UNIVERSIDADE ESTADUAL PAULISTA - UNESP

CAMPUS DE JABOTICABAL

COMPARAÇÃO DOS EFEITOS EM CÃES DO CONSUMO DE B-GLUCANOS EXTRAÍDOS DA PAREDE CELULAR DE ALGAS OU LEVEDURAS

Stephanie de Souza Theodoro

Mestre em Medicina Veterinária

2023

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Stephanie de Souza Theodoro

Orientador: Prof. Dr. Aulus Cavalieri Carciofi

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias - UNESP, Campus de Jaboticabal, como parte das exigências para obtenção do título de Doutora em Medicina Veterinária. Área: Clínica Médica Veterinária.

2023

T388c

Theodoro, Stephanie de Souza
Comparação dos efeitos em cães do consumo de b-glucanos extraídos da parede celular de algas ou leveduras / Stephanie de Souza Theodoro. -- Jaboticabal, 2023
75 f. : il., tabs.

Tese (doutorado) - Universidade Estadual Paulista (Unesp), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal
Orientadora: Aulus Cavalieri Carciofi

1. B-glucano. 2. Levedura. 3. Algas marinhas. 4.Cães saudáveis. I. Título

.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca da Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal. Dados fornecidos pelo autor(a).

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TÍTULO DA TESE: COMPARAÇÃO DOS EFEITOS EM CÃES DO CONSUMO DE β-GLUCANOS EXTRAÍDOS DA PAREDE CELULAR DE ALGAS OU LEVEDURAS

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Jaboticabal, 07 de junho de 2023

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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. Concluiu o ensino médio no ano de 2008 e iniciou sua graduação em 2009 na Universidade Federal de Pelotas – UFPel, concluindo em 2014. No período de março de 2015 a 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 (área de Clínica Médica Veterinária) com ênfase em Nutrição e Doenças Nutricionais de Cães e Gatos sob, também, orientação do Prof. Dr. Aulus Cavalieri Carciofi, no qual sua pesquisa foi focada no uso de parede celular de levedura na dieta para cães e seus efeitos na modulação imune e ação nos produtos de fermentação, sendo concluído em 2019. No mesmo período deu início ao doutorado na mesma instituição e programa, com avaliação de B-glucanos na dieta para cães e efeitos imunes, inflamatórios, pós prandial e microbiota. Ainda realizou doutorado sanduíche na Universidade da California, em Davis, no ano de 2022 sob orientação da profa. Dr. Andrea Fascetti.

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

DEDICATÓRIA

À ***Família Theodoro,*** em especial ao meu anjinho **Silvia Helena *(in memorian)***, que nunca mediram esforços, amor e compreensão para a realização de mais este sonho.

DEDICO

AGRADECIMENTOS

Agradeço inicialmente à Força Superior que nunca me deixou em falta. Ele que soube entender minhas limitações e sempre me presentou com o que há de melhor das pessoas.

À minha pequena, grande família, Pai, Tia Silvia, Vó Edna, Vô Geraldo, minhas irmãs Sophia e Katherine, Rose e Karen. Nestes quatro anos fui ainda mais ausente e vocês mais pacientes. Muito obrigada por me apoiarem, comemorem cada passo, serem o meu colo e dividirem esse sonho comigo. Nada seria possível sem vocês! Amo vocês!

Tia Bel, mais uma conquista por você! Obrigada por me permitir ser sua filha e lutar a cada dia ao meu lado. Você é essencial em cada passo que dou. Eu te amo!

Tio Kojak, obrigada por ser essa fonte de inspiração em minha vida. Obrigada por me tornar quem eu sou hoje. Te amo muito!

Prima Michele, nós sabemos o quanto é bom poder dividir nossas conquistas. Isso também é por você! Te amo!

Ao professor Aulus por ser um exemplo de pessoa, humildade, inteligência e inspiração. Obrigada por me permitir fazer parte desse time, confiar em mim e me permitir crescer cada dia mais.

A minha melhor amiga, irmã, parceira, confidente e colega de trabalho, Duda, obrigada por ser meu ponto de equilíbrio no meio desse furacão todo. Só a gente sabe realmente o quanto não foi fácil essa conclusão, mas juntas somos capazes de tudo. Obrigada por tornar tudo mais leve.

À família Lab Nutri, todos os pós-graduandos atuais e que já concluíram e aos estagiários, NADA seria possível sem vocês. Obrigada por me ajudarem a cada etapa, acordar cedo, dormir tarde, todo o apoio, os momentos de descontração, de desespero, por TUDO. Cada um de vocês têm um papel excepcional nessa trajetória.

Aos funcionários, Claudinha, Lucas, Elaine, Diego, Kelly, Marcos.. obrigada por dividirem os dias, as conversas, as risadas, os trabalhos e a vida. Tudo só dá certo porque vocês estão juntos. OBRIGADA!

Aos animais do Lab Nutri que são nossa fonte de estudo e de maior demonstração de afeto e carinho. Só vocês são capazes de me trazer a paz em momentos de estressantes.

Aos meus amigos Gilmar, Murillo, Pri, Vitor e Luis, meus sinceros agradecimentos pelos momentos alegres, ajuda nos estudos, desabafos e descontração. Obrigada por estarem sempre presente e por tornarem a minha vida mais leve em Jaboticabal!

À CAPES e a FAPESP (2020/04120-4) pelo meu suporte acadêmico e a Kemin pelo suporte financeiro ao experimento.

À BRF Pet, BRF Ingredients, ADM e Adimax pelo apoio e suporte financeiro ao Laboratório.

À Manzoni pela doação a extrusora.

E as todas as empresas parceiras do Lab Nutri, pelo incentivo a pesquisa em nutrição e ser nossa fonte de estudos permanente.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

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COMITÊ DE ÉTICA

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CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Comparação dos efeitos em cães do consumo de β-glucanos extraídos da parede celular de algas ou de leveduras"**, protocolo nº 001974/20, 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 12 de março de 2020.

Vigência do Projeto	01/04/2020 a 28/02/2023
Espécie / Linhagem	Canina/Beagles
Nº de animais	40
Peso / Idade	10 - 15 kg / 1 - 6 anos
Sexo	Machos e fêmeas
Origem	Laboratório de Nutrição e Doenças Nutricionais de Cães e Gatos

Jaboticabal, 12 de março de 2020.

Fabiana Pilarski

Profª Drª Fabiana Pilarski
Coordenadora – CEUA

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LISTA DE ABREVIAÇÕES

ACK: Ammonium-Chloride-Potassium
BCFA: Branched-chain fatty acids
BLEV / B-Yeast: dieta com β-glucanos de levedura
BA15 / BS15: dieta com β-glucanos de algas marinhas inclusão de 0,155%
BA31 / BS31: dieta com β-glucanos de algas marinhas inclusão de 0,310%
$CaCl_2$: Calcium chloride
CO_2: carbon oxide
CON: Controle / Control
CRP: C-reactive protein
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
g: grama / gram
GALT: Gut-associated lymphoid tissue
GE: Gross energy
H / hr: Hora
H_2O_2: hydrogen peroxide
H_2Od: distillated water
IgA: Imunoglobulina A / Immunoglobulin A
IgG: Imunoglobulina G / Immunoglobulin G
IgM: Imunoglobulina M / Immunoglobulin M
IFN-γ: Interferon gama / Interferon gamma
IL-2: Interleucina 2 / Interleukin 2
IL-6: Interleucina 6 / Interleukin 6
IL-8: Interleucina 8 / Interleukin 8
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
Na_2HPO_4: Disodium phosphate

NaCl: Sodium chloride
NaOH: Sodium hydroxide
NO: nitric oxide
PBS: phosphate buffered saline
PMA: Phorbol myristate acetate
OD: optical density
OM: Organic matter
SCFA: Short chain fatty acid
TGF-β1: fator de crescimento transformador beta 1
Th1-helper: T helper 1
Th2-helper: T helper 2
TNF-α: tumor necrosis factor alpha
VFA: Volatil fatty acids
µg: micrograma / microgram
µl: microlitro / microliter
µM: micromol

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COMPARAÇÃO DOS EFEITOS EM CÃES DO CONSUMO DE β-GLUCANOS EXTRAÍDOS DA PAREDE CELULAR DE ALGAS OU DE LEVEDURAS

RESUMO: Objetivou-se avaliar e comparar os efeitos do β-glucanos extraído da parede celular de levedura com o extraído de algas marinhas sobre a composição da microbiota intestinal, produtos de fermentação nas fezes e parâmetros imunológicos e inflamatórios de cães adultos. Para o estudo, uma dieta controle (CON) foi suplementada com B-glucano extraído de levedura (BLEV; 0,115%) ou B-glucano de algas: BA15 (dose de 0,155% de B-glucano de algas marinhas); BA31 (0,31% B-glucano de algas marinhas). Foram utilizados 32 cães, 8 por ração. Estes receberam as rações por 42 dias, sendo coletados sangue e fezes no início e final do período para análise das citocinas, produção ex vivo de intermediários reativos de oxigênio e nitrogênio, atividade fagocítica de neutrófilos e monócitos, proteína C reativa (PCR), parâmetros lipêmicos, concentração de IgA fecal e calprotectina. No meio deste período foi realizado ensaio de digestibilidade e curva pós-prandial de insulina e glicose. No tempo final, fezes frescas ainda foram coletas para avaliar produtos de fermentação. Os dados foram avaliados por análise de variância e comparados pelo teste de Tukey ($P<0,05$), e quando necessário, análise de covariância foi aplicada. Cães alimentados com B-A31 apresentaram menor IL-2 que B-LEV e menor fagocitose por neutrófilos que os demais tratamentos ($P<0,05$). Cães alimentados com B-A31 tenderam a apresentar maior IL-10 que CON ($P=0,095$). O consumo de B-glucano de algas aumentou a produção de óxido nítrico em cultivo celular e a PCR sérica ($P<0,05$). Cães alimentados com BA15 apresentaram maior digestibilidade aparente de matéria seca, matéria orgânica e energia bruta ($p<0,05$). Cães

alimentados com BLEV tenderam a menor média de glicose e menor tempo para pico da resposta insulínica (p<0,1). A beta diversidade da microbiota fecal de cães na dieta BA31 diferiu das dietas BLEV e BA15. Concluiu-se que B-glucanos de leveduras e algas marinhas apresentaram vias de ações diferentes, pois o B-glucano de algas induziu elevação de PCR, ativação de monócitos medido pela maior produção de óxido nítrico e tendeu a elevar a citocina anti-inflamatória IL-10, sendo que a dieta BA31 apresentou maior capacidade em alterar da microbiota fecal. O β-glucano de levedura tendeu em diminuir a média da concentração de glicose e antecipar o pico da ação de insulina endógena.

Palavras chave: β-glucanos, parede celular de levedura, algas marinhas, microbiota.

COMPARISON OF THE EFFECTS IN DOGS OF THE CONSUMPTION OF β-GLUCANS EXTRACTED FROM ALGAE OR YEAST CELL WALL APPLICATION

ABSTRACT: The objective was to evaluate and compare the effects of β-glucans extracted from yeast cell wall with that extracted from seaweed on the composition of the intestinal microbiota, fermentation products in the feces and immunological and inflammatory parameters of adult dogs. For the study, a control diet (CON) was supplemented with yeast-extracted B-glucan (BLEV; 0.115%) or seaweed B-glucan: BA15 (0.155% seaweed B-glucan dose); BA31 (0.31% Seaweed B-glucan). 32 dogs were used, 8 per ration. They received the diets for 42 days, with blood and feces collected at the beginning and end of the period for analysis of cytokines, ex vivo production of reactive oxygen and nitrogen intermediates, phagocytic activity of neutrophils and monocytes, C-reactive protein (PCR), parameters lipemics, fecal IgA concentration and calprotectin. In the middle of this period, a digestibility test and a postprandial insulin and glucose curve were performed. At the final time, fresh feces were still collected to evaluate fermentation products. Data were evaluated by analysis of variance and compared by Tukey's test ($P<0.05$), and when necessary, analysis of covariance was applied. Dogs fed B-A31 showed lower IL-2 than B-LEV and less phagocytosis by neutrophils than the other treatments ($P<0.05$). Dogs fed B-A31 tended to have higher IL-10 than CON ($P=0.095$). Consumption of seaweed B-glucan increased nitric oxide production in cell culture and serum CRP ($P<0.05$). Dogs fed BA15 showed higher apparent digestibility of dry matter, organic matter and gross energy ($p<0.05$). Dogs fed BLEV tended to have lower mean glucose and shorter time to peak insulin response ($p<0.1$). The beta diversity of the fecal microbiota of dogs on

the BA31 diet differed from the BLEV and BA15 diets. It was concluded that B-glucans from yeast and seaweed showed different pathways of action, as B-glucan from seaweed induced an increase in CRP, monocyte activation measured by increased production of nitric oxide and tended to increase the anti-inflammatory cytokine IL -10, and the BA31 diet showed a greater ability to alter the fecal microbiota. Yeast β-glucan tended to lower mean glucose concentration and anticipate the peak of endogenous insulin action.

Keywords: β-glucans, yeast cell wall, seaweed, microbiota.

CAPÍTULO 1

1. Considerações gerais

Os β-glucanos são definidos como polissacarídeos macromoleculares constituídos por monômeros de D-glicose ligados por ligações β-glicosídicas (Du et al., 2014). Podem ser encontrados em uma variedade de fontes naturais, como leveduras, cogumelos, bactérias, algas, cevada e aveia (Zhu et al., 2015a). Em função de suas diferentes estruturas e pesos moleculares, a atividade biológica dos diferentes tipos de β-glucanos pode variar bastante (Du; Xu, 2014).

O interesse pelo emprego de β-glucanos como aditivo nutracêutico tem aumentado, seja mediante inclusão em alimentos ou produtos farmacêuticos (Zhu et al., 2016; Bai et al., 2019), além de seu emprego em cosméticos e produtos químicos. Como exemplos, em bebidas os β-glucanos são usados para ajustar a consistência da solução devido ao seu efeito espessante e no leite, iogurte e pão como aditivos alimentares para diminuir a ingestão de energia e reduzir o colesterol (Barone Lumaga et al., 2012; Sharafbafi et al., 2014). Além disso, os β-glucanos também são adicionados em algumas fórmulas de medicamentos com vistas à propriedades imunomoduladoras, ação antitumoral, efeito na diabetes e ainda como redutor de colesterol no sangue (Du et al., 2014).

Segundo o Food Marketing Institute (FMI) produtos relacionados à saúde e suplementos alimentares representaram 33,7% do mercado global de β-glucanos em 2014. Os β-glucanos derivados de cereais representam a maior fração. Acredita-se que os β-glucanos derivados de levedura, outra fonte importante do composto, seja a segunda maior fonte, seguida pelos β-glucanos derivados de cogumelos (Bai et al., 2019).

No entanto, além de atuar de forma favorável na produção de alimentos e produtos farmacêuticos, os β-glucanos apresentam muitos benefícios importantes já relatados em humanos e animais: capacidade de eliminar radicais livres, de maneira semelhante aos antioxidantes (Kofuji et al., 2012), atividade imunológica moduladora (Lai et al., 2010; Rieder & Samuelsen, 2012; Shah et a., 2015), atividade antitumoral (Rieder & Samuelsen, 2012), redução sérica de colesterol (EFSA, 2010; Maki et al., 2010; Zhu et al., 2015b), redução da resposta glicêmica e insulinêmica, o que evitaria picos de glicose pós-prandiais (Meyer et al., 2002) e prevenção da obesidade (Bai et al., 2019).

Diante do aumento da população de cães e gatos e do desenvolvimento expressivo do mercado Pet Food, que apresentou crescimento de 24% no ano de 2020 (ABINPET, 2021), observou-se nos β-glucanos uma nova fonte a ser explorada no desenvolvimento de “alimentos funcionais”, ou seja, que além do correto fornecimento de nutrientes pudesse vir a conferir características de controle de riscos de acometimento por doenças crônicas (Zielke et al, 2017).

Dentro de seus diversos mecanismos de ação, os feitos relativos à redução de colesterol e da resposta glicêmica e insulinêmica devem-se à ação do β-glucano como fibra alimentar solúvel, com capacidade de formar soluções viscosas no intestino que reduzem a taxa de assimilação de nutrientes (Wolever et al., 2010). Há evidências crescentes de que a adição de fibra fermentável à dieta altere a função e a estrutura do intestino, modificando a produção de hormônios derivados deste orgão se associando a melhor homeostase da glicose em todo o organismo, mesmo na ausência de doença (Massimino et al., 1998; Schley, Field; 2002). Estas funções têm, de modo geral, sido estudadas mais frequentemente para β-glucanos de origem

vegetal, com ligações beta-1,4-glicosídicas, como na aveia e as doses incorporadas aos alimentos são relacionadas a quantidade de carboidratos da dieta, sendo relatado um intervalo de 0,9 a sete gramas de β-glucanos por 30 gramas de carboidratos (AbuMweis et al., 2016; Ekstrom et al., 2017).

Em medicina veterinária, no entanto, os possíveis efeitos prebióticos (ainda não confirmados), antiinflamatórios e imunomoduladores do β-glucano têm se destacado e representam os objetivos da grande maioria dos estudos publicados. O conhecimento da influência dos prebióticos no tecido linfóide associado ao intestino, derivado de seu potencial influencia na composição e metabolismo da microbiota do trato gastrointestinal é hoje amplamente aceito (Cherbut, 2002; Bodera, 2008; Alexander et al., 2018; Carballo et al., 2019). As mudanças na comunidade microbiana, associadas à melhor produção de produtos de fermentação, em especial de butirato podem modular a produção de citocinas inflamatórias e a resposta imunológica dos animais (Lin et al., 2019; Theodoro et al., 2019). Tem-se, também, estudado as citocinas como biomarcadores fecais de inflamação intestinal (Poullis et al., 2010). Em 2013, Prakash et al. validaram a mensuração de citocinas em fezes de cães saudáveis e concluíram que a detecção das citocinas IL-6, IL-8, IL-10 e TNF-α podem ser úteis como biomarcador não invasivo de inflamação.

O potencial prebiótico dos β-glucanos foi relatado no final dos anos 90 e início dos anos 2000, com indícios de promover seletivamente o crescimento de microrganismos intestinais benéficos, como lactobacilos e bifidobactérias, de acordo tanto com estudos in vitro (Jaskari et al., 1998; Kontula et al., 1998b) como experimentos com animais (Dongowski et al., 2002; Drzikova et al., 2005; Snart et al., 2006). β-glucanos obtidos da cevada, algas marinhas, bactérias e cogumelos foram

incubados com culturas puras de *Bifidobacterium infantis, Bifidobacterium longum e Bifidobacterium adolescentis*. Todos os β-glucanos isolados, independentemente de suas fontes e correspondentes diferenças nas ligações glicosídicas e peso molecular, foram utilizados de modo semelhante à inulina (controle positivo) pelas três espécies de bifidobactérias (Zhao; Cheung, 2011). É possível que haja semelhança nos sistemas enzimáticos e transportadores das diferentes espécies de bifidobactérias investigadas, justificando os resultados encontrados (Lam et al., 2013). No entanto, o mecanismo detalhado de fermentação bacteriana seletiva de β-glucanos permanece incerto e existem desafios de pesquisa para estudos adicionais nessa área.

Os β-glucanos obtidos da levedura *Saccharomyces cerevisiae* têm sido os mais largamente investigados na medicina veterinária (Li et al., 2006; Mo et al., 2017; Wang et al., 2019), destacando-se como composto natural capaz de alterar a imunidade de várias espécies animais (Hiss et al., 2003; Stuyven et al., 2010), inclusive cães (Altug et al., 2010). Como exemplo, em trabalho recente Wang et al. (2019) investigaram a ação de β-glucanos de levedura (*Saccharomyces cerevisiae*) na modulação da microbiota intestinal, estimulação do sistema imunológico e alterações histopatológicas em frangos imunossuprimidos. Foi identificado aumento das populações intestinais de *Bifidobacterium* e *Lactobacillus* em comparação ao grupo controle, aumento das concentrações de IFN- γ e IL-6, diminuição de TGF-β1 e maior proliferação de linfócitos, além de diminuição nas alterações histopalógicas da Bursa. Dessa forma, os autores sugerem que o consumo de β-glucanos de leveduras pode efetivamente aliviar a imunossupressão e regular beneficamente a microbiota do intestino.

Em cães, foi realizado um estudo em ração comercial com o objetivo de avaliar os efeitos da adição de dois β-glucanos de *Saccharomyces cerevisiae* com pureza e processos biotecnológico diferentes. Foram mensurados os níveis de IL-2, alterações na fagocitose, produção de anticorpos e níveis de glicose sanguíneo. Os autores concluíram que os dois produtos apresentaram efeitos imunomoduladores significativos em cães, sugerindo fortemente que a suplementação de ração com esses β-glucanos resulta em melhora das condições biológicas e imunológicas dos cães (Vetvicka, Oliveira, 2014). Em 2010, avaliaram o efeito da administração oral de beta-1,3/1,6-glucanos de *Saccharomyces cerevisiae* na imunidade humoral em cães. Ao final de quatro semanas, o nível sérico total de IgA diminuiu e o nível de IgM aumentou significativamente em relação ao grupo controle. Nenhum efeito foi encontrado no nível de IgG. A concentração de IgA também se apresentou significativamente menor na saliva e nas lágrimas do grupo tratado com β-glucanos. Foi observado ainda que os efeitos desapareceram uma semana após a interrupção da suplementação. Portanto, os autores concluíram que os resultados mostram uma mudança temporária nos perfis imunes durante a suplementação com β-glucanos (Stuyven et al., 2010).

O β-glucano de *Saccharomyces cerevisiae* apresenta, ainda, mecanismo direto de imunomodulação; o composto interage com receptores específicos (dectina-1, receptores do tipo Toll 2/6 e receptor do complemento 3) nos glóbulos brancos do sistema imunológico inato, como macrófagos, neutrófilos, granulócitos e células dendrídicas (Baert et al., 2015; Raa, 2015) presentes na mucosa intestinal promovendo o início de processos bioquímicos ao induzir a cascata de respostas imune inata e adaptativa, como fagocitose, burst oxidativo e produção de citocinas e

quimiocinas (Brown et al., 2003). Oliveira e colaboradores (2019) avaliaram a inclusão de duas moléculas de β-glucanos durante 28 dias em quatro diferentes espécies de vertebrados: cães, leitões, camundongos e pintinhos. Os resultados mostraram comportamentos biologicamente diferentes das duas moléculas, porém ambas estimulam o sistema imunológico em um padrão semelhante nestas quatro espécies. Este achado sugeriu que os vertebrados compartilharam mecanismos semelhantes no reconhecimento dos β-glucanos e confirmou os benefícios dos β-glucanos em diferentes espécies.

Na última década, estudos sobre polissacarídeos de algas marinhas ganharam interesse devido a suas propriedades potenciais (Pomin, Mourão, 2010; Wijesekara et al., 2011; Barahona et al., 2014), tendo-se já demonstrados efeitos benéficos à saúde de animais (Raposo et al., 2016). Na parece celular de *Saccharomyces cerevisiae* o β-glucano apresenta-se como polissacarídeo com ligações β-1,3 e β-1,6 glicosídicas, conferindo a este estrutura ramificada de moléculas de glicose conectadas por ligações β-1,3-glicosídicas, nas quais os pontos de ramificação são ligações β-1,6 (Raa, 2015). Já a alga unicelular *Euglena gracilis* também é uma importante fonte de β-1,3. Quando cultivada aerobicamente no escuro com uma fonte de carbono, há o acúmulo de grandes quantidades de paramilo, um β-glucano insolúvel, não digerível, composto por polímeros lineares de β-1,3-D-glicose de alto peso molecular, depositados no citoplasma como grânulos discóides altamente cristalinos (Barsanti et al., 2001; Monfils et al., 2011). Identificam-se, assim, diferenças entre as moléculas que tornam necessários estudos para melhor caracterizar os efeitos biológicos do β-glucano derivado das algas.

Com o objetivo de reduzir estresse e doenças em psicultura, Yamamoto et al. (2018) avaliaram a ação de β-1,3-glucano produzido a partir de *Euglena gracilis*. Os autores verificaram na espécie *Sciaenops ocellatus* melhora na resposta imune inata de fagócitos, com maior produção intracelular de ânion superóxido e aumento da atividade bactericida. Outro estudo investigou a influência da suplementação alimentar com β-1,3-glucano de *Euglena gracilis* na permeabilidade intestinal e resposta imune de porcos desmamados, infectados experimentalmente com *Escherichia coli* patogênica. Os resultados indicaram que a suplementação de aproximadamente 108 mg/kg diminuiu a diarréia de porcos infectados, melhorando a integridade intestinal. Além disso, também aumentou a resposta imune do hospedeiro contra a infecção por *Escherichia coli*, pois estimulou a ativação das células T e reduziu a inflamação (Kim et al., 2019).

Estes achados apontam a possibilidade do β-1,3-glucano de *Euglena gracilis* apresentar efeito prebiótico, anti-inflamatório ou imunomodulador em cães. Os autores não localizaram, no entanto, estudos conduzidos com esta espécie. Em função da significativa quantidade de informações disponíveis para o β-glucano de *Sacchamoryces cerevisae*, comparações entre a suplementação alimentar destes dois tipos de β-glucano seriam interessantes, permitindo-se adequada avaliação das ações biológicas do derivado da parede celular de algas.

2. Hipótese

Hipotetiza-se que o β-glucano de *Euglena gracilis* seja fermentado pela microbiota do intestino grosso dos cães, promovendo alterações nos produtos de fermentação nas fezes. Fruto destas alterações, bem como da possível interação

direta do β-glucano com o sistema imune do animal, seu consumo resultará em imunomodulação com melhora da imunidade inata dos animais e redução da inflamação intestinal. Estes efeitos serão similares e equivalentes quando comparados ao controle positivo β-glucano de *Saccharomyces cerevisiae*.

3. Objetivo

Com base nas considerações anteriores, são objetivos da presente pesquisa avaliar e comparar os efeitos da inclusão de β-glucanos oriundos da parede celular de algas marinhas (*Euglena gracilis*) com os de β-glucanos obtidos da parece celular de levedura (*Sacchamoryces cerevisae*), sobre a composição da microbiota intestinal, produtos de fermentação microbiana nas fezes e alguns parâmetros imunológicos e inflamatórios de cães alimentados com rações extrusadas.

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CAPÍTULO 2 - β-glucans from *Euglena gracilis* or *Saccharomyces cerevisiae* effect comparison on immunity and inflammatory parameters of dogs

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Short title: β-glucan action on immune and inflammatory parameters.

1. Abstract

The objectives of the present study were to evaluate and compare the effects of the inclusion of β-glucans from the seaweed *Euglena gracilis* with β-glucans obtained from the yeast *Sacchamoryces cerevisae*, on the immunostimulatory and inflammatory activity of healthy adult dogs fed with extruded diets. A control diet (CON) was supplemented with yeast extracted β-glucan (B-Yeast; 0.115%) or seaweed β-glucan: B-S15 (0.155% seaweed β-glucan dose); B-S31 (0.31% Seaweed B-glucan). 32 dogs were used, 8 per ration. They received the rations for 42 days, with blood and feces collected at the beginning and end of the period for analysis of cytokines, *ex vivo* production of reactive oxygen and nitrogen intermediates, phagocytic activity of neutrophils and monocytes, C-reactive protein (CRP), concentration of faecal IgA and calprotectin. Data were evaluated by analysis of covariance and compared by Tukey's test ($P<0.05$). Dogs fed B-S31 showed lower IL-2 than B-Yeast and less phagocytosis by neutrophils than the other treatments ($P<0.05$). Consumption of seaweed β-glucan increased nitric oxide production in cell culture and serum CRP ($P<0.05$) and decreased production of hydrogen peroxide ($P<0.05$). It was concluded that β-glucans from yeast and seaweed showed different pathways of action, as β-glucan from seaweed induced an increase in CRP, monocyte activation measured by increased production of nitric oxide and tended to increase the anti-inflammatory cytokine IL -10.

2. Introduction

A strategy increasingly exploited in production species is the use of food additives to improve the innate immune system, aiming at reducing the incidence of diseases and, thus, overcoming the indiscriminate use of antibiotics and chemotherapy (Pohlenz, Gatlin, 2014; Levine et al., 2018; Yamamoto et al., 2018). The immunostimulant is a compound capable of beneficially modulate the immune and inflammatory system in different species, and thus increasing the resistance of the host (Dawood et al., 2018; Koch et al., 2021). The β-glucans are compounds explored for this function, with interesting results for several species (Soltanian et al., 2009; Zielke et al., 2017).

The β-glucans can be found in a variety of natural sources such as yeasts, mushrooms, bacteria, algae, barley, and oats (Zhu et al., 2015). They have attracted attention because of their broad spectrum of biological activities, including antitumor, immunomodulatory, glycemic control, anti-aging, and anti-inflammatory properties, especially in fish, poultry and humans (Du et al., 2014; Zhu et al., 2016; Bai et al., 2019). However, only few β-glucan structures, in particular the β-1,3/1,6-glucans have immunomodulatory and inflammatory bioactivity as they interact with membrane receptors on immune cells and induce specific biological responses (Raa, 2015; Bai et al., 2019; Murphy et al., 2021).

In this context, β-glucans extracted from *Saccharomyces cerevisiae* cell wall have been the most widely investigated in veterinary medicine (Li et al., 2006; Mo et al., 2017; Wang et al., 2019). In dogs, β-glucans from *Saccharomyces cerevisiae* with different purity and biotechnological processes of obtention stimulated interleukin (IL)-

2 serum concentrations, peripheral cells phagocytic activity, antibody production and modulate blood glucose concentrations (Vetvicka; Oliveira, 2014). In a further study, Oliveira et al. (2019) evaluated the inclusion of two molecules of different purities of β-glucans from *Saccharomyces cerevisiae* (77.3 and 55.7 β-glucans) incorporated into complete and balanced diets for dogs, piglets, mice and chicks. Although differences in biological response among the two molecules were observed, both stimulated the immune system in a similar pattern in all tested species, showing greater production of IL-2, greater phagocytosis index of monocytes and neutrophils and also greater production of antibodies, which suggests that vertebrates shared similar mechanisms of β-glucans recognition and action.

In the last decade, studies on seaweed polysaccharides have gained interest due to their potential biological properties (Pomin, Mourão, 2010; Wijesekara et al., 2011; Barahona et al., 2014). The unicellular alga *Euglena gracilis* is considered a potential source of β-1,3-glucan, especially after the development of large-scale cultivation methods. When it is grown aerobically in the dark with a carbon source, large amounts of paramylon, an insoluble, non-digestible β-glucan composed of high molecular weight linear polymers of β-1,3-D-glucose are deposited in the cytoplasm, as highly crystalline discoid granules (Barsanti et al., 2001; Monfils et al., 2011).

Studies with dried *Euglena gracilis* or purified paramylon/β-1,3-glucan are available in some species. In mice, paramylon was tested in a model of atopic dermatitis skin lesions induced by 2,4,6-trinitrochlorobenzene, and the authors verified a reduction in the development of the skin lesions followed by T-helper 1 and 2 cell responses inhibition (Sugiyama et al., 2010). The supplementation with a fermented *Euglena gracilis*, containing more than 50% of β-1,3-glucan, was investigated through

a self-reported changes in upper respiratory tract infection symptoms in humans. The study provided evidence that the supplementation can reduce or prevent upper respiratory tract infection symptoms, providing immune support and overall health (Evans et al., 2019).

No studies published in dogs with *Euglena gracilis* were found by the authors. Considering the molecular differences between the β-1,3/1,6-glucans from *Saccharomyces cerevisiae*, that presents branched chains (Raa et al., 2015; Sivieri et al., 2022), and the high molecular weight linear polymers of β-1,3-glucans of *Euglena gracilis* (Yamamoto et al., 2018; Murphy et al., 2021), it is expected that these compounds differ in the interaction with immune cells and thus biological effects. Based on these considerations, the objectives of the present study was to evaluate and compare the effects of β-glucans from *Euglena gracilis* and *Sacchamoryces cerevisae*, incorporated in complete and balanced diets on the immunostimulatory and inflammatory activity of healthy adult dogs.

3. Experimental methods

The study was conducted in the Laboratory of Research in Nutrition and Nutritional Diseases of Dogs and Cats “Prof. Dr. Flávio Prada”, 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° 001974/20).

Animals

Thirty-two adult Beagle dogs, eight males and twenty-four females, with 2.9±2.3 years and 10.7±1.4 kg were used. For these animals the mean body condition score was 5 on 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. Animals were randomly distributed to treatments, but each group was homogenized to present similar body weight and age ($P>0.05$; data not shown).

Experimental design

The study included four experimental diets and was conducted in a randomized block design with two blocks of 16 dogs each, four dogs per diet in each block, totaling eight animals (repetitions) per diet (treatment). The blocking factor was time, due to the available structure for research. Each block lasted 84 days, with a 42-day washout (all animals received the control diet) and 42-day treatment period. Immediately before and at the end of each experimental period (period that the dogs eat the treatment diets), the following analyses were conducted: serum and fecal cytokines (TNF-α, IL-2, IL-6, IL-10), fecal calprotectin and immunoglobulin (Ig) A, serum C reactive protein (CRP), phagocytic activity of peripheral monocyte and neutrophils, *in vitro* production of hydrogen peroxide and nitric oxide in cell culture by peripheral neutrophils and monocytes, and Ig G production in cell culture.

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). Food was provided once a day (at three pm). Offered food was weighed, no refused diet was identified for any dog, and the intake was recorded daily. Dogs were weighed weekly, and the amount of food provided adjusted such that animals maintained a constant body weight throughout the study. Water was provided *ad libitum.* During the study dogs were housed in kennels measuring 1.5 m x 3.5 m with a solarium and released daily in a collective playground for exercise and socialization during 4 to 6 hours a day.

Ingredients tested

Two sources of β-glucan were evaluated. A purified source extracted from *Sacchamoryces cerevisae* with 78.4% of β-glucans (MacroGard, Biorigin, Lençóis Paulista, Brazil), and a source constituted by dried *Euglena gracilis* cells with 58.5% of β-glucans (Pralisur, Kemin Industries, Iowa, USA). These β-glucans concentrations were analyzed previous the study.

Experimental diets

A single formulation based on corn grain, poultry byproduct meal, poultry fat and sugarcane fiber was used (Table 1), and balanced for adult dogs according to the nutritional recommendations of the European Pet Food Industry Federation (FEDIAF, 2019). To obtain the experimental diets, the β-glucan sources was included in different amounts in the formulation. The amount added were stablished based on the study of Oliveira et al. (2019), that suggested an amount of 15 mg β-glucan per kg of body

weight per day as an effective dosage for dogs. Considering the expected food intake during the study and the analyzed β-glucan concentration in the products evaluated, the following proportions was included (on an as-fed basis): CON - control diet, without inclusion of β-glucans source; B-Yest - inclusion of 0.115% of β-glucan source from *Sacchamoryces cerevisae* cell wall; B-S15 - inclusion of 0.155% of β-glucan source from *Euglena gracilis*; B-S31 - inclusion of 0.310% of β-glucan source from *Euglena gracilis*.

Table 1. Ingredient and chemical composition of the formulation used on the study.

Ingredients	%
Corn grain	51.6
Poultry by-product meal	32.2
Poultry fat	9.2
Liquid palatant [1]	3.0
Sugarcane fiber [2]	2.0
Vitamin-mineral premix [3]	0.6
Common Salt	0.5
Potassium chloride	0.5
Choline chloride	0.3
Mold inhibitor [4]	0.1
Antioxidant [5]	0.05
L-lysine	0.03
Analyzed chemical composition	%, as fed basis
Dry matter	92.8
Crude protein	28.8
Ash	5.7
Acid-hydrolyzed fat	17.6
Crude fiber	2.2
Calcium	1.0

Phosphorus	0.9

[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 produced at the Extrusion Laboratory of the College of Agrarian and Veterinarian Sciences, São Paulo State University (UNESP), Jaboticabal, SP, Brazil. A single lot of raw materials was used for the four experimental diets. Ingredients were weighed and mixed before being ground in a hammer mill (Tigre, Moinhos Tigre, São Paulo, SP, Brazil), fitted with a 0.8 mm sieve screen size, and extruded in a single-screw extruder (Model Mex-250, Manzoni Industria Ltda, Campinas, SP, Brazil), 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 min and coated with poultry fat and liquid palatant.

Phagocytic activity

Phagocytic activity was measured at the beginning and at the end of the experimental period using a commercial kit (pHrodo *E. coli* BioParticles, Molecular Probes Inc., Oregon, USA). Blood samples were collected by jugular venipuncture and placed in heparinized tubes. Then, 100 µL of each sample was incubated with 20 µL of pHrodo *E. coli* BioParticles, a 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 in a water bath at 37 ºC for 15 min. Next, 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 on each collection day, both tubes with no bioparticles, but one placed on ice and the other kept at 37 ºC. Samples were analyzed using a flow cytometer (FACSCanto, Becton Dickinson Immunocytometry System, Mountain View, CA, USA), and the results were expressed as the percentage of fluorescence signal inside the desired population of neutrophils and monocytes. The target cell population was gated according to its volume and complexity (Neaga et al., 2013).

Cytokines: IL-2, IL-6, IL-10 and TNF-α on blood serum and feces

Blood samples (3 mL) were collected via jugular venipuncture and placed in tubes without anticoagulant. Afterwards, the samples were centrifuged at 3,500 x g for 10 min (5810R; Eppendorf, Hamburg, Germany), and the serum was stored frozen at -80 °C until analysis. Fresh stool samples (maximum 15 minutes after elimination) were collected and immediately frozen at -80 °C during three consecutive days. Prior to the analyzes, an extraction with proteinase inhibitor was performed in a *pool* sample. The amount was estimated 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).

Faecal calprotectin determination

Fresh feces (immediately after elimination) were collected for three consecutive days and frozen -80 °C until extraction. Prior to analysis, an extraction in a dilution 1:10 with proteinase inhibitor was performed in a *pool* sample. To quantify the concentration of calprotectin in the stool extract, a specific ELISA kit (Cloud-Clone Corp, Houston, TX, USA) was used. The reading was performed in an ELISA microplate reader (Awareness Technology, Florida, EUA).

Serum C reactive protein

Three mL of blood were collected by jugular venipuncture. Afterwards, the samples were centrifuged to obtain serum and frozen in a -80 ºC freezer for further analysis. The analysis was performed using a specific ELISA kit (Cloud-Clone Corp, Houston, TX, USA) for dogs according to the manufacturer's recommendations.

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

Blood samples (30 mL) were collected with heparin via jugular venipuncture and added to 4.5 mL of Histopaque 1119 and 3 mL of Histopaque 1077 (Sigma Aldrich, St Louis, MO, EUA) in 15-mL conical centrifuge tubes. Tubes were centrifuged at 700 x g for 30 minutes at room temperature. After centrifugation, two distinct opaque layers separated, the mononuclear and granulocyte cells. Each layer was collected separately, transferred to a 50 mL conical centrifuge tube and washed at least twice with isotonic phosphate buffered saline by centrifugation at 360 x g for 10 min at room

temperature. Erythrocyte lysis was conducted when necessary, using 2 mL of ACK (Ammonium-Chloride-Potassium) solution (0.15 M ammonium chloride; 10 mM potassium bicarbonate; 0.1 mM EDTA) for a maximum of 2 min.

Cells were suspended in complete medium (RPMI 1640, Merck KGaA, Darmstadt, German), added to 40 mg/mL gentamicin and 10% fetal bovine serum, and the concentration was adjusted to $3x10^5$ neutrophils or monocytes/mL. Then, suspensions 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 Scientific, Waltham, MA, USA) for one hour for monocytes to adhere to the well surfaces, then supernatants were carefully discarded with a pipette and complete medium was added to each well.

For H_2O_2 production, a total of 36 wells received 100 µL of sample; 18 of them were maintained as suspensions of non-stimulated cells, and the others 18 wells were stimulated with LPS (10 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 as previously described (Pick; Keisari, 1980; Pike; Mizel, 1981). Buffer solution (100 µL/well) consisting of 7.8 mL distilled water (dH_2O), 0.8 mL of solution A (800 mL dH_2O, 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 dH_2O, 1 g $CaCl_2$), 0.1 mL of solution C (100 mL dH_2O, 1 g $MgCl_2$), 0.1 mL of phenol red (100 mL dH_2O, 1 g phenol red), 0.1 mL of peroxidase (10 mg horseradish peroxidase, 2 mL PBS) and 1 mL of glucose (100 mL dH_2O, 1 g glucose), was added to each well. Phorbol myristate acetate (PMA, 10 µL/well) was added to half of the non-stimulated wells and to half of LPS-stimulated wells and kept at 37 °C in a humidified 5% CO_2 incubator for one hour. Consequently, there were nine replications for each cell condition: nine wells for non-stimulated cells, nine wells for

non-stimulated and PMA, nine wells for LPS-stimulated cells, and nine wells for LPS-stimulated cells and PMA. After one-hour incubation, the reaction was stopped with 10 µL of 1 N NaOH. The absorbance was read in a microplate reader (iMarkMicroplate Absorbance Reader 168-1135, Bio-Rad, Hercules, California, USA) at 595 nm. The results were expressed in µM amounts of H_2O_2/3X10^5 cells. A hydrogen peroxide standard curve was constructed for each plate with a range of 0.25 to 16.00 nM of H_2O_2.

The NO production was assessed by the colorimetric method of the GRIESS reaction (Grenn et al., 1982). The analyses were conducted in nine repetitions for non-stimulated cells and nine repetitions for LPS-stimulated cells (10 µg/well *E. coli Lipopolysaccharide*, Sigma Aldrich, St. Louis, USA), totaling 18 wells per sample. One-hundred µL of GRIESS reagents diluted 1:1 (n-(1-naftil)-etil-enediamin diluted 0.1% in dH_2O, 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. The results were expressed as µM amounts of NO/3x10^5 cells. An NO standard curve was constructed for each plate with a range of 0.78 to 100 µM of NO.

Ig G production in cell culture

The same cell separation process previously described was used for the IgG analysis. Lymphocytes were cultured (3x10^5) in the presence of LPS stimulus (5µg/mL) for seven days and then the supernatant was collected and frozen in -80 °C until

analysis. Dosing was performed using a dog-specific IgG ELISA kit according to the manufacturer's recommendations (Cloud-Clone Corp, Houston, TX, USA).

Fecal Ig A

Fresh feces (immediately after elimination) were collected for three consecutive days. For each period, fecal samples were pooled by dog, and fecal IgA was extracted using a saline solution (Peters et al., 2004). Approximately one 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 an 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.

Statistical analysis

The study considered each individual dog as the experimental unit. The sampling size was established based on the results of immunoglobulin A and interleukin 10 (Theodoro et al., 2019), considering the completely randomized block design. The test power was set at 0.8 (procedure Opdoe of the R software), $\alpha = 0.05$, standard deviation = 149.2, and a standard error of approximately 31.7 for Ig A. The analysis was performed with the sample size procedure of the R software, and a sample size of 8 dogs was obtained. All variables were previously tested for errors normality and homoscedasticity. When necessary, logarithmic transformation (log10) was applied. Data were submitted to analysis of variance considering the effects of block and diet. The data were analyzed using ANCOVA and to reduce variation among replicates the tested differences between end measurements were corrected for baseline measures rather than the changes in outcome variables. This has been shown in intervention trials to increase statistical power (Vickers, 2001; Theodoro et al., 2019). When results of the F-test were significant, multiple comparisons of the means were made using Tukey's test ($P<0.05$). The analysis was conducted using the computer program R (version 4.2.0).

4. Results

Regarding the β-glucans sources tested, and the amount of food eaten by each dog to maintain constant body weight along the study, dogs fed B-Yeast ingested 13.7±2.3 mg of β-glucans/kg/day, B-S15 ingested 14.4±2.9 mg of β-glucans/kg/day, and dogs fed B-S31 27.3±5.7 mg of β-glucans/kg/day. β-glucans sources were well

tolerated by healthy adult dogs during 42 days of consumption, as there were no changes in the clinical condition of the animals.

Dogs fed the B-S31 diet presented lower neutrophil phagocytic index than animals fed the others diet (P<0.05), which did not differ from each other (Table 2). Among the evaluated serum cytokines (Table 3), dogs fed the B-Yeast diet exhibited higher IL-2 concentration than animals fed the B-S31 diet (P<0.05), but similar values in comparison with the CON and B-S15 treatment. No differences were detected for the other cytokines evaluated on serum (P>0.05). The concentration of cytokines in feces was similar between dogs fed the different treatments (P>0.05; Table 4).

Table 2. Monocyte and neutrophils phagocytic index (% of positive cells) of dogs fed diets with the addition of different sources and amounts of β-glucans (mean and standard error of the mean).

	Diets[1]				
Item[2]	CON	B-Yeast	B-S15	B-S31	*p-value*
MO	84.5^{b}±0.9	85.9^{b}±0.9	88.9^{a}±0.9	84.9^{b}±0.9	0.019
NE	78.0^{a}±1.4	81.3^{a}±1.5	79.3^{a}±1.5	72.9^{b}±1.5	0.001

[1] CON = control, without β-glucan addition; B-Yeast = CON added of 0.115% of β-glucan from *Saccharomyces cerevisiae*; B-S15 = CON added of 0.155% of β-glucan from *Euglena gracilis*; B-S31 = CON added of 0.310% of β-glucan from *Euglena gracilis.*
[2] MO = monocytes; NE = neutrophils.
a, b = means in a row without a common superscript letter differ (P<0.05).

Table 3. Serum cytokines concentrations (pg/mL) of dogs fed diets with the addition of different sources and amounts of β-glucans (mean and standard error of the mean).

	Diets[1]				
Item	CON	B-Yeast	B-S15	B-S31	*p-value*
IL-2	71.9^{ab}±25.6	172.7^{a}±27.0	101.7^{ab}±25.6	51.7^{b}±23.2	0.027

IL-6	82.7±28.8	125.9±33.1	60.1±34.1	57.8±28.6	0.756
IL-10	28.5±7.4	35.1±8.0	37.5±7.4	53.0±7.3	0.136
TNF-α*	57.4±26.6	58.6±32.3	17.5±28.8	19.2±26.4	0.499

[1] CON = control, without β-glucan addition; B-Yeast = CON added of 0.115% of β-glucan from *Saccharomyces cerevisiae*; B-S15 = CON added of 0.155% of β-glucan from E*uglena gracilis*; B-S31 = CON added of 0.310% of β-glucan from E*uglena gracilis.*

a, b = means in a row without a common superscript letter differ (P<0.05).

* Values transformed to Log10 for statistical analysis.

Table 4. Cytokines (pg/g of feces, as-is basis) on the feces of dogs fed diets with the addition of different sources and amounts of β-glucans (mean and standard error of the mean).

	Diets[1]				
Item	CON	B-Yeast	B-S15	B-S31	*p-value*
IL-2	62.6±2.9	60.4±2.9	63.9±2.9	63.0±2.9	0.841
IL-6	17.1±1.8	17.3±1.8	20.6±1.8	17.9±1.8	0.519
IL-10	103.4±11.1	118.0±11.0	99.8±10.9	87.8±10.8	0.347
TNF-α	12.7±0.6	12.8±0.6	13.1±0.6	12.3±0.6	0.769

[1] CON = control, without β-glucan addition; B-Yeast = CON added of 0.115% of β-glucan from *Saccharomyces cerevisiae*; B-S15 = CON added of 0.155% of β-glucan from *Euglena gracilis*; B-S31 = CON added of 0.310% of β-glucan from *Euglena gracilis.*

Dogs fed both diets containing *Euglena gracilis* β-glucans presented higher CRP serum concentration than animals fed B-Yeast (P<0.05; Table 5). This may be related to the higher *ex vivo* NO production by peripheral monocytes observed in dogs fed these same diets, in comparison with animals fed the CON and B-Yest treatments (P<0.05; Table 6). For hydrogen peroxide, however, the opposite was verified with lower *ex vivo* production in dogs fed diets containing *Euglena gracilis* than CON and B-Yeast (P<0.05; Table 7). The Immunoglobulin G concentration in cell culture and immunoglobulin A in feces did not differ (P>0.05) between dogs fed the different experimental diets (Table 8).

Table 5. Calprotectin (µg/g of feces, as-is basis) and serum C-reactive protein (CRP; mg/L) of dogs fed diets with the addition of different sources and amounts of β-glucans (mean and standard error of the mean).

	Diets[1]				
Item	CON	B-Yeast	B-S15	B-S31	*p-value*
Calprotectin	0.006±0.0008	0.005±0.0007	0.006±0.0008	0.006±0.0007	0.558
CRP	0.80^{ab}±0.05	0.79^{a}±0.05	0.96^{b}±0.05	0.97^{b}±0.05	0.034

[1] CON = control, without β-glucan addition; B-Yeast = CON added of 0.115% of β-glucan from *Saccharomyces cerevisiae*; B-S15 = CON added of 0.155% of β-glucan from *Euglena gracilis*; B-S31 = CON added of 0.310% of β-glucan from *Euglena gracilis.*

a, b = means in a row without a common superscript letter differ (P<0.05).

Table 6. Nitrogen oxide (µM of NO/3x10^5 cells) production in cell cultures of monocytes and neutrophils from the peripheral blood of dogs fed diets with the addition of different sources and amounts of β-glucans (mean and standard error of the mean).

	Diets[1]				
Item	CON	B-Yeast	B-S15	B-S31	*p-value*
Monocytes					
Only cells	9.3^{b}±10.0	10.1^{b}±10.0	45.9^{a}±9.9	43.4^{a}±9.9	0.018
LPS	8.7^{b}±13.1	11.4^{b}±13.0	56.9^{a}±13.1	49.7^{ab}±13.0	0.030
Neutrophils					
Only cells	3.1±1.2	3.0±1.2	5.8±1.3	4.5±1.3	0.463
LPS	3.1±1.2	2.4±1.2	4.4±1.3	4.9±1.2	0.472

[1] CON = control, without β-glucan addition; B-Yeast = CON added of 0.115% of β-glucan from *Saccharomyces cerevisiae*; B-S15 = CON added of 0.155% of β-glucan from *Euglena gracilis*; B-S31 = CON added of 0.310% of β-glucan from *Euglena gracilis.*

[2] LPS – lipopolysaccharide

a, b = means in a row without a common superscript letter differ (P<0.05).

Table 7. Hydrogen peroxide (µM of H_2O_2/3x10^5 cells) production in cell cultures of monocytes and neutrophils from the peripheral of dogs fed diets with the addition of different sources and amounts of β-glucans (mean and standard error of the mean).

Diets[1]

Item	CON	B-Yeast	B-S15	B-S31	*p-value*
Monocytes					
PMA	3.5[a]±0.3	3.3[ab]±0.3	1.9[b]±0.3	2.2[b]±0.3	0.002
PMA + LPS	3.6[a]±0.4	3.6[a]±0.4	1.6[b]±0.4	2.2[ab]±0.4	0.001
Neutrophils					
PMA	4.8±0.7	4.6±0.7	5.0±0.7	4.4±0.7	0.958
PMA + LPS	5.4±0.7	3.9±0.6	4.2±0.6	3.7±0.6	0.254

[1] CON = control, without β-glucan addition; B-Yeast = CON added of 0.115% of β-glucan from *Saccharomyces cerevisiae*; B-S15 = CON added of 0.155% of β-glucan from *Euglena gracilis*; B-S31 = CON added of 0.310% of β-glucan from *Euglena gracilis.*
[2, 3] LPS – lipopolysaccharide; PMA – phorbol myristate acetate.
a, b = means in a row without a common superscript letter differ (P<0.05).

Table 8. Lipopolysaccharide stimulated Immunoglobulin G concentration in cell culture (ug/mL) and immunoglobulin A (mg/g of feces dry matter) concentration on the feces of dogs fed diets with the addition of different sources and amounts of β-glucans (mean and standard error of the mean).

	Diets[1]				
Item	CON	B-Yeast	B-S15	B-S31	*p-value*
IgG	17.0±2.4	13.6±2.8	13.4±2.5	12.8±2.3	0.551
IgA	12.0±4.3	17.3±4.6	14.2±3.9	13.6±4.4	0.897

[1] CON = control, without β-glucan addition; B-Yeast = CON added of 0.115% of β-glucan from *Saccharomyces cerevisiae*; B-S15 = CON added of 0.155% of β-glucan from *Euglena gracilis*; B-S31 = CON added of 0.310% of β-glucan from *Euglena gracilis.*

5. Discussion

β-glucans are considered immune response modulators and have been used as food additives in prophylactic management in immune and inflammatory responses in different species (Vetvicka et al., 2014; Yamamoto et al., 2018). In the current study it was possible to confirm that some inflammatory and immune parameters evaluated in dogs were affected by the β-glucans intake, but the induced responses differed between the studied sources. It has been reported that differences in the shape,

molecular weight, degree of branching, solubility, and structure of the molecule, as well as the process of extraction determine β-glucans biological activity, which may largely differ between sources and manufactures (Sinthusamran et al., 2018; Murphy et al., 2021; Steenwijk et al., 2021).

In the present study, the *Saccharomyces cerevisiae* β-1,3/1,6-glucan intake by dogs stimulated IL-2 serum secretion, without increasing calprotectin in feces, serum CRP, nor NO, and H_2O_2 *ex vivo* production. Specially the increase in IL-2 (in line with the numerically although not statistically increase of serum IL-6) signalize activation of innate immunity, as previously reported by many studies (Li et al., 2007; Stier et al., 2014; Soltanian et al., 2009; Vetvicka; Oliveira, 2014; Oliveira et al., 2019; Rodrigues et al., 2020). The increased serum IL-2 acts regulating the growth, proliferation, and differentiation of T cells, being IL-2 one of the most important cytokines (Vetvicka; Oliveira, 2014; Oliveira et al., 2019). In addition, IL-2 may also be involved in the stimulation of Natural Killer cells and in the cytotoxicity of monocytes (Gaffen; Liu, 2004; Oliveira et al., 2019). In clinical practice, there are reports of treatments for autoimmune diseases, human immunodeficiency virus (HIV), and chemotherapy for metastatic neoplasms with direct administration of IL-2 (Glaspy, 2002; Natarajan et al., 2002; Gaffen; Liu, 2004). At this moment, no disease have been attributed to IL-2 deficiency or excess (Gaffen; Liu, 2004).

When in the diet, β-1,3/1,6-glucans reaches the small intestine intact and are absorbed by the Peyer's patches, where they are recognized by circulating antigen-presenting cells (dendritic cells or M cells) and presented to other immune cells (Reboldi et al., 2016; Murphy et al., 2021). As a fungal structure, β-1,3/1,6-glucans are recognized as pathogen-associated molecules, initiating the immune response. This

recognition is dependent on identification and binding to different cell membrane receptors (Soltanian et al., 2009; Murphy et al., 2021). The main receptor for β-1,3/1,6-glucans are dectin-1, which recognize its triple helix conformation (Falch et al., 2000), but also lactosylceramide, elimination receptors and the complement receptor 3 can be involved (Ross et al., 1987; Brown et al., 2003; Soltanian et al., 2009). They are present in monocytes, macrophages, dendritic cells, neutrophils and natural killer cells (Li; Wang, 2015; Kardani et al., 2016; Murphy et al., 2021). The association of β-1,3/1,6-glucans with these receptors activates a multiple and complex signaling cascades that induces pathogens phagocytosis, respiratory burst, microbial death, inflammatory processes and the release of chemical mediators that activate and recruit other cells (Medzhitov, 2007; Barsanti et al., 2011).

The phagocytic response of monocytes and neutrophils was significantly influenced by b-glucan consumption in different ways. The B-S15 diet increased monocyte phagocytosis and the B-S31 diet decreased neutrophil phagocytosis ($p < 0.05$), and even though it was not significant, the B-LEV diet also showed a greater response in neutrophils. Phagocytosis is the body's first line of defense when faced with an invading agent. These results demonstrate a significant action of the activity of both b-glucans, which corroborates with several findings in different species and also in dogs (Vetvicka; Oliveira, 2014; Oliveira et al., 2019).

As in the study from Prakash et al. (2013), cytokines IL-6, IL-8, IL-10 and TNF-α were evaluated in dog feces by the ELISA method, showing that it may be a useful technique as a non-invasive biomarker of inflammation in dogs, the authors in the current study tried applying the new technology MULTIPLEX with the same objective, but cheaper and more convenient. However, although the observed effect on serum

cytokines, no effect on its fecal concentration was verified. This lack of effect in this study might be explained by the different stimuli, conditions and methods used by Prakash et al. (2013). The dogs used were all health, and the β-1,3/1,6-glucans intake did not induce an inflammatory condition as calprotectin and CRP did not increase, so may be the effect on fecal cytokines was too low or absent in this situation, and only the serum cytokine concentrations were affected.

The intake of *Euglena gracilis* β-1,3-glucans, on the other hand, did not affect serum or fecal cytokines, highlighting the importance to proper characterize the method of action of the different of β-glucan molecules. These results are supported by the study of Noss et al. (2012), which compared 13 different β-glucan sources using human whole blood cell culture and verified no stimulation of cytokine production induced by *Euglena gracilis* β-1,3-glucan.

However, a significant increase in *ex vivo* NO production was verified in dogs fed diets with *Euglena gracilis* β-1,3-glucans, that was not observed for β-1,3/1,6-glucans. This effect of *Euglena gracilis* β-1,3-glucans was also observed by Bianchi et al. (2015) in Mollusca, suggesting that the activation of a signaling cascade that may enhance the defense system of the organism. The activated system, however, might be different as there was no stimulus for cytokines production, what is normally induced by β-1,3/1,6-glucans dectin-1 recognition (Noss et al., 2012; Stier et al., 2014; Rodrigues et al., 2020). This may suggests that other cell membrane receptors and mechanisms of actions could be involved in β-1,3-glucans recognition, whose molecular structure is linear and unbranched (Schley; Field, 2002; Ishibashi et al., 2019), requiring further studies.

Nitric oxide is considered an inflammatory mediator present in practically all cells in the body, and all immunological parameters can be modulated by its action (Zamora; Billiar, 2000). Under physiological conditions, NO acts as a vasodilator (Wang; Deen, 2003), anti-tumor, immunosuppressant, pro-apoptotic (Zamora; Billiar, 2000), neuromodulator and mediator of smooth muscle relaxation in the cardiovascular system and gastrointestinal tract (Wiley, 2007). Stable NO levels are essential for health, and reports are presented of NO deficits or excesses (Wang; Deen, 2003). During an inflammatory state pro-inflammatory cytokines stimulate NO production in monocytes, macrophages, and neutrophils and, thus, levels in the animal can increase up to 1,000-fold (Sharma et al., 2007; Sproston et al., 2018a). As cytokines did not increase after *Euglena gracilis* β-1,3-glucans intake, one cannot suggest the compound induced an inflammatory status, what is reinforced by the very minor increase observed in serum CRP. The *Euglena gracilis* β-1,3-glucans are much less studied than the *Saccharomyces cerevisiae* β-1,3/1,6-glucan, with few publications available. So, although a biological effect was characterized in the present study, its extension and implications to animals health still requires more studies.

In cell culture, the action of different doses of oat β-glucan (0; 0.1; 0.5; 2.0 and 4.0 mg/mL) was evaluated against the addition of a high concentration of H_2O_2 (8 μM) on the cytotoxic effect on the epithelial cells of the mice intestine and it was concluded that the low concentration of β-glucan does not prevent this toxic effect and in high doses it even potentiates the oxidative cell damage induced by H_2O_2 (Cha; Purslow, 2014). In the face of stimulation with phorbol myristate acetate (PMA), the production of hydrogen peroxide in monocytes decreased in the diets with algal β-glucan in relation to other diets, and in the face of stimulation with LPS, the production of this

metabolite was insignificant in all the diets (data not shown, <0.07 μM H_2O_2 /3x10^5 cells), presenting themselves as an advantage to the effect of these diets in this study.

Another important non-invasive marker of intestinal inflammation is calprotectin. It is a protein responsible for up to 50% of the cytosolic protein of neutrophils and is resistant to bacterial degradation of the colon (Angriman et al., 2007). Due to the fact that our animals were healthy throughout the study, we found no signs that the diets could lead to local intestinal inflammation in the investigated animals.

C-reactive protein is also within the group of proteins used in the diagnosis of inflammation, being a positive acute phase inflammatory protein in dogs (Angriman et al., 2007). It is a sensitive test for the detection of inflammation in clinical practice. An increase was detected in dogs at the infectious diseases (Matijatko et al., 2007), immune-mediated (Bathen-Noethen et al., 2008) or neoplasms (Nielsen et al., 2007). Upper limits between 10 and 20mg/dL have already been described in the literature for dogs (Eckersall et al., 1989; Mylonakis et al., 2011; Muñoz-Prieto et al., 2017), thus, our results were well below the reference range of healthy dogs. However, a significant increase was observed in the diets with algae β-glucans ($p<0.05$) in relation to the control diet and the yeast diet.

There is evidence that CRP is an important regulator of inflammatory processes and not just a marker of inflammation and infection (Sproston et al., 2018b). CRP is related to nitric oxide (NO), which is a short-lived free radical regulator of several biological functions, including vasodilation, neurotransmission, inflammation, and macrophage-mediated immunity (Weiming et al., 2002; Sproston et al., 2018a). This protein exists in two forms that will have opposite biological activities, native PCR and monomeric PCR. In the absence of infection, literature data indicate that both forms

increase the production of NO by monocytes in order to regulate systemic inflammatory processes (Ikeda et al., 2002; Hattori et al., 2003; Sproston et al., 2018a), which may partially explain what was observed in our study.

Also in view of this whole scenario, it was possible to observe that the organism did not identify β-glucan as a pathogen, because in *in vivo* responses without stimuli, CRP was still within safe limits and without changes in fecal cytokines, calprotectin and immunoglobulin A.

For the action of these sources of β-glucans also in adaptive immunity, or memory immunity, we evaluated the production of IgG in lymphocytes cell culture with LPS stimulation for systemic immunity and IgA for local intestinal immunity. No significant effect was found on these defense cells, despite numerically increasing fecal mucosal immunoglobulin concentrations. These results are in agreement with the study by Stuyven et al. (2010) that evaluated the effect of oral supplementation with yeast β-glucan on the antibody response in healthy dogs against Pneumodog® vaccine, on serum IgM and IgG and IgA concentrations in serum, saliva and tears. Another study carried out in healthy dogs presented IgA results close to what this study found in diets with beet pulp as a source of fermentable fiber in elderly dogs and in the diet with soy inclusion as a source of dietary fiber in adult and elderly dogs (Maria et al., 2017).

Some limitations of the present study need to be considered. Only healthy dogs were used, in analysis *ex vivo* the results were significative, but in *in vivo* responses maybe the stimulus was needed for the better responses.

6. Conclusion

Both the β-glucans from yeast and seaweed were able to develop effects in modulating the innate and inflammatory immune system. The *Saccharomyces cerevesiae* β-glucan diet showed cell-mediated activation of the innate immune system with the increase of pro-inflammatory cytokines and the *Euglena gracilis* diets acted on the humoral innate immune system by increasing the production of nitric oxide and acute phase reactant protein, CRP. However, different paths in the recognition of these molecules were observed and further research will be important for a deeper understanding of the action of these sources.

7. References

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CAPÍTULO 3 - What is beyond immunomodulation in yeast or seaweed β-glucans intake in dogs?

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1- Escrito nas Normas da revista Journal of Animal Physiology and Animal Nutrition.

1. Abstract

As β-glucan is a functional and bioactive ingredient capable of reducing the risk of several chronic diseases with increased daily intake, there is still not enough understanding of the mechanism of action of all types of β-glucans in foods for health purposes. Based on these considerations, the objective of the present study was to evaluate and compare the effects of including insoluble β-glucans from the seaweed Euglena gracilis and β-glucans obtained from the yeast Sacchamoryces cerevisae on the apparent digestibility of nutrients, fermentation products and composition microbial activity, lipemic profile and postprandial insulin and glucose curve in healthy adult dogs fed extruded rations. One food formulation was used, with the following treatments: CON – control, without β-glucan addition; B-Yeast – 0.115% of β-glucan from yeast cell wall; B-S15 – 0.155% of β-glucan from seaweed; B-S31 – 0.310% of β-glucan from seaweed (targeting the intake of 15mg (B-Yeast and BS-15) and 30mg (B-S31) of β-glucan/kg/day). Thirty-two beagle dogs were used, 8 dogs per food, distributed in a block design. Blocks lasted 84 days, 42 d of washout and 42 d receiving the experimental diets. Before start and at the end of the experimental diet intake faecal microbiota (250 bp Paired end (PE) Illumina sequencing) was evaluated. After 15 d of diet adaptation, nutrient digestibility was determined, and in the sequence the postprandial insulin and glucose response. At the end of the experimental period faeces collected to evaluate fermentation products and blood for lipemic parameters. Data were subjected to analysis of variance and means compared by Tukey's test. For postprandial glucose and insulin responses, repeated-measures analysis of variance was used. For faecal microbiota, beta diversity was analyzed using the relative abundances of bacterial genera (Aitchison distance) ($P<0.05$, and $P<0.1$ as trend).

Dogs fed BS15 showed higher apparent digestibility of dry matter, organic matter, and gross energy ($p<0.05$). Dogs fed BYeast tended to have lower mean glucose and shorter time to peak insulin response ($p<0.1$). The beta diversity of the fecal microbiota of dogs on the BS31 diet differed from the BYeast and BS15 diets. It was concluded that yeast β-glucan tended to decrease mean glucose concentration and anticipate the peak of endogenous insulin action. The BS31 diet showed greater ability to change the fecal microbiota.

2. Introduction

In the late 1990s, some β-glucans were shown to have prebiotic potential, selectively promoting the growth of beneficial intestinal bacteria such as lactobacilli and bifidobacteria, according to some in vitro studies (Jaskari et al., 1998; Kontula, et al., 1998) and animal experiments (Dongowski et al., 2002; Drzikova et al., 2005; Snart et al., 2006).

In a study carried out by Lam & Cheung (2013), β-glucans obtained from barley, seaweed, bacteria and mushroom sclerotia were incubated with pure cultures of Bifidobacterium infantile, Bifidobacterium longum and Bifidobacterium adolescentis in 24-hour fermentation, in order to evaluate its bifidogenic effect and inulin was used as a positive control. Among the results, it was observed that the action of all β-glucans, regardless of their differences in glycosidic bonds and molecular weight, by all three bifidobacteria, was comparable to that of inulin (Zhao & Cheung, 2011).

Diet is known to influence bacterial composition in the gastrointestinal tract and, additionally, the beneficial effects of bacterial fermentation are also associated with changes in colonic short-chain fatty acids (SCFA). There is strong evidence indicating

that consumption of prebiotic fibers (inulin and oligofructose) increases the proportion of beneficial lactic acid bacteria in the human colon (Gibson et al. 1995; Buddington et al. 1996; Menne et al. 2000) and has been shown to that the fermentability of oat β-glucan is capable of producing SCFA for its potential application as a prebiotic to promote health in humans (Lin et al., 2011).

β-glucan is a functional and bioactive ingredient capable of reducing the risk of several chronic diseases with increased daily intake. Although these claims have been accepted, there is still not enough understanding of the mechanism of action of all types of β-glucans in foods for health purposes (Zielke et al., 2017).

Another mechanism suggested when discussing health benefits is the ability of β-glucan to form viscous gels in the intestine (Papageorgiou et al., 2005; Skendi et al., 2003), which results in delayed gastric emptying, decreasing the enzymatic degradation of starch and hindering the intestinal absorption of digestible carbohydrates (Cummings; Stephen, 2007; Novak; Vetvicka, 2008). This could reduce the rate of absorption of macronutrients (Wang; Ellis, 2014) and result in improved glycemic control and postprandial insulin secretion, since the greater the viscosity of the layer, the lower the levels of glucose and insulin in the blood plasma. (Regand et al., 2009; Liu et al., 2016; Wolever et al., 2020).

In addition to viscosity, molecular weight is also of great importance in the actions of β-glucans. Aoe et al. (2021) compared β-glucans of different molecular weights. According to the authors, high molecular weight β-glucans can inhibit fat absorption, reduce abdominal fat deposition, attenuate the glycemic response and lipid absorption, while low molecular weight β-glucans have high fermentability, which can affect the glycemic response and lipid metabolism by prebiotic actions, such as SCFA

production and improvement of the intestinal microbiota composition. Interestingly, β-glucan with different structural characteristics have beneficial effects resulting from different physiological processes.

Based on these considerations, the objective of the present study was to evaluate and compare the effects of including insoluble β-glucans from the seaweed *Euglena gracilis* and β-glucans obtained from the yeast *Sacchamoryces cerevisae* on the apparent digestibility of nutrients, fermentation products and composition microbial activity, lipemic profile and postprandial insulin and glucose curve in healthy adult dogs fed extruded rations.

3. Experimental methods

The study was conducted in the Laboratory of Research in Nutrition and Nutritional Diseases of Dogs and Cats “Prof. Dr. Flávio Prada”, 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° 001974/20).

Animals

Thirty-two adult Beagle dogs, males and females, with 2.9±2.3 years of age and weighing 10.7±1.4 kg was used. For these animals the mean body condition score was 5 on 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 four experimental diets and was conducted in a randomized block design with two blocks of 16 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 84 days, with a 42 day washout and 42 day treatment period. Before the treatment period, fresh faeces were collected for microbiota, and at the end of experimental period, fresh faeces were collected again for microbiota and fermentation products. After 15 days of consumption of the experimental diets, total faeces collection for digestibility measurement was performed, and after that, blood was collected for postprandial insulin and glucose curve.

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). Food was provided once a day (at three pm). Offered food was weighed, no refused diet was identified, and the intake was recorded. Dogs were then weighed weekly, and the amount of food provided adjusted such that animals maintained a constant body weight throughout the study. Water was provided *ad libitum.* During the study dogs were housed in kennels measuring 1.5 m x 3.5 m with a solarium and released daily in a collective playground for exercise and socialization.

Experimental Diets

A single formulation based on corn grain, poultry byproduct 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, 2020). The experimental diets will be obtained by the inclusion of β-glucans to the formulation. The doses were based on the study by Oliveira et al. (2019), who suggest a dose of 15 mg β-glucan per kg of body weight per day as an effective dose. The raw material source of β-glucan from yeast has 78.4% of beta-1,3/1,6-glucan and that of seaweed has 58.5% of beta-1,3-glucan. Based on the expected consumption of the test foods, and on the analyzed concentrations of actives in the products, they were included in the following proportions (on an as-fed basis): CON - control diet, without inclusion of β-glucans; B-Yeast - inclusion of 0.115% of beta glucan from yeast cell wall; B-S15 - inclusion of 0.155% of beta glucan from seaweed; B-S31 - inclusion of 0.310% of beta glucan from seaweed.

Table 9. Ingredient and Chemical composition of the food used on the study.

Ingredients	%
Corn grain	51.6
Poultry by-product meal	32.2
Poultry fat	9.2
Liquid palatant [1]	3.0
Sugarcane fiber [2]	2.0
Vitamin-mineral premix [3]	0.6
Salt	0.5
Potassium chloride	0.5
Choline chloride	0.3
Mold inhibitor [4]	0.1
Antioxidant [5]	0.05
L-lysine	0.03
Analyzed chemical composition	%, as fed basis
Dry matter	92.8
Crude protein	28.8
Ash	5.7

Acid-hydrolyzed fat	17.6
Crude fiber	2.2
Calcium	1.0
Phosporus	0.9

[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.

Dietary formulations 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 four experimental diets. Ingredients were weighed and mixed before being ground in a hammer mill (Tigre, Moinhos Tigre, São Paulo, SP), fitted with a 0.8 mm sieve screen size, and extruded in a single-screw extruder (Model Mex-250, Manzoni Industria Ltda, Campinas, SP), with an average extrusion capacity of 300 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 min and coated with poultry fat and liquid palatant.

Digestibility protocol, feces production and characteristics

This evaluation followed recommendations and procedures previously described (FEDIAF, 2020). Dogs were individually housed for 5 days in stainless steel metabolic cages, and each contained an 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 120 h, weighed,

and stored frozen at -15℃ until analysis. After the end of the collection period, feces were thawed to room temperature and homogenized, compounding a single sample per dog, and then they were dried in a forced-air oven (320-SE, FANEM, São Paulo, Brazil) at 55℃ for 72 hours. Predried feces and diets were ground in a knife type mill (MOD 340, ART LAB, São Paulo, Brazil) with a 1 mm sieve 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 was measured by muffle furnace incineration (method 942.05), crude protein was estimated 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 analyses were repeated when the variation between replicates was greater than 5%.

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, at the end of each block, fecal samples were collected immediately after elimination for three consecutive days. Fecal pH was determined for 2 g of fresh feces mixed with 6 mL of ultrapure water, using a pH meter (model DM20;

Digicrom Analitica LTDA, São Paulo, Brazil). Approximately 10 g of fresh feces was homogenized and mixed with 30 mL of a 16% (vol/vol) formic acid solution and precipitated to determine the volatile fatty acids (VFA). Next, the mixture was centrifuged (5810R; Eppendorf, Hamburg, Germany) 3 times at 4,500 x g for 15 min at 4℃. The supernatant was retained, 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℃ 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 for 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 of each extract 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).

To determine concentrations of biogenic amines in feces, 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 4ºC (5810R; Eppendorf, Hamburg, Germany) (Vale; Gloria, 1997). The supernatant was filtered with 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 were determined in the supernatant by HPLC (HPLC model LC-10AD; Shimadzu Corporation, Kyoto, Japan).

Faecal microbiota evaluation

For microbiota analysis, fresh stool samples were collected during three consecutive days and immediately frozen in liquid nitrogen and stored in a -80oC freezer for later analysis.

The commercial kit “ZR Fecal DNA MiniPrep®” from Zymo Research was used to extract DNA from the samples following the protocol recommended by the manufacturer. The extracted DNA was quantified by spectrophotometry at 260nm. To assess the integrity of the extracted DNA, all samples were run by electrophoresis on a 1% agarose gel.

A segment of approximately 460 bases of the V3V4 hypervariable region of the ribosomal 16S rRNA gene was amplified using the universal primers described by the methodology. From these amplifications, a metagenomic library was built using the commercial kit “Nextera DNA Library Preparation Kit” by Illumina®. Raw Illumina amplicon sequences were processed, and quality filtered using the Divisive Amplicon Denoising Algorithm (DADA) tool implemented in R (v.3.6.2). Briefly, the reads were filtered for quality, allowing for two and three errors per forward and reverse reading, respectively. As the reverse reads were of poor quality, we moved forward with the pipeline using only forward reads. After calculating error rates, Amplicon Sequence Variants (ASVs) were inferred from direct reads using DADA2's core noise reduction algorithm. After that, the chimeric sequences were removed and the resulting ASVs

were given a taxonomy using the SILVA rRNA gene reference database (v138), and those classified as eukaryotic, chloroplast or mitochondrial were removed from the dataset.

Assessment of postprandial glucose and insulin response

After 20 days of consumption of the treatment diets, the animals were submitted to the determination of postprandial glucose and insulin responses according to Carciofi et al. (2008), with changes in blood collection times: 0 (zero, before meals), 15, 30, 60, 120, 180, 240, 300, 420, 540 and 720 min. On the day of blood collection, after shaving and antisepsis, the cephalic vein was cannulated with an intravenous catheter (Angiocath 20GA x 1.16in., Bekton Dickinson, USA). At time zero, a blood sample was collected to determine glycemia and basal insulinemia. The experimental diet was offered and must be consumed within 15 minutes. Dogs that did not consume at least 90% of the stipulated amount in this period were not evaluated, and the test was repeated the next day. After the consumption of food, the counting of collection times began. All glycemic curves were started in the morning, at the same time.

In each collection, 3.0 mL of blood was taken from each animal and separated into two tubes, one mL in a glucose tube containing 0.05 mL of sodium fluoride anticoagulant (Labtest Diagnóstica S.A., Lagoa Santa, Brazil), centrifuged at 1,300G for 5 min and the plasma separated into two polypropylene tubes and two mL were collected for insulin dosage in a tube without anticoagulant, centrifuged at 1,300 G for 15 min and the serum separated. At the end of each collection, the catheters were washed with saline solution to maintain patency.

Samples for glucose and insulin analysis were frozen at -80 ºC for later analysis

(Reynolds et al., 2006). Plasma glucose concentrations were determined by glucose oxidase tests (GOD-ANA, Labtest Diagnóstica S.A., Lagoa Santa, Brazil), using a semi-automatic glucose analyzer (Labquest model BIO-2000, Labtest Diagnóstica S.A., Lagoa Santa, Brazil). The Quantikine ELISA kit (Human/Canine/Porcine Insulin Immunoassay, R&D Systems, Minneapolis, USA) were used to determine the serum insulin concentration, following the manufacturer's recommendations. The reading was performed in a microplate reader (Biochrom Asys Expert Plus, Biochrom, Cambourne, Cambridge, United Kingdom) using a 450 nm filter.

Serum Lipemic Parameters

Three mL of blood were collected by jugular venipuncture after 30 days of consumption experimental diets. Afterwards, the samples were centrifuged to obtain serum and frozen in a -80 ^{o}C freezer for further analysis. The analysis was performed by spectrophotometry (Spectrophotometer Quick-Lab; Drake Eletronica e Comércio, São José do Rio Preto, São Paulo, Brazil) according to the manufacturer's recommendations.

Statistical Analysis

All variables were previously tested for normality or errors using the Cramer-von Misses test and for homoscedasticity using the Levene test. When necessary, logarithmic transformation (log x + 1) or lambda transformation was applied. Data obtained for nutrient digestibility, fecal parameters, fermentation products and lipemic parameters were submitted to analysis of variance and, when significant, compared by Tukey's test ($P<0.05$). For postprandial glucose and insulin responses, analysis of variance for repeated measures over time was used. For fecal microbiota, 16S rRNA

gene sequencing was performed. Beta diversity was analyzed using relative abundances of bacterial genera (Aitchison distance). Values of $P<0.05$ were considered significant, and $P<0.10$ as a trend. The analysis was conducted using the computer program R (version 3.3.3).

For microbiota statistical analyses, alpha-diversity richness and evenness was estimated from bacterial Genus-level abundances in R, using the phyloseq package. Differences in microbiota alpha-diversity between the diet treatments were tested with generalized linear models (and Tukey contrasts) and set alpha=0.05. For beta-diversity analysis, Aitchison distances were constructed from genus-abundances that were applied a Center Log Ratio transformation. PERMANOVA tests from the vegan package tested whether the gut microbiota of dogs varied with diet treatment at timepoint 0 or 30. These were followed up by Post-hoc comparisons using the pairwise.adonis function from the vegan package.

4. Results

Both β-glucans were well tolerated and safe for healthy adult dogs during 42 days of consumption at the concentrations used, as there were no changes in the clinical condition of the animals during the trial period.

Table 10. Body weight (kg), nutrient intake (g/dog/day) and coefficient of total tract apparent digestibility (%) of nutrients od diets of dogs fed diets with the addition of different sources and amounts of β-glucans (mean and standard error of the mean).

	Diets[1]				
Item	CON	B-Yeast	B-S15	B-S31	*p-value*

Body weight (kg)	10.5±0.8	11.1±1.5	10.5±1.2	10.3±0.9	0.507
Nutrient intake (g/dog/day)					
Dry matter	142.6±8.9	150.0±16.7	140.0±17.8	146.5±21.5	0.668
Coefficient of total tract apparent digestibility (%)					
Gross energy	86.1^{b}±1.7	86.9^{ab}±1.4	88.6^{a}±1.5	85.9^{b}±1.5	0.013
Fat	93.1±1.5	93.5±1.4	94.5±1.1	94.0±0.8	0.181
Organic matter	85.3^{ab}±1.8	86.3^{ab}±1.5	87.7^{a}±1.6	85.1^{b}±1.5	0.024
Dry matter	82.6^{ab}±2.2	83.5^{ab}±2.0	85.3^{a}±1.9	82.2^{b}±1.8	0.024
Crude protein	86.6±1.4	88.1±1.4	88.3±1.8	86.4±1.9	0.076

[1]CON = control, without β-glucan addition; B-Yeast = 0.115% of β-glucan from *Saccharomyces cerevisiae*; B-S15 = 0.155% of β-glucan from E*uglena gracilis*; B-S31 = 0.310% of β-glucan from E*uglena gracilis.*
a, b = means in a row without a common superscript letter differ (P<0.05).

Table 11 - Feces production and characteristics of dogs fed diets with the addition of different sources and amounts of β-glucans (mean and standard error of the mean).

	Diets[1]				
Feces	CON	B-Yeast	B-S15	B-S31	*p-value*
g/dog/day (as is)	67.8±17.4	62.2±14.0	53.7±17.2	65.4±19.8	0.408
g/dog/day (dry matter basis)	26.8±7.1	25.0±5.6	22.0±5.9	26.3±6.2	0.414
Dry matter (%)	39.5±1.5	40.3±2.2	41.4±2.9	40.9±3.6	0.486
Score	3.5±0.5	3.7±0.4	3.6±0.4	3.6±0.5	0.313
pH	6.3±0.2	6.2±0.1	6.2±0.2	6.3±0.2	0.484

[1]CON = control, without β-glucan addition; B-Yeast = 0.115% of β-glucan from *Saccharomyces cerevisiae*; B-S15 = 0.155% of β-glucan from E*uglena gracilis*; B-S31 = 0.310% of β-glucan from E*uglena gracilis.*

Table 12. Volatile fatty acids (mMol/g of dry matter), ammonia and lactic acid concentration (mMol/kg of fry matter) on feces of dogs fed diets with the addition of different sources and amounts of β-glucans (mean and standard error of the mean).

	Diets[1]

Feces	CON	B-Yeast	B-S15	B-S31	*p-value*
Acetic	280.1±67.4	283.6±87.3	279.1±55.2	312.9±40.5	0.460
Propionic	149.8±48.1	148.8±62.5	162.3±37.8	174.8±27.7	0.479
Butyric	43.0±17.0	41.3±20.7	34.6±8.6	38.1±13.5	0.745
Isobutyric	7.8±1.7	7.9±2.2	7.7±1.2	8.0±1.5	0.968
Isovaleric	3.1±0.3	3.1±0.5	3.1±0.2	3.1±0.4	0.988
Valeric	13.7±3.0	13.2±4.0	13.1±1.7	13.3±2.2	0.945
Total SCFA	472.9±125.8	473.6±166.6	476.1±96.0	525.7±80.2	0.713
Total BCFA	24.6±4.8	24.2±6.5	23.9±3.1	24.5±3.9	0.968
VFA	497.5±129.8	497.9±172.4	499.9±98.8	550.2±82.3	0.618
Ammonia	192.4±41.2	186.8±41.3	146.8±50.9	162.2±36.4	0.115
Lactate	3.3±0.5	3.5±0.5	3.3±0.4	3.1±0.4	0.572

[1]CON = control, without β-glucan addition; B-Yeast = 0.115% of β-glucan from *Saccharomyces cerevisiae*; B-S15 = 0.155% of β-glucan from E*uglena gracilis*; B-S31 = 0.310% of β-glucan from E*uglena gracilis.*

Table 13. Basal, mean and maximum glucose and time to glucose postprandial peak of dogs fed diets with the addition of different sources and amounts of β-glucans (mean and standard error of the mean).

	Diets[1]				
	CON	B-Yeast	B-S15	B-S31	*p-value*
Basal glucose (mg/dL)	80.8±5.7	79.5±5.4	79.2±4.8	79.6±6.4	0.947
Mean glucose (mg/dL)	86.7±0.9	84.8±0.9	86.6±1.1	86.7±0.9	0.062
Minimum glucose (mg/dL)	75.9±5.5	76.0±5.5	73.8±4.1	76.5±4.9	0.688
Maximum glucose (mg/dL)	99.0±4.1	96.0±7.4	101.5±4.1	100.3±5.5	0.261
Time to glucose peak (min)	277.5±78.1	247.5±113.1	265.7±97.1	270.0±64.1	0.905
Mean incremental concentration	6.3±1.0	5.3±0.8	7.5±1.1	7.1±1.0	0.055
Peak incremental concentration	17.7±6.2	16.6±5.3	22.5±6.5	22.3±6.1	0.148

[1]CON = control, without β-glucan addition; B-Yeast = 0.115% of β-glucan from *Saccharomyces cerevisiae*; B-S15 = 0.155% of β-glucan from E*uglena gracilis*; B-S31 = 0.310% of β-glucan from E*uglena gracilis.*

[a, b] = means in a row without a common superscript letter differ (P<0.05).

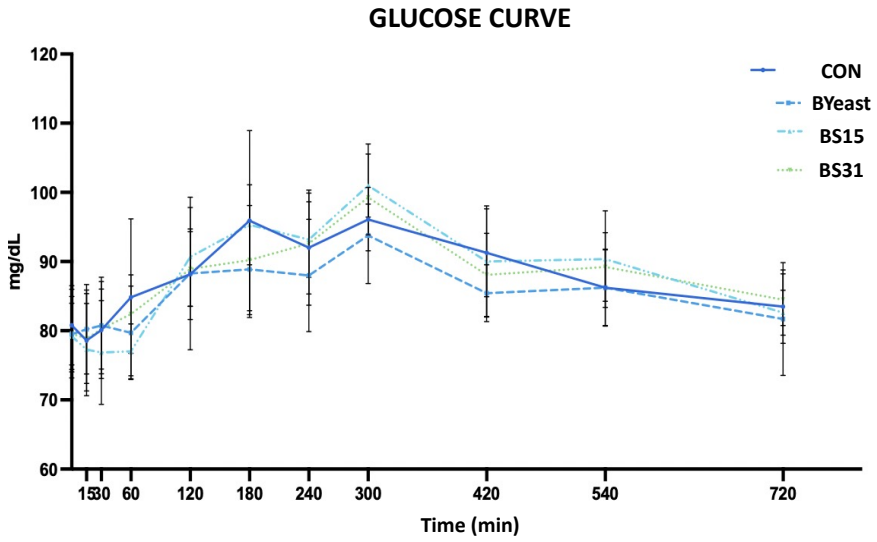

Figure 1: Average postprandial glucose curve of dogs fed diets with the addition of different sources and amounts of β-glucans.

Table 14. Basal, mean and maximum insulin and time to insulin postprandial peak of dogs fed diets with the addition of different sources and amounts of β-glucans (mean and standard error of the mean).

	Diets[1]				
	CON	B-Yeast	B-S15	B-S31	*p-value*
Basal insulin (pmol/L)	41.2±14.7	36.9±11.1	37.2±15.6	35.7±19.8	0.719
Mean insulin (pmol/L)	96.0±7.6	84.2±6.9	88.7±8.9	80.5±8.5	0.255
Minimum insulin (pmol/L)	19.7±4.5	20.9±10.9	17.0±6.7	21.6±11.1	0.646
Maximum insulin (pmol/L)	222.8±50.5	206.8±90.2	216.6±113.1	210.5±124.3	0.989
Time to insulin peak (min)	210.0^{bc}±64.1	154.3^{c}±32.0	270.0^{ab}±55.5	330.0^{a}±78.6	<0.001
Mean incremental concentration	54.9±21.2	50.7±33.6	51.5±30.0	44.8±39.7	0.668
Peak incremental concentration	181.6±48.5	169.9±91.8	179.4±112.5	174.8±119.4	0.995

[1]CON = control, without β-glucan addition; B-Yeast = 0.115% of β-glucan from *Saccharomyces cerevisiae*; B-S15 = 0.155% of β-glucan from E*uglena gracilis*; B-S31 = 0.310% of β-glucan from E*uglena gracilis.*

$^{a, b}$ = means in a row without a common superscript letter differ (P<0.05).

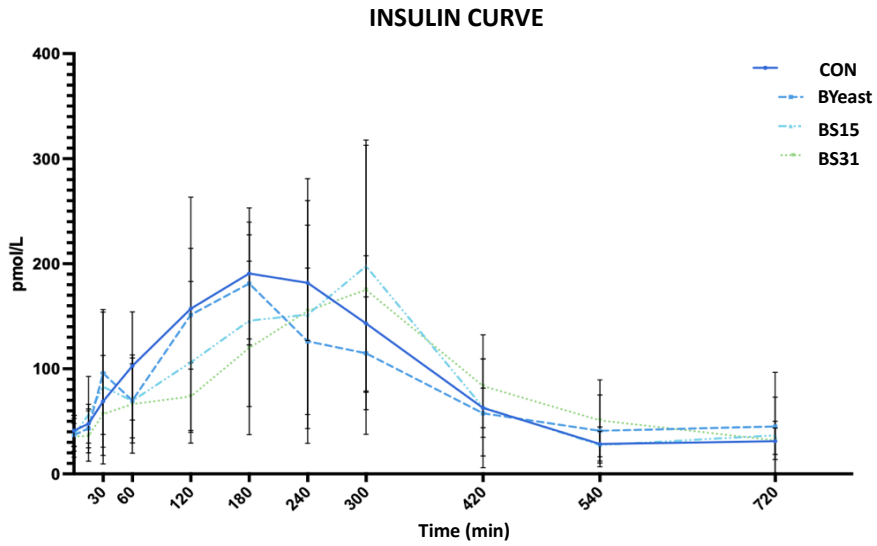

Figure 2: Average postprandial insulin curve of dogs fed diets with the addition of different sources and amounts of β-glucans.

Table 15. Postprandial area under the curve of glucose (mg/dL/hour) and insulin (pmol/L/hour) of dogs fed diets with the addition of different sources and amounts of β-glucans (mean and standard error of the mean).

	Diets[1]				
	CON	B-Yeast	B-S15	B-S31	*p-value*
			Glucose (mg/dL/hour)		
0 - 720	1,067.0±53.9	1,034.8±60.0	1,076.2±31.2	1,068.2±39.3	0.348
0 - 60	81.0±7.3	80.2±5.9	77.3±5.3	80.4±5.5	0.564
60 - 240	272.5±24.8	255.9±19.0	271.1±11.0	266.7±15.5	0.143
240 - 420	281.4±10.5	270.1±16.3	288.1±12.7	283.3±17.9	0.118
420 - 720	432.1±21.8	423.6±26.8	439.8±25.8	437.9±22.6	0.546
			Insulin (pmol/L/hour)		
0 - 720	1,108.4±224.9	1,020.8±2±419.2	885.7±231.0	874.9±292.3	0.419
0 - 60	68.7±23.0	68.9±33.5	67.3±43.9	51.4±31.7	0.688
60 - 240	490.4±122.1	430.8±194.3	362.8±223.7	304.4±194.5	0.180
240 - 420	368.5±125.2	293.0±133.3	359.4±133.7	337.0±133.3	0.524
420 - 720	180.8±38.2	179.0±81.5	187.0±82.4	259.0±145.4	0.244

[1]CON = control, without β-glucan addition; B-Yeast = 0.115% of β-glucan from *Saccharomyces cerevisiae*; B-S15 = 0.155% of β-glucan from E*uglena gracilis*; B-S31 = 0.310% of β-glucan from E*uglena gracilis.*

Table 16. Triglycerides, cholesterol, high-density lipoprotein (HDL), very-low-density lipoprotein (VLDL), low-density lipoprotein (mg/dL) of dogs fed diets with the addition of different sources and amounts of β-glucans (mean and standard error of the mean).

	Diets[1]				
	CON	B-Yeast	B-S15	B-S31	*p-value*
Triglycerides	38.2±6.9	44.5±7.2	45.4±10.1	44.8±9.7	0.265
Cholesterol	203.8±19.4	199.6±25.0	211.1±25.7	198.2±23.1	0.754
HDL	135.1±15.7	139.9±19.7	141.3±23.7	139.6±22.8	0.841
VLDL	7.6±1.4	8.9±1.4	9.1±2.0	9.0±1.9	0.264
LDL	55.9±11.5	50.9±9.9	57.7±11.8	51.3±9.8	0.522

[1]CON = control, without β-glucan addition; B-Yeast = 0.115% of β-glucan from *Saccharomyces cerevisiae*; B-S15 = 0.155% of β-glucan from E*uglena gracilis*; B-S31 = 0.310% of β-glucan from E*uglena gracilis.*

In all diets at the end of the experiment, although not significant, the predominant phylum was Firmicutes with approximately 89% of the fecal microbiota, except for the B-S15 diet which presented 71% of Firmicutes and increased Fusobacteria (20%) (Figure 3A). As for the family, a predominance of Lactobacillaceae and Peptostreptococcaceae was identified. An increase in Fusobacteriaceae on the B-S15 diet and Erysipelotrichaceae on the B-S31 diet is noteworthy, although not significant (Figure 3B). In all diets, the genus Blautia increased in relation to time 0 and in the B-S31 diet, it was also possible to observe an increase in the genus Turicibacter (Figure 3C).

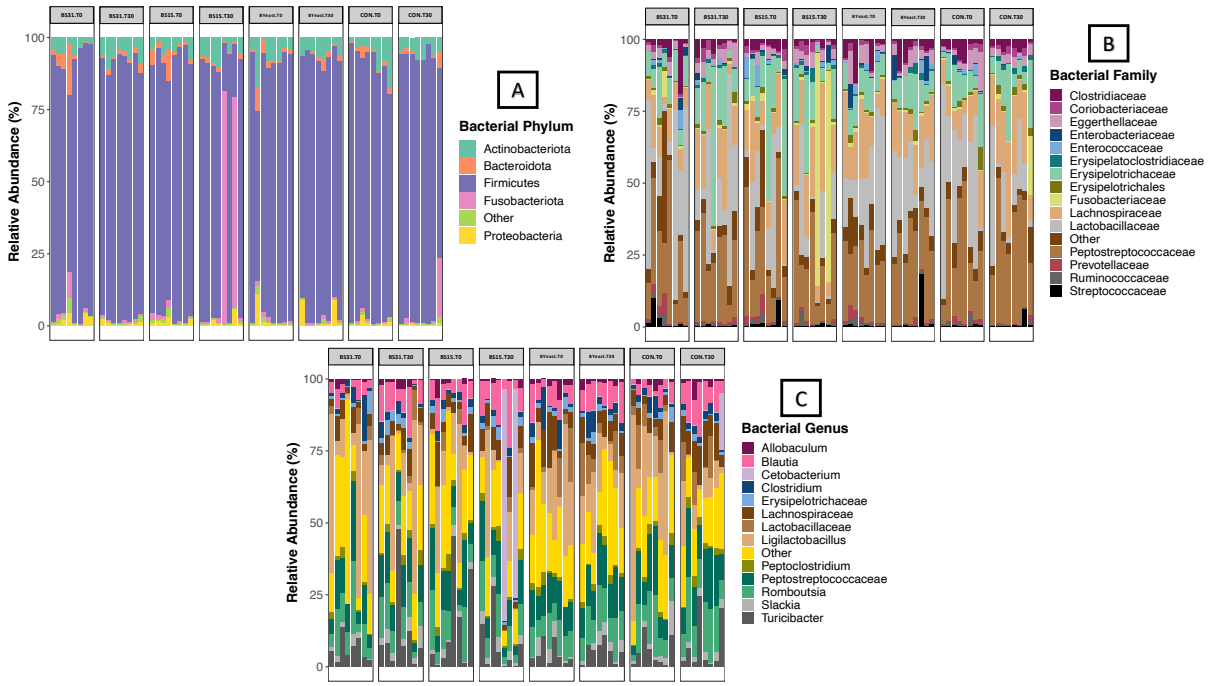

Figure 3: A - Composition of fecal microbiota phylum of dogs fed diets with the addition of different sources and amounts of β-glucans. B - Composition of fecal microbiota family of dogs fed diets with the addition of different sources and amounts of β-glucans. C - Composition of fecal microbiota genus of dogs fed diets with the addition of different sources and amounts of β-glucans.

Although statistical tests for alpha-diversity showed that the differences were not statistically significant, dogs fed the B-S31 diet increased microbiome richness on average (20.5) while dogs fed the other diets showed a decrease in microbiome richness. (B-S15: -49.6; B-Yeast: -80; CON: -71.3) (Figure 4).

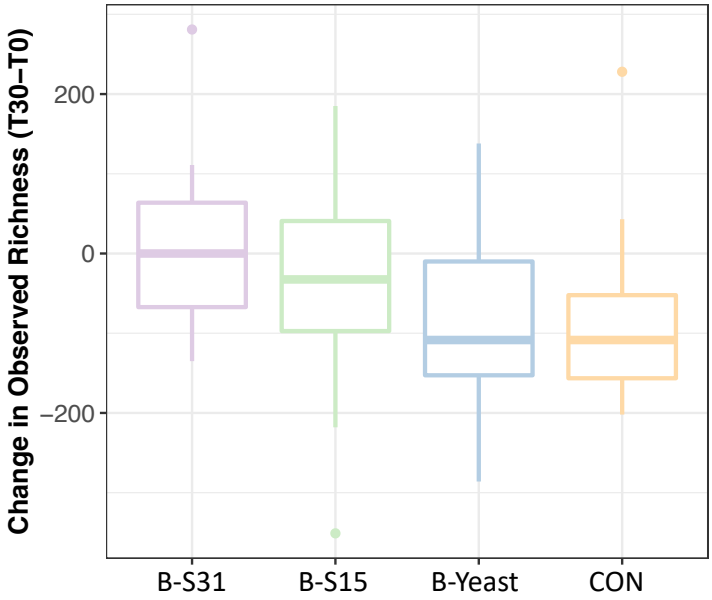

Figure 4: Mean microbiome richness at alpha diversity of dogs fed diets with the addition of different sources and amounts of β-glucans.

Beta diversity was analyzed using the relative abundances of bacterial genera (Aitchison distance). For time zero, showed no differences between the diets, however, after 30 days of consumption of the experimental diets there was a difference, with the diet responsible for 12.6% of the variation in the fecal microbiome (Aitchison distance – R2 = 0.12; P= 0.048). Specifically, the microbiota of dogs that consumed the B-S31 diet is different from dogs that consumed B-Yeast and B-S15 (p=0.044 and p=0.048, respectively).

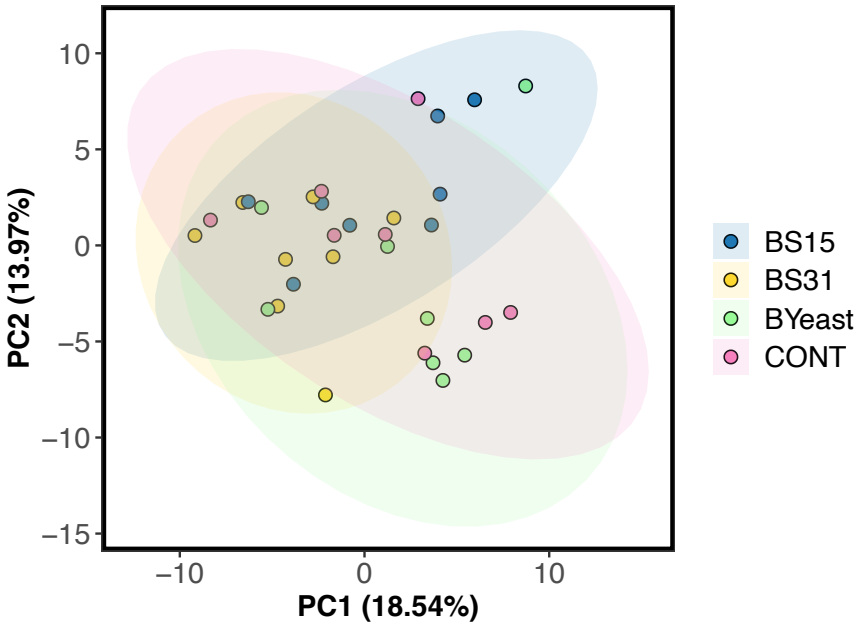

Figure 5: A principal-coordinates analysis plot showing the clustering of samples (Aitchison-distances were used) in time 30 of dogs fed diets with the addition of different sources and amounts of β-glucans.

Also evaluated whether changes in the relative abundances of 26 bacterial genera were correlated with diet. It was identified that dogs fed the control diet had a higher presence of Lachnospiraceae compared to dogs fed the yeast diet ($p<0.05$).

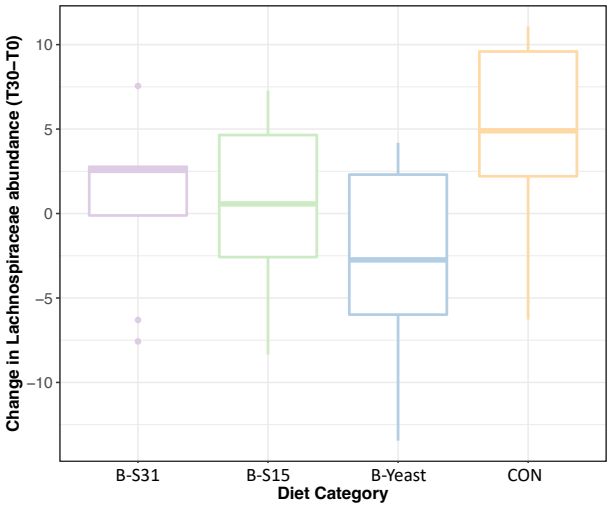

Figure 6: Relative abundance of Lachnospiraceae of dogs fed diets with the addition of different sources and amounts of β-glucans.

5. Discussion

According to Vetvicka, Vetvickova (2010), Carballo et al. (2019) and Oliveira et al. (2019) yeast β-glucans have actions mainly aimed at modulating innate immune activity in several species. Few studies have been carried out directly on the canine species in order to verify the action in the prevention of chronic diseases in healthy animals (Vetvicka; Oliveira, 2014; Ferreira et al., 2022). And even less explored are the activities of seaweed β-glucan, in which only immune effects were found in production species and fish (Yamamoto et al., 2018; Kim et al., 2019).

As already shown, β-glucans were well tolerated in the amounts given to dogs in the present study. Based on the study by Oliveira et al. (2019) stipulated a dose of 15mg β-glucans per kg of body weight per day.

Higher coefficient of apparent digestibility of gross energy, organic matter and dry matter was observed in the BS15 diet compared to the BS31 ($p<0.05$), however both are the same as the control diet. Crude *Euglena gracilis* can become a promising

nutraceutical for the petfood industry because it provides not only paramylon, which is the source of β-glucan, but also high levels of proteins, minerals, polyunsaturated fatty acids and vitamins, making it It is a highly nutritious food additive (Barsanti et al., 2000; Watanabe et al., 2013; Schwarzhans et al., 2015; Yamamoto et al., 2018).

Significant amounts of fiber in the diet can influence blood glucose levels, thereby regulating insulin secretion. In response to this effect, postprandial glucose spikes are avoided and circulating insulin is increased to lower blood glucose concentration. Thus, the intake of dietary fiber by type II diabetic patients seems to be beneficial (Meyer et al., 2002).

According to Vetvicka and Vetvickova (2007), at a dose of 100μg/mouse of yeast-derived β-glucan, no direct effects were found on blood glucose levels in healthy animals. However, in mice with experimentally induced hyperglycemia, there was a significant decrease in glucose concentration. Similar results were observed in dogs (Vetvicka; Oliveira, 2014) fed an extruded diet with two types of insoluble Saccharomyces cerevisiae β-glucans (BG1 - 15mg/kg/day - 68.5% pure and BG2 - 25mg/kg/day – 55.5% pure) for 14 days. In seven days, these dogs with induced hyperglycemia showed a reduction in blood glucose levels in both diets, which suggests that purity and the difference in technology for obtaining β-glucan did not influence the responses.

In this study, a trend towards lower mean glycemia was observed in dogs fed BYeast ($p<0.10$) and a later peak insulin response in seaweed diets, especially in higher dose ($p<0.05$). There is still much controversy about this mechanism, but one hypothesis is that these β-glucans have the ability to bind to water and form viscous gels in the gastrointestinal tract, thus causing a slower digestion and absorption of

macronutrients and slower release. of glucose (Novak, Vetvicka, 2008; Wang, Ellis, 2014; Liu et al., 2016; Wolever et al., 2020).

Along the same lines, despite the literature presenting several studies in which the ingestion of β-glucans had an effect on the concentrations of cholesterol, triglycerides, HDL, LDL and VLDL (Wolever et al., 2010; Whitehead et al., 2014; Ciecierska et al. al., 2019); most of these studies have focused on the response to the effects of (1-3)(1-4)β-glucans from oats and barley (Bai et al., 2019). Since we did not know the actions of (1,3)β-glucans in dogs, these results were evaluated, but no significant differences were observed in these parameters in any of the diets.

Also, no differences were identified between the diets regarding the production of feces by the dogs and microbial fermentation products. Insoluble fiber has been reported to be important for colonic peristalsis, and may even increase weight, capture more water, and increase stool viscosity (Praznik et al., 2015; McRorie, McKeown, 2017; Bai et al., 2019). Kuda et al. (2009) identified that β-glucan from the seaweed Euglena gracilis caused an increase in the volume of feces in rats but did not present a fermentative effect on the microbiota. In dogs, these responses were not verified, since all diets presented very close fecal volume in dry matter or natural matter.

In healthy individuals, the concentration of short-chain fatty acids (SCFA) may better reflect systemic absorption than production itself gastrointestinal (Vogt; Wolever, 2003; Raposo et al., 2016); given that fecal levels of fatty acids reflect a balance between production by the colony of bacteria and their intestinal absorption. Raposo et al. (2016) states that the opposite is also valid, after all, an increase in SCFA in feces can occur due to reduced absorption by sick individuals, as is the case of overweight

or obese patients who have other metabolic disorders and thus, a possible relationship with the lowest amount of microorganisms capable of using SCFA for their benefit.

This production of AGCC associated with the composition of the fecal microbiota can be affected by the immune status, but also by the age and the stress condition in which the patient is found, which can also influence the intestinal transit time (Fernandes et al., 2014).

The intestinal microbiota of adult dogs is considered stable in terms of composition, consisting essentially of organisms from the five main phyla Firmicutes, Fusobacteria, Bacteroidota, Proteobacteria and Actinobacteriota (Hand et al., 2013; Honneffer et al., 2017; Pilla and Suchodolski, 2020; Suchodolski et al., 2021), as observed in this study (Figure 3A). Variations between different individuals are frequent, mainly in relation to species composition and number (Garcia-Mazcorro et al., 2012; Hand et al., 2013; Pilla and Suchodolski, 2020). Even considering individual variation, it is possible to identify stability for the presence of some dominant taxa, such as the genera Peptostreptococcaceae, Romboutsia and Blautia (Figure 3C).

Alpha diversity is based on richness and uniformity, where richness is represented by the number of different species that make up a given sample and uniformity is a factor that estimates the similarity and difference between the abundances of species present in the sample. Higher levels of alpha diversity show the presence of a larger genetic repertoire, demonstrating the existence of functionally redundant organisms, whose predominance indicates greater stability of the intestinal microbiota (Moya and Ferrer, 2016). In the present study, treatments were not able to modulate alpha diversity, but affected beta diversity (Figures 4 and 5). With this result, it is understood that the treatments with the inclusion of β-glucans were not able to

modulate the richness and uniformity of the samples, but significantly affected the similarity of the samples, since there was a difference in the number of taxa present between the treatments.

Dietary macronutrients are responsible for directly interfering with the change in bacterial taxa; however, the addition of micronutrients and additives can also generate changes in the function of the microbiota and its metabolites (Pilla et al., 2021). And although the diet has the ability to change the composition of the fecal microbiota and even present differences in the products of microbial fermentation, the changes resulting from diseases that act in the digestive system are of greater magnitude (Pilla; Suchodolski, 2020).

The microbiota of dogs fed BS31 was different from that of dogs fed BYeast or BS15 (Figure 1). The BS31 diet seems to provide greater relative abundance of the Erysipelotrichaceae family, Ligilactobacillus and Turicibacter genera than in BYeast and BS15. The Erysipelotrichaceae family has been associated with high-fat diets in humans and mice (Martinez et al. 2009; Fleissner et al., 2010), while in dogs the increase in this family has been associated with consumption of an extruded diet (Bermingham et al., 2017). This is a diverse family of microbiota (Rajilic-Stojanovic & De Vos, 2014; Bermingham et al., 2017), as in cases of diseases such as inflammatory bowel disease, there are reports of microbiota similar to healthy dogs (Suchodolski et al., 2012; Guard et al., 2015) and cases of acute hemorrhagic disease, there was greater emphasis in this population on healthy animals (Suchodolski et al., 2012). Furthermore, this family has been reported to have a positive correlation with acetate, propionate, and butyrate levels (Bermingham et al., 2017). Specifically, the Turicibacter genus is more closely related to lactate production and therefore may

contribute to butyrate production. In the function of the gastrointestinal tract, these bacteria act beneficially in regulating serotonin levels in the intestine and, consequently, can influence intestinal motility and the general health of the host (Felix et al., 2022). Although in the present study there was no significant increase in these fermentation products, it was the diet with the highest SCFA production (Figure 2) numerically.

Higher relative abundance of Lachnospiraceae was found in the control diet compared to the yeast diet. In studies Isaiah et al. (2017) these same results was reported in healthy animals compared to dogs with exocrine pancreatic insufficiency. Furthermore, there are reports that Lachnospiraceae are typical producers of SCFA, being beneficial for the gastrointestinal health of dogs (Sandri et al., 2017; Gómez-Gallego et al., 2021).

At long last, is important to report that according to Bermingham et al., 2017, caution is suggested in comparisons with other species when interpreting changes in microbial composition in relation to diet.

6. Conclusion

The yeast β-glucan tended to reduce mean glycaemia and to shorter insulin secretion time, possibly modulating glucose metabolism deserving future studies. The diet with higher inclusion of seaweed β-glucan induced larger changes in faecal microbiota, with higher proportion of desirable bacteria related to intestinal health.

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