 a	2,21±0,10	1,48±0,12 a	0,37±0,04 b	4,16±0,20	3,37±0,16	1,05±0,14	2,12±0,27 b	1,33±0,20	18,77±0,75 b
Monensin	2,025±0,17 b	2,26±0,08	0,98±0,10 b	0,55±0,04 a	4,25±0,21	3,47±0,13	1,07±0,16	1,20±0,21 c	0,90±0,17	16,74±0,73 b
CNSL - CO[1]	2,77±0,15 a	2,51±0,09	1,41±0,12 a	0,55±0,04 a	4,50±0,23	3,52±0,13	1,57±0,18	4,00±0,31 a	2,36±0,31	23,23±0,94 a
					Interaction					
Control_Un	2,05±0,21	2,40±0,10	1,30±0,14	0,26±0,05	4,00±0,28 bc	3,75±0,19	1,15±0,22 bc	1,08±0,26	0,00±0,00 c	16,00±0,80
Monensin_Un	1,30±0,20	2,46±0,11	0,83±0,13	0,43±0,06	3,25±0,31 c	3,65±0,17	0,50±0,16 c	0,66±0,22	0,06±0,06 c	13,16±0,81
Control _CH	3,25±0,25	2,03±0,17	1,66±0,21	0,48±0,06	4,33±0,30 abc	3,00±0,26	0,95±0,19 bc	3,16±0,44	2,66±0,33 b	21,55±1,17
Monensin_CH	2,75±0,25	2,06±0,12	1,13±0,14	0,68±0,06	5,25±0,21 a	3,30±0,21	1,65±0,26 ab	1,75±0,35	1,73±0,30 b	20,31±1,03
CNSL - CO _UN	2,15±0,23	2,63±0,15	0,96±0,18	0,45±0,06	4,00±0,35 bc	3,95±0,21	2,30±0,25 a	2,66±0,41	0,06±0,06c	19,18±1,30
CNSL - CO _CH	3,40±0,16	2,40±0,11	1,86±0,14	0,66±0,06	5,00±0,29 ab	3,10±0,14	0,85±0,22 bc	5,33±0,40	4,66±0,47 a	27,28±1,14
					Probabiliy					
Challenge	0,000	0,002	0,000	0,000	0,000	0,000	0,360	0,000	0,000	0,000
Additives	0,002	0,055	0,004	0,005	0,505	0,759	0,030	0,000	0,000	0,000
Additive*Challenge	0,84	0,803	0,134	0,956	0,019	0,438	0,000	0,088	0,000	0,481
Additive*Challenge	0,257	0,735	0,927	0,507	0,101	0,001	0,303	1,000	0,000	0,598

[1] CNSL- Castor oil_ Essential (US Patent N°. 8,377,485; Oligo Basics Ind. Ltda., Cascavel, Paraná, Brazil. [a,b,c,d] Different letters in the same column indicate a significant difference of P ≤ 0.0.5 by Tukey's test

Anexx 3 - ISI histological analysis scores mean and standard error different treatments in samples of ileum of broilers with 21 days of age

Treatmens	Self-blade thickness	Epithelial thickness	Proliferation of enterocytes	Epithelial plasma infiltration	Mixed inflammatory infiltration of lamina propria	Goblets cells	Congestion	Necrosis (apical karyolysis)	Presence of oocysts	Score
					Challenge					
Unchallenged	4,73±0,18	3,35±0,12 b	2,72±0,14	0,50±0,04 b	3,33±0,26	5,15±0,19	2,11±0,20	2,63±0,23	0,00±0,00	24,55±0,79
Challenge	5,48±0,17	3,92±0,11 a	3,44±0,12	0,91±0,05 a	5,75±0,30	4,98±0,20	1,90±0,17	4,05±0,28	0,95±0,13	31,40±0,84
					Additives					
Control	4,75±0,22	3,43±0,14 b	2,83±0,16	0,54±0,05 b	4,58±0,36	4,30±0,21 b	1,85±0,21	2,95±0,32	0,66±0,13	25,99±1,09
Monensin	4,65±0,22	3,46±0,14 b	2,98±0,15	0,58±0,05 b	3,91±0,33	5,42±0,21 a	2,20±0,26	3,00±0,29	0,00±0,00	26,22±0,87
CNSL - CO [1]	5,90±0,21	4,01±0,15 a	3,38±0,17	1,00±0,07 a	5,12±0,39	5,47±0,27 a	1,97±0,22	4,08±0,34	0,76±0,15	31,72±1,10
					Interaction					
Control_Un	3,90±0,31 d	2,96±0,19	2,16±0,22 b	0,43±0,07	2,50±0,42 b	4,55±0,33	1,85±0,31	1,91±0,37 c	0,00±0,00 b	20,28±1,25 d
Monensin_Un	4,90±0,30 bcd	3,23±0,21	2,86±0,23 ab	0,36±0,06	3,58±0,44 b	5,05±0,24	2,35±0,39	3,00±0,41bc	0,0±0,00 b	25,35±1,32 cd
Control _CH	5,65±0,28 ab	3,90±0,18	3,60±0,20 a	0,65±0,07	6,66±0,47 a	4,05±0,28	1,85±0,28	4,00±0,48ab	1,33±0,24 a	31,70±1,45 ab
Monensin_CH	4,40±0,33 cd	d 3,70±0,17	3,10±0,19 a	0,80±0,07	4,25±0,48 b	5,80±0,34	2,05±0,34	3,00±0,43bc	0,00±0,00 b	27,10±1,139 bc
CNSL - CO _UN	5,40±0,32 abc	3,86±0,22	3,13±0,26 a	0,71±0,08	3,91±0,47 b	5,85±0,39	2,15±0,34	3,00±0,43bc	0,00±0,00 b	28,03±1,38 bc
CNSL - CO _CH	6,40±0,26 a	4,16±0,20	3,63±0,22 a	1,28±0,10	6,33±0,59 a	5,10±0,38	1,80±0,29	5,16±0,50 a	1,53±0,28 a	35,41±1,58 a
					Probabiliy					
Challenge	0,003	0,001	0,000	0,000	0,000	0,544	0,425	0,000	0,000	0,000
Additives	0,000	0,006	0,066	0,000	0,046	0,001	0,566	0,017	0,000	0,000
Additive*Challenge	0,001	0,270	0,022	0,096	0,002	0,059	0,850	0,023	0,000	0,002

[1] CNSL- Castor oil _Essential (US Patent N°. 8,377,485; Oligo Basics Ind. Ltda., Cascavel, Paraná, Brazil. [a,b,c,d] Different letters in the same column indicate a significant difference of P ≤ 0.0.5 by Tukey's test

CONSIDERAÇÕES FINAIS

A mistura comercial do líquido da casca de castanha de caju e do óleo de mamona possui potencial para ser utilizada como um promotor de crescimento apresentando resultado de desempenho semelhante ao ionóforo monensina. Trabalhos anteriores relataram que o Essential melhora o desempenho animal possivelmente por apresentar atividade anti-inflamatória e antimicrobiana. No entanto, contrariando as expectativas, o Essential estimulou a resposta imune inata e celular, potencializando o sistema imune do hospedeiro contra o parasita e consequentemente aumentou as exigências de mantença, desviando recursos e reduzindo o desempenho animal na primeira semana após o desafio. Ao mesmo tempo foi efetivo contra o patógeno sendo demonstrado pela menor excreção de oocistos.

Foi possível observar uma redução na população de C.perfringens com a utilização do Essential. Essa redução pode ter ocorrido por dois motivos, primeiro porque o produto apresenta atividade antibacteriana direto, segundo porque pode ter estimulado o sistema imune tornando-o mais responsivo. Neste ponto, há uma limitação da pesquisa descrita, pois o sistema imune e a microbiota foram avaliados em semanas distintas e uma única vez, permitindo apenas fazer especulações baseadas na literatura sobre a sua interação. O ideal teria sido realizar ambas as análises aos 7, 14 e 21 dias após o desafio, observando respectivamente, o pico da inflamação e o seu efeito na microbiota, a fase intermediária quando a microbiota ainda não se reestabeleceu e após, quando o sistema, provavelmente, já tenha recuperado a homeostase.

Esses resultados permitem questionar se realmente o processo de inflamação deva ser considerado um fenômeno altamente indesejável do ponto de vista da produção, já que no presente caso, a recuperação dos animais foi mais lenta, porém ocorreu na mesma grandeza que nos animais que utilizaram o ionóforo. Principalmente com a possibildade da proibição dos antibióticos promotores de crescimento, é preciso ampliar o conhecimento da saúde intestinal e focar em um sistema imune que esteja pronto para atuar quando for necessário. Possivelmente, no futuro, vamos formular rações e tomar decisões

com base em biomarcadores moleculares de saúde intestinal, que podem ser a comunidade microbiana, as interleucinas ou ainda biomarcadores ainda não descobertos.

A realização desta pesquisa, entre outras realizadas durante o doutorado, proporcionou uma experiência na organização de materiais e principalmente de atividades que envolviam um grupo de pessoas para a execução de tarefas que exigiram uma alta carga de trabalho manual Também possibilitoua convivência em diferentes laboratórios com outros focos que não a produção animal, contribuindo para um aprendizado interdisciplinar e uma visão sistêmica do objeto de pesquisa. Além disso, essa rede possibilitou a discussão de metodologias utilizadas e dos resultados obtidos com colegas e professores de diferentes formações, permitindo a construção de hipótese mais sólidas e complementares.

Esse projeto é fruto de uma parceria entre Universidade e indústria, permitindo a realização de um trabalho com grande relevância científica e aplicabilidade técnica. Além de amplificar a discussão da problemática do projeto e lapidar as metodologias para responder os questionamentos encontrados na prática do campo, o projeto mostrou que esta parceria traz benefícios para ambos os setores, público e privado, e deve ser incentivado, principalmente porque resolve um dos grande s problemas d a pesquisa nos países em desenvolvimento que é a falta de recursos materiais.

Neste sentido, este trabalho gerou além da contribuição cientifica e técnica, uma visão de um vasto campo de pesquisa, com muitos questionamentos que podem continuar a serem explorados, sendo um ponto de partida para a minha carreira como pesquisadora.

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APÊNDICES

APÊNDICE A - Instrução aos autores para publicação na Revista Animal Feed Science and Tecnology

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b. NDFom-NDF not assayed with a heat stable amylase and expressed exclusive of residual ash.
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e. ADFom-ADF expressed exclusive of residual ash.
f. ADF-ADF expressed inclusive of residual ash.
g. Lignin (sa)-Lignin determined by solubilization of cellulose with sulphuric acid.
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animal attracts the best research in animal biology and animal systems from across the spectrum of the agricultural, biomedical, and environmental sciences; it is the central element in a collaboration between the British Society of Animal Science (BSAS), the Institut National de la Recherche Agronomique (INRA) and the European Federation for Animal Science (EAAP) and represents the merger in 2006 of three scientific journals: *Animal Science*; *Animal Research*; *Reproduction, Nutrition, Development*.

Scope

animal publishes original cutting-edge research, horizon-scanning reviews, and opinion papers on animal-related aspects of the life sciences at the molecular, cellular, organ, whole animal and production system levels. It is essential reading for all animal scientists interested in biochemistry, microbiology, nutrition, physiology, modelling, genetics, behaviour, immunology, epidemiology, economics, sociology, food science and technology, human health, farming systems, and land-use management, environmental impact and climate change.

Papers will be considered in aspects of both strategic and applied science in the areas of Animal Breeding and Genetics, Nutrition, Physiology and Functional Biology of Systems, Behaviour, Health and Welfare, Livestock Farming Systems and Environment, and Product Quality, Human Health and Well-being. Emphasis is placed on **managed and farm animals** and on the integrative nature of biological systems. The use of laboratory animal models for the benefit of farmed livestock is within the scope. Studies using farm animals with the aim of improving human health are also acceptable if they indicate obvious benefits to farmed livestock. Wild animals which are marginally bred in a few countries or which could be bred in the future, and wild animals raised in captivity are not in scope. Papers dealing with the translation of basic and strategic science into whole animal, and livestock system, impacts on productivity, product quality, the environment and humans (health, nutrition and well-being) will be welcome, as are methodology papers. Papers should be of **international relevance,** appeal to an international readership and not limited to national or regional conditions. The full scope of the journal should be consulted on http://www.animal-journal.eu/scope.htm before submitting a paper.

General specifications for different types of article

Submitted manuscripts should not have been published previously, except in a limited form (e.g. short communication to a symposium or as part of MSc or PhD theses) and should not be under consideration for publication by other journals. Book reviews are not accepted.

All co-authors should agree with the content of the manuscript. Authors must have obtained permission to use any copyrighted material in the manuscript prior to submission. The work described in the

manuscript must comply with ethical guidelines available on the website http://www.animal-journal.eu/ethical_policy.htm and be reported according to "The ARRIVE Guidelines for Reporting Animal Research" detailed in Kilkenny *et al*. (2010)[1] and summarised at www.nc3rs.org.uk.

animal publishes different types of articles:

Research articles
They correspond to a full account of a complete project. The approach can be experimental or theoretical, provided the work has been carried out in a systematic way. Routine studies, descriptive experiments without an experimental design controlled by the author, papers based on repetition of published experiments with other breeds, or in other geographical conditions are discouraged. Articles presenting a detailed description of a new technique are within the scope. Comparison of existing methods is considered, provided similar comparisons have never been published. Research articles, including meta-analyses, should be comprehensive and should include an in-depth discussion. Papers in a numbered series are not accepted unless all are submitted at the same time.

Short communications
Short communications present exceptionally exciting, novel or timely contents. *animal* publishes a limited number of short communications. Their submission will only be accepted based on Editor's judgement, and they will be peer-reviewed in the same way as research papers. Partial data or complete studies with a limited amount of results will not be considered as short communications, and will be handled as research papers.

Review articles
They are invited by the Editorial Board or unsolicited. Review articles have to be contemporary and comprehensive, and add information to published reviews on the same topic; if not the case, they will be rejected immediately by the Editor-in-Chief. Sharp critical analyses of novel data or concepts are encouraged. When relevant, a statistical analysis of data and a meta-analysis approach are recommended (but meta-analyses only are not considered as review articles). Authors of unsolicited review articles are encouraged to question the Editorial Office prior to submission through questions@animal-journal.eu to ask if their paper is within the scope and of interest to the journal.

Invited Opinion papers
They are submitted by invitation of the Management Board of *animal* journal only and are published as open access papers. They are short papers, which aim to inform scientists, industry, the public and policy makers about cutting-edge issues in research or the impact of research. They reflect the opinion of their authors who bear full responsibility of the published paper.

Conference/Symposium papers
The journal will consider for publication the results of original work and critical reviews that are presented at conferences/symposia. Symposium organisers who wish to publish bundles of papers from a symposium/conference in *animal* should first contact the Editor-in-Chief of *animal* journal (questions@animal-journal.eu) for agreement and information on the management of these papers. If the papers do not fit the requested conditions for publication in *animal*, the papers may be referred to *Advances in Animal Biosciences*, a companion publication of *animal* published by Cambridge University Press. Acceptance of such papers will be subject to:

* the content being within the scope of the journal
* the journal standard peer review process

[1] Kilkenny C, Browne WJ, Cuthill IC, Emerson M and Altman DG 2010. Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. PLoS Biology 8, e1000412. doi: 10.1371/journal.pbio.1000412.

Table 1 *Specifications for the different types of article*

Article type	Maximum length (all text except figures)	Maximum number of tables plus figures	Maximum number of references	Additional information
Original research	7 000 words (equivalent to 9 pages in journal)	8	35	
Short communications	3 000 words	3	10	
Reviews	9 500 words (equivalent to 12 journal pages)	10	50	
Opinion papers	1700 words (equivalent to 2 journal pages) or 1 200 if a figure is submitted	1	5	
All article types			5 references per 1000 words	Supplementary material can be proposed and will be made available online

Recommendations for preparation of papers

The responsibility for the preparation of a paper in a form suitable for publication lies with the author. Authors should consult a free issue or a free article of *animal*, available at https://www.cambridge.org/core/journals/animal, in order to make themselves broadly familiar with the layout and style of *animal*. A **style sheet** summarising these indications is available on our website at http://www.animal-journal.eu/documents/Animal_style_template.doc.

Before submitting your manuscript, we strongly recommend that you consult the pre-submission checklist. Manuscripts that do not comply with the directions or that are too long will not be accepted for peer-review. This will ensure that they are judged at peer review exclusively on academic merit. Any deviations from these recommendations will be at the discretion of the Editor-in- Chief.

English

A good quality of written English is required. Spelling may be in British or American English but must be consistent throughout the paper. Care should be exercised in the use of agricultural terminology that is ill-defined or of local familiarity only. If the English is not good enough, the manuscript will be sent back to the authors. Cambridge University Press recommends that authors have their manuscripts checked by an English language native speaker before submission. We list a number of third-party services specialising in language editing and / or translation at: https://www.cambridge.org/core/services/authors/language-services and suggest that authors contact them as appropriate. Use of any of these services is at the author's own expense. The copy-editor will not perform language editing.

Manuscript layout

Manuscripts should be prepared using a standard word processing programme, and presented in a clear readable format with easily identified sections and headings. A style sheet is available on our website at http://www.animal-journal.eu/documents/Animal_style_template.doc.

Manuscript layout directions

- Typed with double-line spacing with wide margins (2.5 cm)
- The lines must be continuously numbered; the pages must also be numbered
- Font Arial 12 should be used for the text, and Arial 11 for tables and references
- The sections should typically be assembled in the following order: Title, Authors,

Authors' full affiliations including department and post/zip codes, Corresponding author, Short title, Abstract, Keywords, Implications, Introduction, Material and methods, Results, Discussion, Acknowledgements, References, Tables, List of figure captions

- The use of small paragraphs with less than 6 to 8 lines must be avoided
- Footnotes in the main text are to be avoided
- The manuscript complies with the section specific requirements set out below

Full title

The title needs to be concise and informative. It should:

(a) arrest the attention of a potential reader scanning a journal or a list of titles;
(b) provide sufficient information to allow the reader to judge the relevance of a paper to his/her interests;
(c) incorporate keywords or phrases that can be used in indexing and information retrieval, especially **the animal species** on which the experiment has been carried out;
(d) avoid inessentials such as 'A detailed study of ...', or 'Contribution to ...';
(e) not include the name of the country or of the region where the experiment took place;
(f) not include Latin names if there is a common name, or abbreviations.

Full title directions

- No more than 170 characters including spaces
- Include "Review:", "Invited review:" or "Animal board invited review:" before the full title if required (see above)
- The title of an invited opinion paper should start with "Opinion paper:"
- The title of a short communication should start with "Short communication:"

Authors and affiliations

The names and affiliations of the authors should be presented as follows:

Example

J. Smith[1,a], P.E. Jones[2], J.M. Garcia[1,3] and P.K. Martin Jr[2] [initials only for first names]
[1]*Department of Animal Nutrition, Scottish Agricultural College, West Main Road, Edinburgh EH9 3JG, UK*
[2]*Animal Science Department, North Carolina State University, Raleigh, NC 27695-7621, USA*
[3]*Laboratorio de Producción Animal, Facultad de Veterinaria, Universidad de Zaragoza, C. Miguel Servet, 177, 50013, Zaragoza, Spain*

[a]*Present address: Dairy Science Laboratory, AgResearch, Private Bag 11008, Palmerston North, New Zealand* (for any author of the list whose present address differs from that at which the work was done)

Corresponding author: John Smith. E-mail: John.Smith@univ.co.uk.

The corresponding author who submits and manages the manuscript during the submission/review process will need to be registered on Editorial Manager. He or she can be different from the corresponding author indicated in the manuscript who will be the correspondent for the published paper.

Short title (max 50 characters including spacing)

Authors should provide a short title (after the corresponding author line) with the same specifications as the full title for use as a running head. If the short title is not appropriate, it could be modified by the Editorial Office, with the author's agreement.

Abstract (max 400 words, single paragraph)

The abstract should be complete and understandable without reference to the paper. It is important to attract the attention of potential readers. The context and the rationale of the study are presented succinctly to support the objectives. The experimental methods and main results are summarised but should not be overburdened by numerical values or probability values. The abstract ends with a short and clear conclusion. Citations, references to tables and figures are not acceptable. Abbreviations used in the abstract have to be defined in the abstract.

Keywords
Keywords are essential in information retrieval and should complement the title with respect to indicating the subject of the paper.

Keyword directions

- Five keywords
- Keywords should be short and specific
- If not in the title, the animal species or type is among the keywords
- The use of non-standard abbreviations in the list of keywords is discouraged

Implications (max 100 words)
Implications must explain the expected impact that the results may have on practice when they will be applied. Impact may be economic, environmental and/or social. Implications should not be limited to presenting the context and objectives, and should not be an "abstract of the abstract". This is written in simple English suitable for non-specialists or even non science readers. The use of non-standard abbreviations is discouraged.

Introduction
The introduction briefly outlines the context of the work, presents the current issues that the authors are addressing and the rationale to support the objectives, and clearly defines the objectives. For hypothesis driven research, the hypothesis under test should be clearly stated. Increasing the knowledge on a subject is not an objective *per se*.

Material and methods
Material and methods should be described in sufficient detail so that it is possible for others to repeat the experiment. Reference to previously published work may be used to give methodological details, provided that said publications are readily accessible and in English.

If a proprietary product is used as a source of material in experimental comparisons, this should be described using the appropriate chemical name. If the trade name is helpful to the readers, provide it in parentheses after the first mention. Authors who have worked with proprietary products, including equipment, should ensure that the manufacturers or suppliers of these products have no objections to publication if the products, for the purpose of experimentation, were not used according to the manufacturer's instructions.

Statistical analysis of results
The statistical analysis of results should be presented in a separate sub-section of the "Material and methods" section. The statistical design and the models of statistical analysis must be described, as well as each of the statistical methods used. Sufficient statistical details must be given to allow replication of the statistical analysis. The experimental unit should be defined (e.g. individual animal, group of animals). Generally, an analysis of variance is preferred to a simple *t*-test. A statistical guide for authors is available on the website at http://www.animal-journal.eu/statistical_instructions.htm. The publication of Lang and Altman (2013)[2] can also be used as a reference.

Statistics directions

- In the text, the level of significance attained is indicated by the following conventional standard abbreviations (which need not be defined): $P > 0.05$ for non-significance and $P < 0.05$, $P < 0.01$ and $P < 0.001$ for significance at these levels. Exact level of statistical significance (e.g. $P = 0.07$) can also be used
- When data are analysed by analysis of variance, a residual error term, such as the pooled standard error, the residual standard deviation (RSD) or the root mean square error (RMSE) is given for each criteria/item/variable/trait in a separate column (or line)
- Treatment means are reported with meaningful decimals. For guidance, the last digit corresponds to 1/10 of standard error

[2] Lang T and Altman D 2013. Basic statistical reporting for articles published in clinical medical journals: the SAMPL guidelines. In Science editors' handbook (ed. Smart P, Maisonneuve H and Polderman A), pp. 175-182. European Association of Science Editors, Exeter, UK. This document may be reprinted without charge but must include the original citation.

- In tables, statistical significance is indicated in a separate column. The *P* values (e.g. $P = 0.07$) are reported or levels of significance are indicated by *, ** and *** for $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively
- In tables, differences between treatments (or comparison of mean values) are indicated using superscript letters with the following conventional standard: a, b for $P < 0.05$; A, B for $P < 0.01$; in most cases, the 0.05 level is sufficient

Results - Discussion
Separation between Results and Discussion is preferred to highlight the interpretation of results. Presentation of Results and Discussion in a single section is possible but discouraged.

Acknowledgements
In this section, the authors may acknowledge (briefly) their support staff, their funding sources (with research funder and/or grant number), their credits to companies or copyrighted material, etc. All papers with a potential conflict of interest must include a description/explanation under the Acknowledgements heading.

References
Citations from international refereed journals or from national refereed journals with at least an English abstract are highly preferred. Citations should be as "international" as possible. Citations from abstracts/conference proceedings, MSc or PhD thesis, technical documents, not English documents which cannot easily be obtained by the reader or which are not peer-reviewed should be minimized. In general, no more than 3 references can be given for the same statement (except for reviews and meta-analyses).

Citation of references. In the text, references should be cited by the author(s) surname(s) and the year of publication (e.g. Smith, 2012). References with two authors should be cited with both surnames (e.g. Smith and Wright, 2013). References with three or more authors should be cited with the first author followed by *et al*. (in italics; e.g. Smith *et al*.). Multiple references from the same author(s) should be as follows: Wright *et al*. (1993 and 1994), Wright *et al*. (1993a and 1993b). Names of organisations used as authors (e.g. Agricultural and Food Research Council) should be written out in full in the list of references and on first mention in the text. Subsequent mentions may be abbreviated (e.g. AFRC). "Personal communication" or "unpublished results" should follow the name of the author in the text where appropriate. The author's initials but not his title should be included, and such citations are not needed in the reference list.

In-text citation directions

- References are cited by the name(s) of author(s) and the year of publication
- Use Doe (2014) or (Doe, 2014) for single authors
- Use Doe and Smith (2014) or (Doe and Smith, 2014) for two authors
- Use Doe *et al*. (2014) or (Doe *et al*., 2014) for three or more authors
- "*et al*." is in italics
- When multiple references are cited, rank them preferably by chronological order using commas and semicolons: (Doe, 1999; Smith and Doe, 2001; Doe et *al*., 2014 and 2015)

List of references. Literature cited should be listed in alphabetical order by authors' names and references should not be numbered. **It is the author's responsibility to ensure that all references are correct**.

Journal article directions

- References from journal articles are formatted as follows:

Author A, Author B, Author CD and Author E Year. Article title. Full Name of the Journal Volume, first-last page numbers.

Examples

- Berry DP, Wall E and Pryce JE 2014. Genetics and genomics of reproductive performance in dairy and beef cattle. Animal 8 (suppl. 1), 115–121.
- Knowles TG, Kestin SC, Haslam SM, Brown SN, Green LE, Butterworth A, Pope SJ, Dirk Pfeiffer D and Nicol CJ 2008. Leg disorders in broiler chickens: prevalence, risk factors and prevention. PLoS ONE 3, e1545.
- Martin C, Morgavi DP and Doreau M 2010. Methane mitigation in ruminants: from

microbe to the farm scale. Animal 4, 351-365.

- Pérez-Enciso M, Rincón JC and Legarra A 2015. Sequence- vs. chip-assisted genomic selection: accurate biological information is advised. Genetics Selection Evolution 47, 43. doi:10.1186/s12711-015-0117-5.
- When the article is online but not yet printed, the right format is:

Zamaratskaia G and Squires EJ 2008. Biochemical, nutritional and genetic effects on boar taint in entire male pigs. Animal, doi:10.1017/S1751731108003674, Published online by Cambridge University Press 17 December 2008.

- No punctuation (i.e. no comma or full stop or semicolon) between the surname and initials of an author, after initials, before publication years, after journal names and before volume numbers
- Include "and" (without comma) before the last author for multiple author references
- All authors' names are provided, do not use "*et al*." in the reference list
- Publication years are included after the author list without parentheses
- No capitals for article titles except initial capital of the first word and words that ordinarily take capitals
- All journal names are given in full (not in abbreviated form) and the initial letter of all main words is capitalised (except little words such as "and", "of, "in", "the", etc.), e.g. Journal of Animal Science
- Issue numbers are not mentioned
- Use "," (not ";") before page numbers
- Page numbers are given in full (e.g. "1488-1496" not "1488-96")

Book directions

- References from books or official reports are formatted as follows:

Author(s)/Editor(s)/Institution Year. Book title, volume number if more than 1, edition if applicable. Publisher's name, City, State (2-letter abbreviation) for US places, Country.

Examples

- Association of Official Analytical Chemists (AOAC) 2004. Official methods of analysis, volume 2, 18th edition. AOAC, Arlington, VA, USA.
- Littell RC, Milliken GA, Stroup WW and Wolfinger RD 1996. SAS system for mixed models. Statistical Analysis Systems Institute Inc., Cary, NC, USA.
- Martin P and Bateson P 2007. Measuring behaviour. Cambridge University Press, Cambridge, UK.
- National Research Council (NRC) 2012. Nutrient requirements of swine, 11th revised edition. National Academy Press, Washington, DC, USA.

- The list of author or editor name(s) and publication years are written as for journal articles (all authors are provided; commas between authors; "and" before the last author where there are two or more authors; full stops after publication years)

Example

- Author A, Author B, Author CD and Author E Year.

- No capitals for book titles except initial capital of the first word and words that ordinarily take capitals
- Detailed publisher information is given and listed as:

Publisher's name, City, State (2-letter abbreviation) for US places, Country.

Please note – if a publisher is based in more than one place, use only the first one. If multiple publishers are list, it is acceptable to use only the first one.

Examples

- AOCS Press, Champaign, IL, USA.
- Cambridge University Press, Cambridge, UK.
- International Organization for Standardization, Geneva, Switzerland.
- FAO, Rome, Italy.

Book chapter directions

- References from chapters or parts of books are formatted as follows:

Author A, Author B, Author CD and Author E Year. Chapter title. In Title of book (ed. A Editor and B Editor), pp. first-last page numbers. Publisher's name, City, State (2- letter abbreviation) for US places, Country.

Example

- Nozière P and Hoch T 2006. Modelling fluxes of volatile fatty acids from rumen to portal blood. In Nutrient digestion and utilization in farm animals (ed. E Kebreab, J

Dijkstra, A Bannink, WJJ Gerrits and J France), pp. 40–47. CABI Publishing, Wallingford, UK.

- The list of authors and publication years are written as for journal articles (all authors are provided; commas between authors; "and" before the last author where there are two or more authors; full stops after publication years)

Example

- Author A, Author B, Author CD and Author E Year.

- No capitals for chapter and book titles except initial capital of the first word and words that ordinarily take capitals
- Detailed publisher information are given and listed as:

Publisher's name, City, State (2-letter abbreviation) for US places, Country.

Please note – if a publisher is based in more than one place, use only the first one. If multiple publishers are listed, it is acceptable to use only the first one.

Examples

- AOCS Press, Champaign, IL, USA.
- Cambridge University Press, Cambridge, UK.
- Editions Quae, Versailles, France.

Proceedings/Conference papers directions

- References from proceedings or conference papers are formatted as follows:

Author A, Author B, Author CD and Author E Year. Paper title. Proceedings of the (or Paper presented at the) XXth Conference title, date of the conference, location of the conference, pp. first-last page numbers or poster/article number.

Please note – If proceedings are published in a journal, the article should be formatted as for a journal article and if they have been published as chapters in a book, the article should be formatted as for a chapter in a book.

Examples

- Bispo E, Franco D, Monserrat L, González L, Pérez N and Moreno T 2007. Economic considerations of cull dairy cows fattened for a special market. In Proceedings of the 53rd International Congress of Meat Science and Technology, 5-10 August 2007, Beijing, China, pp. 581–582.
- Martuzzi F, Summer A, Malacarne M and Mariani P 2001. Main protein fractions and fatty acids composition of mare milk: some nutritional remarks with reference to woman and cow milk. Paper presented at the 52nd Annual Meeting of the European Association for Animal Production, 26-29 August 2001, Budapest, Hungary.

- The list of authors and publication years are written as for journal articles (all authors are provided; commas between authors; "and" before the last author where there are two or more authors; full stops after publication years)

Example

- Author A, Author B, Author CD and Author E Year.

- No capitals for paper titles except initial capital of the first word and words that ordinarily take capitals
- Conference dates are provided in the format: DD Month YYYY, e.g. 10 August 2014
- Conference locations are given and listed as:

City, State (2-letter abbreviation) for US places, Country.

Examples

- Champaign, IL, USA.
- Cambridge, UK.
- Versailles, France.
- Geneva, Switzerland.

Website directions

- References from websites are formatted as follows:

Author(s)/Institution Year. Document/Page title. Retrieved on DD Month YYYY (i.e. accessed date) from http://www.web-page address (URL).

Examples

- Bryant P 1999. Biodiversity and Conservation. Retrieved on 4 October 1999, from http://darwin.bio.uci.edu/~sustain/bio65/Titlpage.htm

- The list of author name(s) and publication years are written as for journal articles (all authors are provided; commas between authors; "and" before the last author where there are two or more authors; full stops after publication years)

Example

- Author A, Author B, Author CD and Author E Year.

- No capitals for document/page titles except initial capital of the first word and words that ordinarily take capitals
- Dates when documents were retrieved are included in the format: DD Month YYYY, e.g. 10 August 2014
- Web-page addresses are provided

Thesis directions

- References from theses are formatted as follows:

Author AB Year. Thesis title. Type of thesis, University with English name, location of the University (i.e. City, State (2-letter abbreviation) for US places, Country).

Example

- Vlaeminck B 2006. Milk odd- and branched-chain fatty acids: indicators of rumen digestion for optimisation of dairy cattle feeding. PhD thesis, Ghent University, Ghent, Belgium.

- The author's name and publication year are written as for journal articles (no punctuation between surname and initials; full stops after publication years)

Example

- Author AB Year.

- No capitals for thesis titles except initial capital of the first word and words that ordinarily take capitals
- Degree levels are provided, e.g. PhD, MSc, etc.
- University names and locations are given and listed as:
- University name, City, State (2-letter abbreviation) for US places, Country.

Examples:

- Louisiana State University, Baton Rouge, LA, USA.
- Cambridge University, Cambridge, UK.

Tables

Tables should be as simple as possible. The same material should not be presented in tabular and graphical form. An indication is given in the text where the table should be inserted. Please refer to the style sheet available at http://www.animal-journal.eu/documents/Animal_style_template.doc.

Table directions

- Each table is on a separate page at the end of the main text (one table per page)
- Tables are typed, preferably in double spacing. Single spacing is possible for long tables
- Tables are numbered consecutively using Arabic numbering. They are referred to as Table 1, Table 2, etc., with capital 'T', no italics
- Each table has its own explanatory caption. The caption is sufficient to permit the table to be understood without reference to the text. The animal species and the experimental treatments or the issue under study are indicated in each caption. The caption does not contain too many details about the protocol or the results
- Tables are created in Word using the table function within the programme (without using tabs). Layout can be portrait or landscape
- Large tables are discouraged in the manuscript but they may be submitted as Supplementary Material
- No vertical lines between columns and no horizontal lines between rows of data
- Generally, variables are in rows and treatments in columns
- Column headings are concise
- Separate columns are included to present the basic statistical results: error terms (preferably residual error terms) and levels of significance
- Row items are organized with main items followed by indented sub-items in order, for instance, to group the criteria which share the same type of measurements or the same unit
- For any(sub-)item, only the first letter of the first word is in capitals
- Units are clearly stated either in the caption (only if a limited number of units are used), or for each (sub-)item. Standard abbreviations for units are used
- Footnotes are referenced using superscript numbers
- All abbreviations used in a table are defined as footnotes (preferred option) or in the

caption

- Treatment means are reported with meaningful decimals. For guidance, the last digit corresponds to 1/10 of standard error
- The number of decimals for the indicators of residual variability (RSD, SEM, RMSE etc.) are either identical to that chosen for mean values or have one more decimal. The choice is consistent in all the tables
- See above (Statistics) for the presentation of statistical results in tables

Figures

Figures should be as simple as possible. The same material should not be presented in tabular and graphical form. An indication is given in the text where the figure should be inserted. Specific guidelines are provided for images (see Image Integrity and Standards).

Figure directions

- Figure captions are all listed on the same page at the end of the main text
- All figures are numbered consecutively in the text. They are referred to as Figure 1, Figure 2, etc., the word 'Figure' being spelled out with capital 'F', no italics
- Captions begin as Figure 1, Figure 2, etc. They are sufficiently detailed to allow the figure to be understood without reference to the text ("Figure 1 Effect of fat source and animal breed on carcass composition in pigs" is preferred to "Figure 1 Carcass composition"). The animal species and the experimental treatments or the issue under study are indicated in each caption. Abbreviations used in each figure have to be defined in the caption and kept to a minimum
- Figures are not inserted in the text. Each figure (without caption) is uploaded separately with **one separate file per figure and no embedded captions in these files**
- Figure size should be readable in a width of approximately 175 mm (i.e. the maximum size of printing over two columns). Easy reading of the figure is required
- Ensure that the font size is large enough to be clearly readable at the final print size (should not be less than 8 point, or 2.8 mm, after reduction). We recommend you use the following fonts: Arial, Courier, Symbol, Times, Times New Roman and ensure that they are consistent throughout the figures. In addition, ensure that any fonts used to create or label figures are embedded if the application provides that option
- Symbols and line types should allow different elements to be easily distinguished (generally, solid symbols are used before open symbols, and continuous lines before dotted or dashed lines)
- Figures are usually supplied as black and white
- Colours can be used in figures if they are essential to understanding the figure. Publication charges are made for colour figures. The cost for reproducing figures in colour within the printed issue is £200.00 / $320.00 per figure
- If figures are to be printed in colour, use CMYK (instead of RGB) colour mode preferably
- The figures should preferably be provided as TIFF or EPS files. Other formats such as MS Word, MS Excel, MS PowerPoint, AI and layered PSD (up to CS3) are permitted, provided that figures have been originally created in these formats and that all the embedded artwork is at a suitable resolution.
- The resolutions for TIFF figures at the estimated publication size must be:
  - for line figures (e.g. graphs) – 1200 dpi (6000 px for 1 column, 8400 px for 2 columns)
  - for figures with different shadings (e.g. bar charts) – 600 dpi (3000 px for 1 column, 4200 px for 2 columns)
  - for half tones (e.g. photographs) – 300 dpi (1500 px for 1 column, 2100 px for 2 columns)
- Images from the internet are unacceptable, as most of them have a resolution of only 72 dpi
- When your drawing/graphics application does not provide suitable 'export' options, please copy/paste or import the graphic into a Word document
- For further information, please refer to the Cambridge Journals Artwork Guide, which can be found online at: http://journals.cambridge.org/artworkguide

Image Integrity and Standards

Any image produced by an instrument (e.g. scanner, microscopy…) with the objective of being used to derive quantitative results is considered as original data, and manuscripts that report images without any quantitative findings are not acceptable. Digitalisation of an image converts the image into numerical values which can be analysed like any other numerical value. The full information may prove important beyond what the author would like to show. Hence images submitted with a manuscript should be minimally processed; some image processing is acceptable (and may be unavoidable), but the final image must accurately represent the original data and exclude any misinterpretation of the information present in the original image. In case original data are being used just to illustrate a point, this should be accompanied by a very clear statement in the manuscript telling the reader this and explaining what is being demonstrated. Please refer to the Office of Research Integrity guidelines on image processing in scientific publication.

Image Integrity and Standards directions

- Image acquisition: Equipment and conditions of image acquisition and processing must be detailed in the Material and Methods section. This includes the make and model of equipment, the acquisition and the image processing software, and the image treatment if any. If you export files from an acquisition device, make sure to use a format with no loss of information and do not file them into a higher resolution than that of acquisition. Authors have the responsibility to archive original images, with their metadata, in their original format without any compression or compressed without loss of information.

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    2) The grouping of images from different parts of the same gel, or from different gels, fields, or exposures must be made explicit by the arrangement of the figure (i.e., using dividing lines) and in the text of the figure legend.
    3) Adjustments of brightness, contrast, or color balance are acceptable if they are applied to every pixel in the image and as long as they do not obscure, eliminate, or misrepresent any information present in the original, including backgrounds. Non-linear adjustments (e.g., changes to gamma settings) must be disclosed in the figure legend.

  For further information, image examples, and more detailed guidance we advise reading What's in a picture? The temptation of image manipulation (reprinted in the *Journal of Cell Biology* (2004) 166, 11-15).

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- The statistical analysis applied to the quantitative data associated with images must clearly define the statistical unit considered (e.g. the animal, the sample…).
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Supplementary material

Authors can include supplementary material in any type of text (research article, review article, short communication, etc.). Supplementary material will appear only in the electronic version. A link to this on-line supplementary material will be included by the Copy Editor at the proof preparation stage. Supplementary material will be peer-reviewed along with the rest of the manuscript. The main text of the article must stand alone without the supplementary material. Supplementary material should be presented according to the instructions for the main text. **It will not be copy-edited and authors are entirely responsible for the presentation of the supplementary material.**

Supplementary material directions

- In the main text, supplementary material are referred to as:

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For example: "The list of references used for the meta-analysis is given in Supplementary Material S1 and Supplementary Table S1 reports etc."

- Supplementary material is submitted along with the main manuscript in a separate file and identified at uploading as "Supplementary File – for Online Publication Only"
- The title of the article and the list of authors are included at the top of the supplementary material
- No line numbering
- Single spacing
- Unlike the figures included in the main text, each supplementary figure has its own title embedded below the figure

Typographical conventions

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As illustrated and detailed above and in the style sheet (see http://www.animal-journal.eu/documents/Animal_style_template.doc), the *animal* conventions apply to (*a*) *Title* of the paper, Authors' names and addresses; (*b*) *Main section headings* such as Abstract, Implications, Introduction, Material and methods, Results, Discussion, Acknowledgements, References; and (*c*) *Subheadings* which can be used at two levels only.

Title and heading directions

- Title – use bold, with an initial capital for the first word only and for words that ordinarily take capitals
- Authors' names – use lower case with initials in capitals (e.g. J. Doe)
- Authors' addresses – use italics
- Headings are left aligned with an initial capital for the first word only, and not numbered
- Main section headings – use bold with no full stop at the end; text follows on the next line (e.g. **Abstract**)
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All non-standard abbreviations are defined at first use separately in the abstract and in the main text, they should be written in **bold capitals at first occurrence**. To facilitate the understanding of the manuscript, the number of abbreviations should be kept to a minimum (not more than 10 non-standard abbreviations is advised). Abbreviations in the short title or in (sub)headings are discouraged.

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- Do not start a sentence with an abbreviation

Table 2 *Abbreviations that do not require spelling out*

Item	Definition
Standard abbreviation	
ACTH	Adrenocorticotropic hormone
ADF	Acid detergent fibre
ADL	Acid detergent lignin
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BLUP	Best linear unbiased prediction
BW	Body weight
CoA	Coenzyme A
CP	Crude protein Dry matter
DM	
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FSH	Follicle-stimulating hormone
GLC	Gas-liquid chromatography
GLM	General Linear Model
HPLC	High performance (pressure) liquid chromatography
IGF	Insulin-like growth factor
IR	Infrared
LH	Luteinising hormone
MS	Mass spectrometry
n	Number of samples
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
$NADPH_2$	Reduced nicotinamide adenine dinucleotide phosphate
NDF	Neutral detergent fibre
NIRS	Near infrared stectrophotometry
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction Pregnant mare serum
PMSG	gonadotropin
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
UV	Ultraviolet
Statistical standard abbreviation	
CV	coefficient of variation
df	degrees of freedom
EMS	expectation of mean square
F	variance ratio
LSD	least significant difference
MS	mean square
P	probability
use ns	$P>0.05$, in tables
use *	$P<0.05$, in tables
use **	$P<0.01$, in tables
use ***	$P<0.001$, in tables
r	simple correlation coefficient
R	multiple correlation coefficient
R^2	coefficient of determination
rSD	residual standard deviation
RMSE	root mean square error
SD	standard deviation
SED	standard error of difference
SEM	standard error of mean
Sy.x	standard error of estimate
χ^2	chi square

The names of the chemicals do not need to be written out in full; chemical symbols are sufficient. Fatty acids are abbreviated using the following rules: cis-18:1 for the sum of cis octadecenoic acids. When isomers are described, the double bond positions are identified by numbering from the carboxylic acid end: c9,t11-18:2; iso-15:0. The terms "omega 3" and "omega 6" are discouraged ed and replaced by "n-3" and "n-6", e.g. 18:3n-3. Trivial names can be used for the most known fatty acids (myristic, palmitic, oleic, linoleic, linolenic) and abbreviations in some cases: CLA for conjugated linoleic acids, EPA for eicosapentaenoic acid, DHA for docosahexaenoic acid. Chemical names and trivial names cannot be mixed in a same table.

Capitals

Capitals directions

- Initial capitals are used for proper nouns, for adjectives formed from proper names, for generic names and for names of classes, orders and families
- Names of diseases are not normally capitalised

Italics

Use italics for:

Italics directions

- Authors' addresses (see above)
- Subheadings (see above)
- Titles for tables (but not captions for figures)
- Most foreign words, especially Latin words, e.g. *ad hoc, ad libitum, et al., in situ, inter alia, inter se, in vitro, per se, post mortem, post partum, m. biceps femoris*

but no italics for c.f., corpus luteum, e.g., etc., i.e., NB, via

- Mathematical unknowns and constants
- Letters used as symbols for genes or alleles e.g. *HbA, Tf D* (but not chromosomes or phenotypes of blood groups, transferrins or haemoglobins, e.g. HbAA, TfDD)

Numerals

Numerals directions

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- Use figures for dates, page numbers, class designations, fractions, expressions of time, e.g. 1 January 2007; type 2
- Dates are given with the month written out in full in the text and with the day in figures (i.e. 12 January *not* 12th January).
- For time use 24-h clock, e.g. 0905 h, 1320 h

Units of measurement

The International System of Units (SI) should be used. A list of units is found at http://physics.nist.gov/cuu/Units/units.html. Recommendations for conversions and nomenclature appeared in *Proceedings of the Nutrition Society* (1972) 31, 239-247. Some frequently used units which are not in the SI system are accepted: l for litre, ha for hectare, eV for electron-volt, Ci for curie. Day, week, month and year are not abbreviated. The international unit for energy (energy value of feeds, etc.) is Joule (or kJ or MJ).

A product of two units should be represented as N·m and a quotient as N/m (e.g. g/kg and not $g.kg^{-1}$). When there are two quotients, present as follows: g/kg per day (not g/kg/day).

Concentration or composition
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