UNIVERSIDADE ESTADUAL PAULISTA - UNESP

CAMPUS DE JABOTICABAL

EFEITO DA SOLUBILIDADE DA PAREDE CELULAR DE *Saccharomyces cerevisiae* SOBRE A DIGESTIBILIDADE, PRODUTOS DE FERMENTAÇÃO MICROBIANA E PARÂMETROS IMUNOLÓGICOS DE CÃES ADULTOS

Stephanie de Souza Theodoro

Médica Veterinária

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Jaboticabal, 08 de março de 2019

DADOS CURRICULARES DO AUTOR

Stephanie de Souza Theodoro – nascida em 20 de Abril de 1990, filha de Paulo César de Almeida Theodoro e Katia de Souza, brasileira e natural de Guarulhos. Iniciou sua graduação em Março de 2009 na Universidade Federal de Pelotas – UFPel e concluiu em Março de 2014. No período de 1º março de 2015 a 28 de fevereiro 2017 foi residente do Programa de Residência em Nutrição e Nutrição Clínica de Cães e Gatos na Faculdade de Ciências Agrárias e Veterinárias (FCAV) UNESP – Campus de Jaboticabal, sob orientação do Prof. Dr. Aulus Cavalieri Carciofi. Em 2017, ingressou no mestrado na FCAV UNESP, no programa Medicina Veterinária (Clínica Médica Veterinária) com ênfase em Nutrição e Doenças Nutricionais de Cães e Gatos sob, também, orientação do Prof. Dr. Aulus Cavalieri Carciofi.

EPÍGRAFE

“Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas Graças a Deus, não sou o que era antes”.

Martin Luther King

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DEDICO

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SUMÁRIO

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Jaboticabal

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Mananoligossacarídeos da parede celular de *Saccharomyces cerevisiae* sobre a digestibilidade, produtos de fermentação microbiana e parâmetros imunológicos de cães adultos"**, protocolo nº 011937/17, sob a responsabilidade do Prof. Dr. Aulus Cavalieri Carciofi, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 11 de abril de 2019.

Vigência do Projeto	07/08/2017 a 28/02/2019
Espécie / Linhagem	Caninos/ Beagles
Nº de animais	24
Peso / Idade	13-15 kgs/ 2-6 anos
Sexo	Machos e fêmeas
Origem	Laboratório de nutrição de cães e gatos

Jaboticabal, 11 de abril de 2019.

Prof.ª Dr.ª Fabiana Pilarski
Coordenadora – CEUA

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EFEITO DA SOLUBILIDADE DA PAREDE CELULAR DE *Saccharomyces cerevisiae* SOBRE A DIGESTIBILIDADE, PRODUTOS DE FERMENTAÇÃO MICROBIANA E PARÂMETROS IMUNOLÓGICOS DE CÃES ADULTOS

RESUMO - Derivados da parede celular de levedura têm sido estudados por seu efeito prebiótico. Recentemente, preparações purificadas e mais solúveis foram desenvolvidas, com vista a aumentar seu efeito fisiológico. Este estudo teve por objetivo avaliar os efeitos da inclusão de dois diferentes extratos da parede celular de leveduras, um convencional (PCL) e outro com maior porcentagem de mananoligossacarídeos solúveis (PCLs) sobre a digestibilidade, produtos de fermentação nas fezes e alguns parâmetros imunológicos de cães adultos hígidos. Foi empregada uma única formulação, desdobrada em três tratamentos: CON – controle, sem adição de parede celular de levedura; PCL – adição de 0,3% de parede celular de levedura convencional; PCLs - adição de 0,3% de parede celular de levedura com elevada solubilidade de mananoligossacarídeos (MOS). Foram utilizados 24 cães beagle adultos, com oito repetições por ração, em delineamento em blocos casualizados. Cada bloco teve duração 30 dias, sendo avaliados no início e no final do período as concentrações séricas de fator de necrose tumoral alfa e interleucinas 6 e 10, produção *ex vivo* de intermediários reativos do oxigênio e nitrogênio por células mononucleares e polimorfonucleares de sangue periférico, capacidade fagocítica de monócitos e neutrófilos e concentração de IgA nas fezes. Adicionalmente, foi avaliada a digestiblidade aparente dos nutrientes, produção e qualidade das fezes, ácidos graxos de cadeia curta e ramificada, lactato, amônia, pH e aminas biogênicas nas fezes. Os resultados foram analisados por análise de variância e as médias comparadas pelo teste de Tukey ($P<0,05$). Para os parâmetros imunológicos, os valores no tempo zero foram empregados como covariável. A inclusão de PCLs reduziu a digestibilidade da gordura da ração ($P<0,05$), aumentou as concentrações de butirato e putrescina e reduziu a de lactato nas fezes dos cães ($P<0,05$), demonstrando que a solubilização da fração de MOS resultou em maior fermentação do composto, alterando o metabolismo da microbiota intestinal de cães. Foi verificada redução da concentração sérica de IL-6 nos cães alimentados com PCLs ($P<0,05$) e tendência de redução para os alimentados com PCL ($P=0,095$), sugerindo redução da atividade inflamatória nos animais. Maior índice de fagocitose foi também observado após 30 dias de consumo da dieta PCL

(P<0,03), sugerindo estimulação da imunidade inata. Como conclusão, os dois compostos demonstraram feitos relevantes nos cães e a solubilização dos MOS da parece celular de levedura modificou sua interação com a microbiota e efeitos biológicos nos animais.

Palavras chave: imunidade, levedura, mananoligossacarídeos, prebiótico

EFFECTS OF THE SOLUBILITY OF YEAST CELL WALL OF *Saccharomyces cerevisiae* ON DIGESTIBILITY, FERMENTATION PRODUCTS ON FECES, AND IMMUNOLOGICAL PARAMETERS OF ADULT DOGS

ABSTRACT - Derivates of yeast cell wall have been studied by its prebiotic effect. Recently, more purified and soluble preparations were developed, attempting to increase their biological actions. This study evaluated the inclusion of two yeast cell wall preparations, one conventional (YCW), and another with higher solubility of the mannan oligosaccharide fraction (YCWs), on their effects on nutrient digestibility, fermentation products on feces, and some immunological parameters of dogs. A single food formulation was used, unfolded on the following treatments: CON – control, without yeast cell wall addition; YCW – addition of 0.3% of a conventional yeas cell wall extract; YCWs – addition of 0.3% of a yeast cell wall extraction with elevated mannan oligosaccharides solubility. Twenty-four adult beagle dogs were used, eight dogs per food, distributed on a randomized block design. Blocks lasted 30 days, and tumor necrosis factor alpha and interleukins 6 and 10, *ex vivo* production of hydrogen peroxide and nitric oxide by peripheral neutrophils and monocytes, phagocytic index, and fecal IgA were evaluated at the beginning and ending of each period. Additionally, nutrient digestibility, feces production and quality, and fermentation products, pH, and biogenic amines were quantified on feces. Results were evaluated by variance analysis and compared by Tukey test ($P<0.05$). For the immunological parameters, the basal values were used as a covariate. The inclusion of YCWs reduced fat digestibility ($P<0.05$), increased the concentration of butyrate and putrescine, and reduced the lactate on feces ($P<0.05$), showing that mannan oligosaccharide solubilization resulted in higher fermentation of this compound, changing the metabolism of the microbiota of dogs. Lower IL-6 on serum was verified for dogs fed the YCWs diet ($P<0.05$), and the concentration of this cytokine also tended to be lower in dogs fed the YCW than the control diet ($P=0.095$), suggesting a reduction on the inflammatory activity of dogs. Higher phagocytic index was verified for peripheral monocytes after 30 days of the intake of the YCW food, indicating better innate immunity. In conclusion, both yeast cell wall derivates presented prebiotic effects on dogs, and the solubilization of the mannooligosaccharide fraction altered its interaction with the gut microbiota and biological effects on animals.

Keywords: immunity, yeast, mannan oligosaccharides, prebiotic

LISTA DE ABREVIATURAS

ACK: Ammonium-Chloride-Potassium
BCFA: Branched-chain fatty acids
$CaCl_2$: Calcium chloride
CO_2: carbon oxide
CON: Controle / Control
DM: Dry matter
E. coli: Escherichia coli
EDTA: Ethylenediamine tetraacetic acid
FBS: Fetal bovine serum
FEDIAF: Fédération Européenne de L'industrie des Aliments pour Animaux Familiers
FOS: Frutoligossacarídeo
g: grama / gram
GALT: Gut-associated lymphoid tissue
GE: Gross energy
GOS: Glucoligossacarídeo
H / hr: Hora
H_2O_2: hydrogen peroxide
H_2Od: distillated water
(IFN)-γ: Interferon gama / Interferon gamma
IL-2: Interleucina 2 / Interleukin 2
IL-6: Interleucina 6 / Interleukin 6
IL-10: Interleucina 10 / Interleukin 10
KCl: Potassium chloride
Kg: quilo
KH_2PO_4: Monopotassium phosphate
LPS: Lipopolysaccharide
M: molar
$MgCl_2$: Magnesium chloride
Min: minuto / minut
mL: mililitro / militer
mm: milímetro / milimeter
mM: micromolar
nM: nanomol
MOS: Mananoligossacarídeos
Na_2HPO_4: Disodium phosphate
NaCl: Sodium chloride
NaOH: Sodium hydroxide
NO: nitric oxide
PBS: phosphate buffered saline
PCL: parede celular de levedura

PCLs: parede celular de levedura solúvel
PMA: Phorbol myristate acetate
OD: optical density
OM: Organic matter
SCFA: Short chain fatty acid
Th1-helper: T helper 1
Th2-helper: T helper 2
TNF-α: tumor necrosis factor alpha
VFA: Volatil fatty acids
YCW: standard yeast cell wall extract
YCWs: yeast cell wall extract with 20% soluble mannan oligosaccharides
µg: micrograma / microgram
µl: microlitro / microliter
µM: micromol

LISTA DE TABELAS

Página

CAPITULO 1 – Considerações Gerais

1. INTRODUÇÃO

O Brasil é o segundo maior país do mundo em população de animais de estimação. Em consequência deste cenário, o mercado pet apresentou desenvolvimento expressivo nas últimas décadas. No ano de 2017, o Brasil alcançou a terceira posição mundial como maior produtor de artigos e serviços para cães e gatos, representando o setor de pet food 68,6% deste faturamento (Abinpet, 2018).

Devido ao fato de os proprietários de cães e gatos apresentarem-se mais conscientes quanto aos benefícios que a alimentação industrializada proporciona, adicionado ainda a um melhor custo/benefício e maior controle da saúde do animal, inúmeros produtos, ingredientes e empresas do setor de nutrição pet surgiram no mercado. Com isso, o foco da nutrição tem sido direcionado para a obtenção de uma dieta completa e balanceada que proporcione maior qualidade e expectativa de vida, com melhor bem-estar pela utilização de ingredientes que desenvolvam a capacidade de resistência as doenças e melhorem a saúde animal.

A utilização de ingredientes que possam melhorar a saúde do animal e prevenir a ocorrência de doenças está diretamente relacionado com o interesse humano, pois a relação próxima entre estas espécies fez com que ambos compartilhassem alterações significativas na qualidade da saúde, principalmente em relação a composição microbiológica, podendo haver facilidade de exposição à agentes patogênicos e influências ambientais nocivas (Grzéskowiak et al., 2015). Assim, tem se procurado entender como a dieta pode influenciar os mecanismos de defesa do organismo e como esta ação é exercida por vários dos nutrientes essenciais e compostos adicionados ao alimento. Alimentos funcionais têm como objetivo melhorar algumas funções do organismo, sendo também usados como imunomoduladores. Essas substâncias podem agir tanto sobre a imunidade celular como humoral, ao aumentar o número de células, melhorar sua função e elevar a produção de anticorpos, dentre outros mecanismos (Zaine, 2010).

Dentre estas substâncias empregadas com o objetivo de promover a

saúde, destacam-se os prebióticos. Estes podem ser definidos como compostos não digeridos por enzimas, sais e ácidos produzidos pelo organismo do animal, mas que são seletivamente fermentados pelos microrganismos do trato gastrintestinal. Tais compostos podem estar presentes nos ingredientes da dieta ou serem adicionados a ela por meio de fontes exógenas concentradas (Pelícia et al., 2004).

Os mananoligossacarídeos (MOS), compostos naturalmente presentes na parede celular de leveduras, são fermentados no intestino de cães gerando produtos de fermentação benéficos, como ácido butírico. Além disso, acredita-se que os MOS apresentam a capacidade de modular o sistema imunológico, a microbiota intestinal e preservar a integridade da superfície de absorção intestinal, ao bloquear a aderência das bactérias patogênicas às células epiteliais da mucosa do intestino.

Nova geração de prebiótico a base de parede celular de leveduras (*Saccharomyces cerevisiae*) foi desenvolvida a partir de processo que resulta em elevada concentração da camada de mananoligossacarídeos solúveis em água no ingrediente. Estas características, elevada concentração e solubilidade, parecem conferir a este produto melhor potencial em modular o ambiente intestinal e a imunidade. Seu processo produtivo promove solubilização das camadas de mananoligossacarídeos da parece celular e reduz o tamanho das partículas. Devido a estes fatores, é possível que este ingrediente apresente atividade prebiótica aprimorada, mas este ainda não foi testado em cães.

2. REVISÃO DE LITERATURA.

Nos dias atuais, os cães e gatos passaram a ter papéis cada vez mais importantes na sociedade, sendo criados especialmente como “membros da família”. Este fenômeno ocorre em decorrência do aumento da expectativa de vida humana, maior número de pessoas idosas, redução do número de filhos, falta de segurança e, ainda, por maior carência afetiva (Russo, 2005; Gomes, 2009). Esse novo modelo de comportamento social fez com que estes proprietários buscassem mais conhecimentos, produtos e serviços que aumentassem a expectativa de vida, ampliassem o bem-estar e

recompensassem o companheirismo dos seus animais de estimação (Bernasconi & Alcântara, 2007).

De mesmo modo, a maior compreensão da população com relação aos benefícios da alimentação industrializada para cães e gatos, incluindo melhor relação custo benefício e maior segurança na saúde dos mesmos tem levado ao aumento do uso de *pet food* no Brasil nos últimos anos (Háfez, 2002). Dentro deste contexto, este maior grau de instrução da população e dos fabricantes têm contribuído para maior utilização de produtos de melhor qualidade, estimulando o desenvolvimento e a produção dos alimentos com maior valor agregado (Ambrozini, 2007).

O desenvolvimento da nutrição de cães e gatos vem seguindo o mesmo padrão observado na nutrição humana. Os conceitos de nutrição estão se expandindo para além do limite de promover saciedade e garantir a sobrevivência do animal, visando maximizar a produção e utilização de alimentos que promovam bem-estar e melhora na saúde, além de reduzir risco de doenças (Fahey, 2003). Mais recentemente, tem-se definido que fornecer cuidados adequados de saúde e dieta nutricionalmente balanceada para animais de companhia é parte da responsabilidade humana de manter o bem-estar dos animais de estimação (Grzéskowiak et al., 2015).

O interesse mútuo na relação entre o ser humano e os animais de companhia trouxe diversos benefícios para as duas espécies. No entanto, essa proximidade fez com que estas espécies enfrentassem desafios comportamentais e na área da saúde muito parecidos. A saúde e o bem-estar dos animais de companhia, tal como dos seus proprietários, depende da composição microbiológica do intestino e da pele. No entanto, como existem diversas composições microbiológicas pode haver facilidade de exposição à agentes patogênicos e influências ambientais nocivas, com isso, é prudente a pesquisa de novas ferramentas para proteger cães e gatos e, ao mesmo tempo, os seres humanos destes patógenos (Grzéskowiak et al., 2015).

A dinâmica inter-relação entre nutrição, imunologia e microbiota do intestino abre oportunidade para melhorar a saúde intestinal, bem como, o status imunológico de cães e gatos (Kroll, 2014). A composição, associada a atividade da microbiota, têm sido relacionada a diversas doenças nos animais, inclusive em seus proprietários (Colliard et al., 2006). O estudo desta inter-

relação pode beneficiar o desenvolvimento de conhecimento mais exato sobre o ambiente intestinal e o estado imunológico de animais de estimação e, por consequência, de seus proprietários.

Durante a formulação de uma ração a escolha dos ingredientes, o processamento industrial, sua composição nutricional e a presença de alguns carboidratos especiais podem ser ferramentas potenciais para fornecer condições ideais de funcionamento ao intestino, frente às alterações que ocorrem durante todas as fases fisiológicas do animal (Kroll, 2014). Muitos ingredientes já foram propostos como provedores de benefícios por alterarem um ou mais processos fisiológicos no intestino grosso e, com a continuidade das pesquisas, provavelmente muitos outros ainda surgirão (Fahey, 2003). O uso destes ingredientes têm como objetivo promover benefícios adicionais aos da nutrição básica, incluindo reduzir os riscos de doenças e proporcionar saúde adequada, devido ao fato de incluírem componentes fisiologicamente ativos (Cutrignelli, 2011). Dentre as pesquisas mais promissoras nesta área se incluem os alimentos funcionais que promovem saúde do trato gastrintestinal, apresentam efeitos antioxidantes ou atuam sobre o metabolismo dos macronutrientes (Fahey, 2003).

Em animais não ruminantes o intestino grosso tem papel importante na absorção e secreção da água e eletrólitos. Alberga, também, importante ecossistema de microrganismos que influencia tanto a saúde como a doença no animal hospedeiro (Adams, 2003). Carboidratos que são indigeríveis por enzimas de mamíferos estão entre os compostos que influenciam a composição e atividade metabólica da microbiota intestinal, sendo, portanto, de interesse para a formulação de *pet food* e alimentos veterinários específicos (Zentek et al., 2002). Neste contexto, inserem-se os prebióticos, compostos não digeridos por enzimas, sais e ácidos produzidos pelo organismo animal, mas que são seletivamente fermentados pelos microrganismos do trato gastrintestinal (Pawar et al, 2017; Rose et al., 2017). Estes compostos podem naturalmente estar presentes nos ingredientes da dieta ou serem adicionados a ela por meio de fontes exógenas concentradas (Pelícia et al., 2004).

De acordo com a definição atualizada em 2004, um prebiótico deve respeitar à três critérios: 1) ser resistente à acidez gástrica e/ou hidrólise por enzimas de mamíferos e absorção intestinal; 2) fermentado pela microbiota

intestinal; 3) estimular seletivamente o crescimento e/ou atividade de bactérias intestinais associadas à saúde e ao bem-estar (Gibson et al., 2004).

Os oligossacarídeos mais estudados quanto a seu efeito prebiótico na alimentação animal são os frutoligossacarídeos (FOS), glucoligossacarídeos (GOS) e mananoligossacarídeos (MOS). O MOS pode ser obtido a partir de parede celular de leveduras, apresentando o Brasil importante destaque como produtor e detentor de tecnologia para a produção de leveduras e produtos derivados da purificação de sua parede celular. A parede celular de leveduras é constituída principalmente por proteínas e carboidratos. Sua fração de carboidratos é constituída por quantidades semelhantes de glicose e manose mais N-acetilglicosamina (quitina). O MOS, usado como aditivo em rações, consiste de fragmentos de parede celular de *Saccharomyces cerevisiae* com uma estrutura complexa de manose fosforilada, glicose e proteína (Furlan, 2004).

Pelo fato de não ser digerido pelo organismo do animal e ser fermentado seletivamente no intestino grosso, o consumo de MOS apresenta como característica estimular o crescimento e/ou a atividade metabólica de bactérias benéficas (Swanson et al., 2002; Gomes, 2009; Pawar et al, 2017). Em função da produção de substâncias antibacterianas e enzimas pela microbiota benéfica, o consumo de MOS inibe a proliferação dos microrganismos nocivos, tais como *Escherichia coli,* Clostridium spp. e *Salmonella*. Ao estimular o crescimento de bactérias benéficas atua, por consequência, indiretamente sobre o sistema imune. Essas bactérias produzem substâncias com propriedades imuno-estimulatórias, interagindo com o sistema imune de várias maneiras, incluindo: produção de citocinas, proliferação de células mononucleares, fagocitose macrofágica e indução de síntese de maiores quantidades de imunoglobulinas, em especial as de classe A (Macfarlane & Cummings, 1999).

O aumento da concentração de produtos de fermentação no cólon é importante para a saúde intestinal e geral do animal. Os ácidos graxos de cadeia curta acetato, propionato e butirato e o ácido lático são considerados fonte de energia extra para o hospedeiro, promovem redução do pH do cólon e com isto inibição de crescimento de microorganismos patogênicos (Gomes, 2009). O ácido butírico é a principal fonte de energia para os colonócitos,

sendo importante regulador do crescimento e diferenciação celular (NRC, 2006), este pode representar até 70% da energia utilizada pela mucosa do cólon. O suprimento de butirato ajuda a manter a integridade da mucosa, desempenhando importante papel na manutenção de um fenótipo celular normal e redução do risco de carcinomas colônicos (NRC, 2006). Os ácidos graxos de cadeia curta ainda favorecem o fluxo sanguíneo e a atividade muscular no cólon, estimulam da produção de mucina e a proliferação dos enterócitos. Assim, embora os produtos da fermentação intestinal de proteínas sejam geralmente considerados prejudiciais à saúde humana e animal, os produtos da fermentação de carboidratos contribuem positivamente na digestão e metabolismo do hospedeiro, além de atenuar os efeitos negativos dos produtos gerados pela fermentação das proteínas (Tellez et al., 2006).

Pesquisas sobre o assunto levaram a identificação e especificação de diferentes açúcares presentes na parede celular de uma cepa específica de *Saccharomyces cerevisiae*, o que resultou no desenvolvimento de uma segunda geração do produto comercial à base de parede celular de leveduras, tornando-se mais solúvel (Biorigin, Lençóis Paulista, São Paulo, Brasil). Este produto apresenta processo de produção particular para maior solubilização das camada, redução do tamanho das partículas e maior exposição do conteúdo de glucanas. Mais purificado do que o PCL convencional, que em realidade trata-se da simples parede celular seca, após remoção do conteúdo celular, esse produto parece reter boa capacidade de ligação com patógenos específicos com bom potencial para "deslocamento" de bactérias pelo trato gastrointestinal, evitando colonização (Spring, et al., 2015). Che et al. (2012) realizaram estudo com suínos infectados com um tipo específico de vírus reprodutivo e respiratório, assim verificaram que esse MOS foi efetivo no aumento da resposta imune. Além disso, a suplementação com mananoligossacarídeos melhorou a utilização de nutrientes nos suínos infectados durante o tempo de 28 a 42 dias pós-inoculação ($P<0,01$). Munyaka et al. (2012) mostraram que essa suplementação da dieta de frangos de corte também reduziu a expressão genética de receptores semelhantes a TLR4, citoquinas IL-12p35, interferom (IFN)-γ e citocinas IL-10 no íleo. Esses mediadores de imunidade fazem parte de duas vias imunes, a Th1-helper e a Th2-helper, e esses resultados observados sugerem ativação de respostas

anti-inflamatórias em frangos de corte. Quando comparadas com resultados obtidos com PCL convencional, o consumo da parede celular de leveduras mais concentrada em atividade biológica (Bio-Mos) induziu melhor desempenho e crescimento de frangos (Hooge et al., 2013). Em um dos poucos estudos com cães, dissertação realizada por nosso grupo de pesquisas, avaliou a inclusão de até 0,08% de frações ativas de mananoproteínas, que também são mais purificadas, em dietas para animais adultos e idosos. O composto apresenta forma de obtenção semelhante à da PCLs, e seu consumo resultou em efeitos imunomodulares benéficos, com aumento da atividade funcional de neutrófilos de cães adultos e idosos, aumento da contagem de linfócitos T de cães adultos e maior resposta cutânea de cães idosos aos estímulos de vacina e fito-hemaglutinina (Kroll, 2014).

No entanto, não está claro ainda como ocorre esta interação direta dessa nova parede celular com o sistema imune. Além disso, a maior parte dos estudos com a espécie canina foram sobre MOS, e verificaram seus efeitos sobre a digestibilidade, produtos de fermentação e características das fezes (Zentek, et al, 2002; Grieshop, et al, 2004; Middelbos et al., 2007). Poucos trabalhos avaliaram os efeitos imunológicos diretamente em cães, que em última instância são as informações mais recentes sobre estes prebióticos. A indústria brasileira tem desenvolvido, também, processos de purificação e separação dos componentes da parede celular de levedura que geram frações mais solúveis e particulares, com melhor potencial de ação imunológica.

3. HIPÓTESE

Acredita-se que a parede celular de leveduras com maior solubilidade da fração de mananoligossacarídeos em relação à parede celular convencional de *Saccharomyces cerevisiae* incorporadas à dieta de cães adultos possa modular, de forma positiva o sistema imune, por meio da absorção intestinal do composto e por promover, também, a síntese de produtos benéficos de fermentação microbiana, como o ácido butírico.

4. OBJETIVO

Com base nas considerações anteriores, objetivou-se avaliar os efeitos da inclusão na dieta de cães adultos de extrato da parede celular de *Saccharomyces cerevisiae* com maior solubilidade da fração de mananoligossacarídeos sobre a digestibilidade, produtos de fermentação microbiana nas fezes e alguns parâmetros imunológicos.

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CAPÍTULO 2 – EFFECTS OF THE SOLUBILITY OF YEAST CELL WALL PREPARATIONS ON THEIR PREBIOTIC PROPERTIES IN DOGS

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ABSTRACT

Derivates of yeast cell wall has been studied by its prebiotic effect. Recently, more purified and soluble preparations have been developed, attempting to increase their biological actions. The inclusion of two yeast cell wall preparations, one conventional, and another with higher solubility of the mannan oligosaccharide fraction were evaluated on their prebiotic effects for dogs. One single food formulation was used, unfolded on the following treatments: CON – control, without yeast cell wall addition; YCW – addition of 0.3% of a conventional yeas cell wall extract; YCWs – addition of 0.3% of a yeast cell wall extraction with high mannan oligosaccharide solubility. Twenty-four adult beagle dogs were used, eight dogs per food, distributed on a block design. Blocks lasted 32 days, and TNF-a, IL-6, IL-10, *ex vivo* production of hydrogen peroxide and nitric oxide by peripheral neutrophils and monocytes, phagocytic index, and fecal IgA were evaluated at the beginning and ending of each period. Additionally, nutrient digestibility, and feces production, quality, and fermentation products were quantified. Results were evaluated by variance analysis and compared by Tukey test ($P<0.05$). For the immunological parameters, the basal values were used as a covariate. The inclusion of YCWs reduced fat digestibility ($P<0.05$), and increased the concentration of butyrate and putrescine, reducing the lactate on feces ($P<0.05$), showing that mannan oligosaccharide solubilization resulted in higher fermentation of this compound, altering the metabolism of the microbiota of dogs. Lower IL-6 on serum was verified for dogs fed the YCWs diet ($P<0.05$), and the concentration of this cytokine also tended to be lower in dogs fed the YCW than the control diet ($P=0.095$), suggesting a reduction on the inflammatory activity of dogs. Higher phagocytic index was verified for peripheral monocytes after 30 days of the intake of the YCW food, indicating better innate immunity. As a conclusion, the solubilization of the mannooligosaccharide fraction altered it interaction with the gut microbiota and biological actions on animals, although both yeast cell wall preparations presented prebiotic effects on dogs.

Key words: biogenic amines, butyrate, interleukins, mannan oligossacharides, monocytes phagocytosis

1. INTRODUCTION

The health of the gut is dependent of a dynamic inter-relationship between gut microbiota and gut nutrition (Holscher, 2017; Bibi et al., 2019), reflecting directly on the immunological status and general health of dogs (Maria et al., 2017; Coman et al., 2019). It is postulated that the intestinal microbiota presents at least three main functions: protection, nutrition and metabolic control (Ballesteros-Pomar; Arnaiz, 2018). The microbiome acts as a barrier with important protective effect against pathogens; performs the fermentation of dietary nondigestible residues and endogenous substances, allowing the production of important nutrients for gut mucosa as the short-chain fatty acids; controls intestinal epithelial cells proliferation and differentiation; contributes to the immune system development and homeostasis (Ballesteros-Pomar; Arnaiz, 2018).

Living on the interaction of the host and their diet, food composition is one of the most important factors for gut microbiota maintenance, structure and function (Delzenne et al., 2013; Holscher, 2017; Townsend et al., 2019). On this regard, yeast cell wall (YCW) may be an important energy sources for intestinal microorganism (Calabrò, et al., 2013), and has been studied as an effective prebiotic for dogs (Gouveia et al., 2013; Spring et al., 2015). Mainly composed by carbohydrates and proteins, their main chemical constituents are mannose, glucose and N-acetylglucosamine (chitin) (Northcote; Horne, 1952; Aguilar-Uscanga; François, 2003). The YCW meets the three essential criteria of a prebiotic (Gibson et al., 2004), been resistant to gastric acidity and hydrolysis by mammalian enzymes and gastrointestinal absorption, is fermented by intestinal microbiota, and selectively stimulate the growth and/or activity of intestinal bacteria associated with health and wellbeing (Fowler et al., 2015; Park et al., 2016; Holscher, 2017).

Among the possible mechanisms implicated on host health, prebiotics such as the derivates of the YCW may promote short chain fatty acid (SCFA) production, colon pH regulation, and competition against pathogens for cell mucosa receptors (Park et al., 2017). Experimental data on animal studies have shown that the gut-associated lymphoid tissue (GALT) may be the primary target of the immunomodulatory effect of prebiotics (Schley; Field, 2002;

Roberfroid et al., 2010), and the enterocytes are key intermediates that transmit signals from the intestinal lumen to the GALT (Roberfroid et al., 2010). Few studies about the prebiotic effects of YCW are available on dogs. An increase in serum lymphocyte concentration and decrease in plasma neutrophils, indicative of an improvement in immunological status was found (Swanson et al., 2002). The SCFA generated after microbial fermentation of the YCW components may also modulate inflammation, since butyric acid may inhibits the production of the proinflammatory cytokines IL-2 and IFN-γ, as well as the acetic and propionic acids may increase the production of the immunoregulatory cytokine IL-10 (Cavaglieri et al., 2003; Kaisar et al., 2017).

Among the last years, specific strains of *Saccharomyces cerevisiae* and special techniques to separate and purify specific components of the cell wall structure has been developed. More purified than the conventional YCW derivates, which are the simple dried cell wall after the cellular content removal, these preparations have higher concentrations of soluble mannooligosaccharides, different particle sizes and higher solubility in water, characteristics that may change the YCW exposition to gut microbiota and the host mucosa, and may induce different biological responses (Vetvicka et al. 2014; Spring et al. 2015). Based on this, the present study evaluated the effects of the incorporation on extruded diets of two preparations of *Saccharomyces cerevisiae* cell wall, with different soluble mannooligosaccharides amounts and different solubilities in water on nutrient digestibility, microbial fermentation products on feces, and some immunological parameters of adult dogs.

2. EXPERIMENTAL METHODS

The study was conducted in the Laboratory of Research in Nutrition and Nutritional Diseases of Dogs and Cats, College of Agrarian and Veterinarian Sciences, Sao Paulo State University (UNESP), Jaboticabal, SP, Brazil. All procedures with animals followed the ethical principles adopted by the Brazilian College of Animal Experimentation and were previously approved by the Ethics Committee on the Use of Animals (protocol n° 011937/17).

Test Products

Two extracts were used, obtained by the industrial purification of specific strains of *Saccharomyces cerevisiae* cell wall (Biorigin, Lençóis Paulista, São Paulo, Brazil). Proprietary preparations of the cell wall resulted in products of different solubilities in water, one with approximately 20% water solubility index, named standard Yeast Cell Wall (YCW), and another with 40% water solubility index, named soluble Yeast Cell Wall (YCWs) (Table 1). Mainly mannan oligosaccharides was solubilized during the process, that results in 2.1% soluble mannan oligosaccharides for the YCW and 22.2% soluble mannan oligosaccharides for the YCWs. The water solubility index was determined (Anderson, 1981).

Table 1. Characteristics of the yeast cell wall derivates used on the study.

Composition	Yeast Cell Wall preparations [2]	
	YCW	YCWs
Protein (%)	31.44	25.40
Moisture (%)	6.94	4.50
Ash (%)	7.72	5.40
pH	4.96	3.60
Mannan oligosaccharides (%)	17.12	23.90
Soluble mannan oligosaccharides[1] (%)	2.10	22.20
Glucans (%)	25.10	24.90
Product water solubility index[1]	19.56	42.63

[1] Determined on Biorigin Laboratory (Lençois Paulistas, Brazil) according to Anderson (1981).

[2] YCW = standard yeast cell wall extract; YCWs = yeast cell wall extract with 20% soluble mannan oligosaccharides.

Animals

Twenty-four adult Beagle dogs, males or females, with 3.5±0.91 years and 11.95±1.12 kg of body weight were used. The mean body condition score of the dogs was 6±1.23, in a scale from 1 to 9 (Laflamme, 1997). Prior to the study, dogs were submitted to physical, hematological, and serum biochemical evaluations by a veterinarian, and all were considered healthy.

Experimental Design

The study included three experimental diets and was conducted in a randomized block design with two blocks of 12 dogs each, and four dogs per diet in each block, totaling eight animals (repetitions) per diet (treatment). The blocking factor was time, due to available structure for research. Each block lasted 32 days: phagocytic activity of peripheral monocyte and neutrophils were evaluated on days 0, 15 and 30; cytokines on peripheral blood, and the *in vitro* production of hydrogen peroxide and nitric oxide in cell culture were evaluated on days 0 and 30; IgA content on feces were evaluated prior to study (feces collected form days -2 to 0) and after 30 days of diet intake (days 30 to 32); total feces collection for digestibility measurement were performed from days 16 to 20; fresh feces collection to analyze fermentation products, pH and biogenic amines were conducted on days 23 to 25.

The amount of food offered was initially calculated considering the food metabolizable energy content, estimated by its chemical composition, and the individual energy requirement of laboratory dogs (NRC, 2006). The daily amount was provided at once a day (10hs), offered and refused food was weighted, and the intake recorded. Dogs were then weekly weighed, and the amount of food provided adjusted to animals maintain a constant body weight throughout the study. Water were provided *ad libitum.* During the study dogs were housed in kennels with 1,5m x 3,5m with a solarium, and released daily in a collective playground to exercise and socialization.

Experimental Diets

A single formulation based on corn grain, poultry by-product meal, poultry fat and sugarcane fiber was used (Table 2), balanced for adult dogs according to the nutritional recommendations of the European Pet Food Industry Federation (FEDIAF, 2017). Sugarcane fiber was used due to its low fermentation (Loureiro et al., 2017), reducing the interference on fermentation products evaluation. The experimental diets were obtained by the addition of the different yeast cell wall extracts, added in replacement of corn (on as-fed basis): CON - control diet, without inclusion of yeast cell wall extract; YCW - inclusion of 0.30% of the product YCW; YCWs - inclusion of 0.30% of the product YCWs.

Table 2. Ingredient and chemical composition of the food used on the study.

Ingredients	%
Corn grain	51.60
Poultry by-product meal	32.21
Poultry fat	9.22
Liquid palatant [1]	3.0
Sugarcane fiber [2]	2.0
Vitamin-mineral premix [3]	0.5
Salt	0.5
Potassium chloride	0.48
Choline chloride	0.32
Mold inhibitor [4]	0.1
Antioxidant [5]	0.04
L-lysine	0.03
Analyzed chemical composition	**%**
Dry matter	93.0
Crude protein	27.4
Ash	9.0
Acid-hydrolyzed fat	17.4
Crude fiber	2.6
Calcium	1.9
Phosporus	1.3

[1] D'TECH 10L, Palatabilizante Líquido, SPF do Brasil Indústria e Comércio Ltda., Descalvado, Brazil.
[2] Vit2be Fiber, Dilumix Industrial Ltda., Leme, Brazil.
[3] Rovimix, DSM Produtos Nutricionais Brasil S.A., Jaguaré, Brazil. Added per kg of food: Vitamin A, 18,750 IU; Vitamin D3, 1,500 IU; Vitamin E, 125 IU; Vitamin K3, 1,5 mg; Vitamin B1, 5 mg; Vitamin B2, 16.25 mg; Pantothenic Acid, 37.5 mg; Vitamin B6, 7.5 mg; Vitamin B12, 45 mcg; Vitamin C, 0,125 g; Nicotinic Acid, 0.0625; Folic Acid, 0.75 mg; Biotin, 0.315 mg; Iron, 0.1 g; Copper, 9.25 mg; Manganese, 6.25 mg; Zinc, 0.15 g; Iodine, 1.875 mg; Selenium, 0.135 mg.
[4] Mold-Zap Citrus, Alltech do Brasil Agroindustrial Ltda., Araucária, Brazil.
[5] Banox, Alltech do Brasil Agroindustrial Ltda., Araucária, Brazil.

Diets were processed at the Extrusion Laboratory of the College of Agrarian and Veterinarian Sciences, Sao Paulo State University (UNESP), Jaboticabal, SP, Brazil. A single lot of raw materials was used for the three experimental diets. Ingredients were weighted and mixed before being ground in a hammer mill (Tigre, Moinhos Tigre, São Paulo, SP) fitted with a 0.8mm sieve screen size and extruded in a single-screw extruder (Model Mex-250, Manzoni Industria Ltda, Campinas, SP), with an average extrusion capacity of

250 kg/h. The temperature of the extruder preconditioner was kept higher than 85°C by direct steam injection. After extrusion, the kibbles were dried in a forced air dryer at 105°C for approximately 20 minutes and coated with poultry fat and liquid palatant.

Digestibility Protocol, Feces Production and Characteristics

This evaluation followed the recommendations and procedures described by FEDIAF (2017). Dogs were individually housed for 5 days in stainless steel metabolic cages with apparatus to collect feces and urine separately. Food consumption was recorded daily, measuring the offered and refused amounts. Feces were quantitative collected at least twice a day for 120h, weighed, and stored frozen at -15°C until analysis. After the end of the collection period, feces were thawed to room temperature and homogenized compounding a single sample per dog, and dried in a forced-air oven (320-SE, FANEM, São Paulo, Brazil) at 55^{o}C for 72 hours. Pre-dried feces and diets were ground in a knife type mill (MOD 340, ART LAB, São Paulo, Brazil) with a 0.8mm sieve screen size for laboratory analysis.

The gross energy (GE) content of diets and fecal samples was determined using a bomb calorimeter (IKA C2000 Basic, IKA-Werke GmbH & Co. KG, Staufen, Germany). Dry matter (DM) was determined by oven-drying the sample (method 934.01), ash by muffle furnace incineration (method 942.05), crude protein using a LECO nitrogen/protein determination (FP-528, LECO Corporation, Saint Joseph, USA; method 990.03), total fat was assessed using the acid-hydrolyzed fat assay (method 954.02), and organic matter (OM) was calculated as DM minus ash. All samples were analyzed in duplicate, and the analysis were repeated when the variation between replicates was greater than 5%.

During collection, the fecal score was determined using the following system (Carciofi et al., 2008): 0 = watery liquid that can be poured; 1 = soft, unformed; 2 = soft, malformed stool that assumes the shape of its container; 3 = soft, formed, and moist stool that retains its shape; 4 = well-formed and consistent stool that does not adhere to the floor; and 5 = hard, dry pellets, which are small and hard masses.

Fecal pH and Fermentation Products

For this evaluation, from days 23 to 25 of each block fresh feces sample was collected immediately after eliminated for three consecutive days. Fecal pH was determined in 2 g of fresh feces mixed with 6 mL of ultrapure water, and the results obtained with a pH meter (model DM20; Digicrom Analitica LTDA, São Paulo, Brazil). Approximately 10 g of fresh feces were homogenized and mixed with 30 mL of a 16% (vol/vol) formic acid solution and precipitated to determine the volatile fatty acids (VFA). Thereafter, the mixture was centrifuged (5810R; Eppendorf, Hamburg, Germany) 3 times at 4,500 x g for 15 min at 4 ^{o}C. The supernatant was obtained, and the pellet was discarded. The short-chain fatty acids (SCFA) and branched-chain fatty acids (BCFA) of the supernatant were determined by gas chromatography (model 9001; Finnigan Corporation, San Jose, CA) as previously described (Erwin et al., 1961). Lactic acid was measured by mixing 3 g of feces with 9 mL of distilled water. This mixture was centrifuged 3 times at 4,500 x g at 4^{o} C for 15 min. The supernatant was obtained, and the pellet was discarded. The analysis of lactic acid was performed by spectrophotometry (Spectrophotometer Quick-Lab; Drake Eletronica e Comércio, São José do Rio Preto, São Paulo, Brazil) (Pryce, 1969). Samples were quantified by comparing them with a standard curve of lactic acid. The concentration of ammonia was assessed in the same extracts prepared for the VFA. The extracts were thawed at room temperature, and 2 mL was diluted into 13 mL of distilled water and submitted to distillation in a nitrogen system (Tecnal TE-036/1; Tecnal Equipamentos Científicos, Piracicaba, São Paulo, Brazil).

The fecal concentrations of biogenic amines were evaluated with five grams of fresh feces was homogenized and added to 7 mL of a 5% trichloroacetic acid solution and then mixed for 3 min by vortex and centrifuged at 10,000 x g for 20 min at 4^{o}C (5810R; Eppendorf, Hamburg, Germany) (Vale; Gloria, 1997). The supernatant was filtered through qualitative filter paper, and the residue was extracted twice using 7 and 6 mL of a 5% trichloroacetic acid solution, separately. Then, the supernatants were filtered and pooled. The final volume obtained was recorded and frozen. Biogenic amine concentrations in the supernatant were determined by HPLC (HPLC model LC-10AD; Shimadzu Corporation, Kyoto, Japan).

Fecal Ig A

Fresh feces (immediately after elimination) was collected for three consecutive days before and 30 days after the intake of the experimental diets. On each period, feces samples were pooled by dog and fecal IgA extricated using a saline solution (Peters, 2004). Approximately 1 gram of feces was weighed and diluted in 10 mL of extraction buffer composed of 0.01 M phosphate-buffered saline (PBS) (pH 7.4), 0.5% Tween (Sigma-Aldrich, St Louis, MO, USA), and 0.05% sodium azide. After homogenization, the fecal suspensions were centrifuged at 1,500 x g for 20 min at 5 °C. Then, 1 mL of the supernatant was transferred to a sterile microtube containing 20 µL of a protease-inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). To remove the residues, samples were centrifuged at 15,000 x g for 15 min at 5 °C, and the supernatants were kept in microtubes at -20 °C until analysis.

The quantification of IgA was performed by ELISA kit for canine IgA determination (Bethyl laboratories, Montgomery, TX, USA). Optical density (OD) was read at 450 nm with a Microplate Reader (MRX TC Plus, Dynex Technology, Chantilly, Virginia, EUA). To calculate the IgA concentration, the OD of the samples was compared to the OD of a standard with a known concentration of IgA. The standard canine IgA sample was provided in the kit, and seven dilutions of the standard were made in order to develop a regression curve between OD and IgA amount. Samples were analyzed in duplicate, and the analysis were repeated when the variation between replicates was greater than 10%.

TNF-a, IL-6 and IL-10 on blood serum

For the analyzes of tumor necrosis factor alpha (TNF-α) and interleukins 6 (IL-6) and 10 (IL-10), on days zero and 30, blood samples (3 mL) were collected via jugular puncture and placed on tubes without anticoagulant. Afterwards, the samples were centrifuged at 3,500 x g for 10 min (5810R; Eppendorf, Hamburg, Germany) and the serum stored frozen at -80 °C until analysis. The dosage was performed using a Luminex kit specific to dogs according to the manufacturer's recommendations (MILLIPLEX MAP ELISA Canine Cytokine / Chemokine Magnetic Bead Panel - Immunology Multiplex Assay - Merck Millipore, St Charles-Missouri-USA).

Phagocytic Activity

The phagocytic activity was measure on days 0, 15 and 30 using a commercial kit (pHrodo *E. coli* BioParticles, Molecular Probes Inc., Oregon, EUA). Blood samples was collected by jugular puncture and placed on heparinized tubes. After, 100 µL of the sample was incubated with 20 µL of pHrodo *E. coli* BioParticles, reagent provided by the commercial kit. For each blood sample two tubes were prepared with the bioparticles, one was placed on ice and the other kept at 37 ºC on a water bath for 15 minutes. Then, the incubated samples were lysed, followed by centrifugation and washing using the proper reagents as recommended by the manufacturer. Two negative control samples were run together in each collection day, both tubes with no bioparticles, but one placed on ice and the other kept at 37 ºC. Samples were analyzed using flow cytometer (FACSCanto, Becton Dickinson Immunocytometry System, Mountain View, CA, EUA) and the results were shown as the percentage of fluorescence signal inside the desired population of neutrophils and monocytes. The target cell population were gated according to its volume and complexity (Neaga et al., 2013).

Determination of hydrogen peroxide (H_2O_2) and nitric oxide (NO) production

Blood samples (6 mL) were collected with heparin via jugular puncture and added to 4.5 mL of Histopaque 1119, and 3 mL of Histopaque 1077 (Sigma Aldrich, St Louis, MO, EUA) in a 15 mL conical centrifuge tubes. Tubes were centrifuged at 700 x g for 30 minutes at room temperature. After centrifugation, two distinct opaque layers was separated, the mononuclear and granulocyte cells, respectively. Each layer was collected separately, transferred to a 50 mL conical centrifuge tubes and washed at least twice with isotonic phosphate buffered saline by centrifugation at 360 x g for 10 min at room temperature. Erythrocyte lysis were conducted when necessary using 2 mL of ACK (Ammonium-Chloride-Potassium) solution (0.15 M ammonium chloride; 10mM potassium bicarbonate; 0.1 mM EDTA) for a maximum of 2 minutes.

Cells were suspended in complete medium (RPMI 1640, Merck KGaA, Darmstadt, German) added with 40 mg/mL of gentamicin and 10% of fetal

bovine serum and the concentration adjusted to 2 x 10^5 neutrophils or monocytes/mL. After, cells were placed in 96-well flat plates (100 µL/well). Mononuclear cells were kept at 37 °C in a humidified 5% CO_2 incubator (Thermo Fisher Cientific, Waltham, MA, EUA) for one hour for the adherence of monocytes in the well, then the supernatant was carefully discarded with a pipette and complete medium were added to each well. For monocytes, one plate was incubated for H_2O_2 production and one for NO production. For neutrophils, the supernatant of H_2O_2 production was used to conduct the NO analysis.

For H_2O_2 production, a total of 24 wells received 100 µL of sample, 12 of them were kept as a non-stimulated cells and the others 12 wells were stimulated with LPS (1 µg/well - *E. coli Lipopolysaccharide*, Sigma Aldrich, St. Louis, USA). Plates were maintained at 37 °C in a humidified 5% CO_2 incubator for 36 hours. The H_2O_2 production was measured according to the method described by Pick and Keisari (1980) and modified by Pick and Mizel (1981). Buffer solution (100 µL/well) consisted of 7.8 mL of distillated water (H_2Od), 0.8 mL of solution A (800 mL H_2Od; 80 g NaCl; 2 g KCl; 2 g KH_2PO_4; 11.5 g Na_2HPO_4), 0.1 mL of solution B (100 mL H_2Od; 1 g $CaCl_2$), 0.1 mL of solution C (100 mL H_2Od; 1 g $MgCl_2$), 0.1 mL of phenol red (100 mL H_2Od; 1 g phenol red), 0.1 mL of peroxidase (10 mg horseradish peroxidase; 2 mL PBS) and 1 mL of glucose (100mL H_2Od; 1 g glucose) was added to each well. Phorbol myristate acetate (PMA; 10 µL/well) was added to a half of the non-stimulates wells and to a half of LPS-stimulated wells and kept at 37 °C in a humidified 5% CO_2 incubator for one hour. Consequently, there were six replications for each cell condition: six wells for non-stimulated cells; six wells for non-stimulated + PMA; six wells for LPS-stimulated cells; and six wells for LPS-stimulated cells + PMA. After one-hour incubation, the reaction was stopped with 10 µL of NaOH 1N. The absorbance was read in a microplate reader (iMarkMicroplate Absorbance Reader 168-1135, Bio-rad, Hercules, California, USA) at 595 nm. Results were expressed as µM of H_2O_2/2x10^5 cells. The hydrogen peroxide standard curve was built for each plate with a range of 0.25 to 16.00 nM of H_2O_2.

The NO assay was assessed by the colorimetric method of the GRIESS reaction (Grenn et al., 1982). The analysis were conducted in six repetitions for non-stimulated cells and six repetitions for LPS-stimulated cells (1 µg/well- *E.*

coli Lipopolysaccharide, Sigma Aldrich, St. Louis, USA), totaling 12 wells per sample. One-hundred microliters of GRIESS reagents diluted 1:1 (n-(1-naftil)-etil-enediamin diluted 0.1% in H_2Od; 1% sulfonamide diluted in 5% H_2PO_4; Sigma Aldrich, St Louis, MO, USA) were added to the supernatant. The absorbance was read in a microplate reader (iMarkMicroplate Absorbance Reader 168-1135, Bio-rad, Hercules, California, USA) at 540 nm. Results were expressed as μM of NO/2x10^5 cells. The NO standard curve was built for each plate with a range of 0.78 to 100 μM of NO.

Statistical analysis

All variables were previously tested for the normality or errors using the Cramer-von Misses test and for the homoscedasticity of the variances using the Levene test. When necessary, logarithmic transformation (log x + 1) was applied. For the immunological parameters, data was submitted to analysis of variance considering the effects of block, animal and diets. Differences among groups was detected at baseline, and due this the time 0 (baseline) was used as a covariate. When the F-test was significant, multiple comparisons of the means were made using Tukey's test. Data obtained for nutrient digestibility, fecal parameters and fermentation products were submitted to analysis of variance and when significant, compared by the Tukey's test ($P<0.05$). Values of $P<0.05$ were considered significant, and $P<0.10$ as a trend. The analysis were conducted using the computer program R (version 3.3.3).

3. RESULTS

Dogs shown proper food intake and maintained a constant body weight throughout the experimental period, with no episodes of food rejection, vomiting, or diarrhea. The food intake did not differ among diets ($P>0.05$). On the digestibility evaluation, DM intake was similar, resulting in similar nutrient intake by the animals (Table 3). The total tract apparent digestibility of nutrients were similar, except for fat digestibility that was lower than CON for dogs fed the YCWs food ($P<0.05$). Feces production, DM, score and pH was also similar among treatments ($P>0.05$), as shown on Table 4.

Table 3. Body weight (kg), nutrient intake (g/dog/day) and coefficients of total tract apparent digestibility (%) of diets for dogs with the additions of different yeast cell wall preparations (mean and standard error of the mean).

Item	Diets[1]			*p-value*
	CON	YCW	YCWs	
Body weight (kg)	11.3±0.5	12.2±0.5	11.5±0.4	0.664
Nutrient intake (g/dog/day)				
Dry matter	160.4±4.0	170.6±4.6	163.2±3.5	0.204
Coefficient of total tract apparent digestibility (%)				
Dry matter	80.4±0.4	79.7±0.6	78.5±1.4	0.341
Organic matter	85.5±0.3	85.0±0.5	84.0±1.1	0.303
Crude protein	79.8±1.0	80.1±1.1	77.7±1.4	0.321
Fat	94.3±0.2[a]	92.4±0.6[ab]	91.6±0.7[b]	0.007
Gross energy	85.3±0.3	84.8±0.6	84.8±1.01	0.820

[1] CON = without yeast cell wall preparations; YCW = standard yeast cell wall extract; YCWs = yeast cell wall extract with 20% soluble mannan oligosaccharides.
[a, b] = means in a row without a common superscript differ (P<0.05).

Table 4. Feces production and characteristics of dogs fed diets with the addition of different yeast cell wall preparations (mean and standard error of the mean).

Item	Diets[1]			*p-value*
	CON	YCW	YCWs	
g/dog/day (as is)	75.6±3.04	81.5±5.4	86.0±6.8	0.404
g/dog/day (dry matter basis)	33.8±0.8	37.3±1.8	37.9±2.8	0.309
Dry matter (%)	45.1±1.4	46.1±1.1	44.5±2.2	0.774
Score	3.6±0.1	3.8±0.1	3.7±0.1	0.442
pH	6.8±0.1	6.9±0.04	6.8±0.04	0.215

[1] CON = without yeast cell wall preparations; YCW = standard yeast cell wall extract; YCWs = yeast cell wall extract with 20% soluble mannan oligosaccharides.

On fermentation products, higher concentrations of butyrate (approximately 25% more) and lower lactate (approximately 64% less) was verified on the feces of dogs fed the YCWs than the other two foods (P<0.05), without other detectable differences (Table 5).

Table 5. Volatile fatty acids (mMol/g of dry matter), and ammonia and lactic acid (mMol/kg of dry matter) on the feces of dogs fed diets with the addition of different yeast cell wall preparations (mean and standard error of the mean).

Item	Diets[1]			*p-value*
	CON	YCW	YCWs	
Acetic acid	230.3±17.7	218.4±10.7	230.0±19.5	0.844
Propionic acid	111.4±8.0	101.9±6.7	108.1±9.8	0.717
Butyric acid*	46.8[b]±1.8	43.7[b]±2.3	55.6[a]±2.5	0.012
Isobutyric acid	10.1±0.5	9.6±0.4	9.5±0.5	0.594
Isovaleric acid	14.3±0.8	12.7±0.7	14.6±1.0	0.218
Valeric acid	2.7±0.4	3.45±0.5	3.4±0.4	0.406
Total VFA[2]	417.9±27.9	389.8±18.0	421.9±31.0	0.647
Total SCFA[3]	390.8±27.0	364.0±17.3	393.7±30.7	0.670
Total BCFA[4]	27.1±1.3	25.8±1.4	28.2±1.6	0.513
Ammonia	194.5±8.8	174.5±7.2	196.1±11.3	0.206
Lactic acid*	2.7[a]±0.3	2.9[a]±0.3	1.7[b]±0.1	0.008

[1] CON = without yeast cell wall preparations; YCW = standard yeast cell wall extract; YCWs = yeast cell wall extract with 20% soluble mannan oligosaccharides.
[2]: VFA = volatile fatty acids.
[3]: SCFA = short-chain fatty acids.
[4]: BCFA = branched-chain fatty acids.
* Values transformed to Log x + 1 for statistical analysis.
[a, b] = means in a row without a common superscript differ (P<0.05).

Only the biogenic amines putrescine, cadaverine, spermidine and phenylethylamine was detected in significant amounts on the dog feces (Table 6). Among them, putrescine was approximately 70% higher for dogs fed the YCWs than for animals fed the other two diets (P<0.05).

Table 6. Biogenic amines concentration (mg/100 g of dry matter) on the feces of dogs fed diets with the addition of different yeast cell wall preparations (mean and standard error of the mean).

Item	Diets[1]			*p-value*
	CON	YCW	YCWs	
Putrescine*	9.5±1.6[b]	10.3±1.9[b]	17.0±3.4[a]	0.002
Cadaverine	4.1±1.4	2.8±0.5	3.6±0.8	0.267
Spermidine	2.2±0.4	2.5±0.4	3.1±0.5	0.455
Phenylethylamine	0.8±0.3	1.0±0.3	1.1±0.2	0.801

[1] CON = without yeast cell wall preparations; YCW = standard yeast cell wall extract; YCWs = yeast cell wall extract with 20% soluble mannan oligosaccharides.
* Values transformed to Log x + 1 for statistical analysis.
[a, b] = means in a row without a common superscript differ (P<0.05).

The fecal concentration of IgA was similar between foods, as presented on Table 7.

Table 7. Immunoglobulin A concentration (mg/g of dry matter) on the feces of dogs fed diets with the addition of different yeast cell wall preparations (mean and standard error of the mean).

Item	Diets[1]			*p-value*
	CON	YCW	YCWs	
IgA*	1.1±0.3	1.18±0.3	1.38±0.3	0.799

[1] CON = without yeast cell wall preparations; YCW = standard yeast cell wall extract; YCWs = yeast cell wall extract with 20% soluble mannan oligosaccharides.
* Values transformed to Log x + 1 for statistical analysis.

Among the evaluated cytokines (Table 8), dogs fed the YCWs diet presented lower IL-6 serum concentration than control (P<0.05), but similar values in comparison with dogs fed YCW. Dogs fed the YCW diet also tended to present lower IL-6 values than control (P=0.092). No differences were detected for the other cytokines evaluated. No differences between diets were verified for the H_2O_2 and NO production for monocytes and neutrophils, as presented on table 9.

Table 8. Serum cytokines concentrations (pg/mL) of dogs fed diets supplemented with different yeast cell wall preparations (mean and standard error of the mean).

Item	Diets[1]			*p-value*
	CON	YCW	YCWs	
IL-6*	21.3[a]±1.5	16.7[ab]±1.3	16.0[b]±1.3	0.037
IL-10	13.5±1.7	15.4±2.0	15.4±1.7	0.688
TNF-α	26.2±3.7	17.2±3.4	23.1±3.6	0.223

[1] CON = without yeast cell wall preparations; YCW = standard yeast cell wall extract; YCWs = yeast cell wall extract with 20% soluble mannan oligosaccharides.
[a, b] = means in a row without a common superscript differ (P<0.05).

Table 9. Hydrogen peroxide (H_2O_2; µM of H_2O_2/2x10^5 cells) and nitrogen oxide (NO; µM of NO/2x10^5 cells) production in cell cultures of monocytes and neutrophils from the peripheral blood of dogs fed diets with the addition of different yeast cell wall preparations (mean and standard error of the mean).

Item	Diets[1]			*p-value*
	CON	YCW	YCWs	
H_2O_2 – Monocytes[2]				
Phorbol myristate acetate	1.9±0.3	2.1±0.3	2.2±0.3	0.817
Phorbol myristate acetate + Lipopolysaccharide	2.1±0.3	2.4±0.3	2.0±0.3	0.550
H_2O_2 – Neutrophils				
Only cells	0.5±0.1	0.5±0.1	0.7±0.1	0.297
Lipopolysaccharide	0.6±0.1	0.7±0.1	0.6±0.1	0.884
Phorbol myristate acetate	4.7±0.8	3.8±0.7	3.9±0.7	0.676
Phorbol myristate acetate + Lipopolysaccharide	3.0±0.5	3.6±0.5	3.0±0.5	0.656
NO – Monocytes				
Only cells	5.1±2.2	6.4±2.2	4.6±2.4	0.821
Lipopolysaccharide	6.4±2.3	6.2±2.3	3.5±2.3	0.837
NO – Neutrophils				
Only cells	7.8±3.6	7.9±3.6	11.5±3.3	0.983
Lipopolysaccharide	10.0±4.6	10.3±4.6	14.7±4.3	0.746

[1] CON = without yeast cell wall preparations; YCW = standard yeast cell wall extract; YCWs = yeast cell wall extract with 20% soluble mannan oligosaccharides.
[2] – For monocytes, only cells and Lipopolysaccharide results were below the detectable

limit of H_2O_2.

The blood monocytes phagocytic index was 37% higher for dogs fed the YCW than the control diet (P<0.05) on day 30, while dogs fed the YCWs food presented intermediary results. The neutrophils phagocytic activity did not differ among diets, been elevated since the beginning of the study (Table 10).

Table 10. Monocyte and neutrophils phagocytic index (% of positive cells) from dogs fed diets with the addition of different yeast cell wall preparations (mean and standard error of the mean).

Item		Diets[1]			*p-value*
		CON	YCW	YCWs	
Neutrophils					
	Day 15	88.8±2.1	90.2±2.1	92.2±2.1	0.411
	Day 30	85.2±1.9	88.6±1.9	87.7±1.9	0.445
Monocytes					
	Day 15	52.4±6.5	46.1±6.5	48.9±6.4	0.739
	Day 30*	54.6^{b}±5.1	74.8^{a}±5.1	64.5^{ab}±5.0	0.030

[1] CON = without yeast cell wall preparations; YCW = standard yeast cell wall extract; YCWs = yeast cell wall extract with 20% soluble mannan oligosaccharides.
* Values transformed to Log x + 1 for statistical analysis.
[a, b] = means in a row without a common superscript differ (P<0.05).

4. DISCUSSION

As previous studies (Middelboss et al., 2007; Gomes et al., 2011; Lin et al., 2019), the use of yeast cell wall preparation was shown to be safe for use in dog diets, since no changes in fecal quality and in the clinical condition of the animals was observed during the experimental period. In both diets of the present study, a low inclusion of the prebiotics was tested (0.3%), without modifying feces production and characteristics. Fat apparent digestibility, however, were reduced after the consumption of the YCWs treatment, an effect that can be attributed to their solubility acting on intestinal tract as the soluble and fermentable fibers, that may interfere on fat absorption on dogs as demonstrated on previous studies (Diez et al. 1998; de-Oliveira et al., 2012).

This effect on fat apparent digestibility was not observed for the conventional YCW preparation, as previously demonstrated on studies with dogs (Middelboss et al., 2007; Gomes et al., 2011). The implications of the observed reduction on fat digestibility should be explored in future studies, including their use on low energy foods, but the magnitude of the fat digestibility reduction was low and their relevance to dog nutrition uncertain.

The experimental diets were formulated with sugarcane fiber, composed approximately by 45.8 % cellulose, 28.1% hemicellulose and 9.3% lignin (Monti et al., 2016), an insoluble and very low fermentable fiber source (Calabro et al., 2013; Loureiro et al., 2017), that was selected to do not interfere on SCFA production. Under this condition, the intake of the more soluble yeast cell wall preparation, higher in soluble mannan oligosaccharides changed the metabolism and fermentation profile of the gut microbiota, evidenced by higher fecal butyrate and putrescine, and lower fecal lactate in comparison to the other treatments. A higher production of some SCFA, and specially of butyrate, is one of the outcomes expected from an effective prebiotic (Gibson et al., 2004; Teng; Kim, 2018; Santos, 2018), suggesting an advantage for the YCWs. It is interesting that the traditional YCW on the current study, more insoluble, did not interfered on fermentation end-products formation, indicating that the solubility of the carbohydrate fractions of the yeast cell wall may be a key factor on the product interaction with the gut microbiota.

In addition to its role as a source of energy for colonocytes, butyrate has been explored for its ability to directly affect cell growth and differentiation and to have a positive effect on reducing cell inflammation (Rivière et al., 2016; Chung et al., 2017; Holscher, 2017). On different cells and animal models butyrate reduced inflammation and improved the barrier function of the gut, reducing the production of proinflammatory cytokines (Inan et al., 2000; Yin et al., 2001; Jiminez, 2017). Therefore, increases on butyrate concentrations are generally associated with an improved health (Tan et al., 2014; Ríos-Covían et al., 2016) and is one of the main objectives with prebiotic supplementations do diets.

Lactate is also produced as a result of carbohydrate fermentation by colon microbiota (Fischer et al., 2012), however, it does not present a cumulative effect as it is a substrate for several bacteria that utilize it producing

propionate and butyrate (Moens et al., 2019). Thus, lactate concentrations may be interpreted considering the rates of production and consumption (Moens et al., 2019), which can justify the lower lactate and higher butyrate concentration for dogs fed the YCWs diet. This altered butyrate to lactate ratio also exemplify the impact of the YCWs on gut microbiota metabolism.

Amines are formed mainly by the decarboxylation of amino acids by the microorganisms of the gastrointestinal tract (Hussein et al., 1999). The fecal concentrations of amines observed on the present study are comparable to previously reported in dogs (Swanson et al., 2002; Flickinger et al., 2003, Propst et al., 2003; Middelboss, 2007), although the interpretation of amines concentration on dog feces are difficult, due to the very limited available information regarding the normal or desired levels (Gomes et al., 2011). On the present study, as the protein source on diets was the same (a possible source of amines), the increased putrescine concentration may be explained by a higher intestinal formation after the intake of the YCWs. Putrescine is produced by the decarboxylation of ornithine and arginine and, in turn, is progressively metabolized to spermidine (Hanfrey et al., 2011). A previous study of our laboratory did not find yeast cell wall effect on fecal amines concentration (Gomes et al., 2011), reinforcing the lack of effect of the YCW diet, and that the soluble mannan oligosaccharides fraction of the YCWs in fact altered fermentation profile of gut microbiota.

Several favorable and harmful physiological processes involve the amines action, especially the polyamines (Kalac, 2014). They are present in all living cells and are necessary for the normal development and repair of intestinal mucosa cells (Wang and Johnson, 1990; Loser et al., 1999). However, its activity has also been associated with the incidence of colorectal cancers (Milovic and Turchanowa, 2003) and high concentrations is related to inflammation, oxidative stress and genotoxicity (Hoyles, 2019). Therefore, a significant reduction of polyamine concentrations in the intestinal lumen is not interesting, since the polyamines depletion (intracellular) directly affects the apoptosis of epithelial cells (Middelboss, 2007), but high amounts may be also undesirable. In a study with dogs of different age groups, higher putrescine, cadaverine, and spermine were verified for old than adult dogs, and higher spermidine was found on feces of dogs fed a diet based on soybean meal, what

was linked to a higher IgA content on feces and a better intestinal health by the authors (Maria et al, 2017).

IgA was evaluated on the present study as this immunoglobulin is an important indicator of the mucosal immunity status (Norris and Gershwin, 2003; Lin et al., 2019), representing an essential factor in the protection against infectious agents, allergens and foreign proteins (Lycke et al., 1999; Wijburg et al., 2006; Zaine et al., 2011; Maria et al., 2017). The main function of secretory IgA is to prevent bacteria and viruses from attaching and invading enterocytes (Mayer, 2000). The evaluation of this immunoglobulin is also of interest to clinicians to access specific responses to antigens or in the diagnosis of IgA deficiency (Norris and Gershwin, 2003). On the present study, no effect of the two preparations of yeast cell wall was observed on fecal IgA concentration. Differing form the present results, a study with calves found an increase on salivary and fecal IgA after the intake of yeast cell wall derivates (Heinrichs et al., 2013). This might be the results of an incomplete immunity on newborns (HogenEsch et al., 2004; Blount et al., 2005), may be allowing to express the effect of this prebiotic. So, it is possible that the lack of immunological challenge on the present study may interfered on the evaluation of the possible effect yeast cell wall preparations on the secretion of this immunoglobulin, as also verified when the prebiotic resistant starch was evaluated for health old dogs (Peixoto et al., 2018).

The reduced IL-6 on serum of dogs fed with the YCWs diet may also be consequent, at least partially, of the higher butyrate formation on intestine. Butyrate appears to be more potent than acetate or propionate in inducing immunomodulatory effects, as it affects the activity of histone deacetylases, which are responsible to decrease the secretion of IL-12 and IL-6 cytokines by dendritic cells and allow dendritic cells to enhance mucosal regulatory T cells (Frei et al., 2015). However, a tendency for lower IL-6 on serum was also verified for dogs fed the YCW diet, that did not alter SCFA fecal concentration. These data corroborate with findings of other researchers (Posadas et al., 2010), which evaluated the action of a yeast cell wall fraction called "mannoprotein", added to a liquid diet for rats with salmonella infection. The authors also found lower expression of TNF-α and IL-6 mRNA in the jejunum, ileum and colon tissues in the treatment groups and concluded that yeast cell

wall derivates may lower the inflammatory response, protecting the intestinal tissue. So, it is also possible to speculate that YCW may have a direct action on intestinal cell reducing proinflammatory cytokines, in a mechanism independent of the SCFA formation.

The increase on peripheral monocytes phagocytic activity on dogs fed the YCW diet was relevant, as it is an important criterion to evaluate innate immunity (Song et al., 2014; Oliveira et al., 2019). Phagocytic cells act on the first line of defense against microorganisms (Gourbeyre et al., 2011) with monocytes being the key mediators of the early inflammatory response to infection. Considering the lack of changes on fermentation products on feces, it is possible to attribute this effect to a direct interaction of the manna oligosaccharide or the b-1.3/1.6 glucan fractions of the YCW with the dendritic cells of the intestinal mucosa (Esteban et al., 2004; Abu-Elala et al., 2013), what could demonstrate the ability of the YCW prebiotic to modulate the immune system by direct mechanisms (Chung et al., 2017). The ability of b-glucans to increase monocytes and neutrophils phagocytic percentage is well demonstrated for several species, including the dog (Vetvicka et al., 2008; Rodriguez-Estrada et al., 2013; Oliveira et al., 2019). However, a direct interaction of the mannan oligosaccharide fraction and the immune system is also described (Torrecillas et al. 2011, Devi, 2019) and cannot be excluded.

Peripheral blood mononuclear and polymorphonuclear (neutrophils) cells are traditionally used to evaluate in vitro responses of blood-derived immune cells to various antigens (Schmitz et al., 2013). Although in the present study cells were stimulated by lipopolysaccharide and phorbol myristate acetate, substantially increasing H_2O_2 and NO production, no diet effect was verified. In several studies about diet intervention, no effect on H_2O_2 and NO production were also reported (Schmitz et al., 2013; Lin et al., 2019). The procedure is laborious, expensive and requires large volumes of blood to make up the right number of cells. In addition, cell sorting can stimulate cells and lead to loss of specific populations, leading to results that may not reflect the condition *in vivo* (Schmitz et al., 2013).

Some limitations of the present study may need to be considered. Only healthy dogs were used, and the time of diet intake was not previously studied to know if it was enough to express the hole effect of the both yeast cell wall

preparations. Due to this, possible differences between groups on the immunological system and microbiota metabolism could not be observed and the long-term effects of the products are not known.

5. CONCLUSION

Under the conditions of the present research, positive immunomodulatory effects were verified for both yeast cell wall preparations. The addition of YCWs to an extruded diet changed intestinal microbiota metabolism, as verified by increased butyrate and putrescine, and reduced lactate. It also reduced inflammatory response, verified by a reduction on serum IL-6 of dogs. The conventional YCW also tended to reduce IL-6, and stimulated the innate immunity verified by an increase on peripheral monocytes phagocytic activity.

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