LETICIA GOMES DE MORAIS AMARAL

EXPRESSÃO DE GENES INTESTINAIS DE SUÍNOS PELO MODELO *EX VIVO*: EFEITO DE ÓLEOS ESSENCIAIS E LINHAGENS GENÉTICAS SENSÍVEIS À SOJA

LAVRAS - MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Zootecnia, área de concentração em Nutrição de Monogástricos, para obtenção do título de Doutora.

Orientador

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Aprovada em 13 de maio de 2015.

Dr. Brian Thomas Richert	*Purdue University*
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Orientador

LAVRAS – MG

2015

À minha mãe.

Apoiando mesmo quando não concorda!

DEDICO

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Muito obrigada!

RESUMO GERAL

A fim de compreender melhor os óleos essenciais e suas associações, foram realizados três estudos. O objetivo do primeiro trabalho foi determinar a expressão gênica intestinal pelo modelo *ex vivo* após a exposição do jejuno suíno a óleos essenciais e verificar o impacto desses óleos no desempenho produtivo de suínos na fase de creche. Para o ensaio *ex vivo*, doze leitões foram submetidos à eutanásia, e segmentos do jejuno foram incubados durante 60 minutos em *Dulbeco's Modified Eagles Medium* com diferentes óleos essenciais. Os tecidos foram lavados com PBS e armazenados a -80ºC com TRIzol para a determinação subsequente da expressão gênica de GAPDH, IAP, SGLT1, GLP-2R, ocludina, IGF1, MUC2 e RelA/p65. A expressão de IAP foi aumentada por CRINA® 700 ($P < 0,05$) em comparação com o carvacrol e cinamaldeído. Carvacrol apresentou tendência ($P < 0,10$) em ter maior expressão de IGF1 do que cinamaldeído. A expressão de GLP-2R tendeu a ser reduzida ($P < 0,10$) em comparação ao cinamaldeído. No desempenho não houve diferença ($P > 0,05$) entre os tratamentos para nenhuma das variáveis avaliadas. O segundo estudo foi realizado para avaliar a morfologia intestinal e a expressão gênica de jejuno de leitões desmamados que recebem uma fonte comercial de óleos essenciais. Animais alimentados com CRINA® na dieta apresentaram menor ($P < 0,05$) altura de vilosidade no jejuno e expressão IGF1. No entanto, estes mesmos animais demonstraram uma expressão mais elevada ($P < 0,05$) de SGLT1. Esses achados levam à conclusão que a composição do CRINA® tem substâncias que podem funcionar de forma antagônica sobre a saúde intestinal de suínos. O objetivo do terceiro estudo foi determinar a expressão gênica pelo modelo *ex vivo* de jejuno suíno a partir de duas linhagens genéticas de suínos (alta e baixa sensibilidade à proteína de soja e amendoim. Os animais da linha de alta sensibilidade apresentaram menor expressão de GLP-2R, ocludina e RelA / p65 ($P <0,05$). Em conclusão, o jejuno de leitões pode expressar de forma diferente GLP2, ocludina e RelA/p65, de acordo com a sensibilidade genética para as proteínas de soja.

Palavras-chave: Aditivo nutricional. Desmame. Expressão genética. Saúde intestinal. Suíno.

GENERAL ABSTRACT

In order to better understand essential oils and its associations, there were performed three studies. The objective of the first work was to determine gene expression in an *ex vivo* model following exposure of porcine jejunum to essential oils and verify the impact of these oils on growth performance of nursery pigs. For the *ex vivo* trial, twelve piglets were euthanized, and segments of the jejunum were removed. Intestinal segments were incubated for 60 minutes in Dulbeco's Modified Eagles Medium with the essential oils treatments. Tissues were rinsed with PBS and stored at -80ºC with TRIzol for subsequent determination of GAPDH, IAP, SGLT1, GLP-2R, Occludin, IGF1, MUC2, and p65/RelA gene expression. Gene expression for IAP was increased by CRINA® 700 ($P < 0.05$) compared to carvacrol and cinnamaldehyde. Carvacrol tended ($P < 0.10$) to have greater IGF1 expression than cinnamaldehyde. Expression of GLP-2R tended to be reduced ($P < 0.10$) by cinnamaldehyde compared to all other essential oils except CRINA700. For growth performance there were no differences ($P > 0.05$) among the treatments for any of the variables evaluated. The second study was conducted to evaluate intestinal morphology and gene expression of jejunum of post-weaned piglets receiving a commercial source of essential oils. Animals fed CRINA diets had lower ($P < 0.05$) jejunal villus height and IGF1 expression. However, these same animals demonstrated a higher expression ($P < 0.05$) of SGLT1. This findings lead us to infer that CRINA's composition have substances that may work antagonistically on pig intestinal health. The objective of the third study was to determine gene expression in an *ex vivo* model of porcine jejunum from these two genetic lines. The pigs from the high reactivity line had lower expression of GLP-2R, Occludin, and RelA/p65 ($P < 0.05$). These responses in the high reactive line may explain why they have the sensitivity to soybean and peanuts, since these genes are involved with intestinal growth, tight junctions and immune system, respectively. In conclusion, the jejunum of piglets can express differently GLP2, Occludin, and RelA/p65, according to genetic sensitivity to soy proteins.

Keywords: Gene expression. Intestinal health. Nutritional additive. Swine. Weaning.

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1 INTRODUÇÃO

O epitélio intestinal é formado por uma camada celular que compreende a maior e mais importante barreira do organismo contra o ambiente externo. Essa barreira atua com uma permeabilidade seletiva, permitindo a absorção de nutrientes, eletrólitos e água, enquanto mantém uma defesa contra toxinas, antígenos e patógenos luminais. Dessa forma, para obtenção de uma adequada saúde intestinal é necessária a garantia da integridade dessa estrutura.

Nesse sentido, estudos de avaliação de marcadores intestinais têm ganhado destaque nos últimos anos. A maior ou menor expressão de genes presentes no epitélio intestinal podem elucidar grande parte dos mecanismos de ação pelos quais componentes da barreira intestinal manifestam suas reações a diferentes condições ambientais, nutricionais ou fisiológicas.

Entretanto, as avaliações destes marcadores intestinais em pesquisas *in vivo* não elucidam com clareza os porquês das respostas encontradas, uma vez que essas estruturas são sensíveis a diversos fatores que não podem ser completamente isolados/controlados em animais, tais como perfil bacteriano, pH, concentração de nutrientes, entre outros.

Sendo assim, novas técnicas de processamento tecidual têm sido desenvolvidas para uma avaliação direta e específica da mucosa intestinal de suínos. Uma destas tecnologias é a avaliação *ex vivo*, pela qual é possível realizar avaliações de expressão gênica intestinal de diferentes substâncias em um mesmo animal. Com isso, é possível reduzir assim a variação de respostas individuais e o número de animais na pesquisa, bem como controlar exatamente a concentração da substância testada diretamente sobre a mucosa.

Através da metodologia de avaliação *ex vivo* é possível realizar pesquisas com suínos tanto com aplicações na nutrição animal (teste de substâncias

substitutas de antibióticos promotores de crescimento), como modelo animal para saúde humana (animais alérgicos a proteínas).

Na nutrição animal uma fase de bastante interesse é o momento pós-desmame, em que os animais enfrentam queda no crescimento e aumento de disfunções intestinais. Geralmente esses fatores são controlados com a utilização de antibióticos promotores de crescimento, porém tais substâncias foram banidas com este propósito na União Europeia e a tendência é que esta prática seja aplicada em todo o mundo. Dessa forma, a busca por substâncias capazes de substituir, mesmo que parcialmente, os antibióticos promotores de crescimento, tem crescido. Entre os aditivos nutricionais com potencial para desempenhar funções melhoradoras de saúde intestinal estão os óleos essenciais. Essas substâncias são obtidas natural ou sinteticamente e apresentam uma gama de ações, como antimicrobiana (carvacrol), antioxidante (timol), estimuladora do sistema imune (cinamaldeído), entre outras.

Com relação a pesquisas em suínos como modelo animal para a saúde humana, essa espécie apresenta grande vantagem em relação às demais, pois sua fisiologia intestinal se assemelha à de humanos anatômica e histologicamente. Sendo assim, as reações observadas em suínos que possuam alergia a proteínas podem ser extrapoladas para as reações observadas em humanos, auxiliando assim na descoberta não somente dos mecanismos de ação pelos quais o processo alergênico desenvolve, como também no desenvolvimento de tratamentos possíveis para essa condição que afeta grande parte da população mundial.

Dessa maneira, o objetivo neste trabalho foi avaliar a expressão de genes intestinais pelo modelo *ex vivo* com aplicações na produção animal (suínos submetidos a diferentes tratamentos com óleos essenciais) e na saúde humana (linhagens genéticas de suínos com diferentes sensibilidades à proteína de soja).

2 REFERENCIAL TEÓRICO

2.1 Marcadores intestinais

O trato digestório é um ambiente de alto metabolismo e dinamicidade. Para manter sua funcionalidade, diversos marcadores intestinais são mais ou menos expressos no sentido de manter a homeostase deste sistema. Os marcadores que compõem o intestino delgado de suínos estão intimamente ligados e relacionados, podendo ser um parâmetro de avaliação da saúde intestinal (MONTAGNE et al., 2007), podem ser, de forma geral, divididos em: tróficos, digestórios e/ou imunológicos.

2.1.1 Marcadores intestinais tróficos

Os marcadores intestinais de ação trófica compreendem muitas funções. Entre os principais marcadores estudados atualmente, podem ser citados o IGF1 (fator de crescimento semelhante à insulina 1 - *Insulin-like Growth Factor* 1), o GLP-2R (receptor do peptídeo semelhante ao glucagon 2 – *Glucagon-like peptide* 2 *receptor*) e a ocludina (proteína transmembrana localizada nas *tight juctions*).

O fator de crescimento semelhante à insulina 1 (IGF1) apresenta influência direta sobre o crescimento intestinal. Esse marcador tem se apresentado sensível a variações nutricionais (ESTÍVARIZ; ZIEGLER, 1997), apresentando características únicas, podendo implicar em funções cruzadas com vias nutricionais, hormonais e inflamatórias.

Já o glucagon-like peptide 2 receptor (GLP-2R) é o receptor específico do GLP-2, hormônio que pode apresentar uma grande gama de ações no intestino dos animais, entre as quais: estímulo do crescimento de mucosa de intestino delgado e grosso pela ativação de IGF1 (DUBÉ et al., 2006), inibição da apoptose de

enterócitos e células da cripta, estímulo do transporte de glicose pelo enterócito bem como a expressão de GLUT-2R, aumento da absorção de nutrientes, inibição do esvaziamento e secreção gástricos, redução da permeabilidade intestinal, estímulo do fluxo sanguíneo intestinal, relaxamento da musculatura lisa intestinal, entre outros. Diante de tantos efeitos específicos e indiretos, esse hormônio pode ser estudado frente ao seu marcador específico, o GLP-2R, sendo que sua expressão pode sofrer estímulo ou inibição frente a variados fatores, tais como: alteração de pH intestinal, nutrientes presentes no lúmen intestinal, estimulação da atividade do epitélio intestinal, etc.

Apesar de inúmeros trabalhos terem demonstrados os efeitos tróficos do GLP-2, os exatos mecanismos pelos quais isso ocorre ainda não foram elucidados. O receptor de GLP-2 não fica localizado essencialmente onde age o hormônio, e sim em células enteroendócrinas (YUSTA et al., 2000), neurônios entéricos (BJERKNES; CHENG, 2001; ORSKOV et al., 2005) e miofibroblastos bubepiteliais (ORSKOV et al., 2005; RAMSANAHIE et al., 2003), sugerindo que o receptor específico de GLP-2 não seria a única via de ação deste hormônio nas ações intestinais tróficas (DUBÉ et al., 2006).

A cascata de ativação do GLP-2R é complexa e desencadeia diversas ações sobre o processo digestivo animal. Uma vez ativado ocorre o aumento da secreção de bicarbonato pela liberação secundária de peptídeo vasoativo intestinal e óxido nítrico (WANG et al., 2011).

Em relação à ocludina, essa proteína é parte da família das claudinas (FURUSE et al., 1998; MORITA et al., 1999) um grupo importante de proteínas ligadoras, sendo essencial para organização funcional e estrutural das *tight junctions* (FANNING et al., 1998). Especificamente, sua atuação nas *tight junctions* compreende a manutenção da integridade e da barreira funcional dessa estrutura (FURUSE et al., 1996; McCARTHY et al., 1996; WONG; GUMBINER, 1997). Alguns autores (ZHANG; GUO, 2009), avaliando a expressão de ocludina

demonstraram que existe a possibilidade de sua expressão ser afetada dieteticamente pelo fornecimento de zinco para suínos.

2.1.2 Marcadores intestinais digestórios

Entre os marcadores intestinais de efeito digestório, podem ser destacados o SGLT1 (transportador de glicose sódio dependente 1 - *Na^+-dependent glucose transporter* 1) e IAP (fosfatase alcalina intestinal – *Intestinal Alkaline Phosphatase*).

O SGLT1 media as funções de cotransporte de sódio e glicose tanto nos rins quanto no intestino, atuando como transportador ativo secundário (BREVES; KOCK; SCHRÖDER, 2007). A expressão desse transportador (somado à expressão de GLUT-2) foi determinada como crucial na absorção e transporte de glicose no intestino delgado (RODRIGUEZ et al., 2004).

Esse marcador intestinal pode ser regulado também pela nutrição da fêmea gestante durante a fase de gestação, em que a extrapolação das exigências recomendadas aumenta a expressão de SGLT1 no jejuno dos leitões, tanto ao nascimento quanto no momento do desmame (CAO et al., 2014). Essa sensibilidade pode ser observada também diante da nutrição direta do animal, sendo que dietas com baixo nível de fósforo reduzem a expressão deste marcador (VIGORS et al., 2014).

Em relação à IAP, sua expressão no intestino delgado é dependente da diferenciação do enterócito, sendo considerada como enzima-chave marcadora nas funções primárias digestivas e absortivas do intestino delgado (HODIN; CHAMBERLAIN; MENG, 1995). As funções fisiológicas da IAP incluem: hidrólise de esteres monofosfato (CARVER; WALKER, 1995), transporte transcelular de solutos (GASSER; KIRSCHNER, 1983) e participação na absorção de lipídeos (ZHANG et al., 1996).

Foi verificada a atividade de IAP no aumento de secreção de bicarbonato de sódio pelo intestino, permitindo a neutralização do ácido gástrico, o qual gera condições ótimas para atividade da IAP (AKIBA et al., 2007). Sabe-se ainda que a IAP apresenta funções de defesa intestinal, como fator detoxificante endógeno contra endotoxinas de lipopolissacarídeos bacterianos endógenos (GEDDES; PHILPOTT, 2008; POELSTRA et al., 1997). Sua regulação pode ser realizada por meios nutricionais (GOLDBERG et al., 2008), inflamatórios (MALO et al., 2006) e hormonais (MALO et al., 2004). Baixo pH reduz a ação da IAP (AKIBA; KAUNITZ, 2014).

Dados recentes destacam que a IAP apresenta algumas propriedades diretas sobre a proteção da barreira intestinal e indiretas na redução de fatores relacionados à inflamação, sendo que sua administração exógena melhora tanto a inflamação intestinal quanto a regeneração tecidual (LALLÈS, 2014).

2.1.3 Marcadores intestinais imunológicos

Entre os marcadores de aspecto imunológico, podem ser destacados o MUC2 (gene que codifica a produção de mucina - *gene encoding mucin production*) e a RelA/P65 (subunidade de NF-κB).

O epitélio do trato gastro-intestinal (TGI) é coberto por uma camada de mucina, considerada principal representante da resposta imune inata (MONCADA; KAMMANADIMINTI; CHADEE, 2003). Mucinas podem ser categorizadas com base em sua localização e estrutura em duas classes principais: MUC2 e MUC3. No intestino delgado, as mucinas secretadas são produzidas pelas células de Goblet e formam um gel mucoso, com predominância de MUC2. Mucinas ligadoras de membrana, especialmente MUC3, são expressas por enterócitos (CHANG et al., 1994; MONCADA; KAMMANADIMINTI; CHADEE, 2003).

A expressão de MUC2 aumenta no cólon de leitões na fase de creche conforme a idade (LIU et al., 2014). Essa expressão é também influencidada pela dieta fornecida aos animais, especialmente com utilização de probióticos, como o *Lactobacillus fermentum* (YU et al., 2008) ou *Lactobacillus plantarum* (MACK et al., 1999), sendo que lactobacilos, em geral, podem afetar diferentemente o sistema imune inato dos animais (YANG et al., 2015)

A RelA/P65 é uma das cinco subunidades do NF-κB, e predomina com o componente P50 (GHOSH; MAY; KOPP, 1998). Quando inativado, o NF-κB é sequestrado no citoplasma pela associação com proteínas inibitórias. Em resposta a uma gama de estímulos estressores (como lipopolissacarídeos, fator de necrose tumoral, interleucinas e infecções virais), pode apresentar uma variedade de sequências nucleares que permite ao NK-κB penetrar no núcleo e ativar a transcrição de diversos genes-alvo (MAY; GHOSH, 1998). Um dos maiores desafios é entender os mecanismos de regulação do crescimento celular pelo NF-κB em resposta ao estresse ambiental e elucidar como o sinal transdutor das vias ativadas e como ativar diferentes respostas celulares (CHEN et al., 2001).

Em situações de desafio imunológico, NF-κB é ativado, sendo que especificamente a RelA/p65 apresentou um comportamento de maior ativação nuclear do que citoplasmática em células intestinais de animais sob infecção com PEDV (vírus da diarreia suína epidêmica - *Porcine epidemic diarrhea virus*), (CAO et al., 2015) levando à discussão de quais seriam os mecanismos de ação desses fatores.

Dessa forma, a avaliação de marcadores intestinais pode ser um canal para melhor compreensão da responsividade deste órgão às diferentes situações expostas. Sendo assim, metodologias que propiciem esta avaliação direta passam a ser uma alternativa de interesse para a pesquisa, especialmente o ensaio *ex vivo*.

2.2 Metodologia *ex vivo* de avaliação intestinal

Diante da necessidade de uma avaliação mais direta e específica de substâncias sobre o epitélio intestinal, a técnica de avaliação *ex vivo* foi desenvolvida (SUGIHARTO; JENSEN; LAURIDSEN, 2012). Com este processo é possível estudar diferentes substâncias em um mesmo indivíduo, partindo do princípio que o tecido intestinal apresenta funcionalidade mesmo após isolado do animal.

Essa técnica consiste em realizar a coleta de tecido intestinal de animais imediatamente após o abate e, no prazo de uma hora, avaliar a reação desse tecido ainda viável sob diferentes tratamentos.

Para isso, os animais devem ser eutanasiados e, imediatamente em seguida, o tecido intestinal deve ser coletado e acondicionado em tubos contendo solução salina e uma mistura antibiótica aquecidos a 37ºC. A mistura antibiótica é utilizada para bloquear instantaneamente a ação das possíveis bactérias presentes no segmento e minimizar os efeitos paralelos aos dos tratamentos a serem avaliados e a temperatura descrita minimiza o choque térmico sofrido pelo tecido.

Cerca de 10 minutos após imergido na solução antibiótica, o tecido é removido e seccionado longitudinalmente para uma suave limpeza do conteúdo com solução salina. Em seguida, é realizada a secção do tecido de modo a obter-se um material de 2,0 cm^2 (Figura 1). As amostras são então incubadas em um meio específico (*Dulbecco's Modified Eagle Medium*) com adição do tratamento específico a ser aplicado. Em casos que o tratamento seja solúvel em água, a incubação pode ocorrer de forma simplificada, em estufa de CO_2 a 37ºC. Entretanto, quado se trata de materiais insolúveis, é necessária a utilização de um agitador que mantenha as amostras em contínua mistura durante toda a incubação.

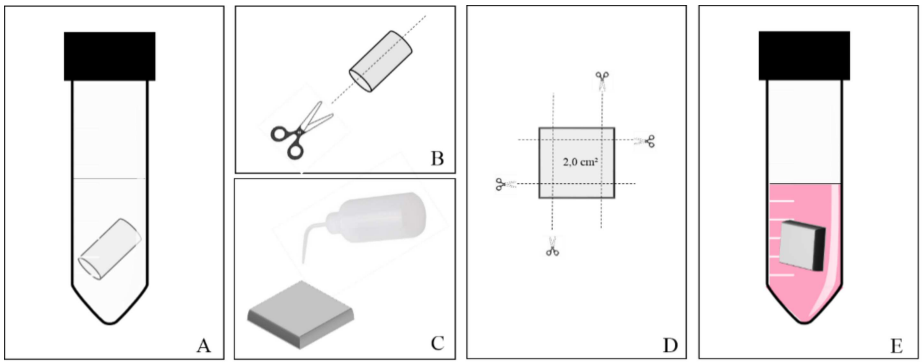

Figura 1 Esquema de processamento de amostras intestinais para procedimento *ex vivo*: A) Amostra em tubos contendo solução salina e mix de antibióticos; B) Secção longitudinal do segmento; C) Enxágue com solução salina para remover conteúdo intestinal; D) Remoção das extremidades do segmento para obter uma amostra com 2,0 cm²; E) Inclusão da amostra em tubo contendo *Dulbecco's Modified Eagle Medium* adicionado do tratamento experimental

Após o período de incubação é realizada a raspagem (Figura 2A) do tecido para obtenção de amostras para isolamento de RNA. O material raspado (Figura 2B) é acondicionado em um tubo contendo TRIzol (500 µL) (Figura 2C) e imediatamente congelado e armazenado a -80 ºC até o momento da análise.

Como toda técnica laboratorial, existem vantagens e desvantagens na sua aplicação. Entre as desvantagens, efeitos tais como interação com a dieta nutricional, efeito do pH intestinal, ou reações frente à população microbiana não são possíveis de serem registrados ou avaliados, mesmo que o desafio bacteriano já tenha sido demonstrado (SUGIHARTO; JENSEN; LAURIDSEN, 2012).

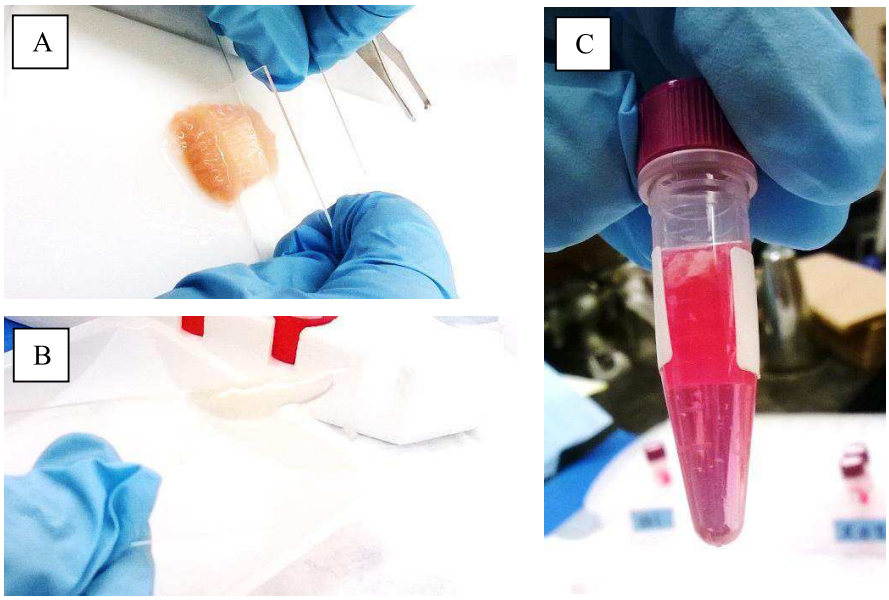

Figura 2 A) Raspagem do tecido após a incubação; B) Material raspado; C) Tubo contendo TRIzol com a amostra coletada

Por outro lado, essa técnica permite isolar o efeito do animal sobre as respostas, uma vez que todos os tratamentos são realizados em um mesmo animal e, naturalmente, permite a utilização de menos animais na pesquisa, atendendo aos requisitos sempre crescentes de bem-estar animal. Além disso, com esse modelo é possível avaliar efeitos exclusivos dos tratamentos sobre diferentes marcadores intestinais, explicitando no nível molecular seus mecanismos e potenciais de ação no organismo animal, muitas vezes direcionando as pesquisas *in vivo*.

Em vista das características desse método, fica claro sua aplicabilidade em pesquisas com óleos essenciais, uma vez que com essa metodologia é possível explorar as ações diretas dessas substâncias individualmente sobre o epitélio intestinal. Essas ações incluem o aumento ou diminuição de marcadores intestinais que representem um possível impacto sobre a saúde intestinal e, em

amplo espectro, desempenho dos animais, especialmente no momento pós-desmame.

2.3 Impactos do desmame na saúde intestinal

A fase de creche é um dos momentos mais críticos do sistema de produção de suínos devido ao estresse do momento da desmama. Este momento fisiológico, pelo qual passam todos os mamíferos, induz mudanças agudas transitórias relacionadas à queda de consumo pós-desmame, seguido de um período de maturação intestinal correspondente à retomada da ingestão de alimento (BOUDRY et al., 2004).

Esse processo causa impactos sobre a imunidade e o consumo alimentar, culminando com a manifestação de doenças e redução na taxa de crescimento (QUADROS et al., 2002). Um dos fatores que contribuem com o aumento no estresse dessa fase é a idade de desmame dos leitões, por volta de 24 dias de vida. Com essa idade, o leitão possui algumas limitações digestivas, como secreção insuficiente de enzimas, ácido clorídrico, bicarbonato e muco, fatores que dificultam a digestão e absorção adequada de nutrientes (LALLÈS; BOSI, 2007).

O desmame cada vez mais precoce implica em considerável queda de desempenho e aumento na incidência de diarreia. Está bem estabelecido que estes sintomas sejam multifatoriais, causados principalmente pelo baixo consumo nos primeiros dias pós-desmame (LALLÈS; BOUDRY, 2004; PLUSKE; HAMPSON; WILLIAMS, 1997). Algumas características fisiológicas foram comparadas por diversos pesquisadores nos últimos anos. Tem sido relatado também que o baixo consumo pós-desmame pode afetar a secreção do hormônio grelina orexígeno, localizado principalmente na mucosa estomacal. Este peptídeo é produzido sob um déficit de energia devido ao jejum, especialmente em animais jovens em fase de rápido crescimento (GILG; LUTZ, 2006).

As alterações no funcionamento gástrico têm recebido menos atenção do que as alterações intestinais, uma vez que o ambiente entérico é um importante segmento do TGI, responsável pela maior parte da digestão, absorção de nutrientes e trocas hídricas e minerais para manutenção da homeostase e suporte de uma microbiota complexa e altamente envolvida com o sistema imune.

As desordens de TGI no pós-desmame são resultado não só das alterações na estrutura e função do TGI, mas também principalmente por mudanças adaptativas à microbiota entérica (KONSTANTINOV et al., 2004) e respostas imunes (BAILEY et al., 2005; STOKES et al., 2004). Todos estes aspectos fisiológicos, microbiológicos e imunológicos do TGI contribuem de forma interativa para o equilíbrio intestinal. Mudanças na composição da dieta ao desmame alteram a composição da microbiota e suas atividades metabólicas. Uma grande quantidade de consequências na fisiologia do TGI tem sido elucidada, mas as influências no sistema imune local ainda são desconhecidas.

Em relação ao sistema imune, o momento do desmame gera grandes mudanças no grau e diversidade de exposição a antígenos ambientais, oriundos do alimento e de organismos potencialmente patogênicos. Sob condições naturais, o desmame é um processo gradual, que se completa por volta de 10 a 12 semanas de idade dos leitões. Mas essa não é a realidade produtiva, em que os animais são desmamados com cerca de três semanas. Como consequência disso, a diarreia pós-desmame comumente acontece e não está relacionada apenas com a idade do leitão, mas sim com o evento do desmame (LALLÈS; BOSI, 2007). Ao contrário do nascimento, o desafio antigênico ao desmame ocorre quando o sistema imune da mucosa está desenvolvido ao ponto de ativar as respostas imunes. Somado a isso, os componentes imunorregulatórios e imunoprotetores do leite materno já não estão presentes. Anticorpos contra os antígenos alimentares já podem ser detectados após o desmame, indicando respostas imunes contra eles. Com o tempo

as respostas assumem uma forma de tolerância, mas não ocorrem imediatamente (BAILEY et al., 2004; MILLER et al., 1994).

Mudanças nos padrões de citocinas intestinais no pós-desmame são caracterizadas por diferenças segmentares ao longo do TGI, e incluem respostas de fase aguda com aumento da expressão gênica de citocinas inflamatórias (IL-1β, IL-6, TNF-α) (PIÉ et al., 2004). Na sequência ocorre uma resposta duradoura que retorna aos níveis normais pré-desmame, exceto o TNF-α, que permanece alto no íleo e cólon. Após o desmame as concentrações teciduais de citocinas anti-inflamatórias e fatores de crescimento TGF-β apresentam-se transitórios da vilosidade para a cripta de duodeno e jejuno (MEI; XU, 2005). Essas mudanças coincidem com as variações na estrutura vilosidade/cripta e nas atividades enzimáticas digestivas. Ainda pode-se afirmar que existem correlações complexas entre o tempo pós-desmame e a densidade de células imunes das vilosidades do jejuno (BROWN et al., 2006).

Diante dessa situação de estresse e alta influência ambiental, nutricional e sanitária sobre o leitão desmamado, faz-se necessário conhecer os principais parâmetros determinantes da saúde intestinal de leitões, especialmente no que se refere aos aditivos nutricionais que podem impactar a saúde intestinal dos animais, como os antibióticos e seus substitutos.

2.4 Desafio da retirada de antibióticos na fase de creche

Antibióticos adicionados às dietas como promotres de crescimento (APC) atuam diretamente no trato digestório de suínos. Eles suprimem microrganismos que competem por nutrientes e produzem substâncias tóxicas não desejáves. Como consequência, é possível obter um ambiente ótimo para a mucosa intestinal, permitindo uma eficiente absorção de nutrientes (FRANÇOIS; MICHEL, 1968). A partir dessa melhor utilização de nutrientes, índices de desempenho como a

conversão alimentar e ganho de peso são comumente melhorados. Além disso, a condição sanitária dos animais recebendo APC que são criados em situações de desafio ambiental torna-se melhor em até 15% do esperado (WENK, 2003).

Entretanto, essas substâncias passaram por banimento como promotoras de crescimento na Europa, diante da pressão de consumidores com base na teoria de que ocorra transferência de bactérias resistentes de animais de produção para humanos. Apesar deste risco de transmissão não ter sido epidemiologicamente comprovado (BAGER, 1997; SCAN, 1996) e estar mais relacionado à má utilização de antibióticos terapêuticos em humanos, o banimento foi realizado e existe a tendência de que essa exigência atinja outros produtores de suínos pelo mundo.

Com isso, é importante o conhecimento de outras substâncias com potencial antimicrobiano para substituir os antibióticos. Diversos aditivos alimentares têm sido avaliados e podem ser subdivididos em: 1) melhoradores da resposta imune, como imunoglobulinas, ácidos graxos ω-3, β-glucanos derivados de leveduras; 2) redutores da população bacteriana patogênica intestinal, como os ácidos orgânicos e inorgânicos, altos níveis de óxido de zinco, óleos essenciais, extratos herbais, alguns tipos de prebióticos, bacteriófagos e peptídeos antimicrobianos; 3) estimuladores da população bacteriana benéfica, como probióticos e alguns prebióticos; e 4) estimuladores da função digestiva, como ácido butírico, frutoligossacarídeos, ácido glucônico, ácido lático, glutamina, arginina, treonina, cisteína e nucleotídeos (LANGE et al., 2010).

Apesar de diversos substitutos terem sido avaliados por diversos centros de pesquisa, não foi encontrada substância capaz de substituir com total satisfação os APC, fazendo com que sejam necessárias ainda mais pesquisas com diferentes compostos capazes de demonstrar tanto efeitos sobre microrganismos, quanto efeitos benéficos sobre o hospedeiro. Nesse cenário de substituição, os óleos

essencias têm ganhado destaque diante das características potenciais que essas substâncias naturais apresentam.

2.5 Óleos essenciais e suas ações sobre a saúde intestinal

Os óleos essenciais são também conhecidos como óleos voláteis e podem estar concentrados em produtos vegetais que contêm componentes aromáticos. Essas misturas de componentes voláteis (principalmente quiterpenos, benzóis e fenilpropanois) exercem diferentes ações biológicas (BASER; BUCHBAUER, 2010), sendo utilizadas em diversas áreas, como perfumaria, higiene, cosméticos, etc. Entretanto, os óleos essenciais são também muito utilizados no tratamento de diversas doenças e suas aplicações medicamentosas tornaram-se muito populares tanto na medicina humana quanto na alimentação animal.

Os efeitos dos diferentes óleos essenciais podem variar não somente devido à sua composição, mas também pela associação de diferentes óleos com efeitos diferentes, ampliando ainda mais as possibilidades de aplicações na nutrição animal.

Entre efeitos dos óleos essenciais descritos na literatura destacam-se: redução da ureia sanguínea (BENEVENGA et al.,1989), aumento da glicose sanguínea (ODLE; BENEVENGA; CRENSHAW, 1989), melhora no metabolismo energético (LEE; CHIANG, 1994), aumento do consumo de ração (ILSLEY et al., 2003), redução da massa microbiana ileal total e aumento da relação lactobacilos e enterobacterias (MANZANILLA et al., 2004).

Óleos essenciais apresentam características que vão além de seu efeito palatabilizante na dieta. Podem estimular as secreções intestinais, apresentar atividades antimicrobianas, coccidiostáticas, anti-helmínticas e anti-inflamatórias, bem como propriedades antioxidantes (WENK, 2003). Carvacrol e timol estão entre os óleos essenciais mais pesquisados. Entretanto, são

encontrados na literatura resultados conflitantes entre os efeitos destes ácidos. Enquanto alguns autores não detectaram resultados antimicrobianos, Manzanilla et al. (2004) e Muhl e Liebert (2007) verificaram que o uso de carvacrol aumentou a presença de lactobacilos e enterobactérias.

Muitas características biológicas dos óleos essenciais estão relacionadas à sua composição e estrutura molecular. Óleos fenólicos (timol, carvacrol e eugenol, por exemplo) são mais propensos a um efeito antioxidante do que óleos mono e sesquiterpenos (ADORJAN; BUCHBAUER, 2010). Existem diversos trabalhos na literatura avaliando óleos essenciais, especialmente a expressão gênica de marcadores imunológicos em linfócitos intraepiteliais intestinais de frangos de corte (KIM et al., 2010) a fim de compreender como tais substâncias desempenham seu papel anti-inflamatório e antibiótico.

Tabela 1 Descrição de diferentes óleos essenciais comumente estudados

Óleo Essencial	Fórmula molecular	Peso Molecular (g/mol)	Nomenclatura científica
Carvacrol	$C_{10}H_{14}O$	150,22	5-isopropil-2-metilfenol
Timol	$C_{10}H_{14}O$	150,22	5-metil-2-isopropilfenol 5-metil-2-(1-metiletil) fenol 2-isopropil-5-metilfenol
Cinamaldeído	C_9H_8O	132,16	trans-3-fenil-2-propenal
Eugenol	$C_{10}H_{12}O_2$	164,20	2-metoxi-4-(2-propenil) fenol 4-alil-2-metoxifenol 4-alilguaiacol

Entre os muitos componentes majoritários de óleos essenciais, o carvacrol apresenta algumas características singulares. Os efeitos antioxidantes e antimicrobianos do orégano (*Origanum acutidens*) são comumente atribuídos ao carvacrol, por ser seu componente majoritário (GOZE et al., 2010). Uma vez isolado e testado separadamente, os efeitos foram superiores ao do óleo de origem (GOZE et al., 2010).

Já o tomilho apresenta um óleo essencial com alta porcentagem de timol, mas também de carvacrol, p-cimene e γ-terpeno. Este óleo apresenta ótima atividade antioxidante (DANDLEN et al., 2010), o que pode alterar conforme o método de extração (VIUDA-MARTOS et al., 2010), prevenindo a peroxidação lipídica. As atividades dos óleos essenciais podem alterar de acordo com sua composição e proporção de componentes (especialmente os compostos por p-cimene, 1,8-cineol, β-felandreno, spatulenol e criptona) e ainda pode ocorrer sinergismo com outros componentes, como o carvacrol (MARTINO et al., 2010).

Outro componente muito estudado dos óleos essenciais é o cinamaldeído. Este componente é isolado do óleo essencial das folhas de canela (*Cinnamomum osmophloeum*) e tem sido identificado como inibidor da secreção de IL-1β e TNF-α na presença de LPS. Este componente isolado também suprime a produção de citocinas a partir do estímulo por LPS (CHAO et al., 2008), além de efeitos principalmente antibacterianos (CHANG; CHEN; CHANG, 2001).

Além destes, o eugenol é um dos componentes de alguns produtos comerciais à base de óleos essenciais. Apesar de ser relatado que sua inclusão de 1000 ppm para suínos na fase de crescimento não afetou o desempenho (YAN; KIM, 2012) esse óleo possui alta atividade antioxidante (HORVATHOVA et al., 2014) e impacto sobre as vias ativadoras de NF-κB (DEEPAK et al., 2015).

Além disso, são encontrados trabalhos que relatam a aplicação de produtos comerciais à base de óleos essenciais (LIU et al., 2013; NEILL et al., 2006) e associações destes (AKYUREK; YEL, 2011; LEVKUT et al., 2011; MANZANILLA et al., 2006; MATHLOUTHI et al., 2012), cujos resultados variam em relação à dose, espécie animal e associação de óleos aplicada, demonstrando a necessidade de conhecer melhor os mecanismos de ação individuais dessas substâncias.

Diante de tantas variações e interações dos diferentes óleos essenciais, conhecer o efeito específico de cada componente majoritário e seu mecanismo de

ação sobre o epitélio intestinal é o início das pesquisas no sentido de entender suas ações e efeitos sobre o desenvolvimento e saúde da mucosa intestinal de leitões. Para isso, a utilização de metodologias como o ensaio *ex vivo* torna-se uma alternativa interessante, não apenas para avaliações relacionadas ao impacto produtivo dessa espécie, mas também com relação à utilização de suínos como modelo animal para humanos.

2.6 Linhagens genéticas de suínos com sensibilidade a proteínas alimentares

Alergia a alimentos é uma condição em crescimento e afeta pessoas de todas as idades por todo o mundo. Este aumento na prevalência de alergias alimentares remete à necessidade de mais pesquisas para melhorar as estratégias de prevenção e tratamento. As reações alérgicas a alimentos podem variar de respostas leves à anafilaxia com risco de vida (BOYCE et al., 2010).

Os modelos animais possuem um grande potencial como ferramentas para ajudar a responder algumas das perguntas difíceis que ainda cercam a epidemia de alergia alimentar. Pesquisa em seres humanos é limitada por preocupações éticas e a possibilidade de reações anafiláticas fatais (BOCK et al., 2001). Este grande interesse estimulou o uso de modelos animais relevantes para prever possíveis gatilhos para a alergia, identificar possíveis mecanismos envolvidos na criação da via alérgica, bem como o teste de tratamentos terapêuticos (HELM, 2003, ALDEMIR; BARS; HEROUET-GUICHENEY, 2009; LEHRER; MCCLAIN, 2009). Com isso, o desenvolvimento de modelos animais para avaliação dos diferentes fatores alergênicos alimentares tem sido benéfico, tanto por permitir pesquisas mais rápidas, como por melhorar a compreensão dos mecanismos de ação envolvidos nos processos alérgicos e, até mesmo, teste de novos tratamentos para essas condições (VAN GRAMBERG et al., 2013).

Os fatores causadores de alergia mais comuns são leite de vaca, ovos de galinha, nozes e amendoins, enquanto menos comumente são observadas alergias à soja, trigo, peixe e ostras (SICHERER; SAMPSON, 2010; WANG; SAMPSON; 2011). Sabe-se que a alergia a alimentos é comum nos primeiros três anos de idade (BOCK, 1987), entretanto tem sido demonstrado que a maioria das alergias que se manifestam no início da vida, como a leite, soja e trigo, eventualmente são superadas. Por outro lado, alergia a amendoim, nozes, peixes e ostras normalmente continuam persistentes por toda a vida (HELM; ERMEL; FRICK, 2003; SICHERER; SAMPSON, 2010; MALONEY et al., 2008).

Os suínos são o modelo animal que apresenta grande vantagem comparado a outros modelos. A fisiologia intestinal dessas espécies é anatomicamente e histologicamente similar a dos humanos, com uma maior e mais diversificada microbiota encontrada em roedores (HELM; ERMEL; FRICK, 2003; HELM et al., 2002; PATTERSON; LEI; MILLER, 2008), apresentando também uma boa resposta imune (BOYCE et al., 2010). Características como essas são extremamente importantes na avaliação da patogênese e resposta imune a alérgenos alimentares.

Modelos de suínos foram previamente utilizados para investigar outras doenças alérgicas, tais como asma. Nessas pesquisas, animais com obstrução das vias aéreas, exibiam eosinofilia, e tardiamente, uma resposta asmática seguinte ao desafio alérgeno das vias aéreas, como tipicamente observada em pessoas asmáticas.

Rupa et al. (2008) realizaram a avaliação de leitões recém-nascidos com alergia induzida com componentes de ovos, sendo utilizadas três ninhadas de leitões Yorkshire, em que os animais foram sensibilizados via intraperitoneal nos dias 14, 21 e 35 com 100 μg de ovomucoide. Os animais foram monitorados durante 1 hora após o desafio para sintomas de alergia, sendo que esta foi validada

diante da reatividade da pele frente a um teste direto em que os animais sob tratamento-controle não apresentaram qualquer resposta.

Entretanto, pesquisas no modelo *in vivo* podem apresentar resultados conflitantes e, em algumas situações, mascarar os efeitos reais da sensibilidade aos alimentos devido a fatores externos, como características individuais dos animais ou fatores ambientais. Sendo assim, a avaliação *ex vivo* de suínos sensíveis à proteína da soja é uma proposta promissora na elucidação dos possíveis mecanismos de ação pelos quais este processo alérgico é estabelecido.

3 CONSIDERAÇÕES GERAIS

A grande variedade de pesquisas com leitões na fase de creche e a constante busca por soluções para os problemas observados durante este período, demonstram que estudos de base são um importante fator para auxiliar no entendimento de mecanismo de ação pelos quais novos aditivos nutricionais atuam sobre o epitélio intestinal.

Dessa forma, a aplicação de metodologias inovadoras, como *ex vivo*, pode ser o caminho para obtenção de respostas concretas sobre os mecanismos de ação pelos quais diferentes óleos essenciais atuam sobre o trato digestório, bem como o canal para novos direcionamentos e aplicações de aditivos na dieta de suínos, tanto com aplicação produtiva, como com aplicação na saúde humana.

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1 Essential oils affect intestinal gene expression using an *ex vivo* procedure, but not growth performance of nursery piglets

ABSTRACT – Essential oils have been shown to improve intestinal health of swine, which is of particular interest during the nursery period. However, the modes of action of essential oils are still relatively unknown. The objective of this work was to determine gene expression in an *ex vivo* model following exposure of porcine jejunum to essential oils and verify the impact of these oils on growth performance of nursery pigs. For the *ex vivo* trial, twelve piglets were euthanized, and segments of the jejunum were removed. Intestinal segments were incubated for 60 minutes in Dulbeco's Modified Eagles Medium with one of the following treatments: control–no supplement; 100 ppm eugenol; 100 ppm thymol; 100 ppm carvacrol; 100 ppm cinnamaldehyde; 100 ppm CRINA® (eugenol, thymol, 2-methoxyphenol, piperne and curcumin); 700 ppm CRINA®. Tissues were rinsed with PBS and stored at -80ºC with TRIzol for subsequent determination of GAPDH, IAP, SGLT1, GLP-2R, Occludin, IGF1, MUC2, and p65/RelA gene expression. For the growth performance trial, 150 weaned pigs were housed in nursery pens with 5 pigs (experimental unit), 5 repetitions and 6 treatments. The treatments included a nursery basal diets (by phase) without any growth enhancer or the basal diet supplemented with 28 ppm of eugenol, carvacrol, thymol, or cinnamaldehyde, or 200 ppm of CRINA®. Weekely growth performance was determined for 40 days post-weaning. Gene expression for IAP was increased by CRINA® 700 ($P < 0.05$) compared to carvacrol and cinnamaldehyde. Carvacrol tended ($P < 0.10$) to have greater IGF1 expression than cinnamaldehyde. Expression of GLP-2R tended to be reduced ($P < 0.10$) by cinnamaldehyde compared to all other essential oils except CRINA700. For growth performance there were no differences ($P > 0.05$) among the treatments for any of the variables evaluated. In conclusion, the evaluation by *ex vivo* procedure, on the proximal jejunum, showed that higher doses of CRINA® (700 ppm) increase the expression of IAP in comparison with essential oils that aren't in it's composition (carvacrol and cinnamaldehyde), but for GLP-2R and IGF1, carvacrol tend to be better than other oils. However, the inclusion in the feed of CRINA, at recommended manufacturer dosage, or individual essential oils, did not affect the nursery piglets growth performance.

INTRODUCTION

Early weaning piglets causes several problems in the swine production industry. These problems decrease the growth performance and are particularly related with intestinal health. Thus, the constant discussion on the ban of growth enhancing concentrations of antibiotics, directs research toward seeking alternative substances that act similarly in relation to the productive benefits but without generating the risk for selection of resistant bacteria.

Essential oils have gained prominence among the alternatives available because they have antimicrobial potential (carvacrol, eugenol – MATHLOUTHI et al., 2012; MICHIELS et al., 2009), antioxidant (thymol – ADORJAN; BUCHBAUER, 2010; DANDLEN et al., 2010), immunomodulatory (cinnamaldehyde - CHAO et al., 2008), among others. On the market, there are available comercial products, as CRINA® (LU et al., 2015; AZAIN et al., 2014) comprising varieties of essential oils that include the association of oils with synergistic effects and applications. With so many compositional variations, animal studies can provide a wide range of biological responses, making the results presented in the literature controversial, requiring further research on the effects of these substances when administered to animals in the nursery phase, as well as the individual responses of these compounds on animal performance. The *in vivo* effects of these essential oils have been evaluated (Si et al., 2006) but only on bacterial effects.

Therefore, it is necessary to evaluate individual essential oil's effects directly on the intestinal epithelium, since *in vitro* studies do not effectively represent their effects on a living organism.

Thus, a method recently developed and which may promote more accurate responses to the effect of additives on the intestinal epithelium is the *ex vivo* study. Through this model it is possible to evaluate the gene expression of

different intestinal markers after exposure to different major components of essential oils. Intestinal markers are responsible for modulating different biological effects. Such responses can be trophic effects (effect on the development of intestinal epithelium), digestive (influence on the digestion and absorption of nutrients) or immunological (impact on immune response of the animal).

The objective of this work was to evaluate the effect of different components of essential oils (eugenol, carvacrol, thymol, cinnamaldehyde) and comercial presentation (CRINA® - DSM) by the *ex vivo* procedure on gene expression of intestinal markers (IAP, IGF1, GLP-2R, MUC2, Occludin, RelA/p65, SGLT1, GAPDH) and growth performance in post-weaned pigs.

MATERIAL AND METHODS

All animal care and handling procedures used in this study were reviewed and approved by Purdue University Animal Care and Use Committee.

Experiment 1

Animals, experimental design and sample, and housing

The *ex vivo* procedure was performed as described previously (SUGIHARTO; JENSEN; LAURIDSEN, 2012). The jejunal samples of twelve crossbred pigs (White line composite: York x Large White x Landrace x Chester White) weaned at 23.8 d of age with initial BW of 6.95 ± 0.40 kg were assigned to be under 7 treatments simultaneously, being 12 replicates per treatment. *Ex vivo* treatments consisted of the following: *Dulbecco's Modified Eagle Medium* (DMEM) as basal treatment; DMEM + 100 ppm of carvacrol (W224502 – Aldrich), DMEM + 100 ppm of eugenol (E51791 – Aldrich), DMEM + 100 ppm of thymol (T0501 – Sigma), DMEM + 100 ppm of trans-cinnamaldehyde (C80687

– Aldrich), DMEM + 100 ppm of CRINA® (DSM - Nutritional Products Inc., Parsippany, NJ), and DMEM + 700 ppm of CRINA®. The 700 ppm for CRINA® was applied considering that this product contains 14% essential oils in its composition, thus providing 100 ppm of essential oils to match the other treatments.

Table 1 Composition of the basal diet for pigs fed for 8 days post-weaning, as fed-basis.

Item	Amount
Ingredient, %	
Corn	35.34
Soybean meal	14.00
Soybean Oil	4.00
Limestone	0.60
Mono-Calcium phosphate	0.10
Vitamin premix[1]	0.25
Trace Mineral premix[2]	0.175
Salt	0.25
Soy concentrate	4.05
Fish Meal	4.50
Dried Whey	22.00
Dried Skim Milk	14.00
L-Lys HCL	0.285
DL-Met	0.27
L-Thr	0.14
L-Trp	0.03
Starch	0.01
Calculated composition	
ME, Mcal/kg	3.57
CP, %	22.87
Total Lys, %	1.72
SID Lys, %	1.51
Ca, %	0.90
P, %	0.67
Available P, %	0.47
Lactose, %	22.54

[1]The vitamin premix provided the following per kg of diet: vitamin A, 6615 IU; vitamin D3, 661.5 IU; vitamin E, 44.1 IU; Menadione, 2.2 mg; vitamin B12, 38.6 µg; riboflavin, 8.8 mg; pantothenic acid, 22.1 mg; niacin, 33.1 mg;
[1]The trace mineral premix provided the following estimated available minerals per kg of diet: Zn, 121.3 mg from zinc oxide; Fe, 121.5 mg from iron carbonate; Mn, 15.0 mg from manganese oxide; Cu, 11.3 mg from copper chloride; I, 0.46 mg from ethylenediamine dihydroiodide; and Se, 0.3 mg as half from sodium selenite and half from organic selenium.

The basal diet was formulated to meet or exceeded NRC (2012) nutrient requirementsfor nursery pigs (Table 1). Pigs were housed in a completely enclosed nursery with slatted floors, a nipple drinker, and five-hole self-feeders per pen. All pigs were allowed ad libitum access to feed and water throughout the experiment period (8 d).

Sample collection

On d 8, all pigs were stunned by CO_2 and euthanized by the cranial vena cava exsanguination for intestine tissue sampling (Figure 1).

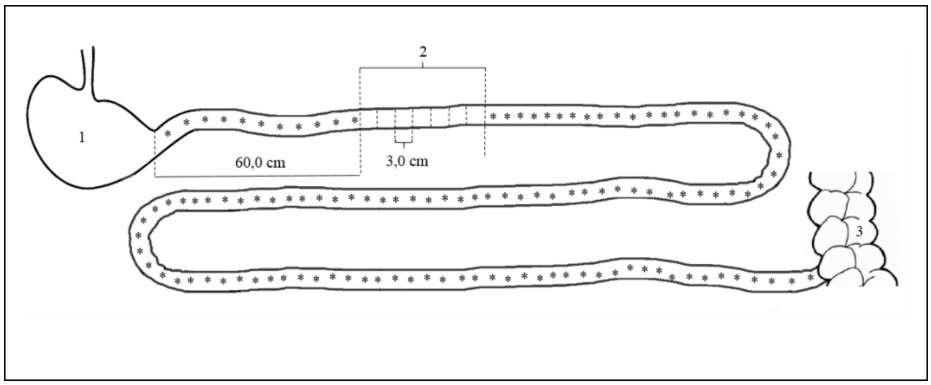

Figure 1 Scheme for sample collection to *ex vivo* trial. *Discard areas; 1) Stomach; 2) First seven jejunum segments for *ex vivo* analysis – 3.0 cm each sample; 3) Cecum

Jejunum samples were collected and placed in tubes containing 25 mL of PBS (saline solution) and 1% of antibiotic (peniciline + streptomycin + neomycin) mix (50 mM of mannitol + 2 mM of Tris-HCl). In order to minimize intestinal tissue stress at collection, the 1% antibiotic solution was kept heated at 37°C. After collection, the samples intended for the *ex vivo* procedure were processed as shown in Figure 2.

Jejunum samples were incubated in DMEM at 37°C for 60 min on rocking platform at the level of 3.5 setting (1.6 seconds per revolution;VWR-200 Rocking

platform, Radnor, PA, USA). Subsequently, mucosal scraping samples for RNA isolation were obtained by scraping the surface area of jejunal segment with a glass slide (Figure 3A, 3B) and placed in tube containing 500 µL of TRIzol reagent (Figure 3C). Scraping samples were snap-frozen in liquid nitrogen and stored at -80 ºC until analysis.

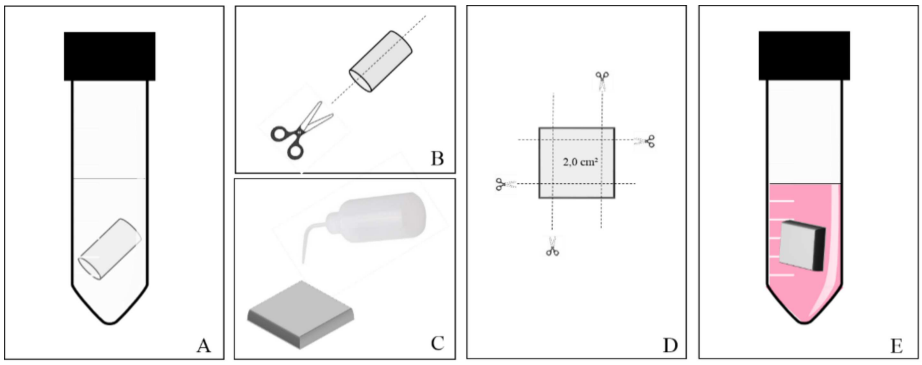

Figure 2 Sample processing scheme for *ex vivo* procedure. A) Sample in 25 mL of PBS and 1% of antibiotic mix; B) Longitudinal segment section; C) Rinsing with saline to remove intestinal content; D) Removal of segment edges to obtain a 2.0 cm² sample; E) Sample inclusion in *Dulbecco's Modified Eagle Medium* plus the specific experimental treatment (control, eugenol, thymol, carvacrol, cinnamaldehyde, CRINA®-100, or CRINA®-700)

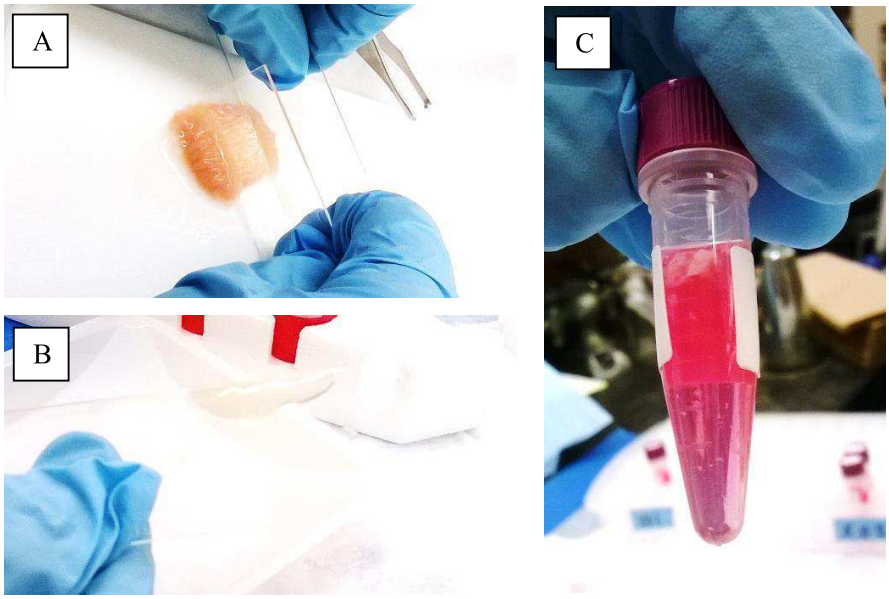

Figure 3 A) Scraping of tissue after incubating; B) Material scrapped; C) TRIzol tube with scrapped sample

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from jejunum mucosa samples using TRIzol reagent (Invitrogen Inc.; APPENDIX I). Total RNA concentration was quantified by using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, Waltham, MA, USA) at 260 nm, and the RNA purity was assessed by examining the ratios of absorbance at 260 (A260) and 280 (A280) nm. All samples had A260/A280 ratio above 1.8. Additionally, RNA integrity was verified by visualization of the 18S and 28S ribosomal RNA (rRNA) bands after ethidium bromide staining in a 1.0% agarose gel (Agarose LE Molecular Biology, DOT Scientific Inc., Burton, MI, USA). The presence of 2 well-defined bands (18S and 28S rRNA) is an indicator of good RNA integrity (Figure 4).

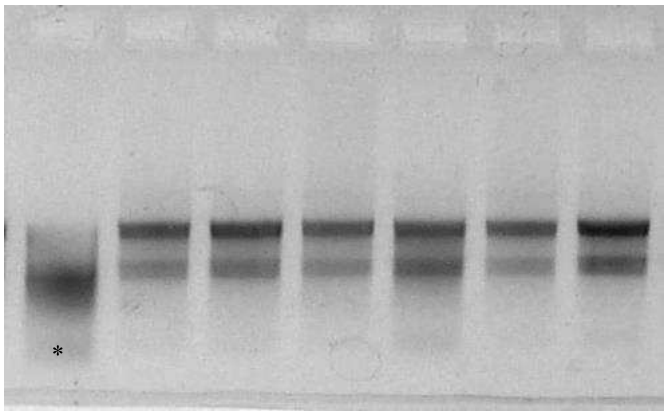

Figure 4 RNA integrity validation extracted from intestinal samples. Lanie 1 features degraded RNA (*).

Total RNA (1 µg) was reverse transcribed into complementary DNA (cDNA; APPENDIX III). Quantitative real-time PCR was performed using a MyiQ real-time PCR detection system (Bio-Rad, Hercules, CA). Primer sequences are shown in Table 2. Amplification was carried out in a total volume of 20 µL containing 10 µL of SYBR Green Supermix (Bio-Rad), forward and reverse primers (0.5 µg/µL each), and 5 µL of cDNA template. Reactions were incubated at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, 52.9 to 59.7°C for 30 s, and 72°C for 30 s. At the end of the PCR, melt curve analysis was conducted to validate the specificity of the primers. A non-template control (only DNAse free water in wells) was run with every assay to have the guarantee of none cross-contamination, and all determinations were performed in duplicate.

Table 2 *Primers* and annealing temperatures (AT) of evaluated genes

Gene[1]		Primer[2*]	AT (ºC)	Reference
GAPDH	F	TCACCATCTTCCAGGAGCG	59.7	Saddoris, Fleet e Radcliffe (2010)
	R	CTGCTTCACCACCTTCTTGA		
IAP	F	CTAAAGGGGCAGATGAATGG	54.4	Lackeyram et al. (2010)
	R	CACCTGTCTGTCCACGTTGT		
IGF1	F	GTGCGGAGACAGGGGCTTTT	53.0	Schedle et al. (2008)
	R	AGATCCGGAAGCAGCACTCA		
GLP-2R	F	ACCTTGCAGCTGATGTACAC	56.6	Petersen, Burrin e Sangild (2001)
	R	GTGTTCTCCAGGTGTGCACG		
MUC2	F	CTGCTCCGGGTCCTGTGGGA	53.0	Pieper et al. (2012)
	R	CCCGCTGGCTGGTGCGATAC		
Occludin	F	ATCAACAAAGGCAACTCT	53.0	Zhang and Guo (2009)
	R	GCAGCAGCCATGTACTCT		
RelA/p65	F	GGAACACGATGGCCACTTG	52.9	Los Santos, San Segundo e Grubman (2007)
	R	AAGAGGACATCGAGGTGTATTTCAC		
SGLT1	F	GGCTGGACGAAGTATGGTGT	53.0	Yang et al. (2011)
	R	ACAACCACCCAAATCAGAGC		

[1]GAPDH: Glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene); IAP: Intestinal Alkaline Phosphatase; IGF1: Insulin-like growth factor 1; GLP-2R: glucagon-like peptide 2 receptor; MUC2: gene encoding mucin production; Occludin: plasma-membrane protein located at the tight junctions; RelA/p65: REL-associated protein involved in NF-κB heterodimer formation; SGLT1: Na+ dependent glucose transporter 1; [2]F: forward; R: reverse; *By IDT (Integrated DNA Technologies®)

The mRNA abundance values for each sample were normalized to GAPDH and the control tissue for each individual pig according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) and the following calculations:

$$\Delta CT_{target\ gene} = CT_{target\ gene} - CT_{GAPDH} \quad (1)$$

$$\Delta\Delta CT_{treatment} = (\Delta CT_{treatment} - \Delta CT_{control})^2 \quad (2)$$

Statistical analysis

Data were analyzed by one-way ANOVA using the MIXED procedure (SAS Inst. Inc., Cary, NC, 2009) and pig served as the experimental unit. The Shapiro-Wilk test was used to analyze the normality followed by the data transformation using the RANK procedure (SAS Inst. Inc., Cary, NC, 2009). Once all the data was transformed, the outliers were eliminated based on standard deviation above 2σ. The model included essential oil treatment, and the residual mean square error was used as the error term. When ANOVA indicated a significant ($P < 0.05$) difference, the means were separated using the Duncan test. Differences were considered statistically significant at $P \leq 0.05$ and trends were considered with values $0.05 \leq P \leq 0.10$.

Experiment 2

Animals, housing, experimental design, and diet

A total of 150 weaned piglets (17.9 days old) with an equal number of gilts and barrows (Duroc x (Yorkshire x Landrace)) and 6.24 ± 1.12 kg of initial BW were selected from the Swine Research Center of Purdue University. The pigs were randomly assigned to treatments in a randomized complete block design with weight as the blocks and pen as the experimental unit. Pigs were housed five animals per pen for the 40 day study. There were a total of 30 pens, 5 repetitions and 6 treatments.

The basal diet for each phase was formulated to meet or exceed NRC (2012) estimates of nutrient requirements of weaned pigs during the 40 day nursery study (Tables 3 and 4). Spray-dried plasma, antibiotics, organic acids, and zinc oxide were not included in the basal control diet. The experimental diets were fed to pigs throughout the experiment. The 6 dietary treatments were the complex

nursery basal control diet (CON) or CON plus 28 ppm of either eugenol (E51791 – Aldrich), thymol (T0501 – Sigma), carvacrol (W224502 – Aldrich), trans-cinnamaldehyde (C80687 – Aldrich), or 200 ppm of CRINA® (DSM - Nutritional Products Inc., Parsippany, NJ). The 200 ppm for CRINA® was applied considering the maximum concentration recommended by the manufacturer and the oils concentration in the CRINA® product (14%) provided a proprietary blend of essential oils at 28 ppm concentration.

Table 3 Composition of the basal diets for nursery pigs fed for 40 days post-weaning, as fed-basis

	Experimental Basal Diets			
Item	Phase 1 (0 to 7 d)	Phase 2 (7 to 14 d)	Phase 3 (14 to 28 d)	Phase 4 (28 to 40 d)
Ingredient, %				
Corn	33.75	37.37	51.28	62.54
Soybean meal	14.00	20.00	26.21	31.34
Soybean Oil	4.00	4.00	3.00	2.00
Limestone	0.33	0.42	0.78	1.18
Mono-Calcium phosphate	0.50	0.54	0.56	1.02
Vitamin premix[1]	0.25	0.25	0.25	0.25
Trace Mineral premix[2]	0.18	0.18	0.18	0.18
Salt	0.25	0.25	0.30	0.35
Soy concentrate	8.00	5.00	-	-
Fish Meal	6.20	5.00	4.00	-
Blood Meal	1.50	1.00	-	-
Dried Whey	30.00	25.00	12.50	-
L-Lys.HCL	0.25	0.25	0.27	0.36
DL-Met	0.27	0.24	0.17	0.16
L-Thr	0.14	0.13	0.13	0.16
L-Trp	0.04	0.03	0.03	0.02
Phytase[3]	0.10	0.10	0.10	0.10
Anthelmintic[4]	-	-	-	0.10
Treatment corn premix	0.25	0.25	0.25	0.25

[1]The vitamin premix provided the following per kg of diet: vitamin A, 6615 IU; vitamin D3, 661.5 IU; vitamin E, 44.1 IU; Menadione, 2.2 mg; vitamin B12, 38.6 µg; riboflavin, 8.8 mg; pantothenic acid, 22.1 mg; niacin, 33.1 mg;
[2]The trace mineral premix provided the following estimated available minerals per kg of diet: Zn, 121.3 mg from zinc oxide; Fe, 121.5 mg from iron carbonate; Mn, 15.0 mg from manganese oxide; Cu, 11.3 mg from copper chloride; I, 0.46 mg from ethylenediamine dihydroiodide; and Se, 0.3 mg as half from sodium selenite and half from organic selenium;
[3]Phytase provided 600 PU/kg, Phyzyme, Danisco Animal Nutrition, Marlborough, Wilts, UK.
[4]Anthelmintic: Banminth® 48(pyrantel tartrate) Phibro Animal Health, Teaneck, NJ, USA.

Pigs were housed in a completely enclosed nursery with plastic slatted floors, and each pen contained a single nipple drinker and 5-hole self-feeder. All pigs were allowed *ad libitum* access to feed and water throughout the experiment period (40 d).

Table 4 Calculated nutrient composition of the basal diets for nursery pigs fed for 40 days post-weaning, as fed basiss

	Experimental Basal Diets			
Item	Phase 1 (0 to 7 d)	Phase 2 (7 to 14 d)	Phase 3 (14 to 28 d)	Phase 4 (28 to 40 d)
Calculated composition				
ME, Mcal/kg	3.49	3.50	3.46	3.41
CP, %	23.79	23.16	20.94	20.31
Total Lys, %	1.72	1.64	1.43	1.38
SID Lys, %	1.58	1.50	1.30	1.25
Ca, %	0.85	0.80	0.80	0.75
P, %	0.75	0.71	0.63	0.58
Available P, %	0.55	0.49	0.38	0.29
Phytase aval. P, %	0.60	0.55	0.45	0.37
Lactose, %	21.00	17.50	8.75	0.00

Drug Treatment

As the animals were under diets without antibiotics, daily they were evaluated for behavior and physical condition. Animals who showed signs of disease, as thin, low feed intake, fuzzy, loose stools, lethargic, or with swollen articulation in their joints were individually treated, as Purdue University Animal Care and Use Committee requests. The animals were treated with ceftiofur, enrofloxacin, banamine, lincomycin, or penicillin, depending on their symptoms.

Growth Performance

Growth performance (average daily gain - ADG, average daily feed intake - ADFI, and feed eficiency - G:F) was determined for each week and for the total experimental trial (0 to 40 d) as the following calculations.

$$ADG_{Wk4} = (BW_{28d} - BW_{21d})/(28 - 21) \quad (3)$$

$$ADFI_{Wk4} = FI_{21\ to\ 28\ d}/(28 - 21) \quad (4)$$

$$G{:}F_{Wk4} = ADG_{Wk4}/AFI_{Wk4} \quad (5)$$

Statistical analysis

In productive performance analysis, pen was used as the experimental unit for ADG, ADFI, and G:F. The Shapiro-Wilk test was used to analyze the normality followed by the data transformation using the RANK procedure (SAS INSTITUTE, 2009). All results were analysed by ANOVA with the GLM procedure (SAS INSTITUTE, 2009) using treatment as the classification factor. The model included essential oil treatment, and the residual mean square error was used as the error term. When ANOVA indicated a significant ($P \leq 0.05$) difference, the means were separated using the Tukey test. Differences were considered statistically significant at $P \leq 0.05$ and trends were considered with values $0.05 \leq P \leq 0.10$.

RESULTS AND DISCUSSION

Gene Expression

A significant difference was observed ($P < 0.05$) in the proximal jejunal segment, wherein the tissues undergoing treatment with CRINA® 700 ppm had higher IAP expression than those under the action of carvacrol and cinnamaldehyde. Regarding the expression of GLP-2R, we observed that eugenol, thymol, carvacrol and CRINA® 100 ppm had greater relative expression than the cinnamaldehyde ($P < 0.10$) treatment. Similar results were observed for the expression of IGF1, which carvacrol had a greater relative expression ($P < 0.10$) than cinnamaldehyde (Table 5).

Table 5 Effect of different essential oils using an *ex vivo* procedure on the relative abundance of mRNA on proximal jejunum

	Treatments[1]						SE	*P*-value
Item	**EUG**	**THY**	**CAR**	**CIN**	**C100**	**C700**		
Trophic Markers								
GLP-2R	1.336^{x}	1.630^{x}	1.601^{x}	0.532^{y}	1.082^{x}	0.810^{xy}	0.361	0.08
IGF1	0.839^{xy}	0.658^{xy}	1.330^{x}	0.473^{y}	0.861^{xy}	0.720^{xy}	0.203	0.09
Occludin	1.204	1.018	1.058	1.131	1.074	1.201	0.167	0.91
Digestive Markers								
IAP	1.548^{ab}	1.759^{ab}	0.860^{b}	1.103^{b}	1.266^{ab}	1.775^{a}	0.310	0.04
SGLT1	0.970	0.850	1.304	0.678	0.840	1.041	0.186	0.26
Immunological Markers								
MUC2	1.001	0.971	1.322	1.175	1.027	0.988	0.153	0.23
RelA/p65	1.420	1.627	1.049	1.344	1.218	1.452	0.182	0.19

^{a,b}Means within each row with different superscript letters are diferente ($P < 0.05$).
^{x,y}Means within each row with different superscript letters tend to be different ($P < 0.10$).
[1]EUG: Eugenol – 100 ppm; THY: Thymol – 100 ppm; CAR: Carvacrol – 100 ppm; CIN: Cinnamaldehyde – 100 ppm; C100: CRINA® 100 ppm; C700: CRINA® 700 ppm.

For gene expression, tissues treated with CRINA® 700 had a higher expression of IAP comparing to carvacrol and cinnamaldehyde could be both

related with CRINA® composition and concentration. Apparently, the essential oils association on CRINA's composition (eugenol, thymol, cinnamaldehyde, 2-methoxyphenol, piperine and curcumin) promoted an synergism actions that were capable of increasing IAP expression. However, not just its composition was determinant, but also its high dosage (700 ppm; 100 ppm of essential oils) used, since CRINA 100 did not stand out.

Few studies in literature related the effects of this specific composition of essential oils on digestive markers. The use of CRINA® for poultry (eugenol, thymol, piperine and curcumin) has demonstrated good results *in vivo*. Giannenas et al. (2014) verify that, in turkeys, there was improvement in performance, acid latic bacteria concentration and reduction of caecal coliforms, as well as improvement in antioxidant condition. Despite this oils effects on bacterial populations , other performance trials did not report improvement in poultry performance (LEE et l., 2003; JANG et al., 2007).

IAP low expression by carvacrol has been previously described in the literature. Levkut et al. (2011), found that oregano oil (mainly composed of carvacrol) reduced expression of IAP on an *in vivo* study with broiler chickens. According to Goze et al. (2010) this results may be explained because of its antioxidant and antimicrobial actions. Apparently carvacrol could reduced the expression of this marker by some effect on enterocyte differentiation or environmental pH reduction, both reasons related with decreasing expression of IAP. As consequence of decreasing this marker, intestinal barrier can have its protecion decreased and tissue regeneration affected, both situations that compromise the intestinal wall functionality.

Regarding to GLP-2R expression, carvacrol showed a tendency of higher expression of this marker. This receptor for the peptide like glucagon performs trophic intestinal actions which comprise the enhacement of nutrient absorption, reduction on intestinal permeability, etc. The low intestinal pH and stimulus of

intestinal activity are causes of its increased expression, related to tissue exposure to carvacrol.

The tendency of carvacrol shows a higher expression of GLP-2R and IGF1 is plausible since this markers are trigger by the same hormone (GLP-2) which enhances intestinal IGF1 expression and secretion (DUBÉ et al., 2006). Increasing ($P < 0.1$) this trophic markers leads the assumption that this oil mode of action is related to enhancing animals intestinal support for metabolism and fuction, which match some *in vivo* results.

On other researchs using this essential oils, Ali (2014) verify that it was capable of reducing *Campylobacter* on caecal contents of broiler chickens and Jamroz and Kammel (2002) decribed an improvement in growth performance. For swine, however, Stoni et al. (2006) verified that this substance is almost all absorbed on intestinal epitelium when added in feed. Yet on other study, Henn et al., (2010) evaluated that the use of oreganum oil for nursery pigs had a good antioxidant and antimicrobial effect, but no effect on growth performance or diarrea control.

Carvacrol also showed a tendency ($P < 0.1$) for higher expression on IGF1 than cinnamaldehyde. Different of an *in vivo* trial, where post-weaned pigs got a comercial product based on anise oreganum oil, citrus peels and chicory (40 ppm of essential oils) added to a diet without antibiotics, that among the reported results, there was a tendency for decreasing ($P < 0.1$) IGF1 expression in ileum (KROISMAYR et al., 2008).

Opposite to carvacrol, cinnamadehyde showed a tendency for reducing the expression of GLP-2R and IGF1. The decreasing of expression of these markers implies a decreasing of digestive and trophic potential of pigs. In the nursery phase, it is important that occurs higher action of factors that contribute positively with digestion and intestinal development, since animals are under a natural stress because of weaning. Cinnamaldehyde tend to play an immunological effect under LPS challenge (CHAO et al., 2008) and shows mainly

antibacterial effects (CHANG et al., 2001), but this oil didn't act on trophic markers directly on intestinal tissue, so it's mechanisms may work through other ways despite the intestinal barrier or these specific markers.

Despite showing an tendency to be diferent on the GLP-2R expression regarding to CRINA 100, it didn't happen regading to CRINA 700. This fact could question the real beneficial effects of the high dosage of CRINA and infer that the action of its compounds reach a negative level for certain markers. With this findings, it remains the expectation of how these essential oils would work facing an *in vivo* situation of post-weaned intestinal environment, and if this impact on trophic and digestive markers make any difference under normal post-weaning conditions.

Growth Performance

On the growth performance trial (Table 6), there were no differences ($P < 0.05$) in any variables evaluated among any of the treatments.

Table 6 Effects of different essential oil sources on nursery pig growth performance

Item[1]	Dietary treatment[1]						CV	SEM	*P*-value
	CON	EUG	THY	CAR	CIN	CRINA			
BW, Kg									
d 0	6.23	6.26	6.25	6.23	6.24	6.25	18.01	0.45	0.7695
d 7	6.47	6.63	6.51	6.64	6.59	6.50	16.35	0.43	0.4082
d 14	7.85	8.02	7.99	8.04	8.12	7.86	13.32	0.43	0.8359
d 21	10.36	10.55	10.62	10.55	10.85	10.39	10.58	0.45	0.6859
d 28	13.32	13.50	13.68	13.88	14.19	13.62	10.18	0.56	0.6418
d 35	17.60	17.93	17.82	17.96	18.41	17.46	9.91	0.71	0.6469
d 40	21.18	21.57	21.36	21.41	21.97	20.95	9.27	0.80	0.6935
Week 1 (d 0 to 7)									
ADG	0.076	0.118	0.081	0.131	0.111	0.079	55.71	0.01	0.4129
ADFI	0.193	0.211	0.172	0.233	0.216	0.191	24.57	0.01	0.4839
G:F	0.32	0.58	0.45	0.55	0.48	0.42	50.89	0.04	0.3867
Week 2 (d 7 to 14)									
ADG	0.196	0.198	0.211	0.200	0.218	0.194	19.24	0.02	0.9281
ADFI	0.259	0.287	0.252	0.271	0.299	0.263	13.64	0.01	0.3654
G:F	0.75	0.69	0.84	0.73	0.73	0.74	13.89	0.02	0.3559
Week 3 (d 14 to 21)									
ADG	0.358	0.361	0.377	0.359	0.390	0.360	11.82	0.02	0.7656
ADFI	0.494	0.532	0.525	0.516	0.559	0.521	10.25	0.02	0.4947
G:F	0.69	0.68	0.72	0.70	0.70	0.69	9.93	0.01	0.9424
Week 4 (d 21 to 28)									
ADG	0.438	0.421	0.436	0.475	0.477	0.462	14.16	0.03	0.6698
ADFI	0.678	0.669	0.654	0.701	0.736	0.707	11.21	0.03	0.4676
G:F	0.65	0.63	0.66	0.68	0.65	0.65	7.98	0.01	0.7262
Week 5 (d 28 to 35)									
ADG	0.598	0.611	0.592	0.583	0.603	0.548	11.71	0.03	0.1527
ADFI	0.898	0.966	0.912	0.923	0.944	0.893	8.64	0.03	0.2118
G:F	0.68	0.65	0.65	0.63	0.64	0.61	7.91	0.01	0.1311
Week 6 (d 35 to 40)									
ADG	0.717	0.728	0.709	0.690	0.712	0.698	10.19	0.03	0.9624
ADFI	1.220	1.162	1.171	1.201	1.239	1.193	9.18	0.04	0.8118
G:F	0.59	0.63	0.61	0.58	0.57	0.59	8.08	0.01	0.5568
Overall (d 0 to 40)									
ADG	0.374	0.344	0.378	0.380	0.393	0.367	7.97	0.01	0.8559
ADFI	0.551	0.589	0.570	0.591	0.616	0.582	8.55	0.02	0.4644
G:F	0.68	0.65	0.66	0.64	0.64	0.63	5.24	0.01	0.3441

[1]BW: body weight (Kg); ADG: average daily gain (Kg); ADFI: average daily feed intake (Kg); G:F: weight gain per feed intake (Kg/Kg).
[1]CON: basal diet; EUG: Eugenol – 28 ppm; THY: Thymol – 28 ppm; CAR: Carvacrol – 28 ppm; CIN: Cinnamaldehyde – 28 ppm; CRINA® 200 ppm (28 ppm essential oils).

This results could be well explained by several reasons. The basal diet composition, hadn't any antimicrobial substance (growth promoter antibiotic, organic acids, zinc oxide, spray-dried plasma, etc). This could be the cause of the high incidence of diarrea on the first week, becoming necessary the water treatment of all animals with tylosin (Tylan® - Elanco Animal Health - 66 mg/L) at d 6 of the experiment for 3 consecutive days. Additionally, the animals were individually treated at any clinical sign of disease, this may have masked any performance results due to the essential oils.

Studies in the literature are found in two lines: essential oils as nutritional additives: it is added to a standard diet; or as a replacement for growth enhancing concnetrations of antibiotics (GEA): where they are added to the diet without growth promoting or antimicrobial substances. Even as nutritional additives as replacements to GEA, few studies have reported significant positive effects on growth performance.

As antibiotic replacer, no differences for growth performance were demonstrated by Neill et al. (2006), evaluating a commercial product with 5% oregano oil (around 75-84% carvacrol and 0.7-4.0% thymol), for piglets in the nursery phase. Similar results were found by Liu et al. (2013) for *Escherichia coli* challenged post-weaned piglets, receiving essential oils in the diet (capsicum oleoresin, garlic botanical, or turmeric oleoresin – 10 ppm). Also for broiler chickens, feeding thymol (250 ppm) and carvacrol (250 ppm) did not show an improvment in growth performance, even when associated with organic acids (AKYUREK; YEL, 2011).

In a 7-d trial with growing pigs, Anderson et al., (2012) did not observed differences in animal performance receiving thymol (67 or 201 ppm) both as GEA replacer nor as a nutritional additive. Additionally, for growing pigs, as nutritional additive (1000 ppm), eugenol or cinnmaldehyde didn't affect the pig growth performance (YAN; KIM, 2012).

As nutritional additive, even the oils in combination (carvacrol, cinnmaldehyde and oleoresin – 300 ppm) were not able to create performance differences (MANZANILLA et al., 2006). A few studies have shown positive effects of essentials oils on performance. One study observed improved weight gain in broiler chickens fed oreganum oil (60% carvacrol, 12% y-terpinene, and 6.4% p-cymene) at 707 ppm (LEVKUT et al., 2011). In a similar study, also with oreganum oil (69.55% carvacrol, 10.57% cymol, and 4.09 thymol), the supplementation of this additive (100 ppm) improved the final weight, weight gain and feed eficiency of broiler chickens when compared to control, which did not have atibiotics included (MATHLOUTHI et al., 2012).

According with the major part of studies, the lack of results on growh performance its very commom. In our study, it can be discussed both by the low suplemmentation of oils (28 ppm) as the absence of any other antimicrobial substances on the diets. Also, the therapeutic antibiotic treatment of the animals, could interfere with our performance results. In this current evaluation, there was an incidence of more than 15 injections for almost all dietary treatments, except animals fed with eugenol (4 shots). This is an interesting result, that lead to the deduce that this oil performed a function on the general health condition of the pigs, possibly related to the antimicrobial effect of this oil against *Escherichia coli* (YAN; KIM, 2012).

CONCLUSIONS

In conclusion, the evaluation by *ex vivo* procedure, on the proximal jejunum, showed that higher doses of CRINA (700 ppm) increased the expression of digestory markers (IAP) in comparison with essential oils that aren't in it's composition (carvacrol and cinnamaldehyde), but for trophic markers (GLP-2R and IGF1) carvacrol tend to be better than other oils. However, the inclusion in the feed of CRINA, at recommended manufacturer dosage, or individual essential oils, did not affect the nursery pigs growth performance.

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ARTIGO 2 Essential oils blend decreases jejunum villus and affect gene expression of nursery pigs

ABSTRACT – The study was conducted to evaluate intestinal morphology and gene expression of jejunum of post-weaned piglets receiving a commercial source of essential oils. Twelve post-weaned piglets were used. These animals received a standard nursery diet with or without CRINA®, a product composed of essential oils, and were euthanized at 7 or is it 7 or 8 days after weaning. Samples from jejunum and ileum were collected to evaluate intestinal morphology (villus height, crypt depth, and villus:crypt ratio) and samples from proximal jejunum for gene expression (GAPDH, IAP, IGF1,GLP-2R, MUC2, Occludin, RelA/p65, and SGLT1). Animals fed CRINA diets had lower ($P < 0.05$) jejunal villus height and IGF1 expression. However, these same animals demonstrated a higher expression ($P < 0.05$) of SGLT1. This findings lead us to infer that CRINA's composition have substances that may work antagonistically on pig intestinal health.

INTRODUCTION

The weaning period is a critical moment in the swine production cycle, causing growth performance decreases and a high incidence of diarrhea (LALLÈS et al., 2004). These problems usually are mitigated by the use of growth enhancing concentrations of antibiotics (GEA) (WENK, 2003), which act in a beneficial way for the animals, but its use has also been investigated regarding the selection of resistant bacterial (BARTON, 2014).

With these discussions, new strategies for replacing GEA has been a focus of research, especially focusing on intestinal health. Among all the alternatives, essential oils have gained prominence because they have antimicrobial potential (carvacrol, eugenol – MATHLOUTHI et al., 2012; MICHIELS et al., 2009), antioxidant (thymol – ADORJAN; BUCHBAUER, 2010; DANDLEN et al., 2010), immunomodulatory (cinnamaldehyde - CHAO et al., 2008), among others. Commercially, there are available products, as CRINA® (DSM - Nutritional Products Inc., Parsippany, NJ) comprising varieties of essential oils that include the association of oils with complementary effects and applications (LU et al., 2015; AZAIN et al., 2014).

However, the results found in the literature are controversial. Essential oils can, both be applied as nutritional additives, added to a standard diet; or as a replacement for GEA, where they are added to the diet without growth promoting or antimicrobial substances. Even as additives or replacements, few *in vivo* studies have reported effects on growth performance. The *in vitro* effects of these essential oils has been evaluated (Si et al., 2006) primarily on their bacterial effects, being the main results found related to *in vitro* assays, describing good antimicrobial effects (DIAZ-SANCHEZ et al., 2015).

On the other hand, the lack of information of oils mode of action, or direct impact on intestinal epithelium make this findings more difficult to understand and explain. Thus, the goal of this study was to evaluate the effects of a commercial source of essential oils (CRINA®) added in feed of post-weaned pigs on morphological parameters and gene expression of trophic (GLP-2R, IGF1 and Occludin), digestive (IAP and SGLT1) and immunological (MUC2 and RelA/p65) intestinal markers in the jejunum of the small intestine.

MATERIAL AND METHODS

All animal care and handling procedures used in this study were reviewed and approved by Purdue University Animal Care and Use Committee.

Animals, experimental design and sample, and housing

There were used twelve crossbred pigs (Large White x Chester White x Yorkshire x Landrace) weaned at 23.3 d of age with initial BW of 6.95 ± 0.40 kg were assigned to be under two treatments, with 12 replicates per treatment. The treatments consisted of the following: nursery basal diet (Non-CRINA) as control treatment; and basal diet + 100 ppm of CRINA® (DSM - Nutritional Products Inc., Parsippany, NJ). All diets were formulated to meet or exceeded NRC (2012) nutrient requirements for nursery pigs (Table 1). Pigs were housed in a completely enclosed nursery with slatted floors, a single nipple drinker, and a five-hole self-

feeder per pen. All pigs were allowed *ad libitum* access to feed and water throughout the experiment period (8 d).

Table 1 Composition of the diets for piglets fed for 8 days post-weaning, as fed-basis

	Experimental Diets	
Item	Non-CRINA	CRINA
Ingredient, %		
Corn	35.34	35.34
Soybean meal	14.00	14.00
Soybean Oil	4.00	4.00
Limestone	0.60	0.60
Mono-Calcium phosphate	0.10	0.10
Vitamin premix[1]	0.25	0.25
Trace Mineral Premix[2]	0.18	0.18
Salt	0.25	0.25
Soy concentrate	4.05	4.05
Fish Meal	4.50	4.50
Dried Whey	22.00	22.00
Dried Skim Milk	14.00	14.00
L-Lys.HCL	0.285	0.285
DL-Met	0.27	0.27
L-Thr	0.14	0.14
L-Trp	0.03	0.03
Starch	0.01	-
CRINA®[3]	-	0.01
Calculated composition		
ME, Mcal/kg	3.57	3.57
CP, %	22.87	22.87
Total Lys, %	1.72	1.72
SID Lys, %	1.58	1.58
Ca, %	0.90	0.90
P, %	0.67	0.67
Available P, %	0.47	0.47
Lactose, %	22.54	22.54

[1]The vitamin premix provided the following per kg of diet: vitamin A, 6615 IU; vitamin D3, 661.5 IU; vitamin E, 44.1 IU; Menadione, 2.2 mg; vitamin B12, 38.6 µg; riboflavin, 8.8 mg; pantothenic acid, 22.1 mg; niacin, 33.1 mg.

[2]The trace mineral premix provided the following estimated available minerals per kg of diet: Zn, 121.3 mg from zinc oxide; Fe, 121.5 mg from iron carbonate; Mn, 15.0 mg from manganese oxide; Cu, 11.3 mg from copper chloride; I, 0.46 mg from ethylenediamine dihydroiodide; and Se, 0.3 mg as half from sodium selenite and half from organic selenium.

[3]CRINA®: commercial product that comprises 14% of essential oils: 13% - mixture of eugenol, thymol and 2-methoxyphenol; and 1% - piperine and curcumin.

Sample collection

On 8-d post-weaning, all pigs were stunned by CO_2 and euthanized by cranial vena cava exsanguination for intestine tissue sampling (Figure 1).

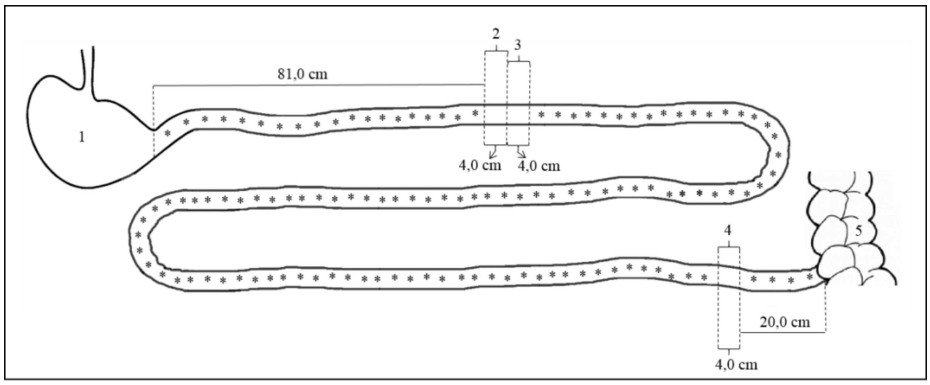

Figure 1 Scheme for sample collection for gene expression and intestinal morphology trial. *Discard areas; 1) Stomach; 2) Jejunal sample for histological analysis – 4.0 cm; 3) Sample for RNA analysis – 4.0 cm; 4) Ileal sample for histological analysis – 4.0 cm; 5) Cecum

Samples of 4.0 cm for intestinal morphology analysis were collected from jejunum (81 cm after stomach) and ileum (20 cm from ileum/cecum junction) of all piglets. Jejunal samples for RNA analysis were collected and immediately scraped with a glass slide (Figure 2A, 2B) and placed in a tube containing 500 μL of TRIzol reagent (Figure 2C). Scraping samples were snap-frozen in liquid nitrogen and stored at -80 ºC until analysis.

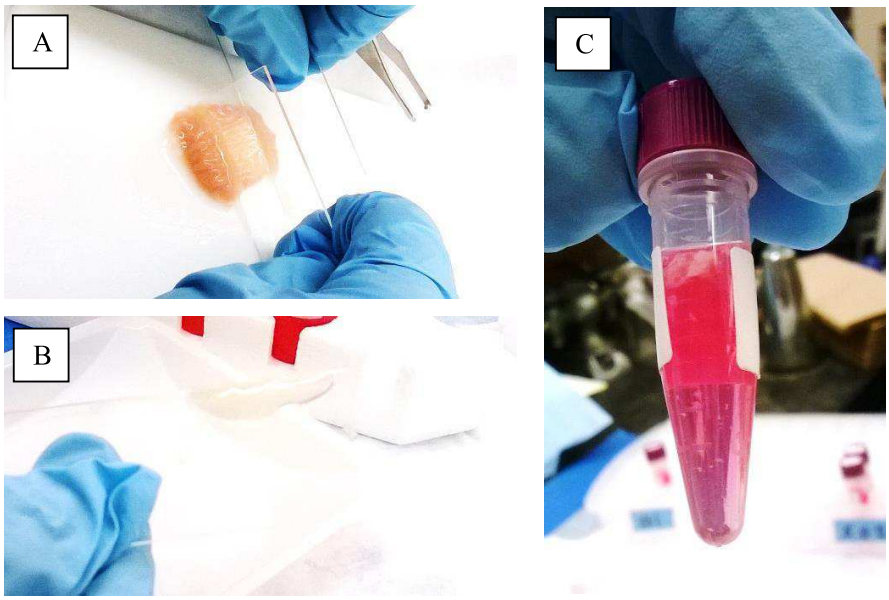

Figure 2 A) Scraping of tissue after incubating; B) Material scrapped; C) TRIzol tube with scrapped sample

Intestinal Morphology Assay

Briefly, freshly cut intestinal sections were rinsed with PBS and then fixed in freshly prepared chilled fixative solution (formaline, 10%). Intestinal segments were dehydrated over a 2-d period using increasing concentrations of ethanol and chloroform. Sections were embedded in paraffin, and cross sections were cut with a microtome approximately 10 µm thick. Sections were stained with hematoxylin and eosin, and morphometric measurements were performed by 1 person (blinded to treatment) using a computer assisted morphometric system (AxioVision V4.8.2.0, Carl Zeiss MicroImaging GmbH Corp., São Paulo, SP). The height and crypt depth of 8 well-oriented villi per sample were measured.

Growth Performance and Liver Weight

Growth performance (average daily gain - ADG, average daily feed intake - ADFI, and feed efficiency - G:F) was determined as the following calculations.

$$ADG_{d8} = (BW_{d8} - BW_{d8})/(8) \quad (1)$$

$$ADFI_{d8} = FI_{d0\ to\ d8}/(8) \quad (2)$$

$$G{:}F_{d8} = ADG_{d8}/AFI_{d8} \quad (3)$$

The liver weights were measure immediately after slaughter and related with pigs body weights at this moment.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from jejunum mucosa samples using TRIzol reagent (Invitrogen Inc.) and the procedure described by the manufacturer (RNeasy Protect Mini Kit – Qiagen®) adapted for this experiment (APPENDIX II). Total RNA concentration was quantified by using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, Waltham, MA, USA) at 260 nm, and the RNA purity was assessed by examining the ratios of absorbance at 260 (A260) and 280 (A280) nm. All samples had A260/A280 ratio above 1.8. Additionally, RNA integrity was verified by visualization of the 18S and 28S ribosomal RNA (rRNA) bands after ethidium bromide staining in a 1.0% agarose gel (Agarose LE Molecular Biology, DOT Scientific Inc., Burton, MI, USA). The presence of 2 well-defined bands (18S and 28S rRNA) is an indicator of good RNA integrity (Figure 3).

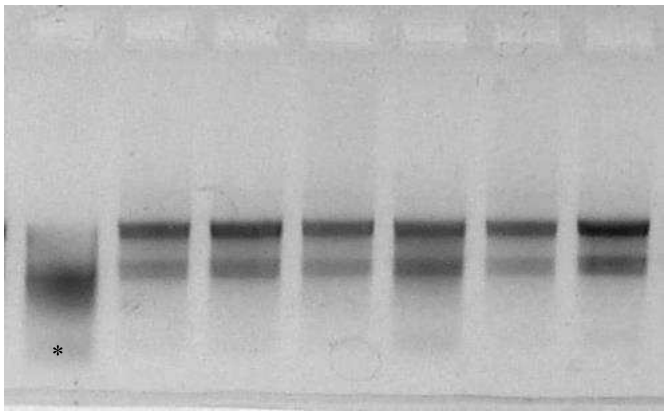

Figure 3 RNA integrity validation extracted from intestinal samples. Lane 1 Features degraded RNA (*).

Total RNA (1 μg) was reverse transcribed into complementary DNA (cDNA; APPENDIX III). Quantitative real-time PCR was performed using a MyiQ real-time PCR detection system (Bio-Rad, Hercules, CA). Primer sequences are shown in Table 2. Amplification was carried out in a total volume of 20 μL containing 10 μL of SYBR Green Supermix (Bio-Rad), forward and reverse primers (0.5 μg/μL each), and 5 μL of cDNA template. Reactions were incubated at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, 52.9 to 59.7°C for 30 s, and 72°C for 30 s. At the end of the PCR, melt curve analysis was conducted to validate the specificity of the primers. A nontemplate control (only DNAse free water in wells) was run with every assay, and all determinations were performed in duplicate.

Table 2 *Primers* and annealing temperatures (AT) of evaluated genes

Gene[1]		Primer[2*]	AT (°C)	Reference
GAPDH	F	TCACCATCTTCCAGGAGCG	59.7	Saddoris, Fleet e Radcliffe (2010)
	R	CTGCTTCACCACCTTCTTGA		
IAP	F	CTAAAGGGGCAGATGAATGG	54.4	Lackeyram et al. (2010)
	R	CACCTGTCTGTCCACGTTGT		
IGF1	F	GTGCGGAGACAGGGGCTTTT	53.0	Schedle et al. (2008)
	R	AGATCCGGAAGCAGCACTCA		
GLP-2R	F	ACCTTGCAGCTGATGTACAC	56.6	Petersen, Burrin e Sangild (2001)
	R	GTGTTCTCCAGGTGTGCACG		
MUC2	F	CTGCTCCGGGTCCTGTGGGA	53.0	Pieper et al. (2012)
	R	CCCGCTGGCTGGTGCGATAC		
Occludin	F	ATCAACAAAGGCAACTCT	53.0	Zhang and Guo (2009)
	R	GCAGCAGCCATGTACTCT		
RelA/p65	F	GGAACACGATGGCCACTTG	52.9	Los Santos, San Segundo e Grubman (2007)
	R	AAGAGGACATCGAGGTGTATTTCAC		
SGLT1	F	GGCTGGACGAAGTATGGTGT	53.0	Yang et al. (2011)
	R	ACAACCACCCAAATCAGAGC		

[1]GAPDH: Glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene); IAP: Intestinal Alkaline Phosphatase; IGF1: Insulin-like growth factor 1; GLP-2R: glucagon-like peptide 2 receptor; MUC2: gene encoding mucin production; Occludin: plasma-membrane protein located at the tight junctions; RelA/p65: REL-associated protein involved in NF-κB heterodimer formation; SGLT1: Na+ dependent glucose transporter 1; [2]F: forward; R: reverse; *By IDT (Integrated DNA Technologies®)

The mRNA abundance values for each sample were normalized to GAPDH according to the $2^{-\Delta\Delta CT}$ method (LIVAK; SCHMITTGEN, 2001) and the following calculations:

$$\Delta CT_{target\ gene} = CT_{target\ gene} - CT_{GAPDH} \quad (4)$$

$$\Delta\Delta CT_{treatment} = (\Delta CT_{treatment} - \Delta CT_{control})^2 \quad (5)$$

Statistical analysis

The data were analyzed by one-way ANOVA using the MIXED procedure (SAS INSTITUTE, 2009) and pig served as the experimental unit. The Shapiro-Wilk test was used to analyze the normality followed by the data transformation using the RANK procedure (SAS INSTITUTE, 2009). Once all the gene expression data were transformed, the outliers were eliminated based on standard deviation above 1.75σ. The model included dietary treatment, and the residual mean square error was used as the error term. Differences were considered statistically significant at $P \leq 0.05$ and trends were considered with values $0.05 \leq P \leq 0.10$.

RESULTS AND DISCUSSION

Morphological differences were observed only on villus height of jejunum samples (Table 3), where piglets receiving diets without CRINA® had higher villus height ($P = 0.0388$).

Other studies that evaluated the use of essential oils on intestinal morphology did not reported differences due to the treatments (KROISMAYR et al., 2008; VUKIC-VRANJER et al., 2013).

There were no differences for organ measures (liver weight and liver/BW ratio) and growth performance (ADG, ADFI and G:F), but there were observed differences for final weight at the end of the 8 post-weaning days, were animals receiving diets without CRINA® demonstrated higher ($P < 0.05$) final weight (Table 3).

Table 3 Intestinal morphology of jejunum and ileum, liver weights and growth performance of pigs (8 days post-weaning) receiving diets with or without CRINA®

Item	Dietary Treatments[1]		CV	SE	*P*-value
	CRINA	Non-CRINA			
Jejunum					
Villus height, µm	218.76	268.37	24.64	11.0	0.0388
Crypt depth, µm	228.22	235.11	23.76	11.1	0.6182
Villus:crypt	0.98	1.18	26.31	0.06	0.0888
Ileum					
Villus height, µm	244.99	259.16	20.52	11.0	0.5147
Crypt depth, µm	175.36	188.90	24.49	9.3	0.3758
Villus:crypt	1.45	1.43	26.55	0.08	0.9033
Organ Measures					
Liver weight, g	185.83	198.75	17.0	0.51	0.2896
Liver/BW, g/Kg	24.87	24.83	10.1	6.67	0.9706
Growth Performance					
BW 14d, kg	7.44	7.98	9.99	0.35	0.0460
ADG, kg	0.067	0.111	40.44	0.02	0.5648
ADFI, kg	0.107	0.141	21.90	0.01	0.3920
G:F	0.59	0.79	26.07	0.04	0.6715

The gene expression results are described on the following figures (Figure 4 to 6). There were no differences between the treatments on the GLP-2R, Occludin, IAP and MUC2 jejunal intestinal markers. However, IGF1 and SGLT1 were influenced by the dietary treatments. Pigs fed the CRINA treatment demonstrated less expression ($P < 0.05$) of IGF1, and more expression ($P < 0.05$) of SGLT1. Regarding to immunological markers, despite the dietary treatment, RelA/p65 was decreased in expression ($P < 0.05$) by both treatments based on normal expression of that gene.

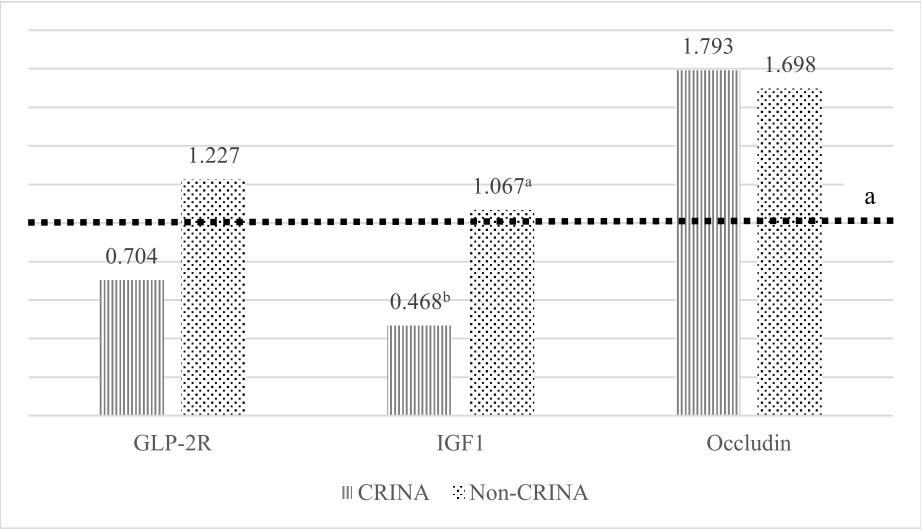

Figure 4 Effect of different dietary treatment on the relative abundance of mRNA of trophic intestinal markers on pigs proximal jejunum. Means with different superscripts differ ($P < 0.05$)

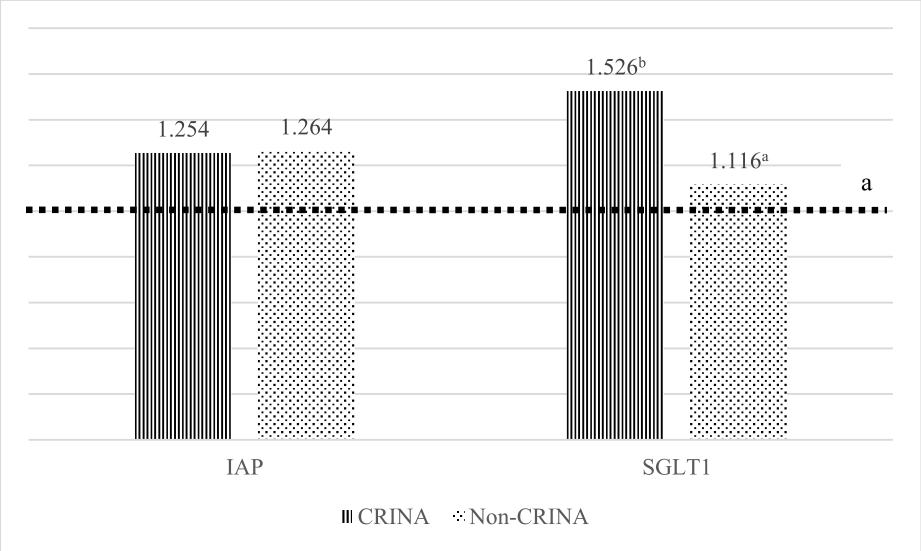

Figure 5 Effect of different dietary treatment on the relative abundance of mRNA of digestive intestinal markers on pigs proximal jejunum. Means with different superscripts differ ($P < 0.05$)

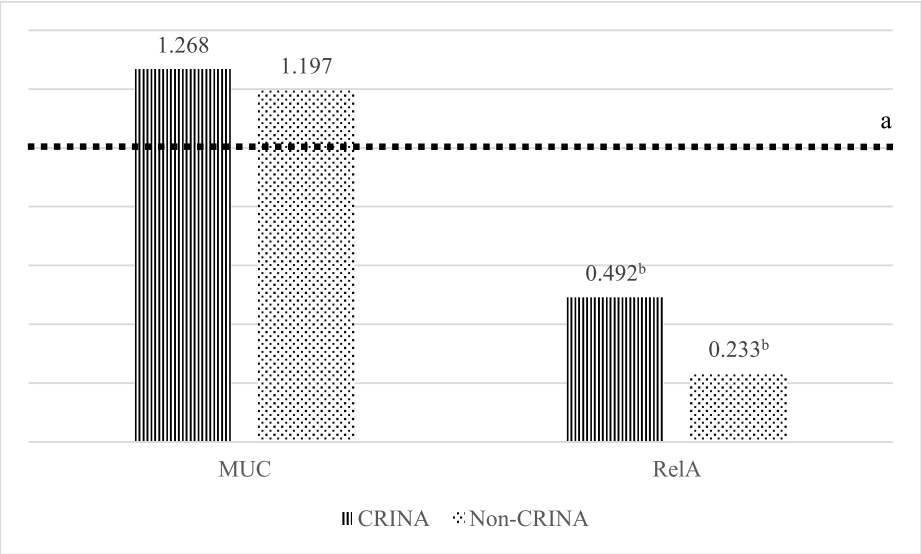

Figure 6 Effect of different dietary treatment on the relative abundance of mRNA of trophic immunological markers on pigs proximal jejunum. Means with different superscripts differ ($P < 0.05$)

The provision of CRINA in the feed for post-weaned pigs caused inverse expression of intestinal markers IGF1 and SGLT1. The higher expression ($P < 0.05$) of SGLT1 leads the idea that CRINA benefits the Na-dependent glucose transport, improving the utilization of available nutrients on intestine lumen and, consequently, may allow for better animal performance.

On the other hand, the reduction of IGF1 expression ($P < 0.05$) can be related to the morphological findings of decreased jejunal villus height, since this growth factor is similar to insulin and shows direct influence on intestinal growth, and it is sensitive to nutritional variations (ESTÍVARIZ; ZIEGLER, 1997).

Some studies that evaluated CRINA® in swine feed described that it can affect the growth performance. Azain et al. (2014) verify only an improvement on performance if associated oils with antibiotics, the same results that Lu et al. (2015) found. With this information, it looks like the impact of this essential oil blend on digestive intestinal markers do not interfere on the nutrient utilization at

a level that could improve performance. Also, it can be possible that this effect may be because the low expression of IGF1.

The IGF1 marker represents an intestinal growth factor that is very important on gut development. During the nursery phase, IGF1 plays a determinant role based on the weaning stress, where animals are under a situation that comprises the good development of the digestive tract (BOUDRY et al., 2004). So, a high expression of this marker would show that the intestine was adapting and adjusting to this situation.

The decreasing of IGF1 can be also related with the diet fed to the animals during the 8 days after weaning. Matteri et al. (2000) verify that addition of low (7%) or no plasma protein in post-weaning diet decreased the serum IGF1 of animals, so this could be related with the feed composition fed in our study, and not just with CRINA® supplementation.

SGLT1 transports dietary sugar from the intestinal lumen to enterocytes. The regulation of this protein is essential for the glucose provision to the body and is important for maintenance of glucose homeostasis. Moran et al. (2010) evaluated the expression of this marker on post-weaned piglets and found that the nutritional contents impact its expression, being animals under higher doses of carbohydrates expressing more SGLT1, but morphometric analyses indicate that the increased expression is not due to a trophic effect. Thus, SGLT1 is the major route for absorption of dietary sugar across the luminal membrane of swine enterocytes and it seems that CRINA® plays an important role in this process, since the carbohydrate levels on the diet remain the same between the treatments.

Other inference that can be made with these results is that it is possible that, despite CRINA shows desirable digestive effects, some issue on its composition could affect negatively the jejunal morphological characteristics and IGF1 expression. Piperine is one of the minor oils present on CRINA's composition and its effects on intestinal epithelium has been described.

Despite the good effect of piperine on oxidative damage (AKYILDIZ et al., 2013), its mode of action increases intestinal permeability (KHAJURIA; THUSU; ZUTSHI, 2002), which could work both for better absorption and as for higher exposure of the surface to antigens. Cardoso et al. (2012) described that piperine (at 60, 120 or 180 ppm) added on broiler chickens diets decreased the jejunal villus height around 40%, being that other possible reason on our results reported here.

Regarding to the immunological markers results, Juul-Madsen et al. (2010) founded that the weaning suppresses a broad spectrum of adaptive immune variables and that this was evident immediately after weaning as well as after a longer period of about one week, but not on innate immune system variables, that seem to be stimulated immediately after weaning. This may explain why RelA/p65 showed low expression that wasn't described for MUC2 (innate response). Independent of the nutritional treatment, RelA/p65 had an overall lower ($P < 0.05$) expression, considering that the post-weaning moment was crucial on this observation and the treatment showed no effect on this issue.

With these results, remains the need of explore other intestinal markers and other dietetary composition for post-weaned pigs fed with essential oils and oil blends, since the information described in this work expose just a few effects of essential oils on intestinal health of nursery pigs.

CONCLUSION

Providing CRINA on post-weaned piglets increase the expression of SGLT1, but reduce the expression of IGF1 and villus height in jejunum, concluding that this product shows a composition of substances that may work antagonistically on pig intestinal health.

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TERCEIRA PARTE – SUÍNOS COMO MODELO ANIMAL PARA HUMANOS

ARTIGO 3 GLP-2R, RelA/p65 and occludin are less expressed in jejunum of piglets with high sensitivity to soy protein and peanuts

ABSTRACT – Swine are an excellent animal model for human research. To better understand the increased high reactivity of people to soybean and peanut proteins, a genetic line of pigs has been selected for seven generations to have either high or low sensitivity to soybean meal based on skin alergy testing reactions (wheel and flair). The objective of this study was to determine gene expression in an *ex vivo* model of porcine jejunum from these two gentic lines. Twenty-four piglets were used, twelve per genetic line. Pigs were weaned at 24 d of age and fed a standard phase 1 diet containg 14% soybean meal (SBM). The pigs were euthanized 8 d post-weaning, and segments of the proximal jejunum were removed. Intestinal segments were incubated for 60 minutes at 37ºC in Dulbeco's Modified Eagles Medium. Tissues were rinsed with PBS and stored at -80ºC with TRIzol for subsequent determination of IAP, SGLT1, GLP2, Occludin, IGF1, MUC2, and RelA/p65 gene expression. The pigs from the high reactivity line had lower expression of GLP-2R, Occludin, and RelA/p65 ($P < 0.05$). These responses in the high reactive line may explain why they have the sensitivity to soybean and peanuts, since these genes are involved with intestinal growth, tight junctions and immune system, respectively. In conclusion, the jejunum of piglets can express differently GLP2, Occludin, and RelA/p65, according to genetic sensitivity to soy proteins.

INTRODUCTION

Allergies to food are growing and affect people of all ages. The development of animal models for testing the various food allergens factors have been beneficial by allowing faster searches and comprehension on mechanisms of action involved in allergic processes, and even testing new treatments for these conditions (VAN GRAMBERG et al., 2013).

The most common food allergy causing factors are cow's milk, chicken eggs, and nuts, peanuts and walnuts, while less common include soy, wheat, fish

and oysters (SICHERER; SAMPSON, 2010; WANG; SAMPSON; 2011). It is known that food allergy is common in the first three years of life (BOCK, 1987), however it has been shown that the majority of allergies that begin early in life, such as milk, soy and wheat, are eventually surmounted. On the other hand, allergies to peanuts, tree nuts, fish and oysters normally continue becoming present throughout life (HELM; ERMEL; FRICK, 2003; SICHERER; SAMPSON, 2010; MALONEY et al., 2008).

Pigs are an animal model that have a big advantage compared to other models. The intestinal physiology of this species is anatomically and histologically similar to humans, with a more diverse microbiota than is seen in rodents (HELM; ERMEL; FRICK, 2003; HELM et al., 2002; PATTERSON; LEI; MILLER, 2008) and also features a fine individual immune responses (BOYCE et al., 2010). Features like these are extremely important in the evaluation of pathogenesis and immune response to food allergens.

As a model for allergy to peanuts, Helm et al. (2002) developed a model using the neonatal pig which had not just a peanut allergy, but also immune and histological patterns similar of allergic patients whose allergic statements found emphasize the ability to be used in the pig with a good animal model for food allergies on human.

However, research on the *in vivo* model can present conflicting results and, in some situations, mask the real effect of treatment due to external factors such as individual characteristics of animals or environmental factors. Thus, this study aimed to compare the gene expression of intestinal markers of piglets with high or low sensitivity to soy protein and peanuts by an *ex vivo* analysis model.

MATERIAL AND METHODS

All animal care and handling procedures used in this study were reviewed and approved by Purdue University Animal Care and Use Committee.

Animals, experimental design and sample, and housing

The *ex vivo* procedure was performed as described previously (SUGIHARTO et al., 2012). The jejunal samples of twelve crossbred pigs (White line composite: York x Large White x Landrace x Chester White) weaned at 23.5 d of age with initial BW of 6.18 ± 1.15 kg wereharvest at 8 days post-weaning for an *ex vivo* study. . Initially there were 9 pigs per pen for each of the 2 pig genetic lines selected for low and high allergic response to soybean and peanut proteins. All 9 pigs per pen were skin tested for allergic response to the soy and peanut proteins on day 7 to ensure the 6 pigs per genetic line that were harvested for the *ex vivo* experiment were indeed allergic reactors in the high line and non-reactors in the low line of pigs harvested. The *ex vivo* assay consisted of the following: *Dulbecco's Modified Eagle Medium* (DMEM) as basal common treatment and as previously described. The basal diet fed to the pigs was formulated to meet or exceeded NRC (2012) nutrient requirements for nursery pigs (Table 1). Pigs were housed in a completely enclosed nursery with slatted floors, a nipple drinker, and a five-hole self-feeder per pen. All pigs were allowed ad libitum access to feed and water throughout the experimental period (8 d).

Table 1 Composition of the basal diet for pigs weaned at 23.5 days of age and fed the diet for 8 days post-weaning, as fed-basis.

Item	Amount
Ingredient, %	
Corn	35.34
Soybean meal	14.00
Soybean Oil	4.00
Limestone	0.60
Mono-Calcium phosphate	0.10
Vitamin premix[1]	0.25
Trace-Mineral premix[2]	0.175
Salt	0.25
Soy concentrate	4.05
Fish Meal	4.50
Dried Whey	22.00
Dried Skim Milk	14.00
L-Lys.HCL	0.285
DL-Met	0.27
L-Thr	0.14
L-Trp	0.03
Starch	0.01
Calculated composition	
ME, Mcal/kg	3.57
CP, %	22.87
Total Lys, %	1.72
SID Lys, %	1.51
Ca, %	0.90
P, %	0.67
Available P, %	0.47
Lactose, %	22.54

[1]The vitamin premix provided the following per kg of diet: vitamin A, 6615 IU; vitamin D3, 661.5 IU; vitamin E, 44.1 IU; Menadione, 2.2 mg; vitamin B12, 38.6 µg; riboflavin, 8.8 mg; pantothenic acid, 22.1 mg; niacin, 33.1 mg;

[2]The trace mineral premix provided the following estimated available minerals per kg of diet: Zn, 121.3 mg from zinc oxide; Fe, 121.5 mg from iron carbonate; Mn, 15.0 mg from manganese oxide; Cu, 11.3 mg from copper chloride; I, 0.46 mg from ethylenediamine dihydroiodide; and Se, 0.3 mg as half from sodium selenite and half from organic selenium;

Sample collection

At 8th day of nursery, all pigs were stunned by CO_2 and euthanized by cranial vena cava exsanguination for intestine tissue sampling (Figure 1).

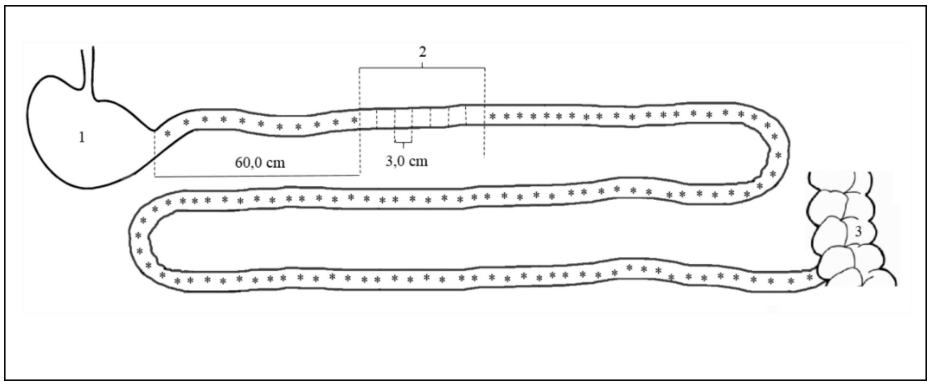

Figure 1 Scheme for sample collection to *ex vivo* trial. *Discard areas; 1) Stomach; 2) Proximal jejunum seven segments for *ex vivo* analysis – 3.0 cm each sample; 3) Cecum

Jejunum samples were collected and placed in tubes containing 25 mL of PBS (saline solution) and 1% of antibiotic (peniciline + streptomycin + neomycin) mix (50 mM of mannitol + 2 mM of Tris-HCl). In order to minimize intestinal tissue stress at collection, the PBS + 1% antibiotic solution was kept heated at 37°C. After collection, the samples intended for the *ex vivo* procedure were processed as shown in Figure 2.

Jejunum samples were incubated in DMEM at 37°C for 60 min on rocking platform at the level of 3.5 setting (1.6 seconds per revolution; VWR-200 Rocking platform, Radnor, PA, USA). Subsequently, mucosal scraping samples for RNA isolation were obtained by scraping the surface area of jejunal segment with a glass slide (Figure 3A, 3B) and placed in tube containing 500 µL of TRIzol reagent (Figure 3C). Scraping samples were snap-frozen in liquid nitrogen and stored at -80 ºC until analysis.

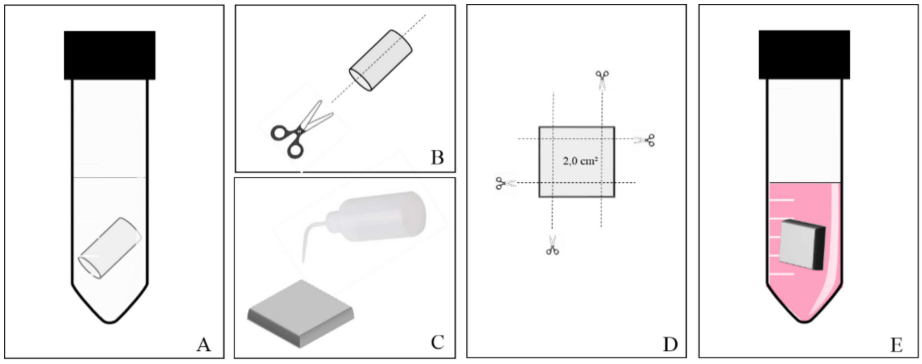

Figure 2 Sample processing scheme for *ex vivo* procedure. A) Sample in 25 mL of PBS and 1% of antibiotic mix; B) Longitudinal segment section; C) Rinsing with saline to remove intestinal content; D) Removal of segment edges to obtain a 2.0 cm² intestinal sample; E) Sample inclusion in *Dulbecco's Modified Eagle Medium*

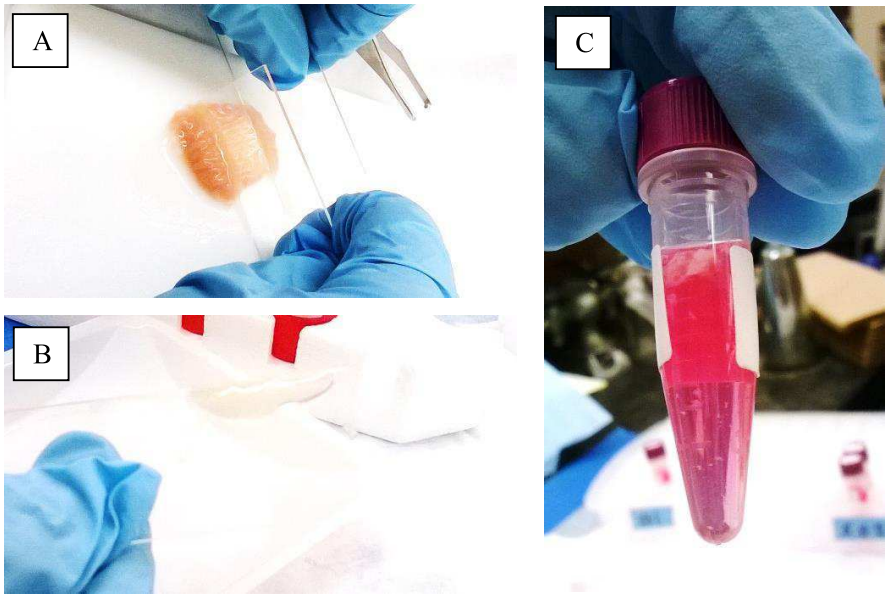

Figure 3 A) Scraping of tissue after incubating; B) Material scrapped; C) TRIzol tube with scrapped sample

Growth Performance and Liver Weight

Growth performance (average daily gain - ADG, average daily feed intake - ADFI, and feed efficiency - G:F) was determined as the following calculations.

$$ADG_{d14} = (BW_{d8} - BW_{d8})/(8) \quad (1)$$

$$ADFI_{d8} = FI_{d0\ to\ d8}/(8) \quad (2)$$

$$G{:}F_{d8} = ADG_{d8}/ADFI_{d8} \quad (3)$$

The liver weights were measure immediately after slaughter and related with piglets body weights at this moment.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from jejunum mucosa samples using TRIzol reagent (Invitrogen Inc.; APPENDIX I). Total RNA concentration was quantified by using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, Waltham, MA, USA) at 260 nm, and the RNA purity was assessed by examining the ratios of absorbance at 260 (A260) and 280 (A280) nm. All samples had A260/A280 ratio above 1.8. Additionally, RNA integrity was verified by visualization of the 18S and 28S ribosomal RNA (rRNA) bands after ethidium bromide staining in a 1.0% agarose gel (Agarose LE Molecular Biology, DOT Scientific Inc., Burton, MI, USA). The presence of 2 well-defined bands (18S and 28S rRNA) is an indicator of good RNA integrity (Figure 4).

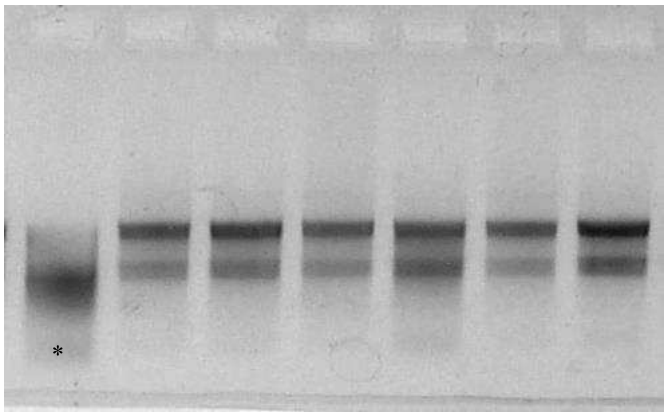

Figure 4 RNA integrity validation extracted from intestinal samples. Lane 1 features degraded RNA(*).

Total RNA (1 µg) was reverse transcribed into complementary DNA (cDNA; APPENDIX III). Quantitative real-time PCR was performed using a MyiQ real-time PCR detection system (Bio-Rad, Hercules, CA). Primer sequences are shown in Table 2. Amplification was carried out in a total volume of 20 µL containing 10 µL of SYBR Green Supermix (Bio-Rad), forward and reverse primers (0.5 µg/µL each), and 5 µL of cDNA template. Reactions were incubated at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, 52.9 to 59.7°C for 30 s, and 72°C for 30 s. At the end of the PCR, melt curve analysis was conducted to validate the specificity of the primers. A nontemplate control (only DNAse free water in wells) was run with every assay, and all determinations were performed in duplicate.

Table 2 *Primers* and annealing temperatures (AT) of evaluated genes

Gene[1]		Primer[2*]	AT (°C)	Reference
GAPDH	F	TCACCATCTTCCAGGAGCG	59.7	Saddoris, Fleet e Radcliffe (2010)
	R	CTGCTTCACCACCTTCTTGA		
IAP	F	CTAAAGGGGCAGATGAATGG	54.4	Lackeyram et al. (2010)
	R	CACCTGTCTGTCCACGTTGT		
IGF1	F	GTGCGGAGACAGGGGCTTTT	53.0	Schedle et al. (2008)
	R	AGATCCGGAAGCAGCACTCA		
GLP-2R	F	ACCTTGCAGCTGATGTACAC	56.6	Petersen, Burrin e Sangild (2001)
	R	GTGTTCTCCAGGTGTGCACG		
MUC2	F	CTGCTCCGGGTCCTGTGGGA	53.0	Pieper et al. (2012)
	R	CCCGCTGGCTGGTGCGATAC		
Occludin	F	ATCAACAAAGGCAACTCT	53.0	Zhang and Guo (2009)
	R	GCAGCAGCCATGTACTCT		
RelA/p65	F	GGAACACGATGGCCACTTG	52.9	Los Santos, San Segundo e Grubman (2007)
	R	AAGAGGACATCGAGGTGTATTTCAC		
SGLT1	F	GGCTGGACGAAGTATGGTGT	53.0	Yang et al. (2011)
	R	ACAACCACCCAAATCAGAGC		

[1]GAPDH: Glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene); IAP: Intestinal Alkaline Phosphatase; IGF1: Insulin-like growth factor 1; GLP-2R: glucagon-like peptide 2 receptor; MUC2: gene encoding mucin production; Occludin: plasma-membrane protein located at the tight junctions; RelA/p65: REL-associated protein involved in NF-κB heterodimer formation; SGLT1: Na+ dependent glucose transporter 1; [2]F: forward; R: reverse; *By IDT (Integrated DNA Technologies®)

The mRNA abundance values for each sample were normalized to GAPDH according to the $2^{-\Delta\Delta CT}$ method (LIVAK; SCHMITTGEN, 2001) and the following calculations:

$$\Delta CT_{target\ gene} = CT_{target\ gene} - CT_{GAPDH} \quad (4)$$

$$\Delta\Delta CT_{treatament} = (\Delta CT_{treatment} - \Delta CT_{control})^2 \quad (5)$$

Statistical analysis

Data were analyzed by one-way ANOVA using the MIXED procedure (SAS Inst. Inc., Cary, NC, 2009) and pig served as the experimental unit. The Shapiro-Wilk test was used to analyze the normality followed by the data transformation using the RANK procedure (SAS Inst. Inc., Cary, NC, 2009). Once all the data were transformed, the outliers were eliminated based on standard deviation above 1.75σ. The model included genetic line, and the residual mean square error was used as the error term. Differences were considered statistically significant at $P \leq 0.05$ and trends were considered with values $0.05 \leq P \leq 0.10$.

RESULTS AND DISCUSSION

There are described on the following tables the gene expression of trophic (GLP-2R, IGF1 and Occludin), digestive (IAP and SGLT1), and immunological (MUC2 and RelA/p65) intestinal markers of proximal jejunum (Table 3) from post-weaned piglets evaluated by *ex vivo* assay, and organ measures and growth performance of piglets during the *in vivo* phase prior to harvest (Table 4).

Table 3 Effect of different swine genetic lines utilizing an *ex vivo* procedure on the relative abundance of mRNA on proximal jejunum.

Item	Genetic Lines[1]		SE	*P*-value
	High	Low		
Trophic Markers				
GLP-2R	1,008	1,462	0,148	0.02
IGF1	0,850	0,762	0,083	0.87
Occludin	0,916	1,310	0,068	0.02
Digestive Markers				
IAP	1,109	1,715	0,128	0.44
SGLT1	0,952	0,962	0,076	0.55
Immunological Markers				
MUC2	1,020	1,145	0,062	0.89
RelA/p65	1,118	1,556	0,074	0.04

[1]High: high reactivity to soy and peanut protein; Low: low reactivity to soy and peanut protein.

Table 4 Effect of different swine genetic lines on liver weights and growth performance of pigs (8 days post-weaning).

Item	Genetic Lines[1]		SE	*P*-value
	High	Low		
Organ Measures				
Liver weight, g	176.80	207.78	0.51	0.0165
Liver/BW, g/Kg	25.62	24.07	6.67	0.1435
Growth Performance				
BW, d8	7.33	8.10	0.35	0.0068
ADG, kg	0.075	0.103	0.02	0.4184
ADFI, kg	0.111	0.138	0.01	0.3300
G:F	0.63	0.75	0.04	0.5077

[1]High: high reactivity to soy and peanut protein; Low: low reactivity to soy and peanut protein.

At the proximal portion of the jejunum, we observed a greater expression of GLP-2R and RelA/p65 in animals with low sensitivity to soy protein and peanuts, and regardless of intestinal segment, these same animals had higher

expression of occludin, and higher liver weight and final body weight for pigswith low sensitivity to soy protein.

The lower expression of RelA/p65 by the high reactor pigs may be involved in the inflammatory tissue issue. This immunological marker is part of a complex immunological activators (NF-kB) involved in several cascades that trigger an immune response. The highest expression of its components refers to a better intestinal protection, which is involved with animals with low sensitivity to soy and peanut proteins.

Likewise, most GLP-2R expression has been discussed. Petersen et al. (2003) reported that GLP-2R expression in the small intestine of piglets naturally decreases from birth until weaning because of oral feed administration, despite the presence of GLP-2 in the plasma increases. This receptor is present in neurons and not across the intestinal epithelium, suggesting that its mechanism of action is also associated with other receptors (SHIRAZI-BEECHEY, et al., 2010).

Despite the implication that GLP-2 inhibits the expression of inflammatory cytokines (IWAI et al., 2015), there are few reports of the relationship of its receptor with this process, and El-Jamal et al. (2014) found that, in humans, the expression of this receptor was down-regulated in intestinal inflammation situations and may be involved with the fact that animals with high sensitivity to the protein have shown a lower expression of this marker.

Furthermore, there is the relationship of the hormone GLP-2 acting in the inflammatory process. Cameron et al. (2003) found that GLP-2 decreases intestinal hypersensitivity reactions in mice with allergy. Later, Yu et al. (2014) demonstrated that GLP-2 can enhance the expression of proteins in jejunal epithelial tight junctions. So possibly the role of this hormone during the *in vivo* period may have influenced the results of occludin, affecting the tight junctions.

Occludin is a protein normally found with tight junctions, epithelial structures that act as regulators of macromolecules on intestinal transit between

the lumen and the interior of the body (LEE, 2015). The lower expression of this and other proteins present in the tight junctions increases the permeability of the structure, predisposing the animal to greater exposure to allergens factors. Factors that may influence the expression of this marker can be nutritional, as described by Zhao et al. (2011), who found that high doses of soy protein in the diet of weanling pigs increases intestinal permeability and reduce the expression of occludin and zonula-ocludens, the same observed by Pan et al. (2013) in relation to claudin.

These results agree with Zhao et al. (2014), who found that the allergenic factor beta-conglycinin reduced linearly the expression of tight junctions markers as it is increasd in the pig diet. Similarly, Chen et al. (2014) observed for mice, demonstrating that food allergies affect the permeability of tight junctions. In a study with humans, Tai et al. (2006) found that allergenic factors such as asthma were able to decrease the expression of this protein ad facilitates their penetration into the lungs and other allergenic factors.

In our results, the animals of the genetic lineage of high sensitivity to soy protein and peanuts, presented low occludin expression, which is probably related to the effects that diets offered in the *in vivo* phase, as the diets had soybean meal (14%), soybean oil (4%) and soy concentrate (4.5%), levels of these ingredients common in most weaning diets and should have been well tolerated by newly weaned pigs. The fact that these animals show allergy to these proteins may not be directly related to the expression of intestinal markers, but this genetic condition affects substantially intestinal factors that contribute to the presentation of allergenic effect.

Despite the higher liver weight of low protein sensitive pigs, there are no differences on liver mass relative to body size, which implies that is not associated with increasing in maintenance cost of these pigs. The higher body weight at 8

days after weaning of these same animals just confirm their ability to respond better to the soy proteins in the diet than high sensitive pigs.

CONCLUSION

Intestinal markers GLP-2R, RelA/p65 and occludin were less expressed in the jejunum of piglets with high sensitivity to soy protein and peanuts. The differences in occludin maybe the most highly related marker tested with the allergic condition these swine genetic lines present.

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ANEXOS

ANEXO I Isolamento de RNA para amostras incubadas

1) Utilize novo tubo contendo 450 µL de TRIzol e adicione uma quantidade de aproximadamente 2 µL de amostra descongelada.
2) Homogenize através de pistão de material plástico até que não reste conteúdo macroscópico (processo não deve durar mais que 10 segundos)
3) Adicione 100 µL de 1-bromo-3-cloropropano (BCP)
4) Misture (vortex) até que atinja coloração homogênea rosa-claro e mantenha em temperatura ambiente por 15 minutos
5) Centrifugue (12.000 rpm – 14.000 G, 4ºC) por 15 minutos
6) Transfira o sobrenadante a um tubo novo e descarte o tubo com TRIzol)
7) Adicione 500 µL de Isopropanol e inverta o tubo cinco vezes
8) Mantenha em temperatura ambiente por 10 minutos
9) Centrifugue (12.000 rpm – 14.000 G, 4ºC) por 15 minutos
10) Descarte o sobrenadante com cuidado para manter o pellet formado no fundo do tubo
11) Adicione 500 µL de etanol (75%) gelado
12) Destaque o pellet do fundo do tubo através de batidas contra a bancada
13) Centrifugue (12.000 rpm – 14.000 G, 4ºC) por 5 minutos
14) Repita as etapas de 10 a 13
15) Remova o etanol e deixe as amostras secarem ao ar (cerca de 10 a 15 minutos)
16) Adicione Água livre de RNAse (RNAse *Free Water*) de acordo com o tamanho do pellet (15 a 50 µL)
17) Spin por 15 segundo e incube a 56ºC por 10 minutos
18) Mantenha as amostras em gelo por pelo menos 10 minutos
19) Armazene as amostras à-80ºC

ANEXO II Isolamento de RNA para amostras frescas

1) Preparar antecipadamente 1% mercaptoetanol em Lysis buffer (10µL/1mL) e DNase (62 µL de RNase free water + 10 µL de DNase + 8 µL de DNase buffer) para cada amostra a ser extraída
2) Identifiqe os tubos para extração e mantenha-os no gelo
3) Adicione 600 µL de 1% Lysis buffer em cada tubo
4) Adicione parte da amostra descongelada nos novos tubos
5) Homogenize com o pistão plástico (10 a 20 segundos)
6) Centrifugue (2.600 G, temperatura ambiente) por 5 minutos
7) Cuidadosamente, transferir o sobrenadante para um novo tubo (utilizar pipeta)
8) Adicionar 600 µL de etanol a 70% e misturar (vortex) – 10 segundos
9) Transfira 650 µLpar o tubo "*spin cartridge*" (SC)
10) Centrifugue (12.000 G, temperatura ambiente) por 15 segundos
11) Descarte o líquido filtrado e coloque o SC no mesmo tubo coletor (TC)
12) Repita as etapas 9 a 11 até que não haja mais amostra a ser processada
13) Adicione 350 µL de Buffer I
14) Centrifugue (12.000 G, temperatura ambiente) por 15 segundos
15) Descarte o líquido filtrado e coloque o SC em um novo TC
16) Adicione 80 µL de DNase mix diretamente na superfície filtrante
17) Incubar à temperatura ambiente por 15 minutos
18) Adicione 350 µL de Buffer I
19) Centrifugue (12.000 G, temperatura ambiente) por 15 segundos
20) Descarte o líquido filtrado e coloque o SC em um novo TC
21) Adicione 500 µL de Buffer II
22) Centrifugue (12.000 G, temperatura ambiente) por 15 segundos
23) Descarte o líquido filtrado e coloque o SC no mesmo TC
24) Repita as etapas 21 a 23
25) Centrifugue (12.000 G, temperatura ambiente) por 60 segundos
26) Descarte o CT e utilize um novo tubo RNase free
27) Adicione 30-100 µL de RNase free water na superfície filtrante
28) Incubar à temperatura ambiente por 60 segundos
29) Centrifugue (12.000 G, temperatura ambiente) por 60 segundos
30) Armazene o RNA purificado em freezer – 80ºC

ANEXO III Síntese de cDNA

1) Calcular a relação RNA/água de acordo com a concentração de cada amostra de RNA de forma a obter 1 ug/mL de RNA em um volume final de 4,75 µL, como descrito no quadro a seguir (Quadro 1):

Quadro 1 Exemplos de cálculo para diluição de RNA

Amostra	ng/mL	ug/µL	µL de RNA	µL de água	Volume total (µL)
Exemplo 1	300,70	0,30070	3,33	1,42	4,75
Exemplo 2	575,51	0,57551	1,74	3,01	4,75

2) Em tubos de 0,2 mL, adicione o volume de água em cada tubo e em seguida o volume de RNA

3) Incube a 70ºC por 5 minutos

4) Resfrie as amostras em gelo por 10 minutos e prepare o mix de cDNA na seguinte proporção (Quadro 2):

Quadro 2 Componentes do mix de cDNA

Reagente de cDNA	µL de reagente por amostra
RT-buffer (5x)	2,00
BSA (1,0 mg/mL)	1,00
dNTPs (10 nmol)	0,50
Oligo-dT (0,1 mg/mL)	1,00
RNAsin	0,25
M-MLV	0,50
Total	**5,25**

5) Misture (vortex) e centrifugue rapidamente o mix e adicione 5,25 µL em cada amostra

6) Spin – vortex – spin cada amostra antes de iniciar o programa cDNA

7) No termociclador que realize o programa de cDNA (aproximadamente 80 minutos)

8) Adicione 90 µL de *Nuclease Free Water* em cada amostra

9) Armazene em temperatura de -20ºC