LETÍCIA DE NADAI MARCON

PRODUTO À BASE DE YACON (*Smallanthus sonchifolius*) MODULA RESPOSTA IMUNE INTESTINAL DE CAMUNDONGOS BALB/c SADIOS E INDUZIDOS À CARCINOGÊNESE COLORRETAL

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ciência da Nutrição, para obtenção do título de *Doctor Scientiae.*

VIÇOSA
MINAS GERAIS – BRASIL
2019

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T

M321p
2019

Marcon, Letícia De Nadai, 1985-
Produto à base de yacon (S*mallanthus sonchifolius*) modula resposta imune intestinal de camundongos BALB/c sadios e induzidos à carcinogênese colorretal / Letícia De Nadai Marcon. - Viçosa, MG, 2019.
xii, 166 f. : il. (algumas color.) ; 29 cm.

Texto em português e inglês
.
Inclui anexos.
Orientador: Maria do Carmo Gouveia Pelúzio.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Alimentos funcionais. 2. Câncer colorretal. 3. *Smallanthus sonchifolius*. 4. Prebióticos. 5. Ácidos graxos de cadeia curta. 6. Imunidade. 7. Célula T reguladora. I. Universidade Federal de Viçosa. Departamento de Nutrição e Saúde. Programa de Pós-Graduação em Ciência da Nutrição. II. Título.

CDD 22. ed. 613.2

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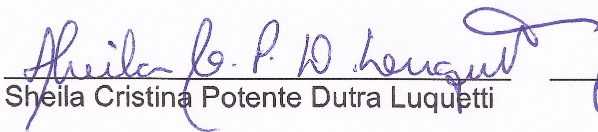
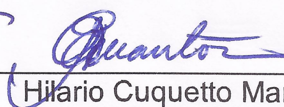
APROVADA: 29 de março de 2019.

Célia Lúcia de Luces Fortes Ferreira
(Coorientadora)

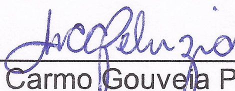
Leandro Licursi de Oliveira
(Coorientador)



Sheila Cristina Potente Dutra Luquetti



Hilário Cuquetto Mantovani



Maria do Carmo Gouveia Peluzio
(Orientadora)

“Somos todos mestres e discípulos uns dos outros”

Dedico esta tese a todos aqueles que tenham interesse genuíno na ciência e na pesquisa, e àqueles que utilizam tais conhecimentos para o bem comum.

AGRADECIMENTOS

Minha gratidão primeiramente a Deus.

À minha família: meus pais Regina Céli De Nadai Marcon e José Luiz Marcon; irmãos Brunella Marcon Fonseca e Marcelo De Nadai Marcon; cunhados Micaeli Marcon e Junior Fonseca; sobrinhas Ana Júlia e Luíza; avós Dirce Marcon e José Marcon, e Vitória De Nadai† e Constantino De Nadai †. Gratidão pelo apoio de vocês, pela presença física e espiritual na minha vida, pelo afeto e orações.

Ao Alan, pelo apoio, companheirismo e amorosidade. Gratidão por você fazer parte da minha vida.

À minha orientadora Profa Maria do Carmo Gouveia Peluzio. Gratidão pelo acolhimento, ensinamentos, contribuições científicas e por viabilizar esta e demais pesquisas sobre o câncer colorretal em nosso departamento.

À minha coorientadora Profa Célia Lúcia de Luces Fortes Ferreira. Gratidão pelos ensinamentos, contribuições científicas e pela generosidade de acolher esta pesquisa e disponibilizar o uso do PBY.

Ao meu coorientador Prof. Leandro Licursi de Oliveira. Gratidão pelos ensinamentos, contribuições científicas, auxílio nas análises imunológicas e moleculares e pela generosidade de acolher esta pesquisa.

Aos colegas de laboratório que se tornaram queridos amigos: Luiz Fernando de Souza Moraes, Bruna Cristina dos Santos Cruz, Poliana Guiomar de Almeida Brasiel, Kelly Aparecida Dias, Milena Frossard Valente, Anny Caroline Messias, Isabela Egídio Ribeiro e Rafael Rezende. Vocês tornaram nossos dias de trabalho mais leves e divertidos. Boas risadas. Saudades!

À Michelle Dias de Oliveira Teixeira pelo auxilio nas análises de biologia molecular.

Aos funcionários: Antônio de Pádua pelo suporte nas atividades do laboratório, e Solange Mara Bigonha nas análises laboratoriais.

Às amigas Ceres Mattos Della Lucia e Adriana Hocayen de Paula. Gratidão pelo companheirismo e amizade.

Aos laboratórios de: Bioquímica Nutricional, Nutrição Experimental e Análises Clínicas, do Departamento de Nutrição e Saúde (DNS/UFV); Culturas Láticas e Frutas e Hortaliças, do Departamento de Tecnologia de Alimentos (DTA/UFV); Imunoquímica e Glicobiologia, do Departamento de Biologia Geral (DBG/UFV); e ao Núcleo de Microscopia e Microanálise (NMM/UFV).

Ao Departamento de Nutrição e Saúde (DNS), ao Programa de Pós Graduação em Ciência da Nutrição (PPGCN) e à Universidade Federal de Viçosa (UFV) pela oportunidade de realizar este doutorado; e à Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela concessão da bolsa.

Àqueles que proporcionaram generosamente seu conhecimento, recursos, disponibilidade, tempo e afeto ao longo destes anos de pesquisa, visando uma produção científica mais humanizada e colaboracionista. Minha gratidão!

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LISTA DE ABREVIATURAS

AGCC	Ácidos graxos de cadeia curta
CCR	Câncer Colorretal
CDT	*Cytolethal distending toxin*
COX 2	Ciclooxigenase 2
DII	Doenças Infamatórias Intestinais
DMH	1,2-dimetilhidrazina
EGFR	Receptor do fator de crescimento epidérmico
FCA	Focos de cripta aberrante
FOS	Frutooligossacarídeos
FOXP3	*Forkhead box P3*
HDAC	Histona desacetilase
IDO	Indoleamina 2,3-dioxigenase
IFN-γ	Interferon-gama
IgA	Imunoglobulina A
IL	Interleucina
iTreg	Células T reguladoras induzidas
LPS	Lipopolissacarídeo
MHC	*Major Histocompatibility Complex*
MPO	Mieloperoxidase
NF-κB	Fator nuclear kappa B
NK	Natural Killer
nTreg	Células T reguladoras naturais
PAMP	*Pathogen-associated molecular pattern*
PBY	Produto a base de yacon
PGE2	Prostaglandina E 2
RORγt	*RAR-related orphan receptor gamma*
IgAs	Imunoglobulina A secretória
STAT3	*Signal transducer and activator of transcription 3*
TCR	Receptor de célula T
TGF-β	Fator de transformação do crescimento beta

TLR	Receptores do tipo Toll
TNF-α	Fator de necrose tumoral alfa
Treg	Células T regulatórias
VEGF	Fator de crescimento endotelial vascular

RESUMO

MARCON, Letícia De Nadai, D.Sc., Universidade Federal de Viçosa, março de 2019. **Produto à base de yacon (*Smallanthus sonchifolius*) modula resposta imune intestinal de camundongos BALB/c sadios e induzidos à carcinogênese colorretal**. Orientadora: Maria do Carmo Gouveia Peluzio. Coorientadores: Célia Lúcia de Luces Fortes Ferreira e Leandro Licursi de Oliveira.

O câncer colorretal (CCR) é o terceiro tipo de câncer mais diagnosticado no mundo. O consumo de prebióticos modula a microbiota e o sistema imune intestinal, promovendo a melhora do CCR. A yacon (*Smallanthus sonchifolius*) é uma fonte conhecida de prebióticos (frutooligossacarídeos (FOS) e inulina) e compostos fenólicos que são benéficos à microbiota intestinal e à modulação da resposta imune intestinal, porém seus mecanismos regulatórios ainda permanecem pouco claros. Nossa hipótese é que a suplementação dietética com um produto à base de yacon (PBY) poderia modular a microbiota intestinal, aumentando a concentração de ácidos graxos de cadeia curta (AGCC) fecal, e a resposta imune intestinal, modificando a população de linfócitos e os fatores de transcrição da resposta imune adaptativa no cólon de camundongos. Portanto, este estudo teve como objetivo investigar os efeitos do consumo do PBY sobre as características intestinais e fecais e sobre o perfil de resposta imune intestinal em camundongos BALB/c sadios e induzidos à carcinogênese colorretal. As lesões pré-neoplásicas foram induzidas em camundongos BALB/c machos por meio de injeção intraperitoneal de 1,2-dimetil-hidrazina (20mg / kg de peso corporal / semana) por 8 semanas. Após a indução, os animais foram divididos em dois grupos que receberam a dieta controle (AIN-93M) (grupo DC, n = 19) ou a dieta PBY (AIN-93M suplementada com PBY, 6,0% FOS + Inulina) (grupo DY, n = 17) por 8 semanas. Paralelamente, camundongos sadios (não induzidos às lesões pré-neoplásicas) foram divididos em dois grupos que receberam a dieta PBY (grupo CC, n = 16) ou a dieta Controle (grupo CY, n = 18) por 8 semanas. Em camundongos sadios, verificamos que o consumo de PBY reduziu a ingestão alimentar, melhorou a umidade e viscosidade fecal, intensificou a concentração fecal dos ácidos graxos acético, propiônico, butírico, isovalérico, valérico e capróico, aumentou o número de células T reguladoras (Treg) e regulou negativamente a

expressão do fator de transcrição RORγt no cólon. Em camundongos induzidos, a dieta PBY não foi eficaz na redução do número de focos de criptas aberrantes (FCA) no cólon, porém, promoveu redução do pH fecal, aumentou a umidade e a viscosidade das fezes, aumentou a concentração dos ácidos graxos fórmico, acético, propiônico, butírico e valérico nas fezes e modulou a expressão de fatores de transcrição no intestino, reduzindo FOXP3 e aumentando RORγt e T-bet, este último ligado ao controle e melhor prognóstico do CCR. Sugere-se que o PBY, durante os estágios iniciais da carcinogênese colorretal, possa contribuir para a integridade da mucosa, promovendo o aumento de AGCC fecal e a modulação da resposta imune no cólon, favorecendo a redução do risco de lesões neoplásicas. Além disso, pode-se inferir que o consumo de PBY por camundongos sadios possa auxiliar na regulação da saciedade, melhora do trânsito intestinal, manutenção da integridade dos tecidos intestinais e aumento da diferenciação das células Treg por meio da regulação negativa da expressão de RORγt, reduzindo consequentemente os processos inflamatórios no cólon.

ABSTRACT

MARCON, Letícia De Nadai, D.Sc., Universidade Federal de Viçosa, March, 2019. **Yacon-based product (*Smallanthus sonchifolius*) modulates intestinal immune response in BALB/c mice healthy and induced to colorectal carcinogenesis**. Adviser: Maria do Carmo Gouveia Peluzio. Co-advisers: Célia Lúcia de Luces Fortes Ferreira and Leandro Licursi de Oliveira.

Colorectal cancer (CRC) is the third most diagnosed type of cancer in the world. The consumption of prebiotics modulates the microbiota and the intestinal immune system, promoting CRC improvement. Yacon (*Smallanthus sonchifolius*) is a known source of prebiotics (fructooligosaccharides (FOS) and inulin) and phenolic compounds beneficial to gut microbiota and intestinal immune response modulation, however, its regulatory mechanisms still remain unclear. We hypothesized that dietary supplementation with a yacon-based product (PBY) could modulate gut microbiota, increasing fecal short-chain fatty acids (SCFA) concentration, and the intestinal immune response, modifying the population of lymphocytes as well as transcription factors that drive host adaptive immune in the colon of mice. Therefore, this study aimed to investigate the effects of PBY intake on intestinal and fecal characteristics and intestinal immune response profile in healthy BALB/c mice and induced to colorectal carcinogenesis. Pre-neoplastic lesions were induced in male BALB/c mice by injecting 1,2-dimethylhydrazine (20mg/kg body weight/week) intraperitoneally for 8 weeks. After induction, the animals were divided into two groups that received the control diet (AIN-93M) (DC group, n=19) or the PBY diet (AIN-93M supplemented with PBY, 6.0% FOS + Inulin) (DY group, n=17) for 8 weeks. In parallel, healthy mice (not induced to pre-neoplastic lesions) were divided into two groups that received the PBY diet (CC group, n= 16) or the Control diet (CY group, n=18) for 8 weeks. In healthy mice, we found that PBY consumption reduced food intake, improved fecal humidity and viscosity, intensified fecal concentration of acetic, propionic, butyric, isovaleric, valeric, and caproic fatty-acids, increased the number of regulatory T cells (Treg cells), and downregulated the expression of RORγt transcription factor in the colon. In induced mice, the PBY diet was not effective in reducing the number of aberrant crypt foci in the colon, however, it promoted reduction of fecal pH, increased the

humidity and the viscosity of feces, increased the concentration of formic, acetic, propionic, butyric and valeric fatty-acid and modulated the expression of transcription factors in the intestine, reducing FOXP3 and increasing RORγt and T-bet, the last linked to the control and better prognosis of CRC. It is suggested that PBY, during the early stages of colorectal carcinogenesis, may contribute to mucosal integrity by promoting fecal SCFA increase and immune response modulation in the colon, favoring risk reduction in neoplastic lesions. Furthermore, it can be inferred that PBY consumption in healthy mice may favor the regulation of satiety, improve intestinal transit, maintain the integrity of the intestinal tissues, and increase Treg cells differentiation by downregulating RORγt expression which consequently reduces inflammatory processes in the colon.

1 INTRODUÇÃO

O câncer colorretal (CCR) é o terceiro tipo de câncer mais diagnosticado no mundo (ARNOLD et al., 2017). Sua ocorrência esta associada a fatores genéticos (STOFFEL; YURGELUN, 2016; KANTH et al., 2017) e hábitos alimentares como a baixa ingestão de fibras, grãos integrais, vegetais e frutas e o alto consumo de carnes vermelhas e processadas, gordura e de bebidas alcoólicas (SCHWABE; JOBIN, 2013; VIEIRA et al., 2017; SCHWINGSHACKL et al., 2018).

Crescentes evidências sugerem a participação da microbiota intestinal na ocorrência do CCR (SCHWABE; JOBIN, 2013). Falhas nos mecanismos de barreira do sistema imune e perda de eubiose estão associadas à carcinogênese direcionada por microrganismos (SCHWABE; JOBIN, 2013). Além disso, existe uma forte associação entre o perfil de linfócitos infiltrantes do tumor à ocorrência e prognóstico do CCR (LEE et al., 2010, 2013). Linfócitos T CD8 e células *Natural Killer* (NK) são associados ao retardo e melhor prognóstico da doença (VIVIER et al., 2012; AMICARELLA et al., 2017; FOUNTZILAS et al., 2018), enquanto que linfócitos Th17 e citocinas deste perfil celular são associados ao pior prognóstico e progressão da doença (DE SIMONE et al., 2015). O papel das células T regulatórias (Treg) ainda é controverso (WOLF et al., 2015; ZHUO et al., 2015). Adicionalmente, a resposta imune do tipo Th1 tem sido associada como benéfica na regressão e melhor prognóstico do CCR (GALON et al., 2006; TOSOLINI et al., 2011).

O consumo de prebióticos contribui para o crescimento de bactérias probióticas (VANDEPUTTE et al., 2017), o que favorece a modulação do tecido linfoide associado ao intestino (MARIMAN et al., 2015) e melhora a atividade celular anti-inflamatória durante o CCR (WALIA et al., 2018). A yacon, uma raiz tuberosa de origem andina, é considerada um alimento prebiótico por ser fonte de frutooligossacarídeos (FOS) e inulina (OJANSIVU; FERREIRA; SALMINEN, 2011). O consumo de yacon é capaz de modular a microbiota intestinal aumentando o número de bifidobactérias e lactobacilos (UTAMI et al., 2013; DE SOUZA LIMA SANT’ANNA et al., 2015), diretamente associados à inibição do crescimento de bactérias patogênicas (VEIGA et al., 2014; SANT’ANNA et al.,

2018). A yacon também contribui aumentando a concentração de ácidos graxos de cadeia curta (AGCC) nas fezes (UTAMI et al., 2013; VAZ-TOSTES et al., 2014; GRANCIERI et al., 2017; SANT'ANNA et al., 2018), melhorando o transito intestinal (Sant'Anna et al., 2015; Geyer, Manrique, Degen, & Beglinger, 2008; Sant'Anna et al., 2018), a saciedade, a perda de peso (GENTA et al., 2009; GOMES DA SILVA et al., 2017), a redução da glicemia (SCHEID et al., 2014; HONORÉ et al., 2018), triglicerídeos e colesterol (HABIB et al., 2011, 2015; ROSELINO et al., 2012) e inibindo a adipogênese (HONORÉ et al., 2018). Além disso, o consumo de yacon apresenta benefícios relacionados à modulação da resposta imune inflamatória, como o aumento da produção de imunoglobulina A secretória (IgAs), interleucinas (IL)-10 e IL-4 séricas (BONET et al., 2010; VAZ-TOSTES et al., 2014; GRANCIERI et al., 2016), atividade antioxidante *in vivo* (HABIB et al., 2015), e aumento da capacidade de fagocitose de macrófagos (PAREDES et al., 2018). Além disso, tem sido relatado que o consumo de yacon promove a regressão de lesões pré-neoplásicas em modelo experimental de carcinogênse colorretal (DE MOURA et al., 2012; GRANCIERI et al., 2017).

Embora os benefícios da yacon estejam diretamente relacionados à modulação da microbiota (Sant'Anna et al., 2015; Utami et al., 2013) e da resposta imune intestinal (BONET et al., 2010), ainda não se conhece o perfil de resposta imune gerado por este prebiótico nas condições de saúde e durante a carcinogênese colorretal. Assim, especulamos se o consumo de um concentrado de yacon (PBY) poderia modular a resposta imune intestinal por alterar fatores de transcrição específicos da resposta imune adaptativa e a população de linfócitos intestinais durante a saúde e carcinogênese do cólon. Dessa forma, o objetivo deste estudo foi investigar os efeitos do consumo do PBY sobre as características intestinais e fecais e sobre o perfil de resposta imune intestinal em camundongos BALB/c sadios e induzidos à carcinogênese colorretal.

2 REVISÃO BIBLIOGRÁFICA

2.1 Câncer Colorretal (CCR)

O câncer colorretal (CCR) é o terceiro tipo de câncer mais diagnosticado no mundo (ARNOLD et al., 2017), sendo considerada a mais importante causa de mortes por câncer entre homens e mulheres nas sociedades ocidentais industrializadas (ARNOLD et al., 2017; SIEGEL et al., 2017). Apesar de multifatorial e complexa, sua etiologia esta associada a fatores genéticos (STOFFEL; YURGELUN, 2016; KANTH et al., 2017) e hábitos alimentares como a baixa ingestão de fibras, grãos integrais, vegetais e frutas e o alto consumo de carnes vermelhas e processadas, gordura e de bebidas alcoólicas (SCHWABE; JOBIN, 2013; VIEIRA et al., 2017; SCHWINGSHACKL et al., 2018). A relação inversa como o alto consumo de grãos integrais, vegetais, frutas e produtos lácteos e baixas quantidades de carnes vermelhas e processadas foi associado ao menor risco para CCR (VIEIRA et al., 2017; SCHWINGSHACKL et al., 2018).

Os estágios da carcinogênese do cólon envolvem alterações bioquímicas, morfológicas, genéticas e epigenéticas da mucosa (TAKAHASHI; WAKABAYASHI, 2004). Dentre as alterações patológicas destacam-se discretas lesões microscópicas conhecidas como focos de criptas aberrantes (FCA) (GUPTA; SCHOEN, 2009; TANAKA, 2009). FCA são considerados lesões pré-neoplásicas que exibem alterações morfológicas como displasia e proliferação anormal de células da mucosa. Podem ser encontrados no cólon de roedores tratados com agentes químicos carcinogênicos (BIRD; GOOD, 2000; DE MOURA et al., 2012; GRANCIERI et al., 2017) e em pacientes com alto risco de desenvolvimento de CCR (KOWALCZYK et al., 2018). Os FCA são facilmente induzidos em roedores após administrações únicas ou múltiplas de 1,2-dimetilhidrazina (DMH) ou de seu metabolito azoximetano (TANAKA, 2009; DE MOURA et al., 2012; GRANCIERI et al., 2017). A identificação e quantificação destes focos é utilizada em estudos voltados para a avaliação de agentes que possam prevenir ou atenuar a carcinogênese do cólon (DE MOURA et al., 2012; LENOIR et al., 2016; GRANCIERI et al., 2017).

2.2 Microbiota intestinal e CCR

Crescentes evidências sugerem a participação da microbiota intestinal na ocorrência do CCR. O fato de que as doenças intestinais não são atribuídas apenas a patógenos, mas também a modificações na microbiota intestinal, é cada vez mais demonstrado na literatura (SCHWABE; JOBIN, 2013; SOMMER et al., 2017). A coexistência simbiótica entre o hospedeiro e a microbiota é dependente da manutenção dos mecanismos de barreira, tais como: epitélio de revestimento da mucosa intacto, produção de muco pelas células caliciformes (JOHANSSON et al., 2008) e sistemas de sensoriamento que detectam e eliminam bactérias invasoras. Células de Paneth, por exemplo, são capazes de regular o número e a localização de bactérias por meio da secreção de peptídeos antibacterianos como RegIIIγ e α-defensinas (SALZMAN; UNDERWOOD; BEVINS, 2007; NESTLE et al., 2009; VAISHNAVA et al., 2011). As barreiras também incluem subgrupos específicos de células imunes abrigadas no tecido linfóide associado ao intestino como os linfócitos Th17 e as células Treg (LITTMAN; RUDENSKY, 2010). A imunoglobulina A secretora (IgAs), produzida pelas células B e transexocitada através do epitélio, é depositada sobre a superfície apical dos enterócitos na mucosa intestinal e constitui um mecanismo de barreira, ligando-se a bactérias luminais e impedindo sua translocação pela barreira epitelial (PABST, 2012; LYCKE; BEMARK, 2017). O microbioma também representa uma barreira funcional (ASHIDA et al., 2012) auxiliando na renovação celular, na produção de mucina e na supressão do crescimento de patobiontes por competição.

Nesse sentido, falhas nos mecanismos de barreira e do sistema imune e perda de eubiose são associadas ao direcionamento da carcinogênese por microrganismos. A translocação bacteriana, por exemplo, é um evento relacionado à falha da função de barreira, que pode ser observada nos locais de iniciação do tumor durante a carcinogênese experimental (GRIVENNIKOV et al., 2012). Além disso, as disbioses, caracterizadas como desequilíbrios da microbiota intestinal, podem favorecer a ocorrência de doenças infamatórias intestinais (DII) e CCR. Essa condição pode ocorrer devido a modificações na dieta, resposta imune inata, inflamações e infecções, afetando a estrutura da

comunidade e a densidade populacional de microrganismos (ARTHUR et al., 2012; HOLMES et al., 2012).

Dentre os mecanismos de indução da carcinogênese do cólon desencadeados pela microbiota destacam-se a produção de genotoxinas como toxina distensora citoletal e a colibactina as quais exercem danos diretos ao DNA causando instabilidade genômica (NEŠIĆ; HSU; STEBBINS, 2004; CUEVAS-RAMOS et al., 2010), e a toxina do *Bacteroides fragilis,* que promove a ativação de STAT3 e da resposta imune do tipo Th17, envolvidas diretamente nas vias de indução do câncer (WU et al., 2009). Além das genotoxinas, metabólitos como espécies reativas de oxigênio (CARINI et al., 2017; KRUK; ABOUL-ENEIN, 2017), o sulfeto de hidrogênio (ATTENE-RAMOS et al., 2006), e ácidos biliares como ácido desoxicólico e ácido quenodesoxicólico (TONG et al., 2008; RIDLON; WOLF; GASKINS, 2016) também têm sido relacionados à promoção do CCR por desencadearem danos ao DNA celular. A fermentação de proteínas também promove a geração de metabólitos potencialmente tóxicos como a amônia, aminas, fenóis, sulfetos e nitrosaminas, diretamente relacionados à ocorrência de DII e CCR (RUSSELL et al., 2011; WINDEY; DE PRETER; VERBEKE, 2012; RIDLON; WOLF; GASKINS, 2016). Por esse motivo, dietas com baixo teor de carboidratos e alto de proteínas aumentam os níveis de metabólitos não desejáveis que a longo prazo podem elevar o risco de doenças no cólon (RUSSELL et al., 2011).

A ativação de receptores do tipo Toll (TLR) por bactérias intestinais, também esta relacionada à indução da carcinogênese no cólon, isso porque vias pró-carcinogênicas como a sinalização intracelular de MyD88 (RAKOFF-NAHOUM; MEDZHITOV, 2007) e a produção de IL-17 e L-23 (GRIVENNIKOV et al., 2012) são desencadeadas neste processo. TLR4 são vistos superexpressos em células tumorais humanas e de camundongos e associados à indução da ciclooxigenase 2 (COX 2), prostaglandina E 2 (PGE2) e ativação do receptor do fator de crescimento epidérmico (EGFR) (FUKATA et al., 2007).

2.3 Células imunes e seu papel no CCR

Existe uma forte associação entre o perfil de linfócitos infiltrantes do tumor e o prognóstico do CCR (LEE et al., 2010, 2013). Linfócitos T CD8 e células *Natural Killer* (NK) são associados ao retardo e melhor prognóstico da doença (VIVIER et al., 2012; AMICARELLA et al., 2017; FOUNTZILAS et al., 2018), enquanto que linfócitos Th17 e citocinas deste perfil celular são associados ao pior prognóstico e progressão do CCR (DE SIMONE et al., 2015). Elevadas densidades de células T reguladoras (Treg) no infiltrado tumoral também têm sido associadas ao pior prognóstico e redução da sobrevida no CCR (DENG et al., 2010; BETTS et al., 2012; BRUDVIK et al., 2012; KATZ et al., 2013; ZENG et al., 2013; LIU et al., 2014). Contudo, há controvérsias quanto ao papel dos linfócitos Th17 e das células Treg durante a carcinogênese do cólon (WOLF et al., 2015; ZHUO et al., 2015).

Evitar a ativação de células T auto reativas, suprimir a resposta imune celular e manter a auto tolerância imunológica, com intuito de prevenir a autoimunidade e danos ao indivíduo é a função base das células Treg (BLUESTONE; ABBAS, 2003; JOSEFOWICZ; LU; RUDENSKY, 2012). Estas células utilizam mecanismos de imunossupressão como a secreção de citocinas inibitórias como IL-10, TGF-β e IL-35, e a citólise por meio de sua atividade citotóxica (VIGNALI; COLLISON; WORKMAN, 2008). Também podem induzir células apresentadoras de antígeno a produzirem a enzima indoleamina 2,3-dioxigenase (IDO), um composto indutor de metabólitos pró-apoptóticos, gerados a partir do catabolismo do triptofano, que culminam na supressão das células T efetoras (FALLARINO et al., 2003). Células Treg também são capazes de responder diretamente a produtos microbianos. Isso porque elas expressam TLR4, um receptor do tipo Toll que se liga à lipopolissacarídeos (LPS) da parede celular de bactérias Gram-negativas. A ligação LPS-TLR4 ativa as Treg aumentando sua sobrevivência e proliferação independentemente das células apresentadoras de antígenos (CARAMALHO et al., 2003).

As células Treg podem ser classificadas quanto à sua origem: Treg naturais (nTregs), originadas no timo, reconhecem antígenos próprios

(SAKAGUCHI, 2004); e Treg adaptativas ou induzidas (iTregs), originadas na periferia, reconhecem antígenos teciduais ou estranhos, incluindo alérgenos, microbiota e antígenos alimentares (DONS et al., 2012; PLITAS; RUDENSKY, 2016). Células Treg se desenvolvem a partir de células T $CD4^+$ virgens após a exposição a estímulos antigênicos na periferia sob condições tolerogênicas. Uma forte sinalização do receptor de célula T (TCR), uma co-estimulação sub-ótima, e quantidades elevadas do fator de transformação do crescimento beta (TGF-β), IL-2 e ácido retinóico favorecem a indução do fator de transcrição FOXP3 (*Forkhead box P3*) nas células T $CD4^+$ virgens (CHEN et al., 2003; JOSEFOWICZ; LU; RUDENSKY, 2012). FOXP3 é o fator de transcrição chave para o desenvolvimento e função das células Treg e tem sido considerado um marcador confiável desta linhagem celular (FONTENOT et al., 2005; HORI; NOMURA; SAKAGUCHI, 2017). A expressão de CD25 é bastante variável nas iTreg, e na ausência de TGF-β tais células podem perder a expressão de FOXP3 (SELVARAJ; GEIGER, 2007). Ambos os tipos de Treg podem se acumular no sitio tumoral e suprimir funções efetoras de células T antígeno específicas do tumor (CURIEL et al., 2004; NUMMER et al., 2007).

Há uma estreita relação entre a diferenciação das células Treg e células Th17, principalmente por causa de uma citocina chave, o TGF-β. Esta citocina em altas concentrações converte células T $CD4^+FOXP3^-$ em células T $CD4^+FOXP3^+$ (ZHU; PAUL, 2008), contudo, na presença de citocinas pró-inflamatórias como a IL-6, o TGF-β leva a expressão do fator RORγt (ZHOU et al., 2008), responsável pela indução de células T secretoras de IL-17 (células Th17) e, ao mesmo tempo, inibe a diferenciação de Treg.

Linfócitos Th17 são associados à progressão do CCR (LI et al., 2014; SHARP et al., 2017). O infiltrado celular neoplásico de pacientes com CCR muitas vezes apresenta células imunes produtoras de citocinas do perfil Th17 (IL-17A, IL-17F, IL-21 e IL-22), fator de necrose tumoral alfa (TNF-α) e IL-6 (LI et al., 2014; DE SIMONE et al., 2015). Tais citocinas também estão elevadas em lesões iniciais do cólon em camundongos com CCR, sendo relacionadas ao aumento da ativação dos fatores de transcrição oncogênicos STAT3 e fator nuclear kappa B (NF-κB) (DE SIMONE et al., 2015). A IL-17 é capaz de

promover a tumorigênese intestinal favorecendo a proliferação de células epiteliais aberrantes (WU et al., 2009; WANG et al., 2014) e a angiogênese por meio da produção do fator de crescimento endotelial vascular (VEGF) (NUMASAKI et al., 2003; CHUNG et al., 2013). Contudo, células Th17 também foram relacionadas à sobrevivência de pacientes com CCR (AMICARELLA et al., 2017). Isso porque tais células foram capazes de recrutar neutrófilos $CD16^{+}$ mieloperoxidase $(MPO)^{+}$ e linfócitos T $CD8^{+}CCR5^{+}CCR6^{+}$ citotóxicos por meio da secreção de IL-8, e das quimiocinas CCL5 e CCL20, respectivamente, nos infiltrados tumorais (AMICARELLA et al., 2017).

O controle e a prevenção do câncer podem ser atribuídos principalmente às células efetoras antitumorais, como as células NK e linfócitos T CD8. Células NK apresentam alta eficácia de destruição de células tumorais e infectadas por vírus, sem a necessidade de uma prévia sensibilização ou reconhecimento de um antígeno específico (VIVIER et al., 2012). As células NK distinguem células normais de anormais por meio de receptores em sua superfície que reconhecem sinais ativadores e inibitórios nas células alvo. Quando ocorre o reconhecimento de ligantes ativadores em combinação com a expressão reduzida de moléculas do MHC classe I em células infectadas por vírus ou células cancerosas ocorre ativação da citotoxicidade das NK e a liberação de citocinas imunoestimuladoras como o interferon-gama (IFN-γ) (PAHL; CERWENKA, 2017). Célula NK também podem responder a padrões moleculares associados a patógenos (PAMP) (OTH et al., 2018) ou a proteases extracelulares (SAINI et al., 2011). Contudo, muitas vezes, as células tumorais conseguem evadir a vigilância das NK por meio da produção de moléculas imunossupressoras e do recrutamento de células Treg, as quais reduzem a atividade tumoricida das NK, na tentativa de manutenção da auto-tolerância imunológica e homeostase (SMYTH et al., 2006; PEDROZA-PACHECO; MADRIGAL; SAUDEMONT, 2013; PAHL; CERWENKA, 2017).

Nas situações em que não ocorre equilíbrio entre imunidade e autoimunidade, como nos tumores, as Treg alteram sua estabilidade para se ajustar a um novo microambiente, o que favorece o escape e a sobrevivência das células tumorais (JOSEFOWICZ; LU; RUDENSKY, 2012; SAVAGE;

MALCHOW; LEVENTHAL, 2013). A secreção de TGF-β pelo tumor é um dos mecanismos que induz a conversão das células T $CD4^+$ periféricas ativadas em Treg $CD4^+CD25^+$, visando à supressão da atividade tumoricida de linfócitos T $CD8^+$ e das células NK (CHEN et al., 2003; NAJAFI; FARHOOD; MORTEZAEE, 2018). Além disso, o tumor secreta citocinas e quimiocinas específicas que atraem mais células Treg para o sítio neoplásico (MÉNÉTRIER-CAUX et al., 2012). Assim, células Treg são induzidas a mediarem a imunossupressão da resposta imune ao tumor, facilitando sua evasão e manutenção (CHEN et al., 2016).

Apesar dos indícios da participação das células Treg na supressão da imunidade antitumoral (LIU et al., 2014; CHEN et al., 2016), estudos clínicos mostraram que o aumento de células Treg no infiltrado tumoral está associado a um bom prognóstico no CCR nos estágios iniciais da doença (estágios I e II) (SALAMA et al., 2009; FREY et al., 2010) e também à presença de tumores menos invasivos (TOUGERON et al., 2013). Adicionalmente, camundongos ($Apc^{Min/+}$) geneticamente predispostos à ocorrência de pólipos intestinais após receberem a transferência adotiva de linfócitos Treg $CD4^+CD25^+$ apresentaram menos adenomas epiteliais no cólon (ERDMAN et al., 2005). As células Treg transferidas foram capazes de aumentar a apoptose e reduzir a expressão genética de COX-2 nos animais que apresentaram regressão tumoral (ERDMAN et al., 2005).

Além disso, especula-se que linfócitos T $FOXP3^+$ possam atuar na supressão de respostas imunes de células T direcionadas a microrganismos, ao invés de respostas contra antígenos associados ao tumor (LADOIRE; MARTIN; GHIRINGHELLI, 2011). Isso mostra que as Treg podem ter um impacto positivo sobre tumores de regiões ricas em patógenos microbianos como o intestino grosso (ERDMAN et al., 2010). Sabe-se que o número e a função das Treg na mucosa são afetados pela presença da microbiota intestinal. *B. fragilis* e certas cepas de *Clostridium*, por exemplo, promovem a diferenciação das células Treg na medida em que fornecem um ambiente rico em TGF-β (ROUND; MAZMANIAN, 2010; ATARASHI et al., 2011). O tratamento diário de camundongos com cepas probióticas de lactobacilos e

bifidobactérias modifica o estado inflamatório dos animais provavelmente por induzir populações de Treg (KARIMI et al., 2009; LYONS et al., 2010).

2.4 Yacon como prebiótico

Considerada um alimento prebiótico por ser fonte de frutooligossacarídeos (FOS) e inulina, a yacon (*Smallanthus sonchifolius*) é classificada como uma raiz tuberosa pertencente à família Asteraceae, originária da região dos Andes. A yacon contém em torno de 40 a 70% de FOS em sua matéria seca (OJANSIVU; FERREIRA; SALMINEN, 2011). FOS são oligossacarídeos de frutose, unidas por ligações β (2→1) ou β (2→6), as quais não são hidrolisadas pelas enzimas do trato gastrointestinal humano. Dessa forma, tais frutanos chegam intactos no cólon e podem ser fermentados pela microbiota intestinal (FERNÁNDEZ et al., 2013).

Ao invés do amido, principal reserva energética das raízes, a yacon possui predominantemente frutanos do tipo inulina como os FOS, e açúcares simples como sacarose, frutose e glicose (15 a 40% na matéria seca) como carboidratos de reserva (OJANSIVU; FERREIRA; SALMINEN, 2011). Contudo, as quantidades destes carboidratos podem ser altamente variáveis, o que se deve a fatores como o cultivar, a altitude, as condições de armazenamento pós-colheita, o tipo de processamento, como a desidratação, por exemplo, e a metodologia utilizada para a quantificação dos seus compostos (DE ALMEIDA PAULA; ABRANCHES; DE LUCES FORTES FERREIRA, 2015). Após a colheita inicia-se o processo de hidrólise dos oligofrutanos o que consequentemente reduz o conteúdo de FOS. Por isso é recomendável o armazenamento à sombra e o estoque em temperaturas mais baixas, evitando-se a exposição das raízes ao sol (GRAEFE et al., 2004).

Quimicamente FOS são classificados como frutanos do tipo inulina, assim como a inulina e oligofrutose (NINESS, 1999). A diferença entre FOS e inulina está no número de moléculas de frutose que compõem as cadeias de polissacarídeos. Na inulina este número varia de 20 a 60, enquanto que nos FOS, cujas cadeias são mais curtas, há entre 2 a 10 moléculas de frutose

(NINESS, 1999; DE ALMEIDA PAULA; ABRANCHES; DE LUCES FORTES FERREIRA, 2015). Estes compostos possuem as mesmas propriedades nutricionais, sendo considerados prebióticos (CORZO et al., 2015). Prebióticos são ingredientes alimentares não digeríveis, porém fermentáveis, que beneficiam o hospedeiro ao estimularem seletivamente a proliferação e/ou a atividade de populações bacterianas desejáveis no cólon (CORZO et al., 2015). Assim, os prebióticos atuam como substrato para o crescimento de bactérias intestinais probióticas como os lactobacilos e as bifidobactérias (GIBSON; ROBERFROID, 1995; ROSSI et al., 2005), modulam a população de patógenos entéricos por favorecer melhor resposta imune protetora (CHEN et al., 2017), e promovem benefícios à saúde tanto em nível intestinal quanto sistêmico (CORZO et al., 2015). Segundo a ANVISA, alimentos contendo FOS somente possuem alegação de propriedades funcionais prebióticas quando seu consumo fornecer no mínimo 5 g de FOS/dia, sendo no mínimo 2,5 g de FOS/ porção do produto, não devendo ultrapassar 30g na recomendação diária do produto pronto (ANVISA, 2019).

Além da yacon, aproximadamente 30 mil espécies de vegetais apresentam frutanos como carboidrato de reserva e são encontrados em espécies de interesse econômico como raízes de alcachofras, aspargos, alho, banana, cebola, trigo e tomate (ROBERFROID, 2007). A autoridade Europeia para a segurança dos alimentos, European Food Safety Authority (EFSA), reconheceu a yacon e seus derivados como seguros para o consumo humano, podendo ser incluída na dieta habitual (OJANSIVU; FERREIRA; SALMINEN, 2011).

O consumo de yacon é capaz de modular a microbiota intestinal aumentando o número de bifidobactérias e lactobacilos (UTAMI et al., 2013; DE SOUZA LIMA SANT’ANNA et al., 2015), diretamente associados à inibição do crescimento de bactérias patogênicas (VEIGA et al., 2014; SANT’ANNA et al., 2018). O aumento do número de bactérias probióticas reflete também no aumento da concentração de ácidos graxos de cadeia curta (AGCC) fecal verificado com o consumo de dietas contendo yacon (UTAMI et al., 2013; VAZ-TOSTES et al., 2014; GRANCIERI et al., 2017; SANT’ANNA et al., 2018). A farinha de yacon (340mg de farinha de yacon/kg de dieta) ofertada à dieta de

camundongos promoveu aumento da proliferação de bifidobactérias e lactobacilos e da produção de sIgA, IL-10 e IL-4 (BONET et al., 2010). Nesse sentido, é possível associar o uso deste prebiótico à melhora da resposta imunológica. A introdução da farinha de yacon (0,14g FOS/kg/dia) à dieta de pré-escolares com idades entre 2 a 5 anos, durante 18 semanas, promoveu o aumento da produção de sIgA fecal e IL-4 sérica (VAZ-TOSTES et al., 2014). Por sua vez, camundongos que consumiram diariamente uma dieta suplementada com FOS proveniente de yacon, durante 30 dias, tiveram significativa redução de IL-1β em culturas de macrófagos e aumento dos níveis de IgA fecal, melhorando a imunidade da mucosa (CHOQUE DELGADO et al., 2012). Também foram descritos benefícios como melhora da atividade antioxidante *in vivo* (HABIB et al., 2015), e aumento da fagocitose de macrófagos (PAREDES et al., 2018) pelo consumo de yacon.

A yacon contribui com a melhora do transito intestinal (Sant'Anna et al., 2015; Geyer, Manrique, Degen, & Beglinger, 2008; Sant'Anna et al., 2018), saciedade, perda de peso (GENTA et al., 2009; GOMES DA SILVA et al., 2017), redução da glicemia (SCHEID et al., 2014; HONORÉ et al., 2018), triglicerídeos e colesterol (HABIB et al., 2011, 2015; ROSELINO et al., 2012) e inibição da adipogênese (HONORÉ et al., 2018). O consumo de yacon também promove a melhora da arquitetura intestinal com o aumento do número, profundidade e bifurcações das criptas intestinais (LOBO et al., 2007; SANT'ANNA et al., 2018), o que favorece a absorção de minerais como cálcio, magnésio e ferro, além de aumentar a deposição de cálcio nos ossos (LOBO et al., 2007, 2014; RODRIGUES et al., 2012).

2.4.1 Yacon, frutanos e CCR

Tem sido relatado que o consumo de yacon promove a regressão de lesões pré-neoplásicas em modelo experimental de carcinogênse colorretal (DE MOURA et al., 2012; GRANCIERI et al., 2017). Ratos quimicamente induzidos ao CCR ao consumirem uma dieta contendo farinha de yacon (20,4% FOS) na proporção de 1% de farinha na dieta, ou uma preparação simbiótica

(yacon 1% + *Lactobacillus casei* 2,5x10^{10} UFC/g de dieta) durante 13 semanas apresentaram redução significativa no número de FCA e adenocarcinomas invasivos (DE MOURA et al., 2012). Da mesma forma, uma dieta suplementada com farinha de yacon (7,5% FOS) oferecida durante 8 semanas a ratos induzidos às lesões pre-neoplásicas com DMH (25mg / kg de peso corporal) mostrou redução de mais de 40% na contagem de FCA (GRANCIERI et al., 2017).

O efeito antineoplásico promovido pelo consumo dos frutanos é atribuído principalmente à modulação da composição e atividade metabólica da microbiota (KUUGBEE et al., 2016; ALMEIDA et al., 2018). Os gêneros *Lactobacillus* e *Bifidobacterium* são os que mostram mais efeitos positivos na prevenção ao CCR em estudos *in vivo* e *in vitro* (WASILEWSKA; ZŁOTKOWSKA; PIJAGIN, 2013; DOS REIS et al., 2017; MENDES et al., 2018) (AMBALAM et al., 2016). Dentre os efeitos anticarcinogênicos dos probióticos estão: inativação de carcinógenos ou agentes mutagênicos (CHONG, 2014); aumento das atividades imunoestimuladoras pela melhora da função de barreira intestinal, indução de moléculas anticarcinogênicas e antioxidantes (CHONG, 2014; GAMALLAT et al., 2016); ativação de células imune como os linfócitos B e T e o aumento da produção de IgA (MANUZAK et al., 2016).

O tratamento com *Lactobacillus rhamnosus* em ratos quimicamente induzidos à carcinogênese do cólon é capaz de promover a redução da incidência e multiplicidade dos tumores, demonstrado pela redução da expressão de β-catenina; induzir a apoptose celular por redução de Bcl-2 e aumento da expressão de p53, Bax e casp3; e melhorar a inflamação por reduzir moléculas pró-inflamatórias como NF-κB-p65, COX-2 e TNF-α (GAMALLAT et al., 2016). Bifidobactérias e lactobacilos podem induzir a secreção de citocinas anti-inflamatórias e favorecer a diferenciação de linfócitos Th2 e Treg por regular positivamente FOXP3 e GATA3 no cérebro e baço de camundongos, assim como inibir a diferenciação de células Th1 e Th17 (SALEHIPOUR et al., 2017). Células Treg podem contribuir na prevenção do câncer, principalmente naqueles onde a inflamação é um fator desencadeador do processo neoplásico, como é o caso do CCR (ALMEIDA et al., 2018). Contudo, após o estabelecimento do tumor, os Treg podem ser direcionadas

para inibição da resposta imune antitumoral, assumindo um papel pró-tumorigênico (NICCOLAI et al., 2017), suportando a ideia de que o uso de probióticos deve ser aplicado na prevenção das fases iniciais do CCR (ALMEIDA et al., 2018).

Nesse sentido, é possível concluir que prebióticos previnem o CCR principalmente por contribuírem com a modulação da microbiota intestinal. As propriedades anticarcinogênicas dos prebióticos como a regulação negativa da expressão de fatores pró-inflamatórios tem relação direta com o efeito bifidogênico, modulação da atividade microbiana e da resposta imune intestinal. Pré e probióticos, dessa forma, trabalham sinergicamente para exercerem seus benefícios à saúde intestinal e do corpo, tornando os simbióticos uma estratégia terapêutica potencial no CCR (KUUGBEE et al., 2016). Lactobacilos e bifidobactérias combinados com prebióticos, como oligofrutose e inulina, atuam na supressão da progressão do tumor por aumentarem a expressão intestinal de fatores relacionados à função de barreira como ZO-1, MUC2, TLR2 e ocludina e reduzir a expressão de COX-2 e TLR4 em ratos (KUUGBEE et al., 2016).

Inulina e FOS adicionados à dieta de camundongos induzidos quimicamente ao CCR são capazes de prevenir a incidência de FCA no colón dos animais (GOMIDES et al., 2014). A efetividade desta prevenção está associada ao aumento da expressão de p16 após o tratamento com FOS (GOMIDES et al., 2014). P16 é uma proteína inibidora de quinase dependente de ciclina supressora da fase G1 da divisão celular, fase em que a célula se prepara para a síntese do DNA. A expressão de p16 está relacionada à supressão de tumores (SHIMA et al., 2011). Adicionalmente, a dieta suplementada com inulina enriquecida com oligofrutose atenuou a carcinogênese colorretal induzida por azoximetano em ratos (FEMIA et al., 2002). Os mecanismos avaliados sugerem que o prebiótico atue no aumento da produção de AGCC, redução da atividade proliferativa das células cancerosas e redução na atividade de enzimas envolvidas na patogênese do cólon como oxido nítrico sintase induzível e COX-2 no tecido tumoral (FEMIA et al., 2002).

O butirato, um tipo de AGCC produzido durante a fermentação bacteriana colônica, têm papel de destaque na diminuição da proliferação celular neoplásica e indução da apoptose (BULTMAN, 2014), demonstrando reduzir FCA e retardar a progressão tumoral em modelos experimentais (POOL-ZOBEL, 2005). Dependendo da concentração do butirato na mucosa ele pode seguir diferentes vias. Concentrações baixas a moderadas de butirato são prontamente metabolizadas nas mitocôndrias dos enterócitos para produzir energia e estimular a proliferação celular. Já em concentrações mais altas, o butirato não metabolizado na via energética é direcionado para o núcleo celular, atuando como um inibidor da enzima histona desacetilase (HDAC) que regula a expressão de genes para inibir a proliferação celular e induzir a apoptose nos colonócitos (BULTMAN, 2014). O butirato pode promover, portanto, a renovação natural do epitélio do cólon como também dos colonócitos cancerosos, os quais por utilizarem a glicose como fonte energética preferencial, também permitem que o butirato se acumule no núcleo e seja utilizado na inibição da HDAC, suprimindo o crescimento tumoral (BULTMAN, 2014).

2.4.2 Produto à Base de Yacon (PBY)

Como uma alternativa para obtenção de FOS, a yacon, considerada a maior fonte vegetal de FOS quando comparada a alimentos fonte como a raiz de chicória e a alcachofra (GRAEFE et al., 2004), passa a ser uma alternativa promissora deste composto bioativo. Contudo, a yacon apresenta alto conteúdo de água, que pode exceder até mais que 70% do seu peso fresco (LACHMAN; FERNÁNDEZ; ORSÁK, 2003), tornando-a altamente perecível e consequentemente limitando sua vida de prateleira. Além disso, a disponibilidade de aquisição sazonal ao longo do ano dificulta sua obtenção (DE SOUZA LIMA SANT’ANNA et al., 2015).

Assim, o desenvolvimento de um produto quimicamente estável, sem alterações no teor de FOS e inulina, e com prolongada vida de prateleira, favorece um acesso contínuo a esta fonte de prebiótico. Nesse sentido, foi

desenvolvido o produto à base de yacon (PBY), como forma de atender os requisitos de conservação de FOS e inulina e facilitar o processo de aquisição deste alimento no mercado. Suas informações de processamento estão resguardadas devido à submissão ao processo de patente (número de registro de patente no Instituto Nacional de Propriedade Industrial - INPI: 014110002964).

Em face da relação entre a microbiota intestinal e o sistema imune no desenvolvimento do CCR, hipotetiza-se que o consumo do PBY possa modular a microbiota intestinal beneficamente e atuar na melhora da resposta imune anti-inflamatória intestinal, alterando fatores de transcrição específicos da resposta imune adaptativa e a população de linfócitos intestinais durante a saúde e carcinogênese colorretal. Dessa forma, o objetivo deste estudo foi investigar as alterações intestinais e o perfil de resposta imune no cólon gerados pelo consumo do PBY em camundongos BALB/c sadios e induzidos quimicamente às lesões pré-neoplásicas do cólon.

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4 OBJETIVOS

4.1 OBJETIVO GERAL

Investigar os efeitos do consumo do produto à base de yacon (PBY) nas características intestinais e fecais e no perfil de respostas imune intestinal em camundongos BALB/c sadios e induzidos à carcinogênese colorretal.

4.2 OBJETIVOS ESPECÍFICOS

- Realizar uma revisão da literatura científica sobre o papel e fenótipo das células T reguladoras no prognóstico do CCR.

- Avaliar o efeito do consumo do PBY em camundongos BALB/c sadios sobre: o consumo alimentar; as características anatômicas e fecais; biomarcadores séricos de função hepática, renal, e perfil lipídico; produção de AGCC; e perfil de resposta imune no cólon.

- Avaliar o efeito do consumo do PBY em camundongos BALB/c induzidos às lesões pré-neoplásicas do cólon sobre: o consumo alimentar; as características anatômicas e fecais; biomarcadores séricos de função hepática, renal, e perfil lipídico; produção de AGCC; formação de lesões pré-neoplásicas; caraterísticas histopatológicas do cólon; e perfil de resposta imune no cólon.

5 RESULTADOS

ARTIGO 1 (Revisão): The role and phenotype of regulatory T cells in colorectal cancer prognostic: are we on the right track?

Submetido em 27/02/2019 à *Clinical Colorectal Cancer* (Fator de impacto 2019: 3.861).

ARTIGO 2 (Original - Publicado): Yacon (*Smallanthus sonchifolius*)-based product increases fecal short-chain fatty acids and enhances regulatory T cells by downregulating RORγt in the colon of BALB/c mice.

Submetido em 12/10/2018; Aceito para publicação em 21/02/2019 pela revista *Journal of Functional Foods* (Fator de impacto 2019: 3.470; CAPES Qualis: A1 (quadriênio 2013-2016).

ARTIGO 3 (Original): Yacon (*Smallanthus sonchifolius*)-based product increases the concentration of fecal short-chain fatty acids and favors the expression of anti-tumor transcription factor T-bet during colorectal carcinogenesis in BALB/c mice.

Submetido em 02/02/2019 à *Nutrition Research* (Fator de impacto 2019: 2.707)

ARTIGO 1 (Revisão)

The role and phenotype of regulatory T cells in colorectal cancer prognostic: are we on the right track?

Letícia De Nadai Marcon[a], Luís Fernando de Sousa Moraes[a], Célia Lúcia de Luces Fortes Ferreira[b], Leandro Licursi de Oliveira[c], Maria do Carmo Gouveia Peluzio[a]

[a]Nutritional Biochemistry Laboratory, Department of Nutrition and Health of Universidade Federal de Viçosa – UFV, Viçosa, Minas Gerais, Brazil.

[b]Institute of Biotechnology Applied to Agriculture & Livestock (Bioagro), Laboratory of Lactic Starter Cultures, Universidade Federal de Viçosa – UFV, Viçosa, Minas Gerais, Brazil.

[c]Immunochemistry and Glycobiology Laboratory, Department of General Biology of Universidade Federal de Viçosa – UFV, Viçosa, Minas Gerais, Brazil.

Co-authors contact details:

Luís Fernando de Sousa Moraes: moraesnando@yahoo.com.br; Leandro Licursi de Oliveira: leandro.licursi@ufv.br; Célia Lúcia de Luces Fortes Ferreira: clferrei@ufv.br; Maria do Carmo Gouveia Peluzio: mcgpeluzio@gmail.com

Corresponding author: Letícia De Nadai Marcon. Present address: Nutritional Biochemistry Laboratory, Department of Nutrition and Health of Universidade Federal de Viçosa – UFV. Av. P.H. Rolfs, s/n. 36570-900. Viçosa, Minas Gerais, Brazil. E-mail adress: leticiadenadai@gmail.com

Abstract

Many studies have reported the correlation between cancer survival and density of tumor-infiltrating regulatory T cells (Treg cells) in colorectal cancer (CRC) patients. Although $FOXP3^+$ T cells are conventionally thought to suppress the anti-tumor immune responses and promote tumor growth, this concept has weaken in regard to CRC as tumor-infiltrating $FOXP3^+$ T cells have been increasingly related to favorable cancer prognosis. This review aimed to investigate the prognostic implication of tumor-infiltrating $FOXP3^+$ T cells in human CRC and to unravel the setbacks involving this Treg cells phenotype. We also highlighted the possible crosstalk between immune system cells, cancer cells, microbiome, and genetic background of CRC subjects. Generally, the role and phenotype of Treg cells may differ according to the clinical stage, genetic background of tumors, and interaction between Treg cells and innate and adaptive immune cells, tumor cells and gut microbiota. Moreover, Treg cells are highly plastic, displaying several phenotypes (subtypes) and hence are able to undergo changes of function and phenotype according to the microenvironment conditions. Therefore, the labeling of $FOXP3^+$ cells alone may not reflect the regulatory role played by Treg cells in the tumor environment. Detailed Treg cells phenotyping and inclusion of the genetic background in CRC prognostic studies are necessary for better indicating the associations between Treg cells and clinical outcomes.

Keywords: Colorectal cancer; Prognosis; Phenotype; Treg cell; FOXP3.

Introduction

Colorectal cancer (CRC) is a deadly disease that arises as result of interaction of genetic and environmental factors.[1] It has been continually ranked as the third most prevalent cancer and the fourth leading cause of cancer-related deaths worldwide.[2] Despite the novel therapeutic approaches, about one million new cases are expected to emerge annually.[3] It has been reported that CRC patients' survival is related to the density of tumor-infiltrating lymphocytes (TILs). Hence, a timely prognosis and monitoring of the immune system response at the tumor's site might be of utmost importance to the survival of a CRC patient.

The primary host immune response against tumor growth and development[4] is essentially initiated by cytotoxic T lymphocytes ($CD8^+$), natural killer cells (NK cells), and helper T-lymphocytes ($CD4^+$),[5] when they leave the bloodstream towards the intratumoral and peritumoral regions[6–8] Although the main host defense has laid on the classical T-helper 1 (Th1) and T-helper 2 (Th2) immune cells, other crucial subsets of helper T-lymphocytes, such as T-helper 17 (Th17), regulatory T cells (Treg cells), and type 1 T regulatory (Tr1) cells, a subgroup of interleukin (IL)-10-producing $FOXP3^-$ Treg cells, have been also identified in and around the tumor microenvironment.[9]

The transcription factor FOXP3 (forkhead box P3) is required to preserve Treg cells lineage integrity and suppressor function.[10,11] Increased densities of tumor-infiltrating $FOXP3^+$ Treg cells have been associated with poor prognosis and correlated with reduced survival in CRC patients.[12–17] Treg cells are responsible to control the host versus tumor immunity and maintain the peripheral self-tolerance by suppressing tumor-specific effector T-cell

responses.[18,19] At first, $CD4^{+}CD25^{high}$ Treg cells might be classified as natural Treg (nTreg, thymic-originated)[20] or adaptive/induced Treg cells (iTreg, peripheral-originated).[21] Both types of Treg cells may accumulate at the tumor site and suppress the effector function of tumor antigen–specific T cells.[22,23]

Spontaneous anti-tumor T cell responses in cancer patients seem to be strongly controlled by Treg cells.[24] The proportion of Treg cells in the bloodstream of CRC patients was reported to be much higher when compared to controls. Furthermore, the number of Treg cells in the colonic tumor mass was also found to be increased when compared to TILs from a non-malignant portion of the colon.[25] Notably, patients with advanced-stage CRC showed higher density of tumor-infiltrating Treg cells than patients in the early-stage of the disease.[26] Although such outcomes reinforce an important role of Treg cells in the progression of CRC, the presence of such cells at the tumor site yields discussions about its benefits in recovery and prognosis of CRC patients.

The fact that the increased number of Treg cells in the bloodstream or in and around the tumor environment of CRC patients has been associated with poor prognosis of the disease.[12–17] has led the scientific community to support approaches to decrease Treg cells in CRC.[25,27] However, it has been disclosed that increased Treg cells infiltration is positively associated with favorable CRC prognosis[28–30] and growing evidences suggest the positive role of Treg cells in the treatment or even in the prevention of CRC.[31–36] Treg cells may benefit the host by abrogating carcinogenesis due to suppression of bacteria-driven inflammation.[29] Thus, in this review, we aimed to shed light on the role of Treg cells in CRC. Do they really have the potential to control CRC or are they mere co-adjuvants in the tumor environment? We also discuss the possible

relationship between immune system cells, cancer cells, microbiome and genetic background on the prognostic significance of Treg cells in human CRC.

Treg cells in a perspective of poor CRC prognosis

Association of Treg cells with poor outcomes in CRC is mainly detected when the density of $FOXP3^+$ cells is increased in the tumor colonic tissue,[15] lymph nodes,[13] and metastatic regions[14,17] (Table 1). Evidences that Treg cells can suppress the tumor-specific immune response have been previously described and related to tumor antigens.[37] For instance, the human onco-fetal antigen, 5T4, is highly expressed on human CRC.[38] Interestingly, the anti-tumor T cell responses to 5T4 was only detected in CRC patients after removal of Treg cells, suggesting that such cells suppress the tumor-specific immune response in CRC patients.[37]

FOXP3 expression has been commonly found in CRC tissues.[15,39] High expression of FOXP3 is positively correlated with gender, tumor malignancy, and lymph node metastasis.[15] Treg cells infiltration seems to be a dynamic process. Treg cells infiltration at the primary tumor site is increased during early-stage of CRC and decreased in the advanced-stage disease.[39] Differently, Zeng and co-wokers (2013)[16] have reported that CRC patients at stage I exhibited the lowest Treg cells count ($CD4^+CD25^+FOXP3^+$), while higher number of Treg cells was observed in patients at stage IV. A significant higher amount of $FOXP3^+$ Treg cells was reported in patients with poorly differentiated tumors when compared to patients with moderate or well differentiated tumors.[16] Serum IL-35 levels were also positively correlated to the number of Treg cells in CRC. The authors suggest that serum IL-35 might be a potent

factor to induce peripheral Treg cells, while cancer cell-derived IL-35 may recruit Treg cells into the tumor microenvironment in CRC patients, thus leading to CRC progression and poor prognosis.[16] IL-35 is an anti-inflammatory cytokine, expressed primarily by Treg cells, that suppress the proliferation and function of effector T-cell and inhibit Th17 cells differentiation. Besides, IL-35 inhibits the anti-tumor T cell responses.[40,41] IL-35 can suppress the proliferation of $CD4^{+}CD25^{+}$ T effector cells *in vitro* via activation of STAT1 and STAT3 pathways.[42] However, contradictory findings demonstrate that IL-35 has an inhibitory effect on the development of colon cancer once the overexpression of IL-35 in colon cancer cells inhibits cell migration, invasion, proliferation and colony formation by inhibiting beta-catenin.[43] Despite of this, anti-inflammatory effects of IL-35 requires additional immune-regulatory cytokines, such as IL-10 and transforming growth factor-beta (TGF-β).[44]

High density of $FOXP3^{+}$ Treg cells in tumor-draining lymph nodes (TDLNs), were shown to be related to tumor progression and immune suppression in CRC patients.[13] In addition, the proportion of $FOXP3^{+}$ Treg cells in TDLNs was positively associated with the disease stage. $CD8^{+}$ T cells function was impaired in TDLNs, but restored after Treg cells depletion.[13] Treg cells in regional draining lymph nodes were also favorable to the immune-suppressive environment of CRC patients and hence to the disease progression. The authors suggest that the proportion of Treg cells in TDLNs might be a relevant prognostic tool in the treatment of CRC and other malignancies.[13] Because $FOXP3^{+}$ Treg cells abrogates the antigen-presenting function of dendritic cells and impair the activation of cytotoxic T cells in

patients with metastatic dissemination of gastric cancer[45] FOXP3 expression is also taken to be involved in lymph node metastasis in CRC patients.[15]

Liver metastasis from CRC is quite frequent.[46] To many patients, surgical resection of colorectal metastases has been proposed.[47] The estimated survival of patients with resected liver metastases is increased to 5 years in 35-50% of cases.[48] Concomitantly, for patients submitted to colorectal liver metastases resection, high number of Treg cells among CD4 or CD8 T cells predicts poor outcome, which leads to a possible conclusion of the immunosuppressive role of FOXP3^{+}.[14] The balance between Treg cells and conventional CD4 or CD8 T cells possibly defines whether the immune response to intrahepatic metastases is effective or not.[14]

The levels of Treg cells-mediated suppression of adaptive anti-tumor immune cells at the time of liver resection purportedly provide valuable prognostic information on metastatic CRC. CD4^{+}CD25^{+}CD127$^{dim/-}$ Treg cells isolated from patients with metastatic CRC, before surgery, mediated suppression of tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) expression, which can be mitigated through the prostaglandin E2 / cyclic adenosine monophosphate pathway.[17] The immune suppression was considerably higher when the disease recurrence is lately developed.[17] CD127$^{(low/-)}$ is the cell surface IL-7 receptor, present in amounts inversely proportional to FOXP3 in Treg cells, used to assess both the level and functional capacity of these cells.[49] Peripheral Treg cells, identified by the CD127$^{(low/-)}$ phenotype, increased frequency and suppressive activity in advanced stage in patients with head and neck carcinoma.[50] The inhibitory effect of anti-tumor immune response promoted by CD127$^{(low/-)}$ Treg cells

correlates with the worse outcome of CRC and contributes to the prognosis of the disease.[17]

Treg cells in a perspective of good prognosis in CRC

Paradoxically to above findings, high Treg cells infiltration is associated with a favorable prognosis in CRC patients[7,28–31,34] (Table 2). In a meta-analysis involving 3972 CRC cases to assess the prognostic effect of FOXP3$^+$ Treg cells in different types of cancer, Shang and co-workers (2015)[51] verified that increased infiltration of FOXP3$^+$ Treg cells was found to be associated with improved overall survival in CRC.

In fact, high infiltration rate of FOXP3$^+$ cells in CRC patients with invasive tumor was associated with a better prognosis.[34] High infiltration rate of intraepithelial CD8$^+$ cells was also associated with a good overall survival, regardless FOXP3$^+$ density at the tumor environment. Nevertheless, in CRC with low infiltration of intraepithelial CD8$^+$ cells, a better overall survival was found in patients with high infiltration of FOXP3$^+$ cells.[34]

High density of tumor-infiltrating FOXP3$^+$ Treg cells in normal colonic mucosa and tumor tissue led to an effective prognostic significance in CRC patients.[28] FOXP3$^+$ Treg cells in tumor tissues correlated with better survival and stronger prognostic significance than CD8$^+$ and CD45RO$^+$ (memory lymphocyte marker). Additionally, FOXP3$^+$ Treg cells in normal tissue were associated with poor prognosis.[28] The inclusion of FOXP3$^+$ Treg cells density in combination with vascular and perineural invasion evaluation may help to improve detection of early-stage CRC.[28]

The role of Treg cells may also differ according to the genetic background of tumors, such as the DNA mismatch repair (MMR)-proficient or MMR-deficient, and hence to the clinical stage.[30] MMR is a highly conserved pathway consisting of repair enzymes that remove base–base mismatches during DNA replication and recombination.[52] Although Frey and co-workers (2010)[30] have hypothesized that high frequency of tumor-infiltrating $FOXP3^+$ Treg cells correlates with poor prognosis and survival in CRC patients, they surprisingly found that high proportion of tumor-infiltrating $FOXP3^+$ Treg cells correlates with improved patient survival in MMR-proficient, but not MMR-deficient patients. Thus, high frequency of tumor-infiltrating $FOXP3^+$ Treg cells is associated with early T stage and independently predicts improved survival in MMR-proficient CRC patients.

Univariate regression analysis of outcome's database of colon and rectal carcinoma cases of two prospective cohort studies (the Nurses' Health Study and the Health Professionals Follow-up Study), showed that densities of $CD8^+$, $CD45RO^+$ and $FOXP3^+$ cells were associated with patient survival.[53] In the multivariate model, tumor-infiltrating $CD45RO^+$ cell density, but not $CD3^+$ (T lymphocyte marker), $CD8^+$ or $FOXP3^+$ cells was associated with microsatellite instability (MSI)-high and survival.[53] In most studies, MSI has been also associated with improved CRC survival.[28,54,55]

In another multivariate model study, Nakagawa and co-workers (2015)[36] have proposed that low peritumoral Treg cells infiltration is an independent predictor of unfavorable cancer-specific survival. Simultaneously, the intratumoral Treg cells counts was not associated with cancer-specific survival. The 5-year disease-free survival and cancer specific survival among patients

with high infiltration of peritumoral Treg cells were 44.2 and 74.8%, respectively. Patients with low infiltration of peritumoral Treg cells presented lower survival (18.9 and 40.3%, respectively). The authors have speculated whether peritumoral Treg cells might suppress inflammation in the tumor microenvironment rather than directly mitigate the cytotoxic activity of T lymphocytes. According to the authors, the neoadjuvant chemotherapy may have influenced such outcomes in these patients.

Around one third of stage II CRC patients show progressive disease,[56] although the high intratumoral $FOXP3^+$ expression, accessed by immunohistochemistry, was associated with improved survival.[35] Independent high intratumoral $FOXP3^+$ Treg cells expression, early tumor stage and absence of lymphovascular tumor invasion or its association were strongly considered as favorable prognostic markers.[35] The authors emphasized the clinical use of $FOXP3^+$ Treg cells immunostaining as an independent good prognostic biomarker.

Clinical outcomes may vary among stage II CRC patients. This might be related to differences in TILs density.[7,57] Lee and co-workers (2010)[7] have demonstrated the prognostic importance of $CD3^+$, $CD45RO^+$, $CD25^+$, and $FOXP3^+$ lymphocytes in stage II CRC patients. Such biomarkers were associated with better disease-free survival. Moreover, $FOXP3^{High}$ was associated with less lymphatic invasion and, probably, with less vascular invasion.[7] It is important to mention that vascular invasion is a putative pathologic marker of CRC invasiveness.[58] The authors concluded that $FOXP3^+$ and $CD45RO^+$ TILs have independent prognostic significance and might help to improve the detection of early-stage CRC. However, in resected stage IV CRC

population, $CD45RO^+$ TILs have been reported as a stronger prognostic value when compared to $FOXP3^+$ or $CD8^+$ at the tumor primary site.[57] Better survival has also been associated with increased densities of primary site $CD8^+$, metastatic site $FOXP3^+$, and $CD45RO^+$ at primary and metastatic sites. Such findings sustain the hypothesis that TILs might modify the tumor microenvironment and, therefore, the tumor invasiveness level.

Treg cells controlling gut inflammation

The intestinal microbiota, highly abundant in the large intestine, has a trend to translocate into the tumor site. The release of pro-inflammatory cytokines by dendritic cells is triggered by bacterial translocation across the mucosal surface, a harmful consequence of increased gut permeability due to tight junction disassembly and/or ulceration caused by the tumor. Such inflammatory anti-microbial response remarkably involves Th17 cell response. These cells can induce angiogenesis by stimulating vascular endothelial growth factor (VEGF) production and inflammatory reaction by releasing cytokines, like IL-17, IL-1, IL-6, and TNF-α, which contribute to tumor promotion. This Th17-cell-mediated pro-inflammatory and tumor-enhancing responses can be mitigated by Treg cells.[29] High amounts of $FOXP3^+$ cells seem to induce effective anti-tumor immune responses. This fact is relevant to associating Treg cells to a better prognosis in human CRC.[34]

In mice, colon polyposis is driven by high densities of microbial cells that accumulate within polyps and trigger local inflammatory responses. Inflammation, local microbial cell densities, and polyp growth are suppressed by IL-10 derived specifically from T cells and Treg cells.[59] High densities of Treg

cells might protect the colon, since they suppress microbiota-induced inflammation and impair cancer development in the colorectal epithelium or avoid tumor growth.[60] In this ground, adjunct probiotic bacteria has been mentioned for underlying the immunomodulatory mechanisms in CRC prevention.[60] Notably, under pro-inflammatory conditions, Treg cells may fail to provide anti-inflammatory protection and instead contribute to a Th17-driven procarcinogenic process. This fact was verified in a "hygiene hypothesis" model proposed by Erdman and co-workers (2010).[61] “Hygienic” mice with a weakened IL-10 and Treg cells-mediated inhibitory loop are highly susceptible to the carcinogenic consequences of elevated IL-6 and IL-17 and show more frequent inflammation-associated cancers. Conversely, gut bacteria-triggered Treg cells have been shown to inhibit cancer even in extraintestinal sites, as in breast and lung.[61] The contradictory observations of Treg cells being associated with good or poor prognosis may be partially understood by the divergent immune functions of this cell modulated according to the previous inflammatory stimuli to which the subject was exposed throughout life.

Probiotics and their products have been shown to contribute greatly to the immune functions modulation and to CRC control. [60,62,63] Oral treatment with *Lactobacillus casei* BL23 in mice modulates host immune responses and significantly protect mice against 1,2-dimethylhydrazine-induced CRC.[62] This protection is associated with the modulation of Treg cells towards a Th17-biased immune response accompanied by the expression of regulatory cytokines (IL-6, IL-17, IL-10 and TGF-β).[62] Also, the conjugated linoleic acid, a bacterial immunoregulatory metabolite with potent anti-carcinogenic effect, and the VSL#3 probiotic, suppressed the colon carcinogenesis in mouse model of

inflammation-driven cancer.[60] The increase of Treg cells ($CD4^{+}CD25^{+}FOXP3^{+}$) and the reduction of Th17 cells ($CD4^{+}IL\text{-}17A^{+}$) due to *L. casei* consumption, improved the duodenal inflammation caused by enterotoxigenic *Escherichia coli* in mice.[64] Therefore, high density of $FOXP3^{+}$ cells in regions rich in bacteria such as the intestines and regions affected by inflammatory processes may contribute to CRC prognosis and might help to understand Treg cells as allies in CRC control.

Lack of association between Treg cells and CRC prognosis

The lack of consistent results between Treg cells and disease prognosis is commonly observed[39,65] (Table 3). According to Loddenkemper and co-workers (2006),[39] survival did not differ between high- and low-infiltrated Treg cells patients. The ratio between Treg cells and other TILs in cancer remains controversial,[51] however it might be useful to predict survival and prognosis in CRC patients.[65,66] A lower $CD3^{+}/FOXP3^{+}$ ratio has been related to worse disease-free survival.[65] On the other hand, the increase of $FOXP3^{+}$ TILs on the tumor mucosa did not associate to clinical outcome.[65] In addition, lower density of intraepithelial $CD3^{+}$ TILs was associated with reduced disease-free survival.[65] The prognostic impact of the $CD3^{+}/FOXP3^{+}$ ratio and $CD3^{+}$ T cells seems to be related to its intraepithelial localization.[65] In contact with tumor cells, $FOXP3^{+}$ T intraepithelial cells suppress the proliferation of other T cells[67] and stimulate antigen-presenting cells to increase the functional expression of indoleamine 2,3-dioxygenase (IDO), which has been associated with reduced $CD3^{+}$ TILs and low patient survival.[68]

CD8$^+$/FOXP3$^+$ T cell ratio was also considered an independent prognostic factor on CRC.[66] The intratumoral CD8$^+$/FOXP3$^+$ T cell ratio was negatively correlated with pathological stages, but positively correlated with disease-free survival and overall survival. In comparison with isolated FOXP3$^+$ or CD8$^+$ T cells, the balance between FOXP3$^+$ and CD8$^+$ T cells is a more sensitive marker for recurrence or survival than the isolated phenotypes.

Studies including the ratio between TILs are not conclusive on the role of Treg cells in the prognosis since they alone did not show any association with the disease prognosis. Moreover, the role of Treg cells may vary in different CRC stages. In early stages of the disease, Treg cells are able to mitigate harmful inflammation[26]. However, in later stages, Treg cells might down-regulate anti-tumor immune responses by suppressing tumor antigen-specific effector T cells[26] (Figure 1). It is assumed that the increased of Treg cells during the late stages of CRC, when it is related to worse prognosis, may be associated with maintenance of the anti-tumor immune response suppression, with upregulated suppressor molecules in Treg cells such as cytotoxic T-lymphocyte associated protein 4 (CTLA-4), inducible T-cell co-stimulator (ICOS), and with increased suppressor cytokines such as TGF-β and IL-10.[69] In contrast, during the early stages of CRC the attenuation of pro-inflammatory response promoted by Treg cells activity leads to the inhibition of the pro-tumor cells activation, such as th17 cells, and tumor growth-stimulating factors, as VEGF. These events prevent or reduce the local tumor growth and in nearby regions such as lymph nodes and adjacent tissues. In addition, the involvement of Treg cells on the suppression of bacterial triggered inflammation contributes to the reduction of mutagenic epigenetic stimuli to the initiation and progression

of CRC sporadic.[70] Therefore, the benefit of Treg cells in CRC may be mainly associated with early stages of the disease. The purported mechanism includes the prevention of the tumor and immune cells activation as well as the attenuation of the inflammation triggered by the pathogenic intestinal microbiota, the latter associated to epigenetic changes.

Treg cells origin in the tumor site

Distinct phenotypic and functional profiles are the grounds of a longstanding debate between the beneficial and unfavorable effects of specific Treg cells subsets on CRC prognosis. $FOXP3^{+}CD25^{high}CD4^{+}$ regulatory T cells present in the tumor may be classified as natural Treg or adaptive/induced Treg. Natural Treg cells are originated during thymocytes development, while iTreg cells arise from activated conventional T cells that upregulate FOXP3 after T-cell antigen receptors (TCR) stimulation in the presence of IL-2 and TGF-β.[21] Moreover, many tumors may secrete TGF-β, which helps the naïve T cell to be transformed into $FOXP3^{+}$ iTreg.[71] Depending on TCR recruitment, both nTreg and iTreg carry distinct regulatory functions, with several differentiated TCR exhibition, and hence different targets: nTreg and iTreg target, respectively, autoantigen and foreign antigen recognition.[72]

There is still a lack of information whether Treg cells originate within the tumors or from blood flow in cancer patients.[73] It has been reported that nTreg are recruited to the tumor site and actively expanded[74,75] or might be a pool of iTreg derived from $CD25^{-}$ cells[76]. In any case, both iTreg and nTreg are likely to contribute to the pool of Treg cells in the tumor microenvironment[77].

Some cancer markers are implicated in the origin of tumor-infiltrating Treg cells.[73] In mice with colon adenocarcinoma, the Treg cells phenotype $CD4^{+}Foxp3^{+}Nrp\text{-}1^{lo}$ (Neuropilin-1, a type-1 transmembrane protein) and $Helios^{lo}$ (a member of the Ikaros transcription factor Family) were suggested of induced origin.[76,78] However, in humans, several phenotypes, such as $CD4^{+}FOXP3^{+}CCR4^{+}CTLA\text{-}4^{hi}$ attributed to Treg cells, are not correlated with their origin.[79]

Different Treg phenotypes in different types of cancer may be related to their activation state and not owing to the existence of different Treg cells sub-populations.[73] The identification of different effector phenotypes of Treg cells,[80,81] through the exposure to different tumor-associated antigens, does not contribute to the identification of nTreg or iTreg, since both are found in the tumor and are exposed to the same antigenic stimuli.[73] However, the detection of the transcription factor BLIMP-1 in Treg cells may contribute to the identification of effector Treg cells derived from the natural pool.[82] In mice, $BLIMP\text{-}1^{+}$ Treg cells were shown to be highly suppressive and BLIMP-1 considered essential for IL-10 production in T cells.[82]

At the genetic level and more specifically at the FOXP3 locus, nTreg cells show a stable DNA hypomethylation pattern, while iTreg show unstable FOXP3 expression, with partial hypomethylation pattern, and may even lose its expression in the absence of signals that enhance FOXP3 induction.[83] Thus, it might be possible that the majority of tumor-Treg cells are nTreg based on their FOXP3 stability. However, the inflammatory pattern of the tumor environment could interfere in the iTreg induction and, consequently, the number of these cells in the tumor milieu.

In CRC, as increased $FOXP3^+$ Treg cells correlate to a good disease prognosis, it is questionable whether there would be any relation to its specific profile or its origin. As Treg cells in the gut-associated lymphoid tissue are easily induced to control inflammation in the presence of microorganisms, it has been speculated that such cells in CRC would be mostly iTreg. Analysis on methylation status might contribute to unravel this observation.[73]

In fact, infiltration of immune cells, tumor type, and disease stage probably impact the prevalent Treg cells phenotype, since the tumor environment shapes the expression of receptors and the activation of Treg cells transcription factors. Consequently, it reflects the composition/subgroups of these cells.[73] Although both iTreg and nTreg in the tumor may be indistinguishable when related to the phenotype, the evaluation of molecular patterns of FOXP3 could be useful to differentiate them[73] and to assist the therapeutic management.

Plastic potential of Treg cells in distinct microenvironments

Treg cells have been shown to be highly modifiable according to the microenvironment where they are exposed.[69] The plastic potential of Treg cells into subgroups of T cells, or the opposite, is verified even in subgroups of inflammatory lymphocytes, such as Th17 cells.[84] An environment containing high amounts of TGF-β and pro-inflammatory cytokines such as IL-6, IL-21 and IL-23 will promote RORγt expression in $TCD4^+$ cells.[85] RORγt is the inducing transcription factor of Th17 lymphocytes differentiation.[85] Whereas a low amount of pro-inflammatory cytokines and high amount of TGF-β will induce

expression of FOXP3 in TCD4 cells.[86] Also on gut inflammation caused by bacteria, intestinal environment modifications may induce changes on FOXP3$^+$ Treg cells phenotype to pro-inflammatory T cells phenotype, such as Th17 cells.[87] Moreover, in hypoxia, FOXP3$^+$ Treg cells can become FOXP3$^+$IL17$^+$ Treg cells, producing IL-17, and displaying a high inflammatory potential and induction of neoplastic cells.[8]

The tumor microenvironment is one of the sites with the greatest instability of mechanisms inducing inflammation. Several cell mechanisms promoting tumor growth are elaborated mainly by neoplastic cells, but also by induced cells to work in favor of the tumor. Such mechanisms contribute to the modification of the tumor milieu and of the characteristics of the cells that reside in this milieu. Regarding Treg cells, they alter their polarity and stability to fit into the new microenvironment, which favors the escape and survival of tumor cells.[88,89] Recruitment of Treg cells to the tumor site occurs by attractive factors such as chemokines CCL3 and CCL4, overexpressed by CRC. This specific target selects or induces the expression of specific receptors on Treg cells surface.[90] The chemokine receptor type 5 (CCR5) expression on Treg cells (CCR5^{hi}FOXP3$^+$) is predominantly seen in human CRC, and appeared to be more suppressive than the phenotype CCR5^{lo}FOXP3$^+$.[90] In addition, cytokines present in the tumor environment contribute greatly to the expression of transcription factors and surface molecules on Treg cells. Besides, these alterations were taken to be associated mainly with the regulatory function. In CRC patients, upregulated CTLA-4 and ICOS in Treg cells were mediated by TGF-β and IL-10 and associated with potent suppressive activity.[91] CTLA-4 promotes T cell suppression by preferentially binding to CD80/86 signaling

molecules over CD28, effectively blocking CD28 co-stimulatory signals required for T cell activation.[92] Furthermore, widely used markers for Treg cells characterization such as FOXP3 and CD25 may be upregulated on non-suppressive T cells driven by activation or inflammation processes.[93] Therefore, due to the high Treg cells plasticity and heterogeneity on the tumor environment, the different phenotypes and their functional representativeness will be discussed in the next section.

Influence of distinct Treg cells phenotypes on CRC prognosis

Evidences that Treg cells are shown as a heterogeneous population were reported in the literature,[69] and this heterogeneous phenotype seems to affect the function and consequently the outcome of the CRC, since different Treg cells populations may have different influences on tumors.[69] FOXP3^{+} T cells are characterized by a group of suppressive and non-suppressive cell subpopulations with different phenotypes, gene expression, and function[94] (Figure 2). Transcription factor FOXP3 has been thought to be a specific marker to differentiate Treg cells from other T lymphocytes populations.[10] Numerous studies have considered FOXP3 as a reliable marker for Treg cells, when analyzed by flow cytometry, immunohistochemistry or RT-PCR.[7,13,95] However, positive labeling for CD25 in CD4 cells is also used for this purpose.[25,96] It is important to note that only CD4^{+}CD25^{hi} T cells may be considered as representatives of Treg cells population, since CD25^{lo} cells contain a heterogeneous pool of both activated cells and Treg cells.[97] Therefore, in order to differentiate Treg cells from non-Treg cells, it is recommended that other characteristic markers of this population, such as CTLA-4 and glucocorticoid-

induced TNFR-related protein (GITR), be used in combination with CD4 and CD25, as none of the markers are specific on their own.[25]

Unlike in mice, FOXP3 expression in humans may not be specific for cells with a regulatory phenotype, but rather a consequence of activation status, since FOXP3 gene expression is not limited to $CD4^+CD25^+$ Treg cells.[98] In fact, FOXP3 not necessarily provide regulatory function in human $CD4^+CD25^+$ T cells,[99] which indicates a lack of homogeneity on the population's suppressive T cells.

Tr1 cells, a subpopulation of IL-10-producing $FOXP3^-$ Treg cells,[9] are induced in the periphery and have a crucial role in promoting and maintaining tolerance. The surface marker, CD49b, and the lymphocyte activation gene 3 (LAG-3) are specific markers for highly suppressive human Tr1 cells.[100] In CRC tissue, high percentage of both Tr1 cells ($CD49b^+LAG\text{-}3^+$) and $IL\text{-}17^+$ cells, but not $FOXP3^+$ Treg cells, was demonstrated to be a predictive marker for poor prognosis in CRC.[33]

In this context, Scurr and co-workers (2014)[91] verified in CRC patients that approximately 30% of intratumoral $CD4^+FOXP3^-$ T cells expressed markers related to regulatory functions, including latency-associated peptide (LAP), LAG-3, and CD25. Interestingly, such subgroup of cells produced IL-10 and TGF-β, and was more suppressive when compared to $FOXP3^+$ Treg cells. Furthermore, the leading regulatory subset of TILs ($CD4^+LAP^+$) did not require FOXP3 for suppression, but instead, expressed TGF-β and IL-10, the two key regulatory cytokines. Authors concluded that the $CD4^+LAP^+$ T-cell population possibly controls anti-tumor immune responses in the local environment. Furthermore, due to the intratumoral Treg cells diversity, therapeutic strategies

that aim to overcome Treg cells activity, and therefore increase antitumor immune responses, also need to consider this novel intratumoral subset of highly suppressive $CD4^+FOXP3^-$ T cells.

The intensity of phenotypic markers expression may also contribute considerably to the analysis on the CRC prognosis. Two distinct $FOXP3^+CD4^+$ T cell subpopulations were implicated in clinical outcomes: $FOXP3^{hi}CD45RA^-$, referred to as effector Treg cells, highly suppressive and functionally stable; and $FOXP3^{lo}CD45RA^-$, do not possess suppressive activity and can secrete pro-inflammatory cytokines.[101] CRC with high infiltration of $FOXP3^{lo}$ T cells showed significantly better prognosis than those with high infiltration of $FOXP3^{hi}$ Treg cells.[101] The fact that it is difficult to distinguish these subpopulations in tumor tissues by immunohistochemistry might have imposed a major confounding factor in earlier studies using this technique to evaluate the clinical significance of $FOXP3^+CD4^+$ T cells in CRC.

In mice, the effector regulatory T cells are described as a potent suppressive subset and have been characterized as BLIMP-1^+FOXP3^+ T cells.[82] These cells co-express effector-like markers and suppression markers.[82] In human CRC, $CD3^+FOXP3^+$BLIMP-1^+ cell infiltration was associated with disease-free survival and related with a better outcome.[102] In this work, the authors speculated that BLIMP-1^+ effector Treg cells may have a direct role in the anti-tumor immune response by suppression of inflammatory responses via IL-10 production.

Additionally, the regulatory $CD8^+CD25^+FOXP3^+$ T cell (T8reg) subgroup has been identified in more than 90% of human CRC specimens, but not from most normal tissue samples.[103] Such subset of cells accumulates within CRC

tissue and presets an activated phenotype and suppressive functions. They can be induced by IL-6 and potentiated by TGF-β1. T8reg correlates with the tumor stage and demonstrates strong immunosuppressive properties that may contribute to tumoral immune escape and disease progression.[103] Nevertheless, T8reg cells comprise only a small fraction of CD8 T cells *in vivo*, and their origin and clinical relevance still remain to be determined.[103]

VEGF is abundant in the tumor microenvironment and suppresses anti-tumor immunity when secreted by several types of tumors.[104] Researchers have shown that VEGF receptor 2 (VEGFR2) is selectively expressed by $CD4^+FOXP3^{high}$ Treg cells.[105] In turn, $FOXP3^+VEGFR2^+$ Treg cells have stronger inhibitory effect of autologous T cell proliferation in relation to $FOXP3^+VEGFR2^-$ cells.[105] Suzuki and co-workers (2013)[32] have reported that low number of intratumoral $FOXP3^+VEGFR2^+$ cells correlates with poor disease-free survival and overall survival. This phenotype is an independent predictive and prognostic factor of disease-free survival and overall survival.[32] Neither intratumoral $FOXP3^+$ cell number nor intratumoral $FOXP3^+VEGFR2^-$ cell number showed correlation with disease-free survival or overall survival.[32] The authors suggest that $FOXP3^+VEGFR2^+$ may be a better predictive Treg cells marker than $FOXP3^+$ alone for recurrence and survival in patients with colorectal cancer.

Pro-inflammatory phenotype Treg cells

Originally, Treg cells produce several suppressive molecules, including TGF-β and IL-10, which may suppress the immune effector T cell function and facilitate the tumor to escape.[5] However, $CD4^+CD25^+FOXP3^+IL\text{-}17^+$ Treg cells

subset was shown to keep the immune regulatory capacity in the peripheral blood stream, although showing pro-inflammatory features.[106] These cells coexpress FOXP3 and RORγt transcription factors, produce IL-17 and strongly inhibit the proliferation of $CD4^+$ T cells.[106] It is speculated whether such phenotype would be a novel therapeutic target on the CRC treatment.[107]

Under certain cytokine environment, $FOXP3^+$ Treg cells are capable to express IL-17.[106] Yang and co-wokers (2011)[8] verified that there are abundant $FOXP3^+IL\text{-}17^+$ T cells in CRC tissue expressing high levels of TGF-β, CXCR3, chemokine receptor 6 (CCR6), and RORγt. Moreover, they found that during hypoxia, a common pathophysiological feature in cancer tissues,[108] $CD68^+$ cell (macrophages) infiltration occurs. Such cells produce CXCL11, a chemokine responsible to attract $FOXP3^+$ Treg cells into tumor tissue.[8] Then, Treg cells become $FOXP3^+IL\text{-}17^+$ cells under hypoxic environment.[8] Furthermore, $FOXP3^+IL\text{-}17^+$ T cells are capable of inducing CRC-associated cell markers (EpCAM, epithelial cell adhesion molecule; ALDH1, aldehyde dehydrogenase 1; CD44s; CD133; CD166) in bone marrow-derived mononuclear cells and driving the cells to be cancer-initiating cells.[8] It is then implied that $FOXP3^+IL\text{-}17^+$ cells have the ability to induce cancer-initiating cell development.

$FOXP3^+IL17^+$ cells might induce tumor development by regulating phosphorylation pathways.[8] It is proposed that Akt and MAPK pathway plays a crucial role in tumor growth. Akt overexpression in colon adenocarcinoma cell lines demonstrate tumorigenic cell behaviors, as increased cell survival, proliferation, invasion, and angiogenesis.[109] The levels of activated MAPKs are also commonly increased in CRC.[110] Interestingly, hyperphosphorylation of Akt and MAPK in cancer cells seems to be stimulated by $FOXP3^+IL\text{-}17^+$ T cells.[8]

It is important to mention that pro-inflammatory phenotypes are not always related to pro-inflammatory activity. FOXP3$^+$IL-17$^+$ T cells in human CRC tissue, positive to CD4, CD25, CCR6, TGF-β, IL-10 and IL-6, showed the ability to suppress tumor-specific CD8$^+$ T cells.[107] Nevertheless, no significant correlation between the frequency of FOXP3$^+$IL-17$^+$ cells and the cancer stage was noticed.[107] Moreover, tumor-infiltrating FOXP3$^+$ cells are not always Treg cells. Instead, they might be conventional T cells temporarily expressing FOXP3 when TCR is activated.[111] Overall, FOXP3 should not be considered as a single functional Treg cells marker[112] since biological properties of FOXP3$^+$ T cells seem to be influenced by other phenotypes on tumor microenvironment.

Treg cells interaction with immune cells: changing roles

Colorectal tumors are infiltrated by a heterogeneous collection of inflammatory cells such as T cells, dendritic cells, macrophages, neutrophils and mast cells.[113] The role of immune cells is highly influenced by the cytokines and chemokines produced in the tumor environment.[114] Much effort has been done to unravel the role of these cells in cancer and their influence on the tumor environment. In addition, the association with disease stages, prognosis and survival is essential for targeting the most appropriate treatments.

Plasticity is a characteristic of Treg cells[69] which seem to be strongly influenced by innate and adaptive immune cells and the tumor immune environment. This plasticity is responsible for CRC delay or progression and depends on cell type interactions (Figure 3). In CRC patients, Nagorsen and co-workers (2007)[115] reported relationship between dendritic cells and Treg cells. In epithelial and stromal tumor tissues, tumor-infiltrating dendritic cells (TIDCs)

showed a positive correlation with Treg cells infiltration and survival in CRC patients.[115] The survival of patients was significantly better in high TIDCs infiltration than in low TIDCs infiltration. They also detected the lack of response of systemic T-cell against tumor-associated antigens in patients with high TIDCs, which purportedly indicates that they primarily induce Treg cells to reduce specific T-cell responses.[115] Increased number of tumor infiltrating CD3$^+$ T cells and CD83$^+$ dendritic cells, as well as increased serum CCL4 and serum IFN-γ levels, represent relevant markers of favorable disease outcome in CRC patients.[116] It was indicated that CD83$^+$ dendritic cells (DC) infiltration may be beneficial indicator for the prognosis of CRC patients, whereas the decreased infiltration of DC and other immune cells in the tumors and the increased serum IL-8 levels is associated with worse prognosis.[116] On the other hand, the ability of CD11b$^+$TIDCs to mediate tumor cell death by TNF-related apoptosis-inducing ligand (TRAIL) may be inhibited by CD4$^+$CD25$^+$ Treg cells.[117] These Treg cells were seen to control the TRAIL-dependent cytotoxicity of tumor-infiltrating DCs in CRC mice abrogating the ability CD11b$^+$ TIDCs to express TRAIL.[117]

Mesenchymal stromal cells (MSCs) exert immunomodulatory effects in the CRC microenvironment favoring the angiogenesis, invasion and metastasis.[113] [113] Although it is currently not fully understood how tumor cells escape from immune control[69] it seems that MSCs-immune cell interactions may interfere on the activity of T-cells, dendritic cells, natural killer cells and macrophages, contributing to tumor growth and allowing the tumor escape from the immune cells action.[113] Moreover, soluble factors released in exosomes by MSCs and tumor cells can promote tumor cell proliferation, migration and angiogenesis.[113] However, in very early stages of the tumor, MSCs may also

act in preventing of inflammation of induced colon cancer development, decreasing IL-6 and phosphoSTAT3 signaling and reducing DNA damage.[113] Nevertheless, in later stages, MSCs recruited to the tumor by factors such as nuclear factor (NF)-κB, chemokine receptor type 4 (CXCR4), stromal cell derived factor (SDF)1, monocyte chemotactic protein (MCP)-1 and vascular cell adhesion molecule (VCAM)-1 promote tumorigenesis and the dampening of anti-tumor immunity.[113]

Nevertheless, in a colitis-associated CRC (CAC) model, induced by azoxymethane and dextran sulfate sodium, MSCs injected intravenously could migrate to colon tissues and induce differentiation of Treg cells, thus suppressing colitis and CAC development.[118] Such tumor suppressive effect was mainly characterized by longer colon length, decreased tumor numbers, and decreased expression of Ki-67, a cellular proliferation marker. In both colon and serum, MSCs abrogated the release of pro-inflammatory cytokines (IL-1α, IL-1β, IL-5, IL-6 and IL-12).[118] Besides, secretion of TGF-β by MSCs enhanced the induction of Treg cells from naïve T cells *in vitro*.[118] In addition, MSCs was also able to activate SMAD2 signaling. The authors concluded that MSCs may secrete TGF-β to induce differentiation of Treg cells via SMAD2 and then suppress inflammation-dysplasia-carcinoma progression in CAC.[118]

Tumor-associated macrophages (TAM) are usually the most abundant immune population in the tumor-microenvironment and are recruited to tumor site by several molecules, such as the chemokines CCL2 and CCL5, VEGF, TGF-β and colony stimulating factors (GM-CSF and M-CSF).[119] Tumor-associated macrophages have already been reported as beneficial in CRC.[120] Low macrophages infiltration, for instance, is often observed in more advanced

CRC. In turn, high macrophage infiltration along with the tumor front is associated with improved survival, indicating that TAM may act against malignant cells in CRC patients.[120,121] However, in a CRC mouse model, TAM was found to recruit $CCR6^{+}$ Treg cells to tumor mass and promote its development by enhancing CCL20 chemokine production[122] while the selective removal of TAM was able to inhibit tumor progression.[122] A typical biological feature of macrophages is their ability to express distinct functional programs in response to different environment, as happen in tumor site.[123] There are two macrophages subpopulations: M1 macrophages, characterized by expression of nitric oxide synthase 2 and Th1 cytokines and chemokines, considered to have anti-tumor immunity role; and M2 macrophages, expressing arginase, scavenger receptors and Th2 cytokines, carrying anti-inflammatory functions.[124] Nevertheless, it is worth noting that some studies have failed to specify such phenotypes, which would be valuable for the comprehension of macrophage role in the tumor environment.

Treg cells and mast cells (MC) are both abundant in CRC tumors. MC are related to poor outcome, partially due to the release of mediators that promote angiogenesis and recruitment of pro-inflammatory cells.[125,126] The interaction between Treg cells and MC is known to promote immune suppression, autoimmunity or even loss of Treg cells functions. In both human and murine CRC, such interaction, although leading to a potently immune suppressive Treg cells, might also induce a pro-inflammatory Treg cells profile.[127] In this situation, Treg cells restrain IL-10 and become more susceptible to express IL-17, switching from suppressing to promoting MC expansion and degranulation. Thus, MC are able to induce Treg cells to switch

function and intensify of inflammation in CRC, without losing T-cell-suppressive properties.[127]

In resected patients, the functional interdependence between myeloid-derived suppressor cells (MDSCs), T cells and cancer cells in CRC pathogenesis was verified by OuYang and co-wokers (2015).[128] They showed that CRC cells may promote the expansion of MDSCs ($CD33^{+}CD11b^{+}$) *in vitro* and *in vivo*. Tumor-induced MDSCs express high levels of immune inhibitory molecules, including TGF-β, IDO, IL-10, inducible nitric oxide synthase, NADPH oxidase-2, and arginase 1, and may suppress T cell proliferation and promote CRC cell growth through oxidative metabolism, by generation of nitric oxide and reactive oxygen species, but not via TGF-β signaling or iTreg cells ($CD4^{+}CD25^{high}FOXP3^{+}$).[128] Therefore, these tumor-induced MDSCs promote tumor cell growth through cell-to-cell interaction with tumor cells and suppression of T cell anti-tumor immunity.[128]

Cancer cells influence Treg cells in the tumor milieu

Tumor microenvironment composition is of utmost importance to its suppression or growth. How tumor cells communicate with their microenvironment is widely crucial for tumor growth. Specifically, the crosstalk between tumor cells and infiltrating lymphocytes may influence tumor development and patient prognosis.[129] Cancer cells are able to secrete cytokines and chemokines into the tumor microenvironment leading to an immunosuppressive milieu and hence tumor progression. In this context, Treg cells can be recruited to participate in the microenvironment responses. For instance, IL-35 released from CRC cells may attract Treg cells

($CD4^+CD25^+FOXP3^+$) and other immunosuppressive cells into the tumor microenvironment favoring CRC progression associated with poor prognosis.[16]

Colorectal cancer cell-derived extracellular vesicles (CRC-EVs) have an important role in tumor growth by altering immune cells among cancer cells. Yamada and co-workers (2016)[130] evaluated the role of CRC-EVs on T cell function. CRC-EVs were enriched with TGF-β1, which induced a phenotypic reprogram of T cells to Treg-like cells due to upregulation of specific genes (FOXP3, CTLA-4, LAG-3, IL-10, Perforin-1, and Granzyme B), activation of TGF-β/SMAD signaling, and abrogation of SAPK signaling.[130] CRC-EVs-induced-Treg-like cells promoted tumor-growth *in vitro* and *in vivo*.[130] The authors suggest that inhibiting EV-secretion by tumor cells and removing CRC-EVs could be a potential therapeutic strategy.

Kim and co-workers (2013)[95] reported that the positive FOXP3 labeling in tumor tissues from CRC patients may lead to different prognosis depending on the type of cell evaluated. High FOXP3 expression in cancer cells was related to poor prognosis when compared to patients with low FOXP3 expression.[95] Conversely, low and high FOXP3 level in tumor-infiltrating Treg cells did not induce significant differences in overall patient survival. This type of cancer cells showed a significant direct correlation with the expression of both immunosuppressive IL-10 and TGF-β.[95] The authors suggested that FOXP3 expression by cancer cells followed by secretion of immunosuppressive cytokines into the tumor microenvironment may favor the tumor to induce immunological destruction. In addition, an inverse correlation between the number of $FOXP3^+$ Treg cells and $FOXP3^+$ cancer cells on CRC patients suggests an anti-proliferative effect of TGF-β on Treg cells. Thus, disease

progression may be stimulated by FOXP3 expression mediated by cancer cells rather than by Treg cells.

Genomic influence on CRC prognosis

CRC is caused by genetic mutations on oncogenes, tumor suppressor genes and genes related to DNA repair mechanisms. The CRC molecular classification is based on pathogenic mechanisms namely chromosomal instability, MSI and CpG island methylator phenotype.[131] Chromosomal instability may contribute to 85% of CRC occurrence and 15% are related to either epigenetic or genetic alterations, as seen by deficiencies in the DNA mismatch repair system.[132,133] Defects in the DNA MMR machinery often lead to MSI, a condition in which repetitive DNA sequences named microsatellites accumulate gene mutations.[134] Some specific genes may be mutated on CRC such as c-MYC, KRAS, BRAF, PIK3CA, PTEN, SMAD2 and SMAD4. Likewise, important pathways such as WNT, MAPK/PI3K, TGF-β, TP53 may be affect.[131]

Sporadic or hereditary CRC with MSI is often characterized by inflammatory lymphocytic infiltration and associated with a better outcome than microsatellite stable (MSS) CRC, which reflects a more effective immune response.[133,135] In this context, a higher density of $CD3^+$, $CD8^+$, $CD45RO^+$, and T-bet^+ T lymphocytes was observed in MSI CRC than in MSS CRC patients, whereas the number of Treg cells $FOXP3^+$ remained unchanged among the groups.[136] Moreover, MSI CRC was related to specific cytokines expression, such as CCL5, CXCL10, and CXCL9, which participate in the Th1 response and recruitment of memory $CD45RO^+$ T cells.[136] Therefore, the role of adaptive immunity in MSI CRC is suggested to favor CRC prognosis.[136] Additionally,

intraepithelial $CD25^+$ T cells were increased in MMR-deficient tumors, whereas stromal $CD25^+$ T cells were higher in MMR-proficient cases.[65] The same pattern was not noticed for $FOXP3^+$ Treg cells, suggesting that $CD25^+$ effector T lymphocytes may undergo expansion and activation within the epithelia of MMR-deficient tumors.[65]

The predominance of specific cell type, such as Th1 lymphocytes, in the tumor microenvironment associated with different molecular characteristics (MSI, CpG island methylator phenotype status, and BRAF and KRAS mutational status) may influence T cells recruitment and activity. High number of infiltrating Th1 lymphocytes is strongly related to improved prognosis in CRC patients.[137] Th1 lymphocytes are more highly infiltrated in BRAF mutated tumors than BRAF wild-type tumors, whereas the opposite has been reported for KRAS mutated tumors.[137] In BRAF mutated tumors there are higher levels of the Th1-attracting chemokine CXCL10 and reduced levels of CCL22 and TGF-β1, stimulating Th2/Treg cells recruitment and polarisation.[137] In addition, MSI, CpG island methylator phenotype-high and BRAF mutated tumors display the increase infiltration of Th1 lymphocytes, and thus are associated with improved prognostic.[137] This indicates that T-bet, the Th1 immune response transcription factor,[138] may be a valuable marker in the clinical setting. Th2 and Treg cells were not associated with any prognostic.[137]

A connection between the Beta2-microglobulin (*B2M*) mutation status of Lynch syndrome-associated CRC and the density of $FOXP3^+$ T cells was analyzed by Echterdiek and co-workers (2015).[139] Lynch syndrome represents an ideal human model to study the concept of immunoediting. It describes how continuous anti-tumoral immune surveillance of the host eventually leads to the

selection of tumor cells that escape immune cell recognition and destruction. *B2M* mutations are known to completely mitigate HLA class I-mediated antigen presentation, a crucial mechanism of tumor cell recognition and elimination by cells of the adaptive immune system.[140] It may be then inferred that immune cell infiltration of the colonic mucosa is associated with tumor phenotype. In patients with *B2M*-mutant tumors, lower density of FOXP3$^+$ T cells in the normal colonic mucosa around the tumor was noticed, whereas higher infiltration of FOXP3$^+$ T cells was verified in patients with no such mutation (*B2M*-wild type tumors).[139] High numbers of FOXP3$^+$ T cells in the colonic mucosa are related to the occurrence of *B2M*-wild type tumors and therefore still capable of antigen presentation via HLA class I.[139] The absence of immunosuppressive Treg cells favors the outgrowth of less immunogenic *B2M*-mutant tumor cells.[139] This finding reinforce the immunoediting concept in human solid cancer development and demonstrate a critical role of the immune milieu in normal colonic mucosa and thus in disease progression.

Treg cells inhibition in cancer immunotherapy

The depletion of Treg cells during tumor progression has been reported to promote tumor-specific immune responses which may be efficient to eradicate established tumors.[141,142] Besides activating tumor-specific effector T cells and enhancing anti-tumor immune response, immune therapies such as monoclonal antibodies, vaccines and drugs aim to deplete, decrease and attenuate their suppressive activity in tumor tissues.[141] Cell surface molecules such as CTLA-4,[143] GITR,[142,144] CCR4,[145] PD-1,[146] CD25,[147] and more recently TIM-3.[148] are being used as targets to block immune functions of Treg cells

during immunotherapies. Monoclonal antibodies (mAb) targeting the T-cell receptor GITR in mouse act by depleting and destabilizing the intratumoral Treg cells population, preferentially targeting a highly activated subpopulation of $CD44^{hi}ICOS^{hi}$.[144] Anti-GITR mAb shifts Treg cells populations to enable immune attack on tumors, favoring a more functional phenotype in intratumoral $CD8^{+}$ T cells by downregulation exhaustion markers PD-1 and LAG-3.[144] In preclinical tumor models, the modulation of GITR/GITR ligand (GITRL) axis has been shown to provide the desired biological outcome inhibiting Treg cells function while activating $CD8^{+}$ T effector cells.[142]

Colorectal cancer treatment is based on neoadjuvant chemoradiotherapy (CRT) consisting of neoadjuvant radiotherapy associated with chemotherapy using 5-fluorouracil. It aims to reduce tumor volume and the local recurrence after surgery.[149,150] Both chemotherapy and radiotherapy may enhance anti-tumor immunity, as for example, by selective depletion of Treg and cancer cells.[150,151] The treatment may differ among individuals.[150] It is speculated whether Treg cells density during pre-CRT or post-CRT can predict subsequent treatment response, since tumor-infiltrating $FOXP3^{+}$ Treg cells were associated with poor outcome in many studies.[13,15] This was proven in the work of McCoy and co-workers (2015),[150] when they demonstrated that a low stromal $FOXP3^{+}$ Treg cells density in post-CRT was associated with pathological complete response (when there is no remaining viable tumor cells in the surgical specimen on histopathologic assessment), and improved long-term outcome in locally advanced rectal cancer. However, prior to CRT, no association was observed between tumor-infiltrating Treg cells density and

clinical outcome in CRC patients, indicating that Treg cells density prior to CRT does not predict response to neoadjuvant CRT in rectal cancer.[152]

It is worth noting that, when discussing immunotherapies, it is important to take into account that the immune system acts on a dynamic process, and the success of any inhibitory agent will depend on the tumor microenvironment, the patient's immune response and the pharmacodynamics of the agent used.[142] In addition, it should be assessed to what extent the Treg cells marker inhibition could contribute to cancer regression, since the long-term depletion of Treg cells may trigger autoimmune diseases.

Thus, selective deletion of tumor antigen-specific Treg cells or effector Treg cells could be a strategy to avoid elimination of all Treg cells. Treg cells appear to exert T cell suppression in an antigen-selective manner, recognizing distinct set of few tumor antigens.[24] Some, but not all of T cell responses are kept under the control of tumor-associated antigens-specific Treg cells. This explains why even after Treg cells depletion, as in non-specific Treg cells depletion, there still may be kept the suppression of effector / memory T cell responses. Thus, it is suggested that the selection of antigens according to preexisting T cell responses may be an alternative to success for cell depletion with the possibility for enhancing the efficacy of immunotherapies. Additionally, selected sets of tumor-associated antigens for tumor vaccinations that induce optimal effector T cell responses but minimal Treg cells activity, may improve the efficacy of vaccination protocols.[24] Likewise, the deletion of effector Treg cells highly suppressive, prevalent in the tumor site,[101] rather than all FOXP3+ T cells, could lead to a rational elimination of these cells, avoiding autoimmune disorder. However, despite the success of immunotherapy, depleting $FOXP3^+$

cells from cancer patients should be considered with caution.[112] The fact that Treg cells are able to attenuate pro-carcinogenic inflammatory immune response on colon[29] must be taken into account particularly in the early stages of CRC.

Limitations of the use of FOXP3 alone and viable solutions for Treg cells labeling

Considering that FOXP3 expression occurs in an unstable manner and that it is highly affected by the tumor environment, this phenotype should not be evaluated alone for Treg characterization. Eventually, FOXP3 may not be expressed in some regulatory subpopulation, or may be lost, or even may be induced according to the microenvironment. Absence of FOXP3 in Tr1 cells ($CD49b^+LAG\text{-}3^+$)[100] does not restrain them from performing suppressor functions as, for example, producing IL-10.[9] In addition, TILs regulatory subset ($CD4^+LAP^+$) expresses high suppressive activity without requiring FOXP3.[91]

FOXP3 expression may also occur in $CD4^-$ cells as $CD8^+CD25^+$ cells which play suppressive functions.[103] On the other hand, the stimulation *in vitro* of human T cells $CD4^+$ and $CD8^+$ promotes FOXP3 and CD25 expression without generating suppressor or regulatory functions.[99] $CD4^+FOXP3^+$ cells associated with VEGFR2 positive labeling have a strong suppressor effect than $FOXP3^+VEGFR2^-$ cells.[66] Also, FOXP3 T cells associated with BLIMP-1 positive labeling are described as effector regulatory T cells suppressive.[82,102] In contrast, FOXP3 expression associated with inflammatory markers such as IL-17 has been shown in cells with suppressor functions,[107] whereas $FOXP3^+IL\text{-}17^+$ cells may contribute to the development of cancer-initiating cells in CRC.[8]

FOXP3 has also been found in tumor cells of CRC patients,[95] thus in tumor tissue analyzes, the lack of association with T cell marker would hamper the correct cell specification. Thereby, FOXP3 as a marker alone makes the comprehension difficult concerning the functional role and action mechanism of the cell type investigated. Therefore, we encourage researchers to associate FOXP3 with conventional Treg cells markers such as CD4, CD25, CTLA-4, GITR, IL-10 and TGF-β. Seemingly to unconventional markers such as CD8, CD49b, LAG-3, LAP, VEGFR2, BLIMP-1 and IL-17, which could greatly aid in uncovering the regulatory functions of these highly plastic cell population.

Conclusion

Colorectal cancer is a deadly disease of public health significance. The detection of intratumoral Treg cells has been a criteria for CRC prognosis due to their immunosuppressive and anti-inflammatory functions. $FOXP3^+$ T cells are used as standard marker for Treg cell but this concept has been weakened on the grounds of the plasticity of this population in CRC microenvironment. More recently, as indicated in this review, in order to better associate Treg cells and clinical outcomes, lead to suggest that the labeling of $FOXP3^+$ T cells alone may not reflect a regulatory role played by Treg cells in the tumor environment. Then it became necessary to include detailed Treg cells phenotyping as well as genetic background in the prognostic of CRC. In fact, the detection of specific Treg cells subgroups that produce crucial factors for tumor immunosuppression, such as IL-10 and TGF-β, initially taken as $FOXP3^+$ T cells lead to highlight the role of $FOXP3^-$ T cells, that might exhibit more immunosuppressive activity than the regulatory $FOXP3^+$ T cells. Moreover, the production of pro-

inflammatory cytokines by FOXP3$^+$ T cells has emphasized the dual role of such cells. In addition, cancer cells also manipulate the tumor microenvironment by production of cytokines and transcription factor leading to tumor progression. Therefore, according to the type of the cell that interact with Treg cells, they are induced to alter their phenotype and function, then acquiring either an immunosuppressive or pro-inflammatory profile. As consequence, there are different responses in CRC progression. Thus, we suggest not to use the conventional phenotype attributed to Treg cells (FOXP3$^+$) alone to characterize such population, but rather the association of FOXP3$^+$ with other phenotypes and functional markers such as CD4, CD8, CD25, CTLA-4, GITR, CD49b, LAG-3, LAP, VEGFR2, BLIMP-1, IL-10, TGF- β and IL-17. In addition, we suggest considering positive the Treg cells association (anti-inflammatory profile) with improved prognosis in CRC, at least in early stages.

Acknowledgements

The study was supported by the National Council for Scientific and Technological Development - CNPq, Coordination for the Improvement of Higher Education Personnel - CAPES, Minas Gerais Research Foundation - FAPEMIG, and Universidade Federal de Viçosa - UFV.

Disclosure

There is no conflict of interest.

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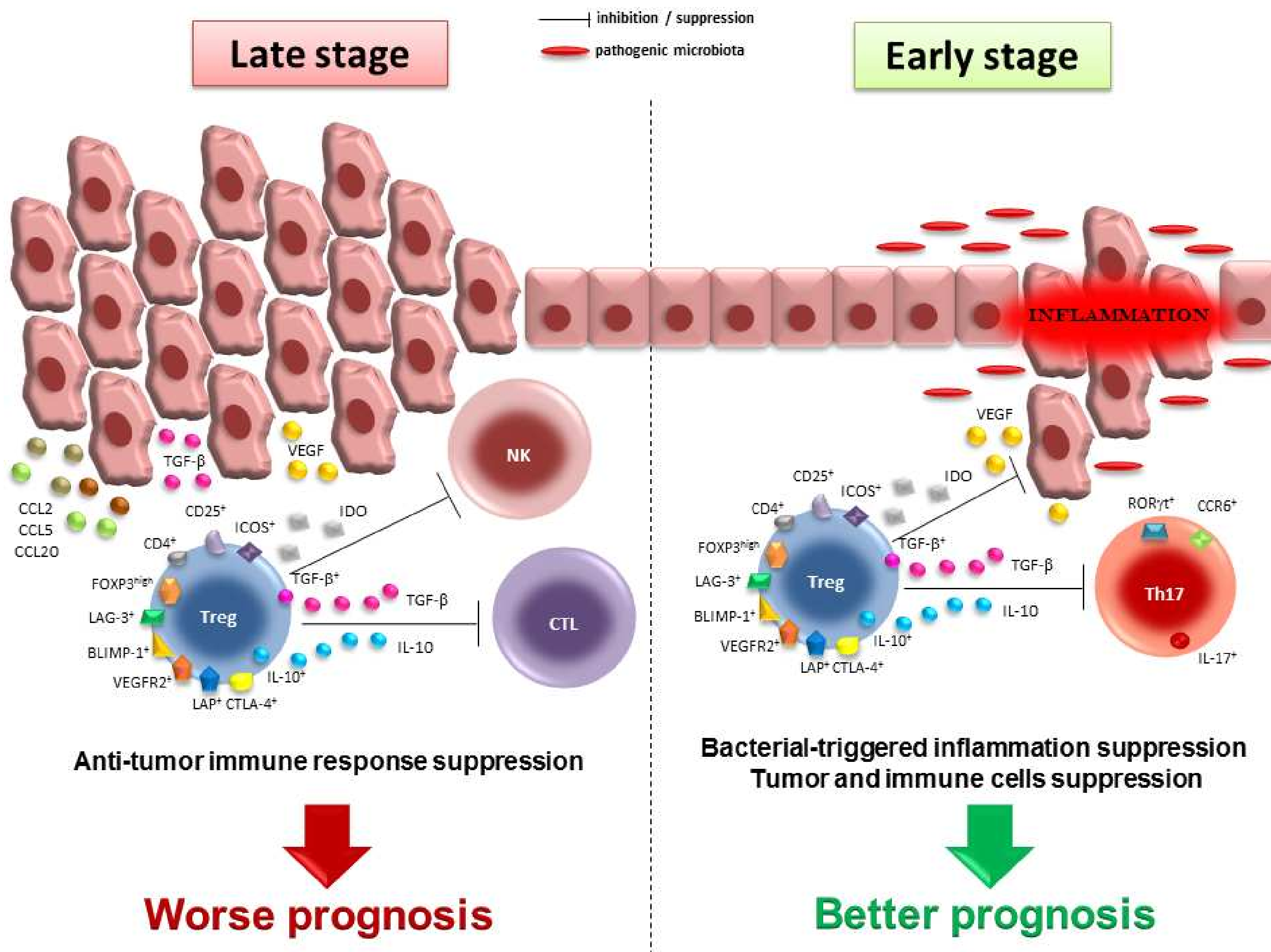

Figure 1. Proposed mechanism for Treg cells role on early and late stages of CRC and their association with prognosis. During the CRC late stage, tumor factors such as chemokines and cytokines induce Treg cells to upregulate suppressor molecules such as CTLA-4, CD25, LAG-3, VEGFR2, LAP, BLIMP-1, ICOS, TGF-β and IL-10. These Treg cells changes play an anti-tumor immune response suppression inhibiting T lymphocyte and NK cells immune response. Such events are related to worse prognosis. In contrast, during the CRC early stage, pro-inflammatory response triggered by intestinal microbiota induces Treg cells to upregulate suppressor molecules, which in turn inhibiting T lymphocyte response, such as Th17 lymphocyte, and tumor cell activity. Bacterial-triggered inflammation suppression by Treg cells decreases the mutagenic epigenetic stimuli which could lead to initiation and progression of sporadic CRC. Further, Treg cells may prevent or reduce local tumor growth and in nearby regions such as lymph nodes and adjacent tissues. Such events are related to better prognosis of the disease.

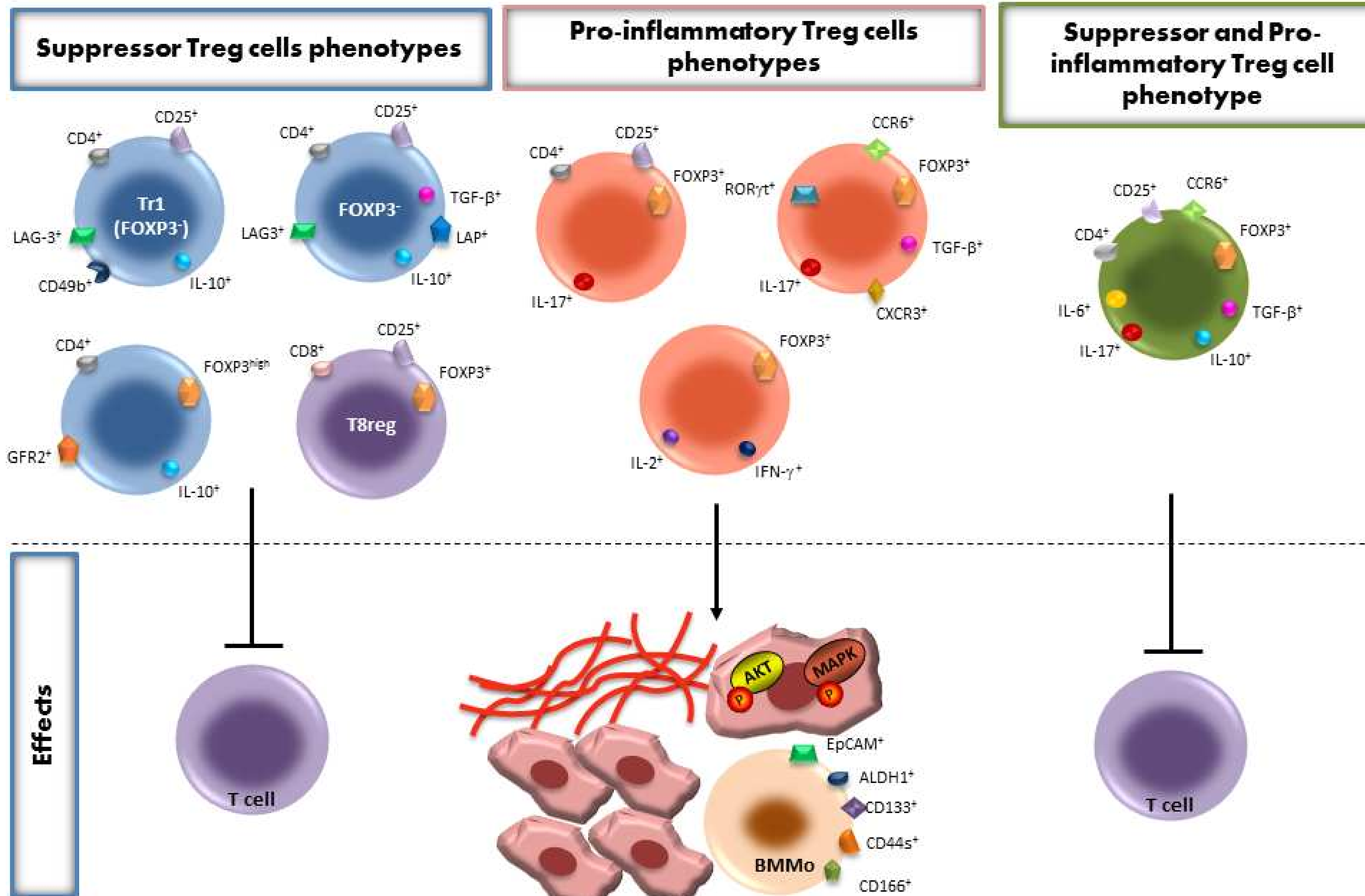

Figure 2. Treg cells phenotypes with either suppressor or pro-inflammatory role on CRC. Treg cells exhibiting suppressor phenotype may express or not FOXP3 and promote the maintenance of immune tolerance, besides inhibiting T cell activity and proliferation. Another Treg cells type, subset $CD8^+$, demonstrates suppressive influence over T cells. Treg cells with pro-inflammatory phenotype may express IL-17 and secrete pro-inflammatory cytokines (IL-2 and IFN-γ). They are capable to induce CRC-associated cell markers, such as CD133, CD44s, CD166, epithelial cell adhesion molecule (EpCAM), and aldehyde dehydrogenase 1 (ALDH1) in bone marrow-derived mononuclear cells (BMMo), which stimulates those cells to be cancer-initiating cells. $FOXP3^+IL17^+$ cells have the ability to increase phosphorylated Akt and MAPK in cancer cells and induce colony formation, thus increasing cell survival, proliferation, invasion, and angiogenesis. Some pro-inflammatory phenotypes are not always related to pro-inflammatory activity. $FOXP3^+IL17^+CD4^+CD25^+CCR6^+TGF\text{-}\beta^+IL\text{-}10^+IL\text{-}6^+$ cells, for instance, may suppress tumor-specific $CD8^+$ T cells and have no correlation to cancer stage.

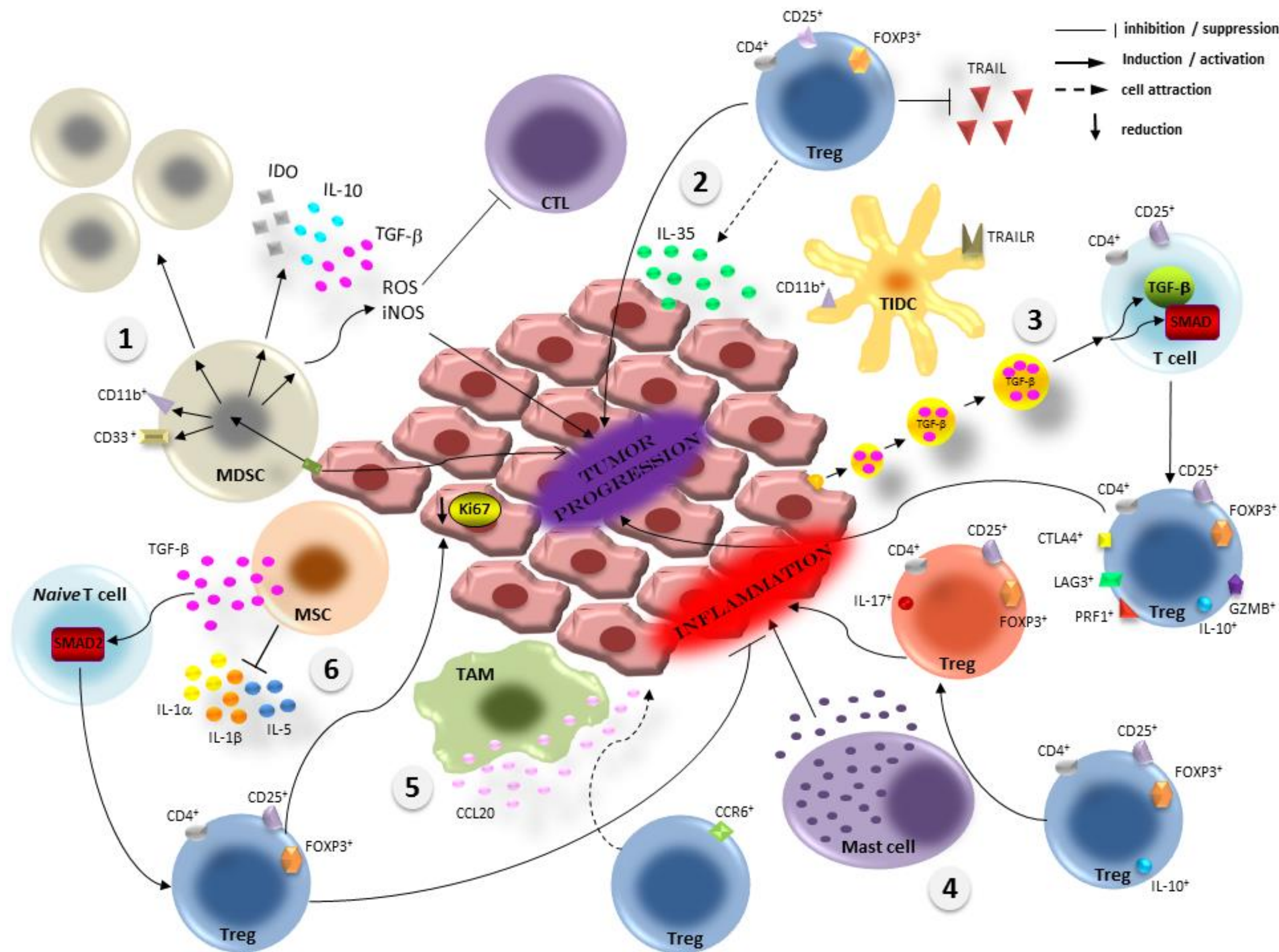

Figure 3. The crosstalk between immune system and cancer cells modulates Treg cells on colorectal cancer. 1) Tumor-induced myeloid-derived suppressor cells (MDSCs) promote CRC cell growth via direct interaction with tumor cells (cell-to-cell contact) and suppress T cell proliferation through oxidative metabolism (NO and ROS generation). As a result, MDSCs are expanded and hence express higher levels of immune inhibitory molecules, such as TGF-β, IDO, IL-10, and iNOS. 2) IL-35 released by CRC cells may recruit Treg cells into the tumor microenvironment and promote CRC progression. Treg cells inhibit the anti-tumor immune response and the death of tumor cells through inhibition of TNF-related apoptosis-inducing ligand (TRAIL) on CD11b$^+$ tumor-infiltrating dendritic cells (TIDCs). 3) CRC cell-derived extracellular vesicles (CRC-EVs) enriched with TGF-β1 induce phenotypic alteration of T cells into Treg-like cells (FOXP3, CTLA-4, LAG3, IL-10, PRF1 and GZMB) through activation of the TGF-β/SMAD signaling. CRC-EVs-induced-Treg-like cells have remarkable tumor-growth promoting activity. 4) Mast cells (MC) induce Treg cells to switch function, shut down IL10, and express IL17, which leads to inflammation in CRC without losing T-cell–suppressive properties, besides promoting MC expansion and degranulation. 5) Tumor-associated macrophages (TAMs) may recruit CCR6$^+$ Treg cells to tumor mass and promote its development via enhancing the production of CCL20. 6) Mesenchymal stem cells (MSCs) inhibit inflammatory cytokines (IL-1α, IL-1β, IL-5, IL-6 and IL-12) and secrete TGF-β to induce differentiation of Treg cells via SMAD2, thus suppressing inflammation and decreasing Ki-67 expression on CRC cells.

Table 1. Main findings related to the prognostic value of Treg cells density in a poor outcome of human CRC.

CRC stage / Sample	Tissue	Methods of Treg cells detection	Phenotype of Treg cells	Main Findings	Prognostic value of Treg cells density	Reference
CRC TNM* stage (I – III) / n=12	Peripheral blood; lymph nodes	FC and ELISPOT assays	$CD4^+CD25^+FOXP3^+$	↑ Treg cells on peripheral blood and mesenteric lymph nodes; Treg cells depletion → ↑ $CD4^+$ T cell response; ↑ IFN-γ.	Treg cells inhibit TAA-specific immune response.	38
CRC AJCC stage (I - IV) / n=34	TDLNs; peripheral blood; tumor tissue	FC	$CD4^+CD25^+FOXP3^+$	↑ $FOXP3^+$ Treg cells in TDLNs → ↑ disease stage, ↓ $CD8^+$ T cells function; Treg cells depletion → ↑ $CD8^+$ T cell response.	$FOXP3^+$ Treg cells in TDLNs correlate with disease progression.	13
Metastatic CRC / n=18	Peripheral blood	FC	$CD4^+CD25^+CD127^{dim/-}$ cells	Treg cells → ↑ suppression of anti-tumor immune responses (*in vitro*) prior resection of hepatic tissue metastatic → recurrent disease; ↑ Treg cells-mediated suppression → ↑ recurrent disease.	Treg cells-mediated suppression may influence in recurrence disease.	17
CRC TNM* stage (II - III) / n=62	Peripheral blood; colonic tissue	IHC and FC	$CD4^+$ $FOXP3^+$ and $CD3^+CD4^+CD25^+FOXP3^+$	↑ FOXP3 → inhibition of anti-tumor immune response; Inhibition of anti-tumor immune responses prior to colon resection → tumor recurrence; Post to tumor resection → normal FOXP3 levels.	Treg cells contribute to disease progression.	12
CRC TNM* stage (I - IV) / n=50	Tumor tissue	FC	$CD4^+CD25^+FOXP3^+$	↑ IL-35 → ↑ Treg cells induction → CRC progression; ↑ Treg cells → poorly differentiated tumor and stage IV; ↑ IL-35 → promotes CRC progression and poor	Treg cells were associated with CRC progression and poor prognosis.	16

				prognostic; Post to tumor resection → ↓ IL-35.		
Metastatic CRC / n=188	Hepatic tissue metastatic	TMA and IHC	$FOXP3^+$ alone**	↑ FOXP3:CD4 and FOXP3:CD8 ratios → ↓ OS; ↑ intratumoral Treg → ↑ clinical risk score; ↑ CD4 T cell → ↑ OS and recurrence-free survival; ↑ CD8:CD3 ratio → ↑ OS.	↑ Treg cells predicts poor outcome.	14
CRC Dukes staging (A - D) (UICC) / n=63	Tumor tissue; peripheral blood	IHC and FC	$FOXP3^+$ alone** and $CD4^+CD25^+FOXP3^+$	↑ FOXP3 in CRC tissues and peripheral blood; ↑ $FOXP3^+$ → ↑ gender, ↑ stage and ↑ lymph node metastasis.	↑ FOXP3 expression correlates with tumor malignancy.	15

Abbreviations: ↑ higher; ↓ lower; AJCC, American Joint Committee on Cancer; CRC, colorectal cancer; FC, Flow cytometry; IHC, Immunohistochemistry; OS, Overall survival; PGE_2, prostaglandin E_2; TAA, tumor-associated antigens; TDLNs, Tumor draining lymph nodes; TIL, tumor-infiltrating lymphocyte; TMA, tissue microarrays.

* TNM Staging System is based on the extent of the tumor (T), the extent of spread to the lymph nodes (N), and the presence of metastasis (M). TNM was developed and is maintained by the AJCC and the Union for International Cancer Control (UICC).

** Labeling using only FOXP3 as a way to characterize Treg cells.

Table 2. Main findings related to the prognostic value of Treg cells density in improved outcome of human CRC.

CRC stage / Sample	Tissue	Methods of Treg cells detection	Phenotype of Treg cells	Main Findings	Prognostic value of Treg density	Reference
CRC AJCC stage (II - III) / n=967	Tumor tissue	TMA and IHC	$FOXP3^+$ alone**	↑ $FOXP3^+$ Treg cells in tumor tissue → improved survival; ↑ $FOXP3^+$ Treg cells in normal mucosa → worse prognosis.	↑ $FOXP3^+$ Treg cells in CRC was associated with survival.	28
CRC (MMR proficient / deficient) / n=1420	Tumor tissue	TMA and IHC	$FOXP3^+$ alone**	↑ $FOXP3^+$ Treg cells in MMR-proficient → early T stage prevalence, ↑ 5-years survival rate; ↑ $FOXP3^+$ Treg cells in MMR-deficient → absence lymph node involvement, absence of vascular invasion, ↑ 5-years survival rate.	↑ $FOXP3^+$ Treg cells in MMR-proficient CRC predicts improved disease-specific survival.	30
CRC AJCC stage II / n=87	Tumor tissue	IHC	$FOXP3^+$ alone**	Intraepithelial $CD3^+$, $CD45RO^+$, $CD25^+$, and $FOXP3^+$ TILs → ↑ DFS; ↑ $CD45RO^+$ and FOXP3+ → 100% of OS in 5-years.	$FOXP3^+$ TILs predict survival in the early CRC stage.	7
CRC AJCC stage (II - III) / n=1000	Tumor tissue	TMA and IHC	$FOXP3^+$ alone**	↑ SPARC and FOXP3 in CRC; ↑ SPARC and ↑ FOXP3 in stage II CRC → good disease outcome and better long-term CSS; ↑ FOXP3 in stage II CRC → ↓ adjuvant chemotherapy; ↑ CD8 and CD45RO in stage II CRC → good disease	FOXP3 associates with better disease outcome in stage II CRC and predicts survival.	31

				outcome and better CSS.		
CRC UICC (I - IV) / n=88	Tumor tissue	IHC and IF	$CD3^+FOXP3^+$ and $CD3^+FOXP3^+$ $VEGFR2^+$	↓ $FOXP3^+VEGFR2^+$ → ↓ DFS and OS; $FOXP3^+$ cell and $FOXP3^+VEGFR2^-$ cell → do not correlate with DFS or OS.	$FOXP3^+VEGFR2^+$ cells, better than $FOXP3^+$ alone, predict good prognosis and survival.	32
CRC TNM* stage (I - IV) / n=108	Tumor tissue	IHC	$FOXP3^+$ alone** and $CD49b^+LAG\text{-}3^+$ (Tr1 cells)	Tumor tissue → ↓ T-bet$^+$, ↑ IL-17$^+$, ↑ $FOXP3^+$, ↑ $CD49b^+$, and ↑ LAG-3$^+$ cells; ↑ $CD49b^+$ and LAG-3$^+$ (Tr1 cells) and ↑ IL-17$^+$ cells → disease progression; $FOXP3^+$ Treg cells and Th1→ do not correlate with clinic-pathological characteristics and survival.	Tr1 cells, but not $FOXP3^+$ Treg cells, predict a poor prognosis.	33
CRC stage (I - IV) / n=426	Tumor tissue	IHC	$FOXP3^+$ alone**	↑ intraepithelial $CD8^+$ → better outcome; ↑ $FOXP3^+$ cells in tumor invasive front and center → better prognosis.	↑ $FOXP3^+$ predicts a better prognosis.	34
CRC UICC stage II / n=820	Tumor tissue	TMA and IHC	$FOXP3^+$ alone**	↑FOXP3 Treg cells, ↓ tumor stage, absence of lympho-vascular tumor invasion → good prognostic; $FOXP3^+$ Treg immunostaining → good prognostic biomarker in stage II CRC.	↑$FOXP3^+$ predicts improved survival in stage II.	35
Metastatic CRC / n=162	Hepatic tissue metastatic	IHC	$FOXP3^+$ alone**	↓ peritumoral Treg cells→ ↓ CSS in patients undergoing hepatic tissue resection; Intratumoral Treg cells→ do not correlate with CSS.	↓ peritumoral Treg cells is an unfavorable survival predictor.	36

Abbreviations: ↑ higher; ↓ lower; AJCC, American Joint Committee on Cancer; CSS, cancer specific survival; DFS, disease-free survival; IHC, Immunohistochemistry; MMR, mismatch repair; OS, overall survival; SPARC, Secreted Protein Acidic and Rich in Cysteine; TMA, tissue microarrays; Tr1, type 1 T regulatory; UICC, International Union against Cancer; VEGF, vascular endothelial growth factor.
* TNM Staging System is based on the extent of the tumor (T), the extent of spread to the lymph nodes (N), and the presence of metastasis (M). TNM was developed and is maintained by the AJCC and the Union for International Cancer Control (UICC).
** Labeling using only FOXP3 as a way to characterize Treg cells.

Table 3. Main findings related to the prognostic value of Treg cells density in absence of association in outcome of human CRC.

CRC stage / Sample	Tissue	Methods of Treg cells detection	Phenotype of Treg cells	Main Findings	Prognostic value of Treg cells density	Reference
CRC UICC stage (I - IV) / n=40	Tumor tissue	IHC	$CD3^+FOXP3^+$	↑ Treg cells in stromal tissue and in limited disease compared to metastatic CRC; Survival → did not differ with Treg cells infiltration.	Treg cells infiltration do not correlate with survival.	40
CRC TNM* stage (II - III) / n=160	Tumor tissue	IHC and IF	$FOXP3^+$ alone** and $CD4^+FOXP3^+$	↑ Intraepithelial $FOXP3^+$ → poor tumor differentiation; ↓ Intraepithelial $CD3^+/FOXP3^+$ cell ratio and ↓ $CD3^+$ T cells → ↓ DFS; $FOXP3^+$ cell → do not correlate with prognostic.	$FOXP3^+$ TILs density do not predict clinical outcome alone.	66
CRC TNM* stage (I - IV) / n=94	Tumor tissue	IHC	$FOXP3^+$ alone**	↑ $FOXP3^+$ cells → ↑ lymph node metastasis, ↑ TGF-β; $FOXP3^+$ cells → do not correlate with survival; ↑ $CD8^+$ T/$FOXP3^+$ cell ratio → ↑ DFS and OS.	$FOXP3^+$ cells alone do not correlate with survival.	67
CRC UICC stage (I - IV) / n=65	Tumor tissue	FC, IHC, IF and RT-qPCR	$FOXP3^+$ alone**, $CD4^+FOXP3^+$, $CD4^+CD25^+FOXP3^+$, $FOXP3^+$ $L\text{-}10^+$, $FOXP3^+$ $TGF\text{-}\beta^+$	↑ FOXP3 in cancer cells → poor prognosis; FOXP3 in Treg cells → do not correlate with survival.	FOXP3 expression in cancer cells, but not in Treg cells, contributes to disease progression.	95

Abbreviations: ↑ higher; ↓ lower; DFS, disease-free survival; FC, Flow cytometry; IF, immunofluorescence; IHC, Immunohistochemistry; OS, overall survival; RT-qPCR, Real-time quantitative reverse transcription-PCR; UICC, International Union against Cancer.
* TNM Staging System is based on the extent of the tumor (T), the extent of spread to the lymph nodes (N), and the presence of metastasis (M). TNM was developed and is maintained by the AJCC and the Union for International Cancer Control (UICC).
** Labeling using only FOXP3 as a way to characterize Treg cells.

ARTIGO 2 (Original - Publicado)

Yacon (*Smallanthus sonchifolius*)-based product increases fecal short-chain fatty acids and enhances regulatory T cells by downregulating RORγt in the colon of BALB/c mice

Journal of Functional Foods 55 (2019) 333–342

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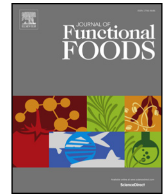

Yacon (*Smallanthus sonchifolius*)-based product increases fecal short-chain fatty acids and enhances regulatory T cells by downregulating *RORγt* in the colon of BALB/c mice

Letícia De Nadai Marcon[a,*], Luís Fernando de Sousa Moraes[a], Bruna Cristina dos Santos Cruz[a], Michelle Dias de Oliveira Teixeira[b], Thais Calcagno Vidon Bruno[a], Isabela Egídio Ribeiro[a], Anny Caroline Messias[a], Célia Lúcia de Luces Fortes Ferreira[c], Leandro Licursi de Oliveira[b], Maria do Carmo Gouveia Peluzio[a]

[a] *Nutritional Biochemistry Laboratory, Department of Nutrition and Health, Universidade Federal de Viçosa – UFV, Viçosa, Minas Gerais, Brazil*
[b] *Immunochemistry and Glycobiology Laboratory, Department of General Biology, Universidade Federal de Viçosa – UFV, Viçosa, Minas Gerais, Brazil*
[c] *Institute of Biotechnology Applied to Agriculture & Livestock (Bioagro), Laboratory of Lactic Starter Cultures, Universidade Federal de Viçosa – UFV, Viçosa, Minas Gerais, Brazil*

ARTICLE INFO

Keywords:
Yacon
Fructooligosaccharides
Prebiotic
Treg cell
Short-chain fatty acids
RORγt

ABSTRACT

Although yacon (*Smallanthus sonchifolius*) is a known source of prebiotics (fructooligosaccharides (FOS) and inulin) and phenolic compounds beneficial to gut microbiota and intestinal immune response modulation, its regulatory mechanisms still remain unclear. Therefore, we investigated whether the consumption of a yacon-based product (PBY) modulates the population of intestinal lymphocytes as well as transcription factors that drive host adaptive immune responses. For this purpose, BALB/c male mice were fed either a standard AIN-93M diet or AIN-93M diet supplemented with PBY (6.0% FOS + Inulin) for 8 weeks. We found that PBY consumption in mice reduced food intake, improved fecal humidity and viscosity, intensified fecal short-chain fatty acid production, increased the number of regulatory T cells, and downregulated the expression of RORγt transcription factor in the colon. Thus, it can be inferred from the findings that PBY consumption improves satiety and mucosal integrity, and possibly favors anti-inflammatory immune responses in the colon.

1. Introduction

Yacon (*Smallanthus sonchifolius*) is a tuberous root rich in phenolic compounds and considered prebiotic due to its high fructooligosaccharides (FOS) and inulin content (Choque Delgado, Thomé, Gabriel, Tamashiro, & Pastore, 2012; Russo, Valentão, Andrade, Fernandez, & Milella, 2015). FOS and inulin are fructose oligomers composed mainly of α (2 → 1) or β (2 → 6) linkages which are not hydrolyzed by human digestive enzymes thus remain intact in the colon (Fernández et al., 2013). As a result, these fructans serve as fermentable substrates for probiotic bacteria (Gibson & Roberfroid, 1995).

The consumption of yacon has been reported in the literature to increase the number of *Bifidobacteria* and *Lactobacilli* (de Souza Lima Sant'Anna, Rodrigues, Araújo, de Oliveirado Carmo Gouveia Peluzio, & de Luces Fortes Ferreira, 2015; Utami et al., 2013) and concurrently inhibit the growth of pathogenic bacteria (Sant'Anna et al., 2018; Veiga et al., 2014). Interestingly, the number of probiotic bacteria directly reflects the production of fecal short-chain fatty acids (SCFA) (Grancieri et al., 2017; Sant'Anna et al., 2018; Utami et al., 2013; Vaz-Tostes et al., 2014). Yacon consumption also improves intestinal transit (de Souza Lima Sant'Anna et al., 2015; Geyer, Manrique, Degen, & Beglinger, 2008; Sant'Anna et al., 2018), satiety and body weight (Genta et al., 2009; Gomes da Silva et al., 2017). In addition, it confers antiobesity properties by inhibiting adipogenesis (Honoré, Grande, Gomez Rojas, & Sánchez, 2018). Other metabolic benefits have been commonly reported such as glycemia control (Honoré et al., 2018; Scheid, Genaro, Moreno, & Pastore, 2014), decrease in total cholesterol and triglycerides levels, as well as improvement in high-density lipoprotein-

Abbreviations: PBY, yacon-based product; FOS, fructooligosaccharides; SCFA, short-chain fatty acids; RORγt, Retinoic acid receptor (RAR)-related orphan receptor gamma

* Corresponding author at: Nutritional Biochemistry Laboratory, Department of Nutrition and Health of Universidade Federal de Viçosa – UFV, Av. P.H. Rolfs, s/n. 36570-900, Viçosa, Minas Gerais, Brazil.
E-mail address: leticiadenadai@gmail.com (L.D.N. Marcon).

https://doi.org/10.1016/j.jff.2019.02.039
Received 12 November 2018; Received in revised form 20 February 2019; Accepted 21 February 2019
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cholesterol levels (Habib, Honoré, Genta, & Sánchez, 2011; Habib, Serra-Barcellona, Honoré, Genta, & Sánchez, 2015; Roselino et al., 2012).

Yacon consumption is capable of improving intestinal architecture and cellularity despite regulating the number and depth of intestinal crypts (Lobo, Colli, Alvares, & Filisetti, 2007; Sant'Anna et al., 2018), which in turn increases the intestinal absorption of calcium, magnesium and iron, and calcium deposition in bones (Lobo et al., 2007; Lobo, Gaievski, De Carli, Alvares, & Colli, 2014; Rodrigues et al., 2012). Regarding immunological benefits, yacon intake increases secretory immunoglobulin A (IgA) production in feces (Choque Delgado et al., 2012; Grancieri et al., 2016), serum cytokines related to anti-inflammatory processes, such as interleukin (IL)-10 and IL-4 (Bonet et al., 2010; Grancieri et al., 2017; Vaz-Tostes et al., 2014), and phagocytic activity of macrophages (Paredes et al., 2018). Furthermore, it has been reported that yacon consumption promotes the regression of pre-neoplastic lesions in experimental models of colorectal carcinogenesis (de Moura et al., 2012; Grancieri et al., 2017), and it presents a high in vivo antioxidant activity (Habib et al., 2015).

Evidently, the health benefits of yacon consumption are mainly attributed to the modulation of the intestinal microbiota (de Souza Lima Sant'Anna et al., 2015; Utami et al., 2013) and intestinal immune response (Bonet et al., 2010), however the regulatory mechanisms involved in these events remain unclear and unexplored. Studies have shown that probiotic bacteria may induce anti-inflammatory responses through the activation of regulatory T cells (Treg cells) (Roselli et al., 2009) and the attenuation of a pro-inflammatory microenvironment through reduced IL-17 and IL-23 production (Ghadimi, Helwig, Schrezenmeir, Heller, & de Vrese, 2012; Ichiyama et al., 2008; Tanabe, 2013).

Treg cells play an important role in immune response regulation owing to the release of cytokines and immune modulating factors that prevent exacerbated and auto immune responses (Josefowicz, Lu, & Rudensky, 2012). Alternatively, T-helper-17 (Th17) cells seem to release pro-inflammatory cytokines, such as IL-17A, IL-17F, IL-21, IL-22, tumor necrosis factor α (TNF-α), and IL-6, associated with inflammatory bowel disease and colorectal cancer progression (Cătană et al., 2015; De Simone et al., 2015). The immune cell phenotype is targeted by specific transcription factors such as FOXP3, the key transcription factor for Treg cells differentiation and RORγT, the crucial Th17-lineage transcription factor. Moreover, two important adaptive immunity pathways are generated according to specific transcription factors: T-bet, a hallmark of the Th1 cell lineage development, is responsible for interferon-gamma regulation (IFN-γ), IL-2, and TNF-α genes (Szabo et al., 2000); and GATA-3, a Th2 cell lineage-promoter factor, produces cytokines, such as IL-4 e IL-5, IL-6, IL-9 and IL-13 (Szabo et al., 2000; Zhang, Cohn, Ray, Bottomly, & Ray, 1997; Zheng & Flavell, 1997).

It is hypothesized that the consumption of PBY, a source of FOS and inulin, is able to modulate the intestinal microbiota of mice and consequently increase the production of SCFA in feces. In addition, we speculate that the modulation of the microbiota and the fermentation products generated by the consumption of PBY could alter intestinal immune response pathways, favoring an increase in the number of Treg cells and attenuating the expression of immune response transcription factors responsible for the activation of pro-inflammatory responses. Given the above, we aimed to investigate the immunomodulatory response triggered by PBY consumption in BALB/c male mice.

2. Material and methods

2.1. Animals and experimental design

Thirty-four BALB/c male mice were obtained from the Central Bioterium (Health and Biology Science Center) of Universidade Federal de Viçosa, Brazil. The animals were housed at the Experimental Nutrition lab in a temperature-controlled room (22 ± 2 °C) with a 12-hour light/dark cycle and had *ad libitum* access to water and food. The animal protocol was approved by the Ethics Committee on Animal Experimentation of Universidade Federal de Viçosa, Brazil, under the process number 30/2016. Upon arrival, the animals were randomly assigned to two experimental groups: CC, control diet (n = 16), and CY, the group fed diet supplemented with PBY (n = 18) (Table 1). The diets were offered *ad libitum* for 8 weeks. Subsequently, the mice were anesthetized using 3% isoflurane and blood samples were collected from the *retro*-orbital sinus. The mice were euthanized by cervical dislocation and their tissues and feces were harvested for analysis.

Table 1
Composition of AIN-93 M diet for control and PBY diet.

Ingredients (g/100 g)	Control diet (g/kg)	PBY diet (g/kg)
Casein	155.5	145.3
Dextrinized starch	155	148.2
Sucrose	100	0
Soybean oil	40	40
Fiber (microfine cellulose)	67	0
Mineral mix	35	35
Vitamin mix	10	10
L-Cystine	1.8	1.8
Choline bitartrate	2.5	2.5
Cornstarch	303	263.6
*PBY	0	353.6
Distilled water	130.2	0
Total weight (g)	1000	1000
Total energy (kcal)	3214	3064

* Centesimal composition and digestible content of carbohydrate, inulin and FOS on PBY in 100 g of product: Fructose: 15.25 g; Glucose: 8.59 g; Sucrose: 6.35 g; FOS: 12.81 g; Inulin: 4.16 g; Total carbohydrate: 45.49 g; Fibers: 1.99 g; Humidity: 36.82 g; Ashes: 3.39 g; Lipids: 0.21 g; Protein: 2.89 g. PBY, yacon-based product.

2.2. Yacon-based product and experimental diet

Yacon was purchased from a local market in Viçosa – Minas Gerais, Brazil. The yacon-based product was processed according to the methodology proposed by Rodrigues et al. (2012) currently undergoing a patent application process (PI 1106621-0). The chemical composition of PBY (carbohydrates, proteins, fats, fiber, ash and humidity) was determined according to the AOAC methodology (AOAC, 1997). FOS and inulin content of the PBY diet was determined by High Performance Liquid Chromatography (HPLC) using a BIO-RAD brand HPX-87p column (lead stationary phase) whose mobile phase is purified water.

The experimental purified diets were based on the AIN93-M diet, as recommended by the American Institute of Nutrition (Reeves, Nielsen, & Fahey, 1993). PBY diet was supplemented with 6.0% FOS + Inulin from PBY (Table 1), as suggested by Paula et al. (2012). Casein, sucrose, dextrinized starch, starch, and fiber in the control and PBY diets were adjusted, aiming to obtain similar amounts of carbohydrates, lipids, proteins, fibers and calories. The diets were made in pellets and stored at −20 °C for a maximum period of thirty days before consumption.

The human equivalent amount of dietary PBY consumed by the BALB/c male mice was calculated using the body surface area normalization method as previously described (Reagan-Shaw, Nihal, & Ahmad, 2007). PBY supplementation was 353,565 g PBY/kg of diet. In our study, the average daily consumption was 6 g per mouse. This is equivalent to 2121 mg of PBY daily for an adult mouse of 45 g, which approximately corresponds to 47 g PBY/kg body mass/day. Taking into account that the average weight of an adult human is 60 kg, the equivalent average daily consumption per day for humans is 229 g.

2.3. Body weight and dietary intake

To evaluate the weight loss/gain of the animals, individual body

weight was recorded weekly using a digital weighing scale. Dietary intake was determined based on the diet offered (g) minus diet waste. This quantification was done every 3 to 5 days during the 8 week dietary intervention period using a digital weighing scale. The data represent diet consumption per cage (7 to 10 animals/cage). Diet consumption (g) was corrected by humidity loss (fresh diet weight (g)/diet weight (g) in the cage per day).

2.4. Fecal characteristics

Fresh excreted feces were harvested and stored at −80 °C for pH determination. For each animal, an aliquot of feces was diluted in distilled water (1:10), homogenized, and the pH was measured with a duly calibrated digital pH meter (Hexis ultra Basic UB-10®) in a temperature-controlled room for an adequate amount of time for pH stabilization (Bedani et al., 2011).

Fecal humidity was determined using approximately 110 mg of moist feces. The feces were weighed in petri dishes, previously dried in an oven for 24 h at 105 °C. Afterwards, the material (petri dish + feces) was placed in a desiccator until it reached ambient temperature, and was later weighed for the determination of humidity using Eq. (1) (Cecchi, 2007).

$$\text{humidity}(\%) = (\text{Initial Weight}^* - \text{Final Weight}^*) \times 100/(\text{Sample Weight}) \quad (1)$$

*Initial and final weight: weight of the dishes containing the samples, before and after drying, respectively.

In the last week of the experiment, fresh feces were harvested and used for fecal score. The following scale was considered: 1. Firm or normal feces consistency; 2. Viscous non-diarrhea feces; 3. Watery feces characteristic of diarrhea (De Freitas et al., 2006, with modifications).

2.5. Anatomical characteristics

After euthanasia, the organs (liver, spleen, small intestine, cecum, colon, abdominal adipose tissue and kidney) were harvested, washed in phosphate-buffered saline and weighed using a semi-analytical weighing scale. Hepatosomatic index was obtained by dividing liver weight by body weight of the animal. Colon length was measured from the end of the cecum to the end of the rectum on a flat surface using a millimeter ruler.

2.6. Analysis of serum biomarkers

After euthanasia, the blood of the animals was collected from the *retro*-orbital sinus and centrifuged at 1190 × g/10 min/4 °C. The serum markers: total cholesterol, triglycerides, gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST) alanine aminotransferase (ALT), albumin, alkaline phosphatase, creatinine, and urea were assessed by specific colorimetric assays (Bioclin®, Brazil) using a clinical chemistry analyzer BS-200 (Mindray®). The results are expressed as mean ± SEM.

2.7. Fecal SCFA quantification

SCFA quantification was performed according to the method of Smiricky-Tjardes, Grieshop, Flickinger, Bauer, and Fahey (2003) with some modifications. 50 mg of frozen feces, which was previously weighed and thoroughly vortexed with deionized water (950 µL) was used. While being incubated on ice for 30 min, the samples were homogenized every 5 min for 2 min. The samples were centrifuged (10,000*g*, 30 min, 4 °C) three times and the supernatants were collected. The final supernatant from each sample was filtered through a 0.45 µm membrane and transferred to vials. SCFA were measured by high performance liquid chromatography - HPLC (Shimadzu®) using an Aminex HPX 87H column (300 × 7,8 mm, Bio-rad®, Rio de Janeiro, Brazil) at 32 °C with acidified water (0.005 M H2SO4) as eluent at a flow rate of 0.6 mL/minute. The products were detected and quantified by an ultraviolet detector (model SPD-20A VP) at 210 nm. Standard curves of formic, acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic and caproic acids (SUPELCO®) were constructed. The results were expressed as µmol SCFA/g feces.

2.8. Determination of leukocytes by immunophenotyping

Leukocytes were quantified and characterized in the mucosa of the large intestine as previously described (Belkaid, Jouin, & Milon, 1996) with some modifications. The colon was removed and washed in ice-cold PBS (NaCl: 0.85%, NaH_2PO_4: 0.023%, $NaHPO_42H_2O$: 0.15%, pH 7.2), cut into small fragments and incubated in cell culture medium, DMEM, pH 7.2 (Sigma-aldrich ™) for 90 min at 37 °C, the suspension was centrifuged three times at 42*g* for 5 min in order to harvest the supernatant, and lastly at 543*g* for 10 min. After the last centrifugation, the remaining pellet was resuspended with PBS buffer (100 µL, pH 7.2). Cell viability was assessed with Trypan blue exclusion and the cells were counted in a Neubauer chamber. The obtained leukocytes were incubated with the following antibodies, according to manufacturer's instructions: anti-CD4 (PeCy5), anti-CD25 FITC-conjugated, anti-CD196 (anti-CCR6) PE-conjugated, anti-CD49b (anti-PanNK) APC-conjugated, anti-CD8 PECy7-conjugated (Biolegend, San Diego, CA, USA). The leukocytes (1×10^4 events) were acquired (FACSVerse™ and BD FACSuite software; BD Biosciences PharMingen San Jose, CA, USA) according to size (forward scatter) and granularity (side scatter). One or two stains were used to identify TCD4 lymphocytes ($CD4^+$), TCD8 lymphocytes ($CD8^+$), regulatory T cell ($CD4^+CD25^+$), Th17 lymphocytes ($CD196^+$) and *Natural Killer* cells ($CD49b^+$). The results are expressed as mean ± SEM of the percentage of each subpopulation of antibodies specifically colored within the gated cells.

2.9. Real-time PCR

Total RNA was extracted from the whole colon using Trizol reagent (Invitrogen™, Carlsbad, CA, USA) according to manufacturer's instructions. cDNA was synthesized using 5 µg of RNA through an RT reaction in a specific kit (GoScript™ Reverse Transcription System, Promega, Madison, WI, USA). Random primer and RNAse free water were added to the sample and heated for 5 min at 70 °C. Subsequently, a mixture containing the reverse transcriptase dNTPs and ribonuclease inhibitor was added to the sample and heated for 60 min at 37 °C. Real-time PCR quantitative mRNA analyses were performed in an Applied Biosystems® 7500 Real-Time PCR System. Sybr green (Ludwig, Biotec), sense primer, and antisense primer (400 nm/reação), DNase free water and the sample (250 ng cDNA/µl) were added to each reaction. Standard PCR conditions were used during the reading (7500 software V2.3, Applied Biosystems®). The sequences of murine primers (Integrated DNA Technologies®) were as follows: FOXP3, sense: 5′-AGG AGC CGC AAG CTA AAA GC- 3′, antisense: 5′-TGC CTT CGT GCC CAC TGT-3′; RORγt, sense: 5′-GGA GCT CTG CCA GAA TGA CC-3′, antisense: 5′-CAA GGT TCG AAA CAG CTC CAC-3′; Tbet, sense: 5′-AGC AAG GAC GGC GAA TGT T-3′, antisense: 5′-GGG TGG ACA TAT AAG CGG TTC-3′; GATA3, sense: 5′-CAA TCT GAC CGG GCA GGT-3′, antisense: 5′-CAG AGA CGG TTG CTC TTC CG-3′; GAPDH, sense: 5′-TCA ACA GCA ACT CCC ACT CTT CCA-3′, antisense: 5′-ACC CTG TTG CTG TAG CCG TAT TCA-3′.mRNA values were calculated according to the constitutive GAPDH gene on the basis of the ΔΔCt algorithm.

2.10. Statistical analysis

The results were expressed as mean ± SEM. The data were analyzed using GraphPad Prism (version 6.0). The means were evaluated by the Kolmogorov-Smirnov normality test, and the groups with a

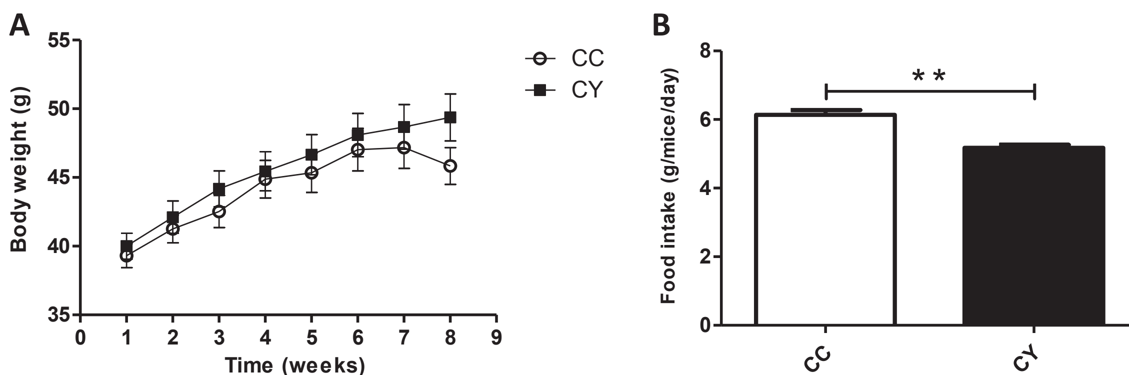
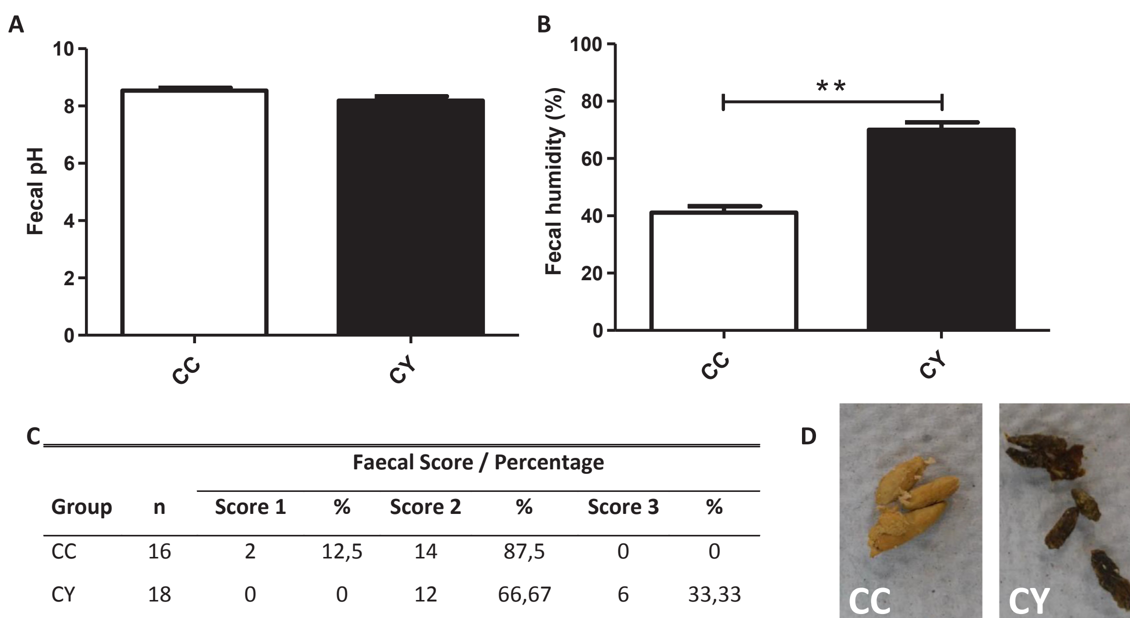


Fig. 1. Effect of PBY diet on weight gain and food intake in BALB/c mice during 8 weeks of dietary supplementation. (A) Average weight gain of animals over the weeks of dietary supplementation. The data are expressed as mean ± SEM (n = 16 to 18 mice/group). (B) Mean food intake (g/mice/day) of PBY diet or control diet consumed by BALB/c. The data are expressed as mean ± SEM of each experimental group (means referring to the group/cage consumption pool (4 pools/week). Statistical difference between groups were analyzed by the unpaired Student's *t*-test or Mann Whitney, (*) p < 0.05, (**) p < 0.001. CC, control diet; CY, PBY diet.



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Group	n	Faecal Score / Percentage					
		Score 1	%	Score 2	%	Score 3	%
CC	16	2	12,5	14	87,5	0	0
CY	18	0	0	12	66,67	6	33,33

Fig. 2. Effect of PBY diet on fecal characteristics in BALB/c mice during 8 weeks of dietary supplementation. (A) Fecal pH; (B) Fecal humidity percentage; (C) Fecal Score (Score 1: normal to firm consistency; Score 2: viscous non-diarrheal consistency; Score 3: watery consistency characteristic of diarrhea). The results of fecal score are represented as number of animals in each score and their percentage into the group (n = 16 to 18/group); (D) Feces color after dehydration. The data of fecal pH and fecal humidity are expressed as mean ± SEM (n = 10 mice/group). Statistical difference between groups were analyzed by the unpaired Student's *t*-test or Mann Whitney, (*) p < 0.05, (**) p < 0.001. CC, control diet; CY, PBY diet.

normal distribution were tested using the unpaired Student's *t*-test. The samples that did not follow a normal distribution were tested by the Mann-Whitney test. Statistical differences were considered for $p < 0.05$ (*) or $p < 0.001$ (**).

3. Results

3.1. PBY diet reduces food intake without weight gain

During the 8 week dietary intervention, there was no difference in the body weight gain of the animals (Fig. 1A). However, food intake was lower in the groups that received the PBY diet ($p < 0{,}001$) (Fig. 1B), suggesting a possible satiety in the animals who received this diet.

3.2. PBY diet increases fecal humidity and viscosity

Fecal characteristics were evaluated in order to verify the possible interferences of PBY diet on feces production and intestinal transit. There was no difference in the fecal pH of the animals treated with the PBY diet when compared to the control diet group (Fig. 2A). However, fecal humidity was higher in animals fed the PBY diet ($p < 0{,}001$) (Fig. 2B). Predominantly, fecal score of the PBY group was classified as stage 2 (viscous non-diarrhea feces), and stage 3 (watery feces characteristic of diarrhea) (Fig. 2C).

3.3. PBY diet increases intestine weight without changing the anatomical characteristics of visceral organs

Colon length and weight were higher in the PBY diet group ($p < 0{,}001$) (Fig. 3A and B). The same was observed for the weight of the cecum ($p < 0{,}001$), and the small intestine ($p < 0{,}05$) (Fig. 3C and D). No differences were found in the weight of the liver, spleen, abdominal adipose tissue and kidneys. The same was observed for the hepatosomatic index (Fig. 3E–I).

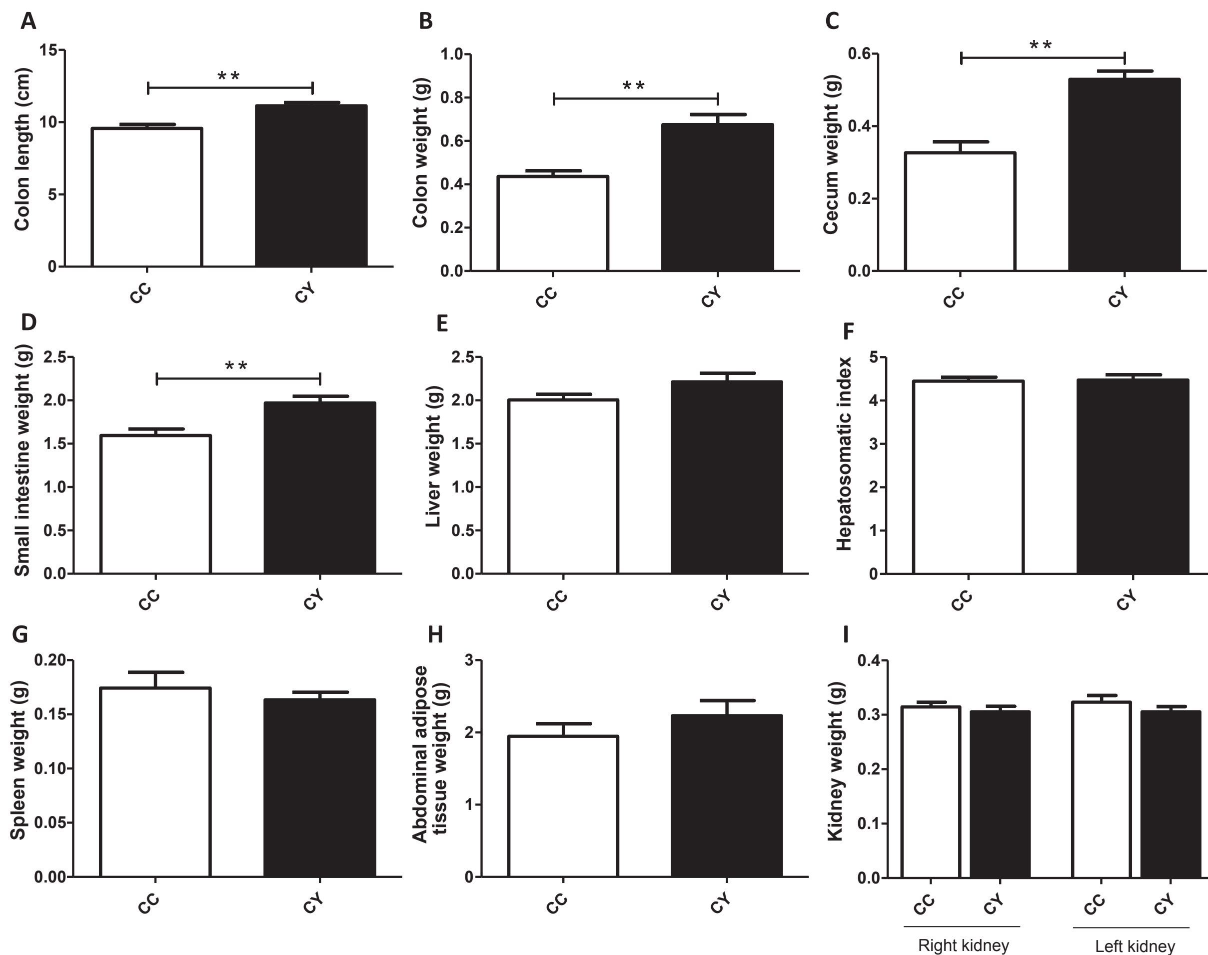

Fig. 3. Effect of PBY diet on the anatomical characteristics of visceral organs of BALB/c mice during 8 weeks of dietary supplementation. (A) colon length (cm); (B) colon weight (g); (C) cecum weight (g); (D) small intestine weight (g); (E) liver weight (g); (F) hepatosomatic index; (G) spleen weight (g); (H) abdominal adipose tissue weight (g); (I) kidney weight (g). The data are expressed as mean ± SEM (n = 16 to 18 mice /group). Statistical difference between groups were analyzed by the unpaired Student's *t*-test or Mann Whitney, (*) $p < 0.05$, (**) $p < 0.001$. CC, control diet; CY, PBY diet.

3.4. PBY diet does not alter the biomarkers of liver function; however, it increases serum triglycerides

Serum biomarkers were evaluated in order to verify alterations in liver and kidney functions, and lipid profile. There were no changes between the groups in relation to serum levels of GGT, AST, ALT, creatinine, alkaline phosphatase, and total cholesterol (Fig. 4). Nonetheless, an increase in triglycerides and albumin was found as well as a reduction in serum urea of the group fed the PBY diet ($p < 0,05$).

3.5. PBY diet increases the fecal short-chain fatty acids (SCFA)

Intestinal bacteria activity was evaluated by fecal SCFA concentration The PBY diet group presented higher levels of acetic ($p < 0,001$), propionic ($p < 0,05$), butyric ($p < 0,001$), isovaleric ($p < 0,05$), valeric ($p < 0,05$) e caproic ($p < 0,05$) fatty-acids compared to the control group (Fig. 5).

3.6. PBY diet increases Treg cell number in the colon

Immunophenotyping of immune cells in the colon was performed to evaluate the profile of intestinal lymphocytes. The analysis showed an increase in the percentage of Treg cells ($CD4^+CD25^+$) in the PBY diet group ($p < 0,05$) compared to the control diet group (Fig. 6). There was no significant difference among the groups in relation to the percentage of $CD4^+$ and $CD8^+$ cells, $CD8^+/CD4^+$ ratio, NK cells, and Th17 lymphocytes.

3.7. PBY diet reduces RORγt expression in the colon

Specific transcription factors of adaptive immune response were evaluated to verify the intestinal immune response pattern. A lower expression of RORγt transcription factor was detected in the colon of animals fed the PBY diet compared to the control diet group ($p < 0,05$) (Fig. 7). There was no significant difference in FOXP3, GATA-3, and T-bet transcription factors in the colon of the mice.

4. Discussion

Yacon has been associated with intestinal microbiota modulation due to its high soluble fiber content, FOS, and inulin. The benefits are related to the increment of *Bifidobacterium* and *Lactobacillus*, and bacterial metabolites, such as SCFA (Caetano et al., 2016). Furthermore, yacon consumption has been demonstrated to attenuate colon cancer in experimental models (de Moura et al., 2012; Grancieri et al., 2017). Although the immunomodulatory role of yacon is suspected to favor increased secretory fecal IgA production (Grancieri et al., 2016), inflammatory cytokine reduction (Choque Delgado et al., 2012), increased phagocytic activity of macrophages (Paredes et al., 2018), as well as possibly decreasing the number of pathogenic bacteria in colon (Sant'Anna et al., 2018), its immune response profile in the intestine is still unknown.

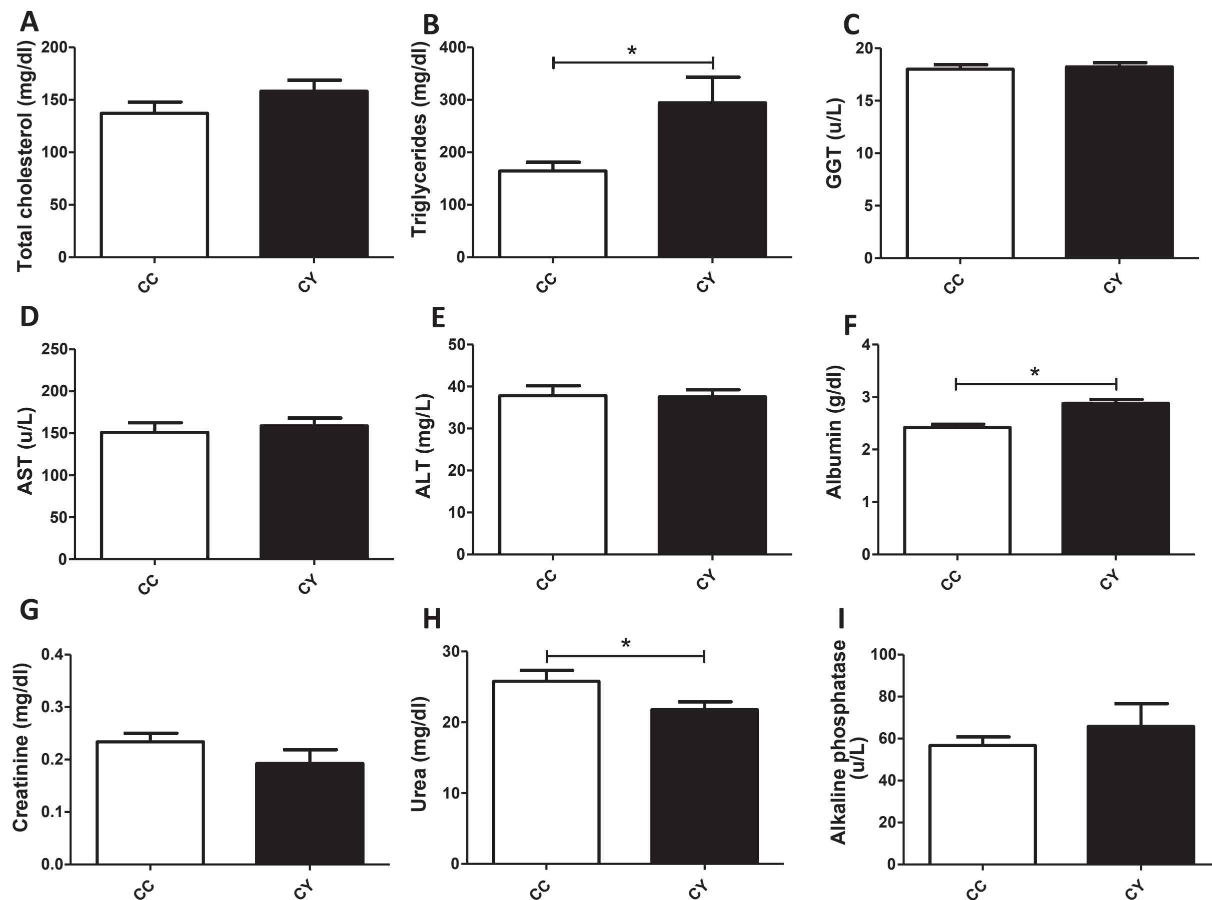

Fig. 4. Effect of PBY diet on serum biomarkers level in BALB/c mice during 8 weeks of dietary supplementation. (A) total cholesterol (mg/dl); (B) triglycerides (mg/dl); (C) GGT (gamma-glutamyl transferase) (u/L); (D) AST (aspartate aminotransferase) (u/L); (E) ALT (alanine aminotransferase) (mg/dl); (F) albumin; (G) creatinine; (H) urea; (I) alkaline phosphatase. The data are expressed as mean ± SEM (n = 10 mice/group). Statistical difference between groups were analyzed by the unpaired Student's *t*-test or Mann Whitney, (*) $p < 0.05$, (**) $p < 0.001$. CC, control diet; CY, PBY diet.

In our study, the diet supplemented with PBY, containing soluble fibers, increased fecal humidity and viscosity, without altering fecal pH. It is known that bacterial fermentation favors the reduction of fecal pH, leading to water retention in the intestinal lumen in order to preserve intraluminal osmotic pressure (Le Blay, Michel, Blottière, & Cherbut, 1999). Furthermore, the osmotic effect provided by the fructans increases the absorption of Ca and Mg due to the optimization of paracellular transport. This transport is favored by increased solubilization of minerals attributed to high amount of fluid in the colon (Bongers & Van den Heuvel, 2003; Lobo et al., 2007). However, we did not observe any reduction in fecal pH, this is probably related to the reabsorption of fatty-acids along the colon, resulting in less acidic feces.

FOS and inulin are initially fermented in the cecum (Roberfroid, 2007), leading to the production of bacterial metabolites, which stimulate cell proliferation. Among these metabolites, butyrate is highlighted, which is the main source of energy for the colonocytes, helping in the maintenance and integrity of the mucosa (Le Blay et al., 1999). The trophic effect in the mucosa, reflects an increase in the weight and length of the intestinal tissue. An increase in the weight of the cecum, colon and small intestine, and the length of the colon was verified in our study, suggesting a potent trophic action caused by the PBY diet, which may be linked to the higher production of fecal SCFA, also observed in our results.

Regarding serum biomarkers, there were no alterations in the levels of GGT, AST, ALT, creatinine, FA, or total cholesterol between the groups. However, higher concentration of TG in the PBY diet group was found. This increase may be attributed to free fructose present in the PBY diet. The increase of TG, total lipids, and low-density lipoproteins in the blood may occur after the consumption of diets with fructose when compared to diets with complex carbohydrates and other sugars since greater activity of lipogenic enzymes in the liver can increase the synthesis of glycerol and fatty-acids in the same (Barreiros, Bossolan, & Trindade, 2005). It is possible that the amounts of fructose ingested by the animals in the PBY group may have deviated their metabolism for lipid synthesis.

The reduction of serum urea in the PBY diet group may be related to the reduced ingestion of proteins, as this group presented a significant reduction in dietary intake. Moreover, fiber in the PBY diet might favor the lower absorption of proteins due to increased intestinal transit. This fact may be confirmed by the feces consistency of the animals fed the PBY diet, classified as viscous - watery feces.

PBY diet also induced an increase in the concentration of acetic, propionic, butyric, isovaleric, valeric, and caproic fatty-acids in the feces of the animals in the PBY diet group. This increase in SCFA may be attributed to the prebiotic role of PBY, capable of stimulating *Lactobacillus* and *Bifidobacterium* growth, and consequently generating these acids as end products of fermentation. According to the literature, the SCFA produced in the largest quantity by the fecal microbiota is acetic acid, followed by propionate, and butyrate (Cummings, Pomare, Branch, Naylor, & Macfarlane, 1987). However, in the present study this trend was only relevant in the feces of animals fed the control diet, while in the PBY diet group, a higher production of butyric acid, followed by acetic, and propionic acid, was verified respectively. Carbohydrates which are not absorbed in the small intestine can be used by the intestinal microbiota to generate acetate, being the main route by which the body obtains energy from undigested carbohydrates

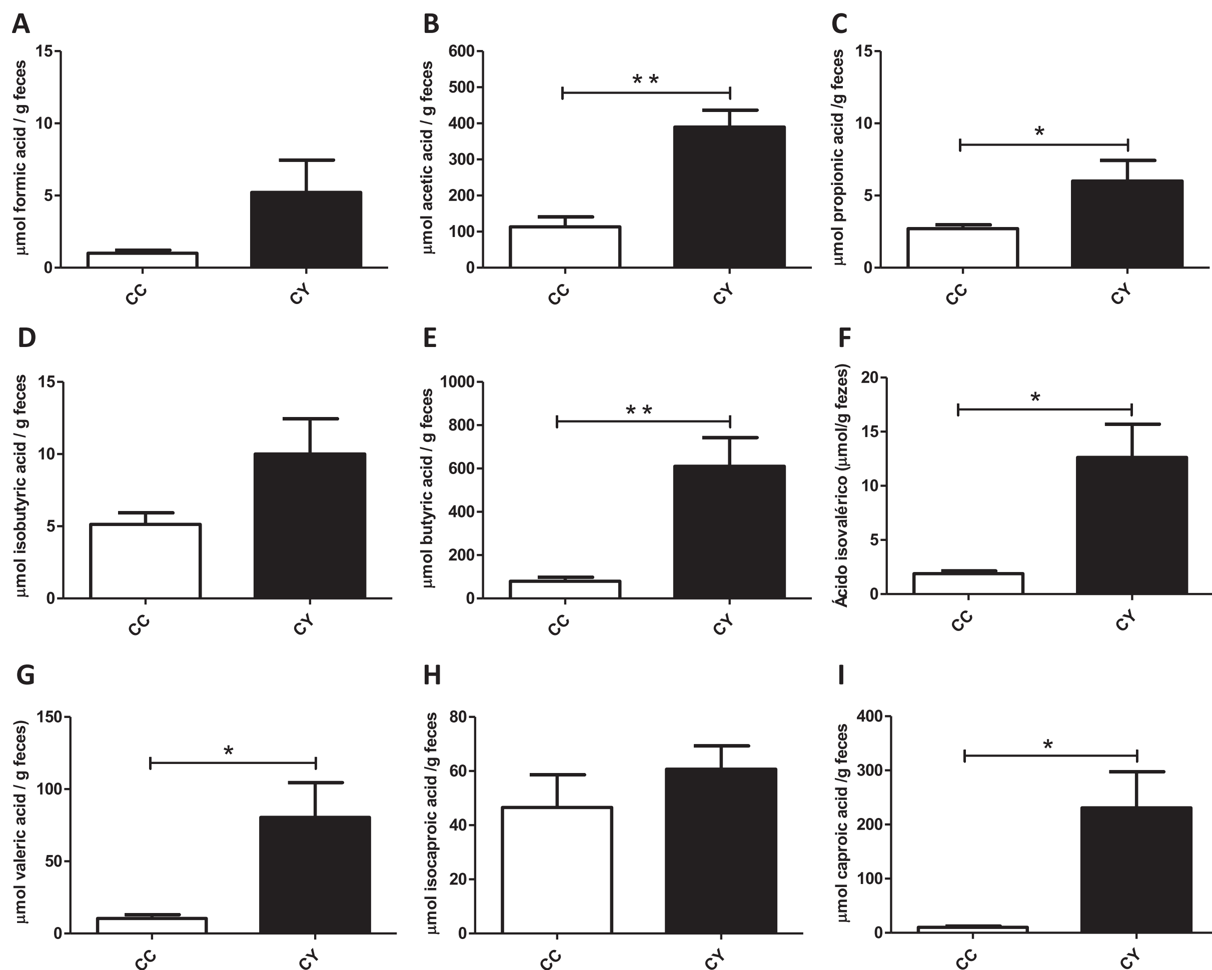

Fig. 5. Effect of PBY diet on fecal SCFA concentration in BALB/c mice during 8 weeks of dietary supplementation. (A) formic acid (μmol); (B) acetic acid (μmol); (C) propionic acid (μmol); (D) isobutyric acid (μmol); (E) butyric acid (μmol); (F) isovaleric acid (μmol); (G) valeric acid (μmol); (H) isocaproic acid (μmol); (I) caproic acid (μmol). The data are expressed as mean ± SEM (n = 4 to 10 mice/group). Statistical difference between groups were analyzed by the unpaired Student's *t*-test or Mann Whitney, (*) $p < 0.05$, (**) $p < 0.001$. CC, control diet; CY, PBY diet.

(Salminen et al., 1998). Almost all the heterotrophic anaerobic eubacteria in the intestine can produce acetate (Flint, 2006). However, butyrate is considered the most significant SCFA in relation to human health, because it may promote a trophic action in the mucosa, and an apoptotic action in cancer cells (Williams, Coxhead, & Mathers, 2003), promoting the reduction of pre-neoplastic lesions. Caproic acid is also capable of reducing the viability of *in vitro* HCT-116 cancer cells to up to 80% (Narayanan, Baskaran, Amalaradjou, & Venkitanarayanan, 2015).

Branched-chain fatty acids (BCFA), such as isobutyrate and isovalerate acids are formed through the fermentation of amino acids, valine and leucine, respectively (Macfarlane & Gibson, 1995). It is worth mentioning that protein fermentation is greater than carbohydrate fermentation in the descending colon, due to the availability of substrate, and increased pH. Therefore, the quantity of BCFA in the descending colon is higher than that of SCFA (Cummings et al., 1987). As observed in our study, PBY diet consumption promoted a significant increase of isovaleric acid, but not significant for isobutyric acid. This fact may be attributed to the possible increase of proteins and amino acids in the colon, which were not absorbed in the small intestine.

Furthermore, SCFA have been demonstrated to influence satiety, due to their interference in the production of leptin, a hormone released by the white adipose tissue, whose signaling pathway defects are associated with severe obesity, hyperphagia, infertility, and immune system disorders (Hoyles & Wallace, 2010). Studies using mice showed that SCFA (C2 – C6), and BCFA (C4 – C6) act in the production of leptin in the adipocytes through the G protein-coupled receptor GPR41, shown to be the agonists of this receptor (Xiong et al., 2004). Thus, the consumption of the PBY diet may be directly related to the increased production of SCFA (acetic (C2), propionic (C3), butyric (C4), isovaleric, and valeric (C5)), which might modulate the production of leptin in the animals.

In this study, a significant increase in the percentage of Treg cells in the colon of animals fed the PBY diet was found, as well as a reduction in *RORγt* expression. Treg cells produce IL-10 as a mechanism to modulate immune response, which promotes inflammatory response control, inhibiting hyperactivation of cells that could cause tissue damage (Laidlaw et al., 2015; Zhu & Paul, 2009). Furthermore, modulation of the immune response by the Treg cells indicates that there are no stimuli for Th17 cells differentiation (Korn, Bettelli, Oukka, & Kuchroo, 2009), as verified in our study. During the early stages of TCD4^{+} cells differentiation there is the expression of both *RORγt and FOXP3* (Ichiyama et al., 2008). However, Treg or Th17 cells differentiation are affected by the amounts of TGF-β and pro-inflammatory cytokine milieu, on one hand, high IL-6 and TGF-β promote *RORγt* expression, and on the other hand, low pro-inflammatory cytokines and high TGF-β, induce *FOXP3* expression (Zhu & Paul, 2009). Therefore, an anti-inflammatory environment promotes an immune response appropriate for Treg cells differentiation.

In the present study, *RORγt* reduction might explain the lack of alteration in the Th17 cells. Despite the increase of Treg cells in the PBY diet group, we did not verify an increase in the FOXP3 transcription

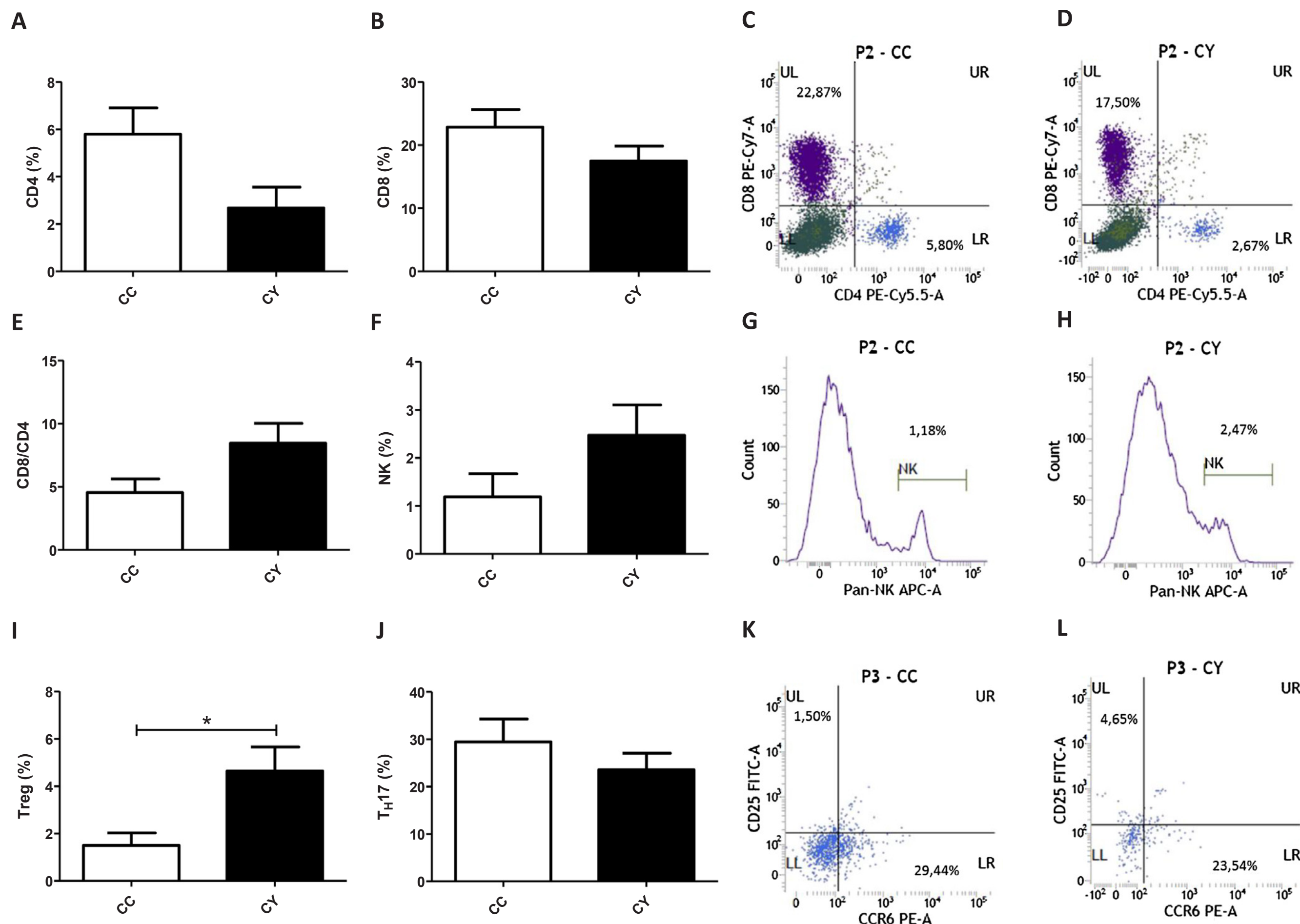

Fig. 6. Effect of PBY diet on the phenotypic profile of lymphocytes in the colon of BALB/c mice during 8 weeks of dietary supplementation. (A) % $CD4^+$ cells; (B) % $CD8^+$ cells; Flow cytometry plots from forward scatter/side scatter-gated lymphocytes cells marked with anti-CD8 (PE-Cy7) on the upper left side quadrants (% CD8 cells) and anti-CD4 (PE-Cy5) on the lower right side quadrants (% CD4 cells) on CC (C) and CY groups (D); (E) % $CD8^+/CD4^+$ ratio; (F) % NK cells (Pan NK^+ cells); Histogram plots of gated lymphocytes cells marked with anti-Pan-NK (APC) on CC (G) and CY groups (H); (I) % Treg cells ($CD4^+CD25^+$ cells); (J) % Th17 cells ($CD4^+CCR6^+$). Flow cytometry plots from gated $CD4^+$ cells marked with anti-CD25 (FITC) on the upper left side quadrants (% Treg cells), and anti-CCR6 (PE) on the lower right side quadrants (% Th17 cells) on CC (K) and CY groups (L). The results are represented as mean ± SEM of each experimental group (n = 5 to 6/group). Statistical difference between groups were analyzed by the unpaired Student's *t*-test or Mann Whitney, (*) $p < 0.05$, (**) $p < 0.001$. CC, control diet; CY, PBY diet.

factor. Furthermore, there were no changes in T-bet and GATA-3 expression, which suggest a local immune balance. Therefore, we propose a possible mechanism underlying the health benefits of PBY. PBY, a prebiotic source of FOS and inulin, increases the proliferation of probiotic bacteria which enhances SCFA production through fermentation. These SCFA might modulate transcription factors responsible for adaptive immune responses in the colon, downregulating RORγt and, therefore, reducing the stimuli required for Th17 cell differentiation, which favors Treg cell induction. Higher Treg cells conduce to reduced activation of pro-inflammatory cells, and consequent formation of an anti-inflammatory microenvironment. Thus, PBY supposingly prevents and attenuates inflammatory processes by increasing SCFA concentration, negatively modulating RORγt and increasing Treg cells in the colon.

5. Conclusion

PBY diet reduced food intake in the animals, suggesting its important and beneficial role in satiety due to soluble fiber content and SCFA production, which possibly interfere with leptin production. PBY diet also increased fecal humidity and viscosity, favoring intestinal transit. Furthermore, the observed increase in Treg cells and reduction in RORγt transcription factor, indicate that PBY is beneficial for the modulation of inflammatory processes and immune cells. Therefore, the consumption of a diet containing PBY may favor the regulation of satiety, improve intestinal transit, maintain the integrity of the intestinal tissues, and increase Treg cells differentiation by downregulating *RORγt* expression which consequently reduces inflammatory processes.

6. Ethics statement

All the trials and experimental protocols were approved by the Ethics Committee on Animal Experimentation of Universidade Federal de Viçosa, Brazil, under process number 30/2016 and were performed according to the ethical guidelines of the European Community (Directive 2010/63/EU).

Acknowledgement

The study was supported by the National Council for Scientific and Technological Development - CNPq, Coordination for the Improvement of Higher Education Personnel - CAPES, Minas Gerais Research Foundation - FAPEMIG, and Microscopy and Microanalysis Division (NMM) of Universidade Federal de Viçosa - UFV.

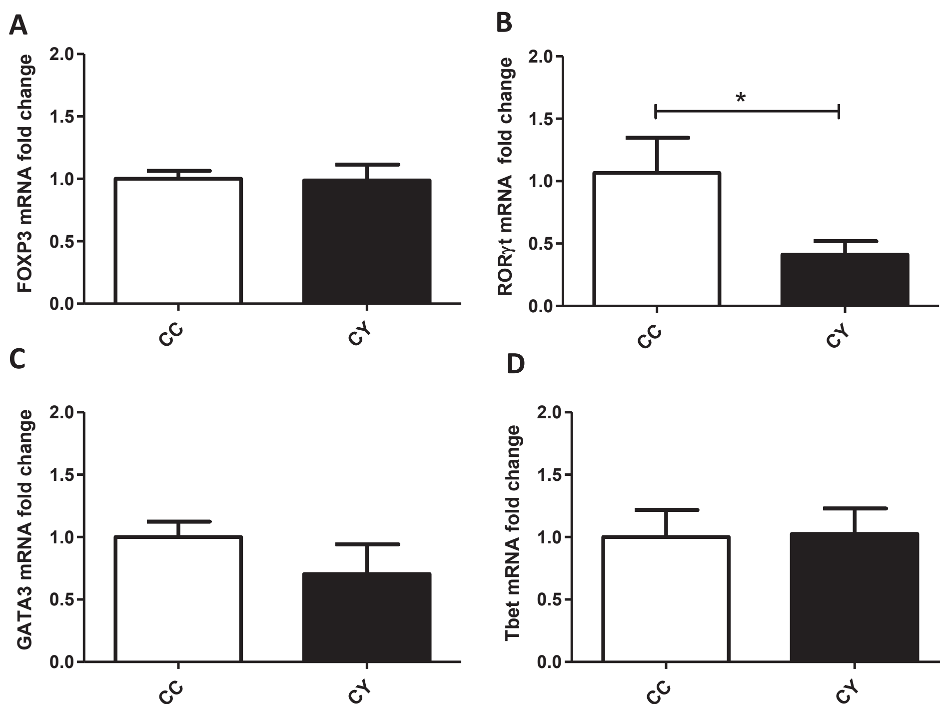

Fig. 7. Effect of PBY diet on the transcription factor expression of immune response on the colon of BALB/c mice during 8 weeks of dietary supplementation. Real-time PCR analysis of the colonic gene expression of FOXP3 (A), RORγt (B), GATA-3 (C) and T-bet (D). Gene expression was calculated in relation to the constitutive GAPDH gene and presented as a relative variation of the control group. The data are expressed as mean ± SEM (n = 4 to 6 mice/group). Statistical difference between groups were analyzed by the unpaired Student's *t*-test or Mann Whitney, (*) $p < 0.05$, (**) $p < 0.001$. CC, control diet; CY, PBY diet.

Conflict of interest

There is no conflict of interest.

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ARTIGO 3 (Original)

Yacon (*Smallanthus sonchifolius*)-based product increases the concentration of fecal short-chain fatty acids and favors the expression of anti-tumor transcription factor T-bet during colorectal carcinogenesis in BALB/c mice

Letícia De Nadai Marcon[a], Luís Fernando de Sousa Moraes[a], Bruna Cristina dos Santos Cruz[a], Michelle Dias de Oliveira Teixeira[b], Antônio Frederico de Freitas Gomides[c] Célia Lúcia de Luces Fortes Ferreira[d], Leandro Licursi de Oliveira[b], Maria do Carmo Gouveia Peluzio[a]

[a]Nutritional Biochemistry Laboratory, Department of Nutrition and Health, Universidade Federal de Viçosa – UFV, Viçosa, Minas Gerais, Brazil.

[b]Immunochemistry and Glycobiology Laboratory, Department of General Biology, Universidade Federal de Viçosa – UFV, Viçosa, Minas Gerais, Brazil.

[c]Institute of Biological Sciences, Universidade Federal de Juiz de Fora, Governador Valadares, Minas Gerais, Brazil

[d]Institute of Biotechnology Applied to Agriculture & Livestock (Bioagro), Laboratory of Lactic Starter Cultures, Universidade Federal de Viçosa – UFV, Viçosa, Minas Gerais, Brazil.

Corresponding author: Letícia De Nadai Marcon. Present address: Nutritional Biochemistry Laboratory, Department of Nutrition and Health of Universidade Federal de Viçosa – UFV. Av. P.H. Rolfs, s/n. 36570-900. Viçosa, Minas Gerais, Brazil. Tel.: +55 31 3899 2111. E-mail adress: leticiadenadai@gmail.com

Abbreviations

ACF, Aberrant Crypt Foci;

AIN, American Institute of Nutrition;

ALT, Alanine Aminotransferase;

AST, Aspartate Aminotransferase;

CRC, Colorectal Cancer;

DMH, 1,2-dimethylhydrazine;

FOS, Fructooligosaccharides;

FOXP3, Forkhead Box P3;

GGT, Gamma-glutamyl Transferase;

HDAC, Histone Deacetylase;

HPLC, High Performance Liquid Chromatography;

IFN-γ, Interferon-Gamma;

IL, Interleukin;

NK, Natural Killer;

PBS, Phosphate-Buffered Saline;

PBY, Yacon-Based Product;

RORγt, Retinoic-Acid-Receptor (RAR)-Related Orphan Receptor Gamma;

SCFA, Short-Chain Fatty Acids;

sIgA, Secretory Immunoglobulin A;

Th17, T-helper 17 cell;

TNF-α, Tumor Necrosis Factor-Alpha;

Treg, Regulatory T cell.

Abstract

Colorectal cancer (CRC) is the third most diagnosed type of cancer in the world. The consumption of prebiotics modulates the microbiota and the intestinal immune system, promoting CRC improvement. We hypothesized that dietary supplementation with a yacon-based product (PBY-*Smallanthus sonchifolius* concentrate) rich in fructooligosaccharides (FOS) and inulin could induce the modulating gut microbiota and the intestinal immune response promoting the improvement of CRC. This study aimed to investigate the impact of PBY consumption on intestinal and fecal characteristics and immune response profile in BALB /c mice during colorectal carcinogenesis. Pre-neoplastic lesions were induced in male BALB/c mice by injecting 1,2-dimethylhydrazine (20mg/kg body weight/week) intraperitoneally for 8 weeks. After induction, the animals were divided into two groups that received the control diet (AIN-93M) or the PBY diet (AIN-93M supplemented with PBY, 6.0% FOS + Inulin) for 8 weeks. The PBY diet was not successful in reducing the number of aberrant crypt foci in the colon, however, it promoted reduction of fecal pH, increased the humidity and the viscosity of feces, increased the production of formic, acetic, propionic, butyric and valeric short-chain fatty acid (SCFA) and modulated the expression of transcription factors in the intestine, reducing FOXP3 and increasing RORγt and T-bet, the latter linked to the control and better prognosis of CRC. It is suggested that PBY, during the early stages of colorectal carcinogenesis, may contribute to mucosal integrity by promoting fecal SCFA increase and immune response modulation in the colon, favoring risk reduction in neoplastic lesions.

Keywords: Colorectal cancer; Yacon; Prebiotic; Short-chain fatty acid; Immune response; Transcription factor.

1. Introduction

Colorectal cancer (CRC) is the third most diagnosed type of cancer in the world [1]. CRC etiology is associated with genetic factors, eating habits, immune response and intestinal microbiota composition [2–4]. There is a strong association between the profile of tumor-infiltrating lymphocytes with the prognosis of CRC [5,6]. CD8 T lymphocytes and Natural Killer (NK) cells are related to disease delay and better prognosis [7–9]. The Th1 type immune response also has been shown to be beneficial in the regression and better prognosis of CRC [10,11]. On the other hand, Th17 cells and cytokines of this cellular profile are associated with worse prognosis and disease progression [12], whereas the role of regulatory T cells (Treg) is still contradictory [13,14].

Evidence suggests that the consumption of prebiotic foods contributes to the selective growth of probiotic bacteria [15] which favours the modulation of the gut-associated lymphoid tissue [16,17] and improves the anti-inflammatory activity associated with CRC [18]. Yacon, a tuberous root of Andean origin, is considered a prebiotic food because it is a source of fructooligosaccharides (FOS) and inulin [19]. The consumption of yacon is able to modulate the intestinal microbiota, increasing the number of bifidobacteria and lactobacilli [20,21] which are directly associated with the inhibition of the growth of pathogenic bacteria [22,23]. Yacon also contributes to the increased production of fecal short-chain fatty acids (SCFA) [17,21,24–26], improvement in intestinal

transit [20,27], satiety [17], weight loss [28], reduction of glycemia [29], triglycerides and cholesterol [30,31]. In addition, yacon consumption has benefits regarding modulation of inflammatory response, with increased production of fecal secretory immunoglobulin A (sIgA), interleukin (IL)-10 and IL-4 serum [26,32,33] and in the regression of pre-neoplastic lesions during colorectal carcinogenesis in animals [24,34]. Recently our research group verified the role of yacon in the modulation of intestinal immunity by favoring the increase of the percentage of Treg cells in the colon associated to the attenuation of the inflammatory profile by the decrease of the expression of the transcription factor RORγt. [17].

Although the benefits of yacon are directly related to the modulation of the microbiota [21] and the intestinal immune response [17,33], it is yet unknown which immune response profile this prebiotic is able to promote in the colon during the carcinogenic process. Thus, we speculated whether yacon consumption could modulate the intestinal immune response by altering transcription factors specific to the immune response and the population of intestinal lymphocytes during experimental colorectal carcinogenesis. Therefore, the objective of this study was to investigate the impact of PBY consumption on intestinal and fecal characteristics and immune response profile in BALB /c mice during colorectal carcinogenesis.

2. Methods and materials

2.1. Animals and experimental design

Thirty-six BALB/c male mice nine-week-old were obtained from the Central Bioterium (Health and Biology Science Center) of the Universidade Federal de Viçosa, Brazil. The animals were housed at the Experimental Nutrition lab in a temperature-controlled room (22±2°C) with a 12-hours light/dark cycle and had *ad libitum* access to water and food. Animal protocol was approved by the Ethics Committee on Animal Experimentation from the Universidade Federal de Viçosa, Brazil, under the process number 30/2016, and were performed according to the ethical guidelines of the European Community guidelines (Directive 2010/63/EU). Upon arrival, mice were induced to pre-neoplastic colorectal lesions by intraperitoneal injection of 1,2-dimethylhydrazine (DMH) (Sigma, Saint Louis, USA) (20 mg/kg body weight) once a week during 8 weeks [35]. DMH was dissolved in 0.9% saline solution containing 1 mM EDTA and 10 mM sodium citrate, pH 8 [36]. After, animals were randomly assigned to two experimental groups: DC, the group receiving control diet (n=17), and DY, the group receiving diet supplemented with PBY (n=19) (Table 1). The sample size was calculated by comparing two groups according to quantitative variables for paired samples [37], based on aberrant crypt data in the study by de Moura and co-workers (2010) [34]. Diets were offered *ad libitum* for 8 weeks. Subsequently, mice were anesthetized using 3% isoflurane and blood was collected from the retro-orbital sinus. Mice were euthanized by cervical dislocation and tissue and feces were harvested for analysis (Figure 1).

2.2. Yacon-based product (PBY) and experimental diet

Yacon was purchased from a local market in Viçosa – Minas Gerais, Brazil. The PBY was processed according to the methodology proposed by Rodrigues and co-workers (2012) [38], which is currently going through patent request PI 1106621-0. The chemical composition of PBY (carbohydrates, proteins, fats, fiber, ash and humidity) was determined according to the AOAC methodology (AOAC, 1997). FOS and inulin content in the PBY were determined by High Performance Liquid Chromatography (HPLC) with a BIO-RAD brand HPX-87p column (lead stationary phase) using purified water for the mobile phase.

The experimental purified diets were based on the AIN93-M diet, as recommended by the American Institute of Nutrition [40]. PBY diet was supplemented with 6.0% FOS + Inulin from PBY (Table 1), as suggested by Paula and co-workers (2012) [41]. Casein, sucrose, dextrinized starch, starch, and fiber adjustments were made to the control diet and the PBY diet, aiming to obtain similar amounts of carbohydrates, lipids, proteins, fibers and calories. The diets were made in pellet format and stored at -20°C for a maximum period of thirty days before consumption.

The calculation of the human equivalent amount of dietary PBY consumed by BALB/c male mice was performed by using the body surface area normalization method as previously describe [42]. PBY supplementation was 353.56g PBY/kg of diet. In our study, the average daily consumption was 6g per mouse. This is equivalent to 2,121g of PBY* for an adult mouse of 45g daily, which approximately corresponds to 47g PBY/kg body mass/day. Taking into account that the average human adult weight is 70kg, it is thus converted to

229g per day for humans. (* Corresponding to 360mg of FOS+Inulin (272mg of FOS and 88mg of inulin)

2.3. Body weight and dietary intake

To evaluate the weight loss/gain of the animals, individual body weight was recorded weekly using a digital weighing scale. Dietary intake was determined based on the diet offered (g) subtracting the diet waste. The quantification was done every 3 to 5 days during the 8 weeks of dietary intervention on a digital weighing scale. The data represented diet consumption per cage (7 to 10 animals/cage). The diet consumption (g) was corrected by the diet humidity loss ratio (fresh diet weight (g)/diet weight (g) in the cage per day).

2.4. Fecal characteristics

Fresh excreted feces were harvested and stored at -80ºC for pH determination. For each animal, an aliquot of feces was diluted in distilled water (1:10), homogenized, and the pH was measured with a digital pH meter (Hexis ultra Basic UB-10® duly calibrated) in a temperature-controlled room for an adequate amount of time for pH stabilization [43].

Fecal humidity was determined using approximately 110mg of moist feces. The feces were weighed in petri dishes, previously dried for 24 hours at 105ºC. Afterwards, the material (petri dish + feces) was placed in a desiccator until it reached ambient temperature, and later weighed for the determination of the humidity by means of equation 1 [44].

Equation 1: humidity (%) = (Initial Weight* - Final Weight*) x 100 / (Sample Weight)

*Initial and final weight: weight of the dishes containing the samples, before and after drying, respectively.

In the last week of the experiment, fresh feces were harvested and assessed according to method used by De Freitas and co-workers (2006) [45] with some modifications to the fecal score: 1. Firm or normal feces consistency; 2. Viscous non-diarrheal feces; 3. Watery feces characteristic of diarrhea.

2.5. Anatomical characteristics

After euthanasia, the organs (liver, spleen, small intestine, cecum, colon and abdominal adipose tissue) were excised, washed in phosphate-buffered saline (PBS) (NaCl: 0.85%, NaH_2PO_4: 0.023%, $NaHPO_42H_2O$: 0.15%, pH 7.2) and weighed using a semi-analytical weighing scale. The hepatosomatic index was obtained by dividing the liver weight by the body weight of the animal. The colon length was obtained by measuring from the end portion of the cecum to the end portion of the rectum on a flat surface using a millimeter ruler.

2.6. Serum biomarkers analysis

After euthanasia, blood of the animals was collected from the retro-orbital sinus and centrifuged at 1190 x g/10 minutes/4°C. The serum markers: total cholesterol (mg/dl), triglycerides (mg/dl), gamma-glutamyl transferase (GGT) (u/L), aspartate aminotransferase (AST) (u/L), alanine aminotransferase (ALT)

(mg/L), albumin (g/dl), alkaline phosphatase (u/L), creatinine (mg/dl), and urea (mg/dl) were assessed by specific colorimetric assays (Bioclin®, Brazil) using a clinical chemistry analyzer BS-200 (Mindray®).

2.7. Fecal SCFA quantification

SCFA quantification was assessed according to the method used by Smiricky-Tjardes and co-workers (2003) [46] with some modifications. 50 mg of frozen feces, which was weighed and thoroughly vortexed with deionized water (950 µL) was used. While incubated on ice for 30 minutes, the samples were homogenized every 5 minutes for 2 minutes. Samples were centrifuged (10,000 x g, 30 minutes, 4°C) three times and the supernatants were collected. The final supernatant from each sample was filtered through a 0.45 µm membrane and transferred to vials. SCFA were measured by High Performance Liquid Chromatography - HPLC (Shimadzu®) on an Aminex HPX 87H column (300 x 7,8 mm, Bio-rad®, Rio de Janeiro, Brazil) at 32°C with acidified water (0.005 M H2SO4) as eluent at a flow rate of 0.6 mL/minute. The products were detected and quantified by an ultraviolet detector (model SPD-20A VP) at 210 nm. Formic, acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic and caproic acids (SUPELCO®) standard curves were performed. Results were expressed as µmol SCFA/g feces.

2.8. Aberrant Crypt Foci (ACF) counts

After removal, the large intestine was washed in PBS solution, opened along the mesenteric margin, placed in paraffin plates with the mucous facing

the top of the plate, and fixed in Carson's formalin [47] for 24 hours. Following fixation, flat colon of animals were equally divided into three segments (proximal, medium, and distal) and stained with 0.1% methylene blue for 2 minutes to quantify aberrant crypt foci under a BX-60 light microscope (Olympus, Tokyo, Japan) with a magnification of 200X. The ACF were counted across the mucosal surface of the large intestine by three independent observers. The ACF categorization was based on the number of aberrant crypts per focus: foci with fewer than or equal to three crypts (ACF≤3) and foci with more than three crypts (ACF>3) [48].

2.9. Histopathological score

Paraffin-embedded colonic gut tissues were sectioned at 5-μm thickness and subjected to hematoxylin–eosin (H&E) staining. Each colonic section was scored as previously described by Kang and co-workers (2017) [49]. The score system was based on three independent parameters: severity of inflammation (0–3), depth of injury (0–3) and crypt damage (0–4). The summation of these scores provides a total histopathological score with 0 being a normal tissue and 10 being the most extensive/severe disease symptoms. The assessment of histopathological score was performed using a BX-60® light microscope (Olympus, Tokyo, Japan) at 200x magnification. Three sections per animal that covered the length of 500 μm of the proximal colon were stained, and their evaluation was in a blinded manner.

2.10. Determination of leukocytes by immunophenotyping

Leukocytes were quantified and characterized in the colon mucosa as previously described [50] with some modifications. The colon was removed and washed in ice-cold PBS, cut into small fragments and incubated in cell culture medium, DMEM, pH 7.2 (Sigma-aldrich™) for 90 minutes at 37°C, the suspension was centrifuged three times at 42 x g for 5 minutes in order to harvest the supernatant, and lastly at 543 x g for 10 minutes. After the last centrifugation the remaining pellet was then resuspended with PBS buffer (100 µL, pH 7.2). Cell viability was assessed with Trypan blue exclusion and cells were counted in a Neubauer chamber. The obtained leukocytes were incubated with the following antibodies, according to the manufacturer's instructions: anti-CD4 (PeCy5), anti-CD25 FITC-conjugated, anti-CD196 (anti-CCR6) PE-conjugated, anti-CD49b (anti-PanNK) APC-conjugated, anti-CD8 PECy7-conjugated (Biolegend, San Diego, CA, USA). Leukocytes (1×10^4 events) were acquired (FACSVerse™ and BD FACSuite software; BD Biosciences PharMingen San Jose, CA, USA) according to size (forward scatter) and granularity (side scatter). One or two stains were used to identify TCD4 lymphocytes ($CD4^+$), TCD8 lymphocytes ($CD8^+$), regulatory T cells ($CD4^+CD25^+$), Th17 lymphocytes ($CD4^+CD196^+$) and Natural Killer cells ($CD49b^+$). The results are expressed as mean ± SEM of the percentage of each subpopulation of specifically stained cells within each selected gated.

2.11. Real-time PCR

Total RNA was extracted from the whole colon using Trizol reagent (Invitrogen™, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized using 5µg of RNA through a reverse transcriptase reaction using a specific kit (GoScript™ Reverse Transcription System, Promega, Madison, WI, USA). Random primer and RNAse free water were added to the sample and heated for 5 minutes at 70ºC. Subsequently, a mixture containing the reverse transcriptase dNTPs and ribonuclease inhibitor was added to the sample and heated for 60 minutes at 37ºC. Real-time PCR quantitative mRNA analyses were performed on the Applied Biosystems® 7500 Real-Time PCR System. Sybr green (Ludwig, Biotec), the primer sense, and the primer antisense (400nm/reação), DNase free water and the sample (250ng cDNA/µl) were added at each reaction. Standard PCR conditions were used during the reading (7500 software V2.3, Applied Biosystems®). The sequences of murine primers (Integrated DNA Technologies®) were as follows: FOXP3, sense: 5'-AGG AGC CGC AAG CTA AAA GC- 3', antisense: 5'-TGC CTT CGT GCC CAC TGT-3'; RORγt, sense: 5'-GGA GCT CTG CCA GAA TGA CC-3', antisense: 5'-CAA GGT TCG AAA CAG CTC CAC-3'; Tbet, sense: 5'-AGC AAG GAC GGC GAA TGT T-3', antisense: 5'-GGG TGG ACA TAT AAG CGG TTC-3'; GATA3, sense: 5'-CAA TCT GAC CGG GCA GGT-3', antisense: 5'-CAG AGA CGG TTG CTC TTC CG-3'; GAPDH, sense: 5'-TCA ACA GCA ACT CCC ACT CTT CCA-3', antisense: 5'-ACC CTG TTG CTG TAG CCG TAT TCA-3'. mRNA values were calculated according to the constitutive GAPDH gene on the basis of the ΔΔCt algorithm.

2.12. Statistical analysis

The results were expressed as mean ± SEM. Data were analyzed using GraphPad Prism version 6.0. Mean values were tested by the Kolmogorov-Smirnov normality test, and the groups with a normal distribution were tested using unpaired Student's t-test. Groups that did not show normal distribution were tested using the Mann-Whitney test. Statistical differences were considered when the $p < 0.05$ (*) or $p < 0.001$ (**).

3. Results

3.1. PBY diet does not alter diet intake or body weight of the animals

There was no difference in the weekly weight gain of the animals during the period of the pre-neoplastic lesion induction (1st to 8th week) or during the dietary intervention period (9th to 16th week) (Figure 1A). There was also no change in the average dietary intake between groups over the period of dietary treatment (Figure 2B).

3.2. PBY diet reduces fecal pH and increases fecal humidity

In order to verify the possible interferences of the PBY diet on the physical-chemical characteristics of the feces, the pH, humidity and fecal score of the animals were evaluated. Fecal pH was lower in the animals treated with the PBY diet ($p < 0.05$) (Figure 3A). Fecal humidity was higher in the group receiving the PBY diet compared to those receiving the control diet ($p < 0{,}001$)

(Figure 3B). In proportion to the humidity, the classification of the fecal score in the animals treated with the PBY diet was classified in stages 2 (viscous non-diarrheic stools) and 3 (aqueous feces characteristic of diarrhoea) (Figure 3C). The induced animals that received the control diet were only classified in stage 2.

3.3. PBY diet increases bowel and adipose tissue weight

The PBY diet promoted increased colon ($p < 0.05$), cecum ($p < 0.001$), small intestine ($p < 0.05$) and abdominal adipose tissue weight ($p < 0.05$) when compared to animals treated with the control diet (Figure 4B-E). There was no significant change in colon length, liver and spleen weight or hepatosomatic index between groups (Figure 4A, F-H).

3.4. PBY diet increases albumin and alkaline phosphatase serum

Analyses of serum biomarkers were performed in the animals in order to verify possible alterations of hepatic and renal function and lipid profile. There was no change in total cholesterol, triglycerides, GGT, AST, ALT, creatinine or urea serum between the groups (Figure 5A-E, GH). However, animals from the group treated with the PBY diet had increased albumin ($p < 0.05$) and alkaline phosphatase ($p < 0.05$) compared to the group fed with the control diet (Figure 5F and 5I).

3.5. PBY diet increases SCFA concentrations

In order to evaluate the intestinal bacterial activity, concentrations of SCFA in the feces of the animals were verified. The feces of the animals treated with the PBY diet had higher concentrations of the formic ($p < 0.001$), acetic ($p < 0.05$), propionic ($p < 0.05$), butyric ($p < 0.001$) and valeric ($p < 0, 05$) acids when compared to those treated with the control diet (Figure 6A-C, E and G). There was no difference in isobutyric, isovaleric, isocaproic and caproic acid concentrations between the groups (Figure 6D, F, H and I).

3.6. PBY diet does not change the number of aberrant crypt foci

In both groups a greater number of ACFs categorized as ≤ 3 ACFs were observed in relation to the ACFs containing > 3 aberrant crypts (Figure 7A-C, E). Within the category of ≤ 3 ACFs, a greater number of ACFs were found in the proximal colon, followed by the medial and finally the distal (Figure 7A-C). There was no significant difference in the total aberrant crypt count (Figure 7D) or categorized (≤ 3 ACFs and> 3 ACFs) per colon segment between animals receiving the PBY diet or control diet (Figure 7A-C, F).

3.7. PBY diet does not alter the histopathological score of the colon

In both groups, DMH-induced colorectal carcinogenesis promoted histopathological changes in the colon at crypt level (Figure 8B), inflammation (Figure 8C), depth of damage (Figure 8D), and histopathological score (Figure 8A). Animals treated with control diet presented a prevalence of damage in the

crypts that compromised 2/3 of the crypt regarding its base, characterized by an oval aspect, where the crypt light does not reach the intestinal lumen (Figure 8E). Also, animals treated with PBY diet presented similar damages, being a mean impairment of 1/3 crypts in relation to their base. Inflammatory infiltrates were found in both groups and were minimally expressive (Figure 8E). The areas affected by the damage are mostly restricted to the mucosa.

3.8. PBY diet does not alter the percentage of lymphocytes in the colon

As a way of evaluating the profile of the lymphocyte population of the intestine, immunophenotyping of the colon immune cells was performed. There was no significant change in the percentages of $CD4^+$ and $CD8^+$ T cells, Treg cells ($CD4^+CD25^+$), Th17 cells ($CD4^+CCR6^+$), NK cells and CD4/CD8 ratio between the two types of dietary treatments (Figure 9A-L). However, there was a reduction of 30.4% on the percentage of $CD4^+$ T lymphocytes, an increase of 37.7% on $CD8^+$ T cells, a 156.9% increase on the ratio of $CD8^+/CD4^+$, and an increase of 84.8% of Treg cells in the colon of the animals treated with the PBY diet compared to the animals that consumed the control diet.

3.9. PBY diet increases expression of RORγt and T-bet in the colon

In order to verify the pattern of intestinal immune response in the colon, specific transcriptional factors of the adaptive immune response were evaluated. Animals treated with the PBY diet showed increased expression of RORγt ($p < 0.05$), T-bet ($p < 0.05$) and GATA-3 ($p < 0.05$) and reduction of

FOXP3 ($p < 0,05$) when compared to the group that received the control diet (Figure 10A-D). However, GATA-3 expression should not be considered biologically relevant because of the very low amount in both groups (DC: 0.023; DY: 0.046).

4. Discussion

Yacon has been associated with its beneficial potential in relation to the regression of pre-neoplastic lesions in experimental CRC [24,34], mainly because of its high content of FOS and inulin. These benefits are related to the increase in the number of probiotic bacteria in the colon and corresponding metabolites such as SCFA [51]. Despite the promising role of yacon on the modulation of the immune system, favouring the production of sIgA [26,32,33], reducing the number of pathogenic bacteria in the colon [20], increasing fecal SCFA and enhancing Treg cell by downregulating RORγt in healthy BALB/c mice [17], the profile of the immune response that this prebiotic is capable to yield in the colon during the carcinogenic process is yet unknown. Thus, this study sought to investigate the immunological mechanisms that permeate the response to the treatment of pre-neoplastic colon lesions using a yacon concentrate (PBY).

In this study, a reduction in fecal pH and an increase in the humidity and viscosity of feces of the animals treated with the PBY diet were observed. Dietary supplementation with yacon has already been shown to reduce fecal pH in rodents [24], and improve the consistency of feces and intestinal transit in humans [20]. In obese individuals, yacon consumption also improve satiety

sensation and decrease body weight [28]. However, it was not find a significant difference in dietary intake and body weight between the groups during the period of dietary intervention. In anatomical analyses, increased colon, cecum and small intestine weight in animals treated with the PBY diet are purportedly due to the yacon's trophic potential on the intestine. Yacon is able to increase the number and depth of intestinal crypts [23,52] and generate SCFA through bacterial metabolism, which act as energetic substrate for colonocytes [53], favouring intestinal trophism.

The PBY diet did not influence the concentrations of markers for hepatic, renal function and lipid metabolism, however, an increase of albumin and alkaline phosphatase in the animals treated with PBY diet was observed. Increased albumin may be beneficial for animals with induced carcinogenesis, as albumin levels decrease during inflammation, and is considered a negative reagent of the acute phase [54]. In addition, albumin as a carrier of fatty acids in the bloodstream [55] might have increased in response to high fecal concentration of SCFA. The increase in alkaline phosphatase can be attributed to its role in osteogenesis [56], as yacon promotes an increase in intestinal calcium absorption and bone deposition [52].

The consumption of the PBY diet promoted increased concentrations of formic, acetic, propionic, butyric and valeric SCFA in the feces of the animals. Such an increase can be attributed to the prebiotic role of yacon, which contains high concentrations of FOS and inulin [57], capable of stimulating the growth of probiotic bacteria such as lactobacilli and bifidobacteria [20,21]. The use of yacon flour has already been shown to increase propionate and butyrate concentrations in the fecal content of rodents [24]. As well as to having a trophic

action on the mucosa, butyrate has an apoptotic action on cancer cells [58]. SCFA, mainly butyric acid, and to a lesser extent propionic acid, are proposed as histone deacetylase (HDAC) inhibitors [59,60]. HDAC inhibitors can trigger histone hyperacetylation, allowing chromatin to open and activate gene transcription. This process promotes the expression of silenced genes related to apoptosis and cell cycle arrest, contributing to the suppression of colon cancer cell growth [61,62]. The SCFA-induced cell growth blockade is associated with increased expression of the p21 cell cycle inhibitor and down regulation of cyclin B1 in colon cancer cells [59]. In addition to these mechanisms, the role of the SFCA- SFCA receptor axis in the suppression of bacterial invasion, chronic inflammation and intestinal carcinogenesis associated with inflammation has recently been demonstrated [63].

In the present study, we investigated the formation of ACF, considered to be pre-neoplastic lesions and thus previous evidence of CRC development [48]. DMH-induced colorectal tumorigenesis in male BALB/c mice resulted in a greater amount of ACF in the proximal colon, followed by the medial and a lesser number in the distal, in both induced groups, with the majority of the crypts categorized as foci containing a maximum of 3 aberrant crypts. Induction also compromised the structure of the intestinal mucosa, affecting the architecture of intestinal crypts and leading to the formation of inflammatory infiltrates. The treatment of the animals with the PBY diet did not alter the number of aberrant crypts in the colon, and although it caused slight improvement in crypt structure it was not able to significantly modulate the parameters related to the histopathological score. In studies were DMH-induced animals fed with yacon-containing diets ACF count was reduced in the colon

[24,34]. Yacon flour, containing 20.4% FOS, added to the diet at a ratio of 1%, offered for 13 weeks after induction, significantly reduced the lesions in rats [34]. Similarly, a diet supplemented with yacon flour (7.5% FOS) offered for 8 weeks to DMH-induced rats (25mg / kg body weight) showed more than a 40% reduction in the ACF count [24]. The lack of reduction in ACF in the present study might have been attributed to factors such as: the lower percentage of FOS and inulin in the diet (6%); the short period of dietary intervention (8 weeks); and the onset of dietary intervention protocol, after induction of lesions and not before or during the establishment of the disease. This outcome reinforces the functional role of yacon which stands in decrease of risk disease and not treatment.

Regarding the immune cells present in the colon, there were no differences in the populations of $CD4^+$ and $CD8^+$ T lymphocytes, Treg cells ($CD4^+$ $CD25^+$), Th17 cells ($CD4^+$ $CCR6^+$), NK cells and CD4 / CD8 ratio, between groups. Despite this, there was an increase of 156.9% in the $CD8^+/CD4^+$ T cells ratio on the animals fed with the PBY diet, which denotes a prevalence of $CD8^+$ T cells on $CD4^+$ T cells. $CD8^+$ T lymphocytes are strongly related to their anti-tumor role in CRC [9]. In the other side, the Th17 lymphocytes are involved with the production of inflammatory cytokines such as IL-17A, IL-17F, IL-21, IL-22, tumor necrosis factor-alpha (TNF-α) and IL-6, associated with tumour progression [12]. The increase of 84.8% in the Treg cells percentage might be attributed to the modulatory role of PBY able to increase these regulatory population and to promote the anti-inflammatory profile of adaptive immune response on the colon, recently described by our research group [17]. However, the role of Treg cells is still controversial in CRC,

as they are related to both tumor progression [64–66] and to the better prognosis of CRC [67–69]. Treg cells play a regulatory role in the immune response, secreting cytokines and immunity modulating factors [70], able to suppress the effector function of cytotoxic T lymphocytes and NK cells, which act in the elimination of tumor cells [71]. Thus, Treg cells could not only mediate the immunosuppression of the tumor immune response [72], but also attenuate the inflammatory immune response, especially when triggered by bacteria in the intestine [67].

A reduction of FOXP3 expression and increased expression of RORγt and T-bet in the animals treated with the PBY diet was also observed. FOXP3 is the key transcription factor in Treg cell differentiation. In turn, RORγt is the inducing transcription factor of Th17 lymphocytes differentiation [73] expressed in TCD4 cells in situations opposite to FOXP3 expression. An environment containing high amounts of transforming growth factor-β (TGF-β) and pro-inflammatory cytokines such as IL-6, IL-21 and IL-23 will promote RORγt expression, whereas a low amount of pro-inflammatory cytokines and high amount of TGF-β will induce expression of FOXP3 [73]. The reduction of FOXP3 in the colon of the CRC-induced animals fed the PBY diet might be indication of a reduction of Treg cells, occasionally beneficial since such cells may be involved in the suppression of the anti-tumor immune response [72]. In addition, cancer cells may express FOXP3 [74], generating a confounding factor in the overall tissue analysis. Therefore, it is possible that the FOXP3 decrease might indicate a positive result of the PBY in the diet. On the other hand, increased expression of RORγt did not alter the differentiation of Th17 cells but

may be related to the prevalence of inflammatory infiltrates and the lack of alterations in the histopathological score and ACF count.

The T-bet transcription factor initiates the development of the Th1 cell line which secretes interferon-gamma (IFN-γ), the main cytokine of this lineage. It also produces IL-2 and TNF-α [75]. GATA-3 is the specific transcription factor for Th2 cell line development, which secretes IL-4, key cytokine, in addition to IL-5, IL-6, IL-9 and IL-13 [75–77]. It is widely recognized that IFN-γ producing Th1 cells, as well as CD8 T lymphocytes, play an important role in the inhibition and death of tumor cells [78]. Moreover, Th1 cells are essential for the proliferation of cytotoxic T lymphocytes, since they require IL-2 produced by Th1 cells for its proliferation [79]. In addition, Th1 cells have already been shown to be associated with better CRC prognosis [10,11]. Increased T-bet expression in animals receiving the PBY diet suggests a possible beneficial effect on tumour progression as well as prognosis of the disease.

In summary, supplementation with PBY as a form of treatment was not able to reduce the number of pre-neoplastic colonic lesions. However, the PBY diet promoted the reduction of fecal pH, increased the humidity and the viscosity of feces, which could help reduce the time the intestinal wall is exposed to carcinogens from the diet. The PBY diet also promoted a significant increase in the production of formic, acetic, propionic, butyric and valeric SCFA, a direct indication of probiotic microbial activity. The increase of these SCFA could contribute to the activation of cancer suppressor genes. In addition, the consumption of the PBY diet reduced the expression of the FOXP3 transcription factor and increased the expression of RORγt and T-bet, the latter being related to the activation and proliferation of CD8 T lymphocytes, and better CRC

prognosis. We might infer that PBY contributes to the targeting of the anti-tumor immune response, at least on early stages of CRC carcinogenesis. It is suggested that PBY as a functional food might contribute to the risk reduction for neoplastic lesions mainly by modulating the SCFA production and immune response in the colon.

Acknowledgements

The study was supported by National Council of Scientific and Technological Development - CNPq, Coordination for the Improvement of Higher Education Personnel - CAPES, Research Support Foundation of the State of Minas Gerais - FAPEMIG, and Microscopy and Microanalysis Division (NMM) of the Universidade Federal de Viçosa - UFV. The authors declare no conflict of interest.

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Table 1.Composition of AIN-93M diet for control and PBY diet.

Ingredients (g/100g)	Control diet (g/kg)	PBY diet (g/kg)
Casein	155.5	145.3
Dextrinized starch	155	148.2
Sucrose	100	0
Soybean oil	40	40
Fiber (microfine cellulose)	67	0
Mineral mix	35	35
Vitamin mix	10	10
L-Cystine	1.8	1.8
Choline bitartrate	2.5	2.5
Cornstarch	303	263.6
*PBY	0	353.6
Distilled water	130.2	0
Total weight (g)	1000	1000
Total energy (kcal)	3214	3064

* Centesimal composition and digestible content of carbohydrate, inulin and FOS on PBY in 100g of product: Fructose: 15.25g; Glucose: 8.59g; Sucrose: 6.35g; FOS: 12.81g; Inulin: 4.16g; Total carbohydrate: 45.49g; Fibers: 1.99g; Humidity: 36.82g; Ashes: 3.39g; Lipids: 0.21g; Protein: 2.89g. PBY, yacon-based product.

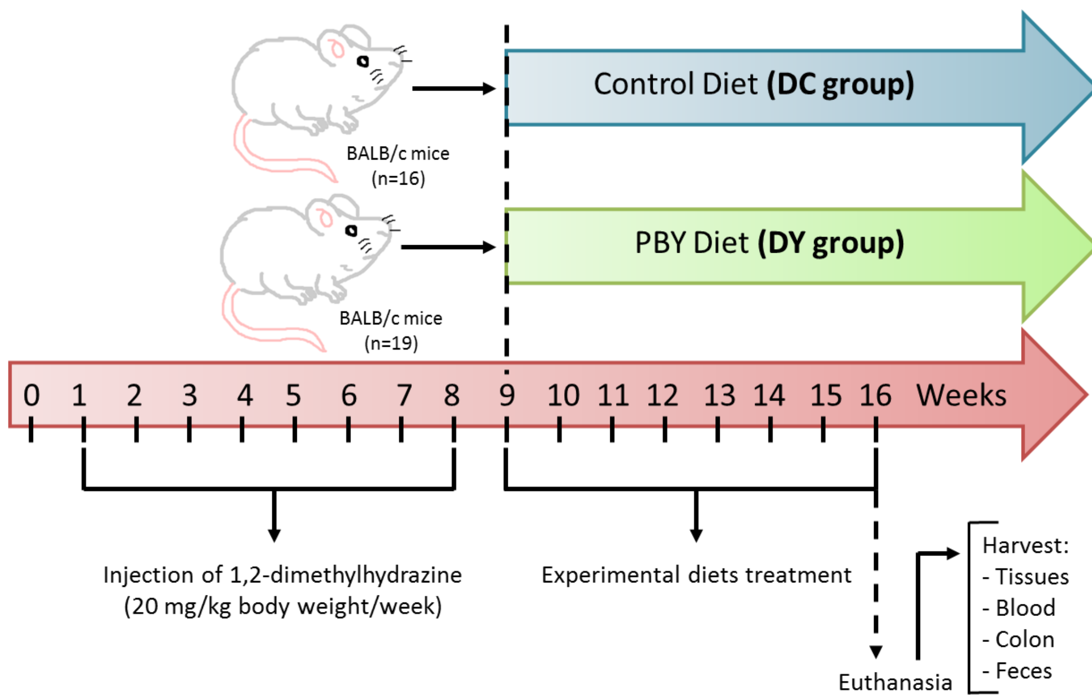
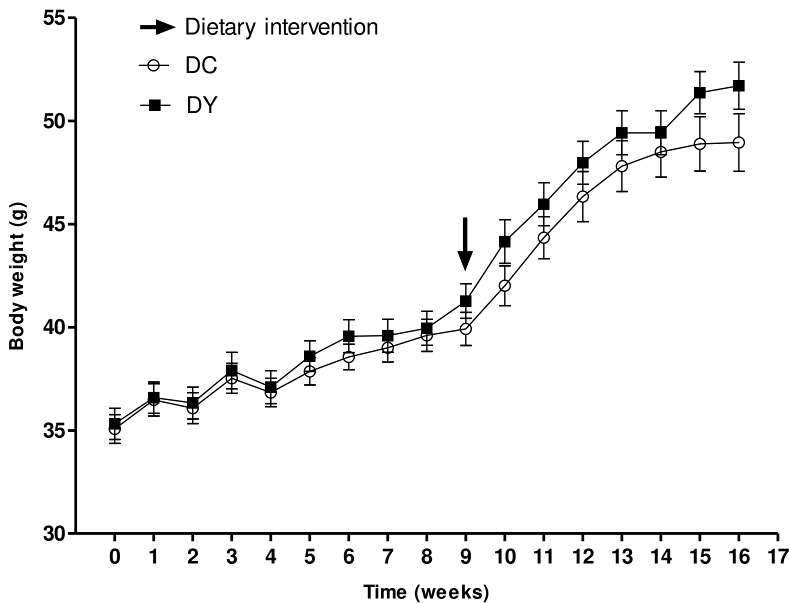


Figure 1. Experimental design.

A



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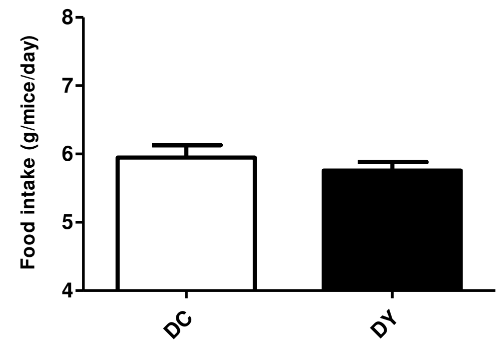
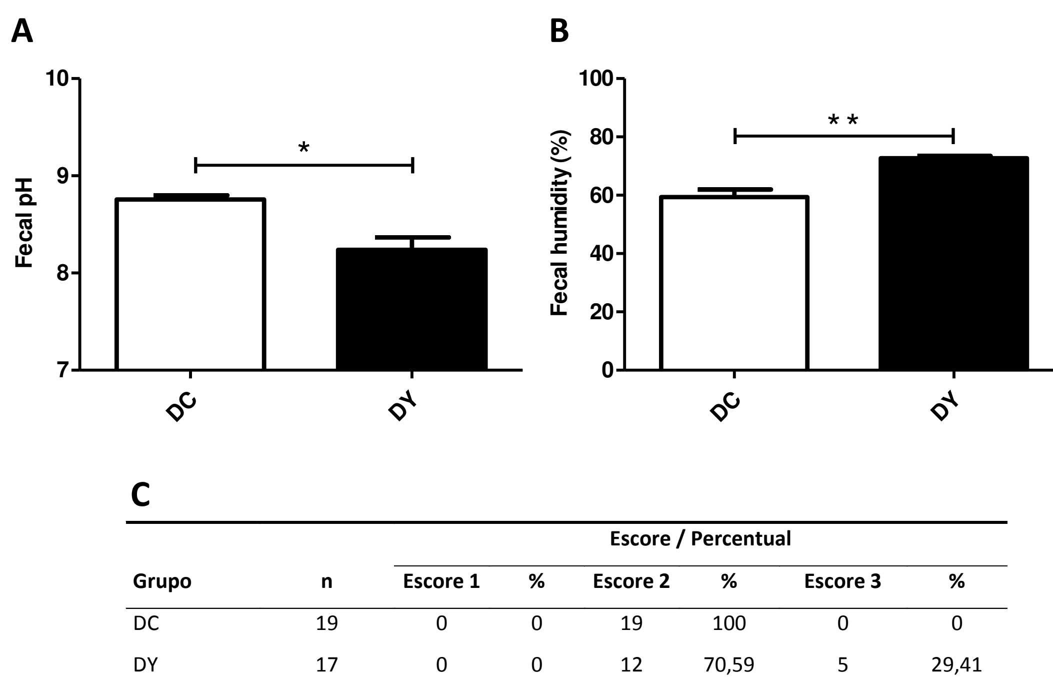


Figure 2. Weight gain and food intake in BALB/c mice induced to pre-neoplastic colorectal lesions and submitted to 8 weeks of dietary treatment. (A) Average weight gain of animals over the weeks. The data are expressed as mean ± SEM (n = 17 (DC); n = 19 (DY)). (B) Mean food intake (g/mice/day) of PBY diet or control diet consumed by BALB/c. The data are expressed as mean ± SEM of each experimental group (means referring to the group / cage consumption pool (4 pools / week). Statistical difference between groups were analyzed by the unpaired Student's t-test or Mann Whitney, (*) $p < 0.05$, (**) $p < 0.001$. DC - Animals exposed to DMH and receiving diet Control; DY - Animals exposed to DMH and receiving PBY diet.



		Escore / Percentual					
Grupo	n	Escore 1	%	Escore 2	%	Escore 3	%
DC	19	0	0	19	100	0	0
DY	17	0	0	12	70,59	5	29,41

Figure 3. Fecal characteristics in BALB/c mice induced to pre-neoplastic colorectal lesions and submitted to 8 weeks of dietary treatment. (A) Fecal pH; (B) Fecal humidity percentage; (C) Fecal Score (Score 1: normal to firm consistency; Score 2: viscous non-diarrheal consistency; Score 3: watery consistency characteristic of diarrhea). The results of fecal score are represented as number of animals in each score and their percentage into the group (n = 17 (DC); n = 19 (DY)); The data of fecal pH and fecal humidity are expressed as mean ± SEM (n = 10 mice/group). Statistical difference between groups were analyzed by the unpaired Student's t-test or Mann Whitney, (*) $p < 0.05$, (**) $p < 0.001$. DC - Animals exposed to DMH and receiving diet Control; DY - Animals exposed to DMH and receiving PBY diet.

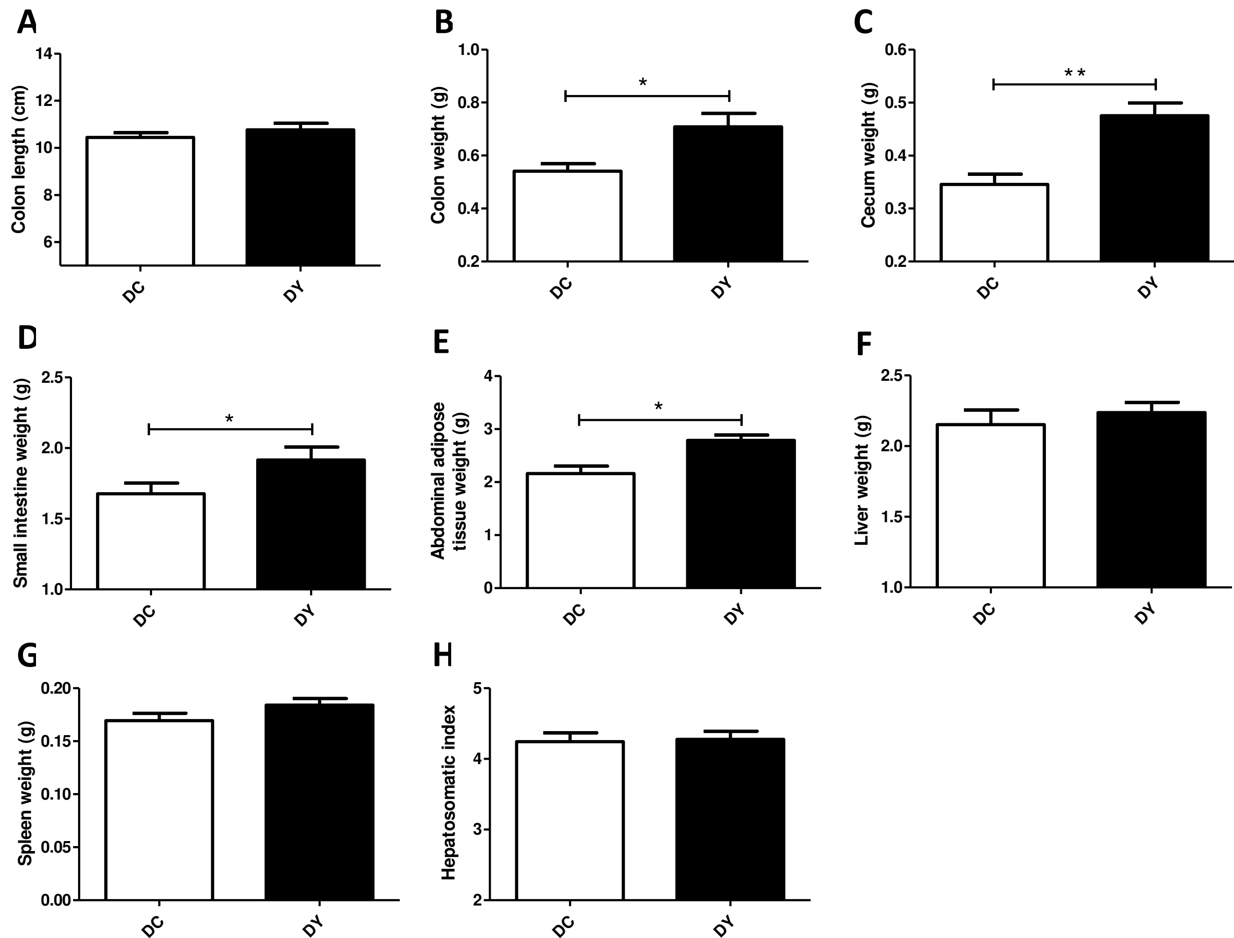


Figure 4. Anatomical characteristics of visceral organs of BALB/c mice induced to pre-neoplastic colorectal lesions and submitted to 8 weeks of dietary treatment. (A) colon length (cm); (B) colon weight (g); (C) cecum weight (g); (D) small intestine weight (g); (E) abdominal adipose tissue weight (g); (F) liver weight (g); (G) spleen weight (g); (H) hepatosomatic index. The data are expressed as mean ± SEM (n = 17 (DC); n = 19 (DY)). Statistical difference between groups were analyzed by the unpaired Student's t-test or Mann Whitney, (*) $p < 0.05$, (**) $p < 0.001$. DC - Animals exposed to DMH and receiving diet Control; DY - Animals exposed to DMH and receiving PBY diet.

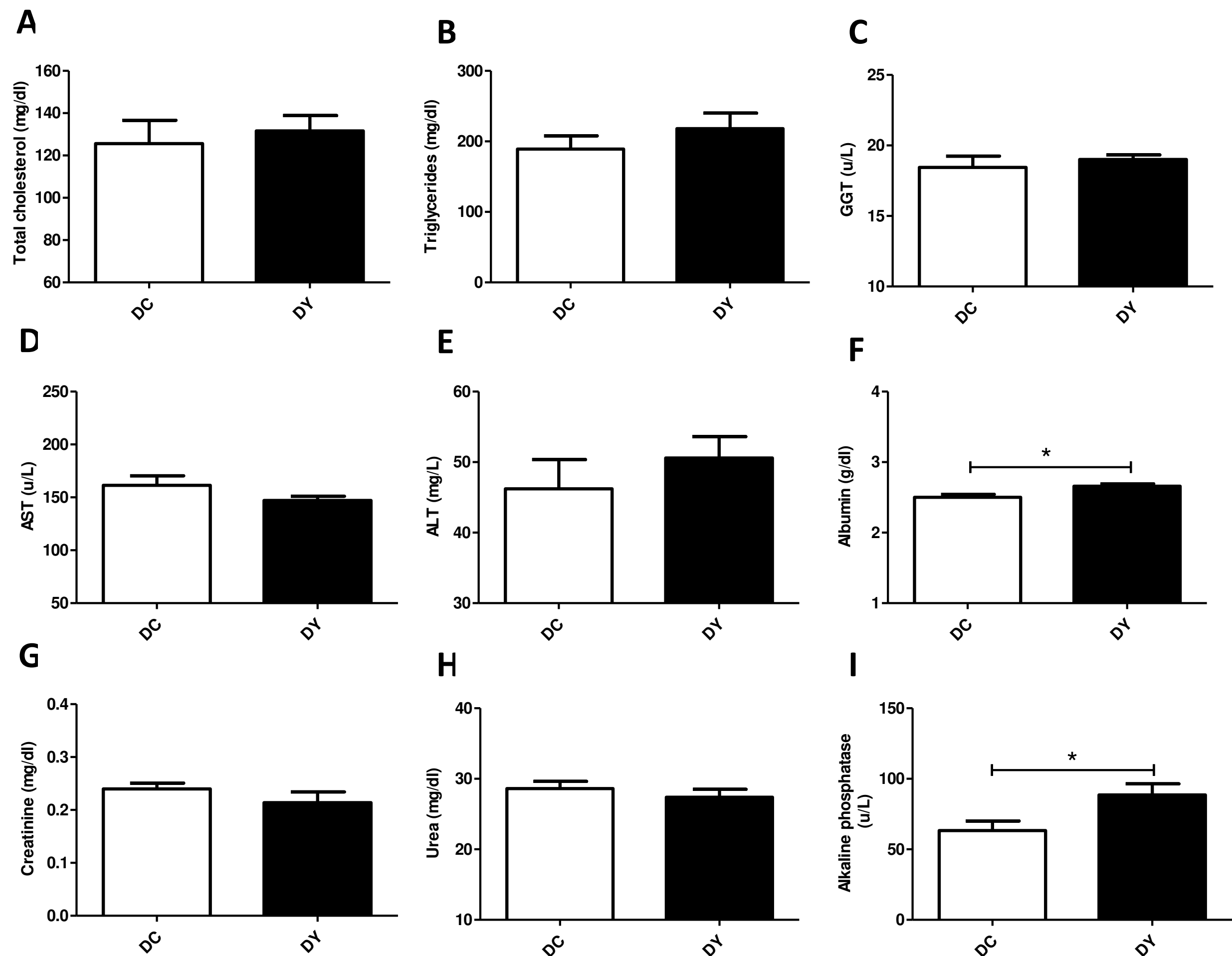


Figure 5. Serum biomarkers level in BALB/c mice induced to pre-neoplastic colorectal lesions and submitted to 8 weeks of dietary treatment. (A) total cholesterol (mg/dl); (B) triglycerides (mg/dl); (C) GGT (gamma-glutamyl transferase) (u/L); (D) AST (aspartate aminotransferase) (u/L); (E) ALT (alanine aminotransferase) (mg/dl); (F) albumin; (G) creatinine; (H) urea; (I) alkaline phosphatase. The data are expressed as mean ± SEM (n = 10 mice/group). Statistical difference between groups were analyzed by the unpaired Student's t-test or Mann Whitney, (*) $p < 0.05$, (**) $p < 0.001$. DC - Animals exposed to DMH and receiving diet Control; DY - Animals exposed to DMH and receiving PBY diet.

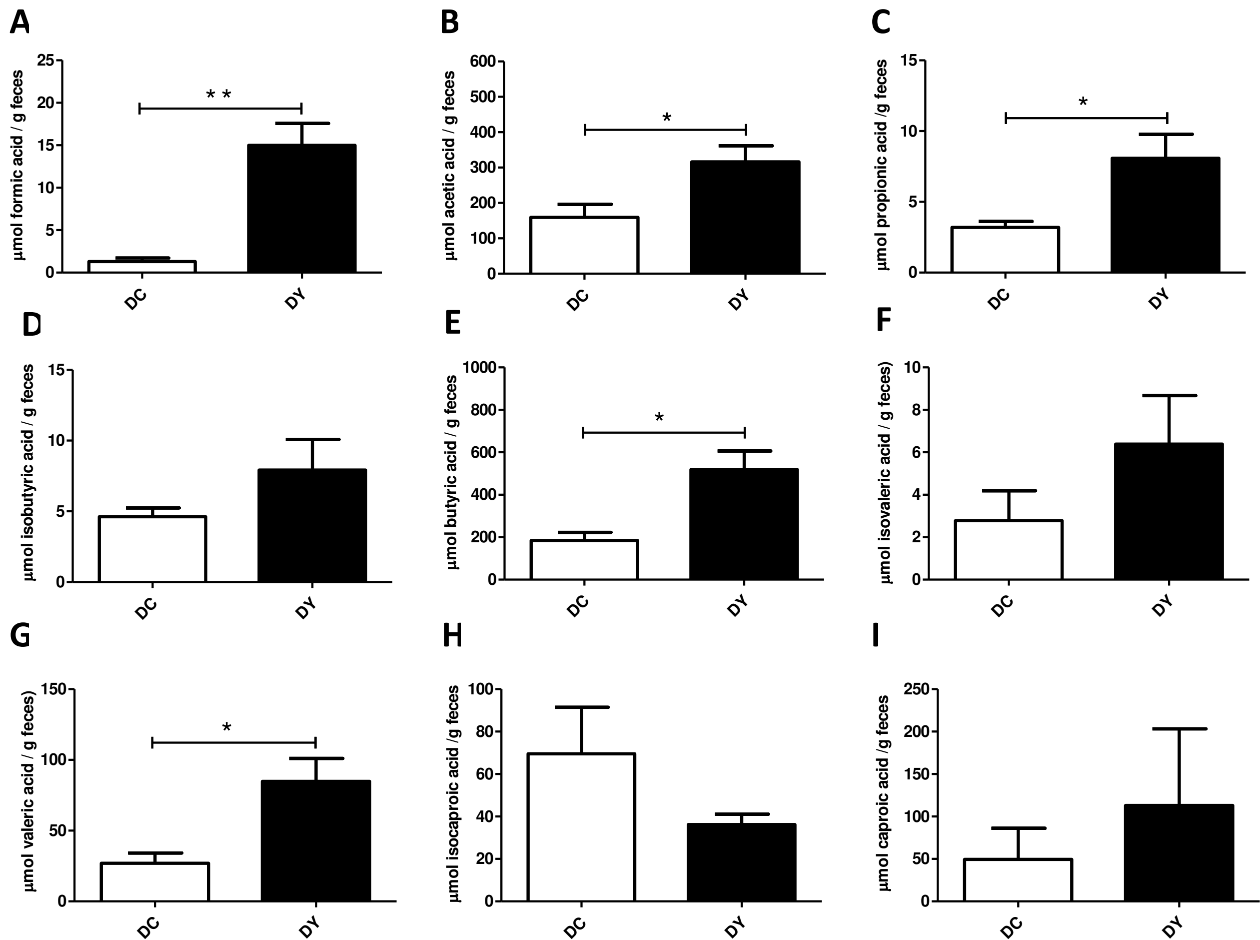
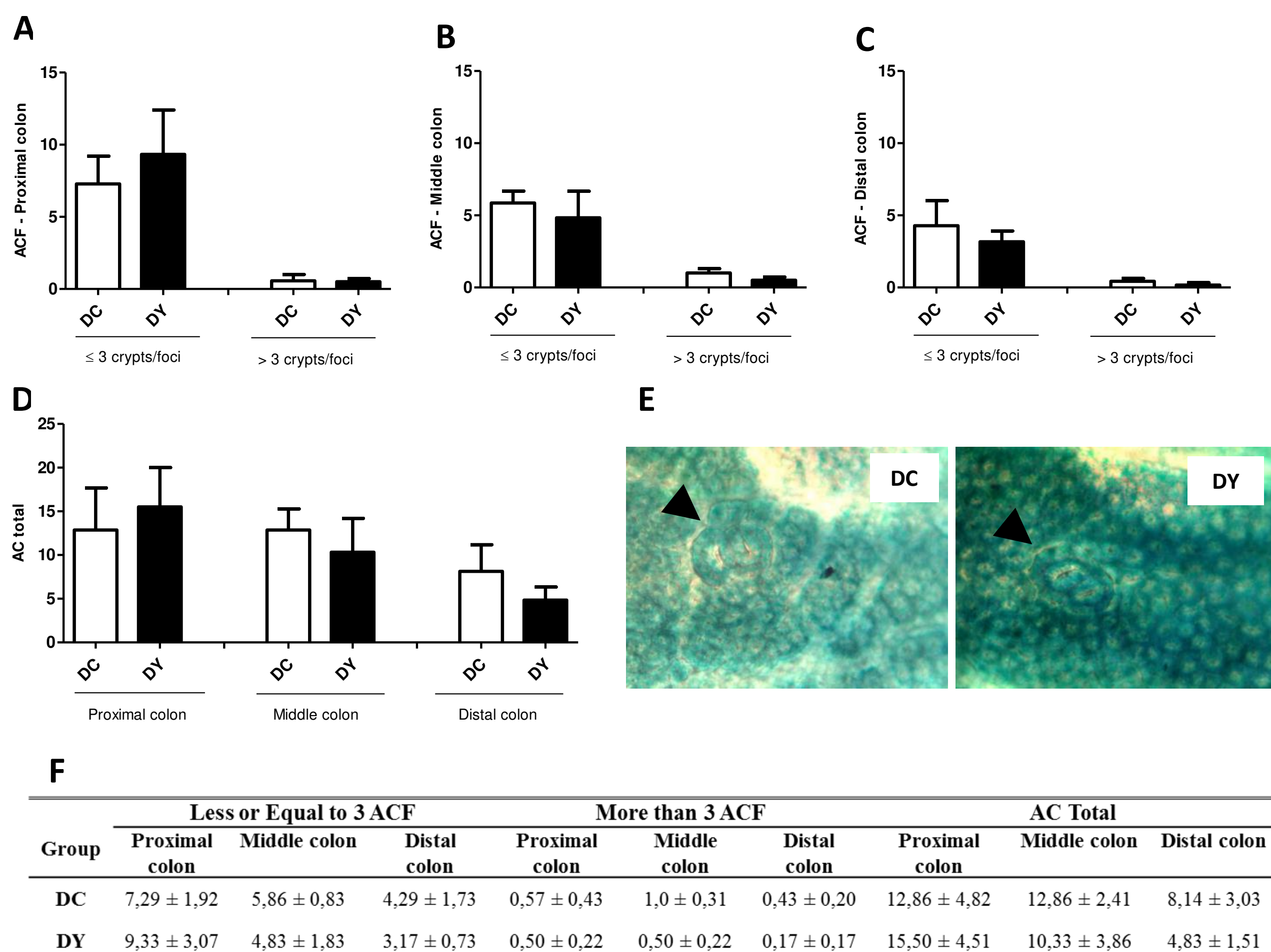


Figure 6. Fecal SCFA concentration in BALB/c mice induced to pre-neoplastic colorectal lesions and submitted to 8 weeks of dietary treatment. (A) formic acid (µmol); (B) acetic acid (µmol); (C) propionic acid (µmol); (D) isobutyric acid (µmol); (E) butyric acid (µmol); (F) isovaleric acid (µmol); (G) valeric acid (µmol); (H) isocaproic acid (µmol); (I) caproic acid (µmol). The data are expressed as mean ± SEM (n = 10 mice/group). Statistical difference between groups were analyzed by the unpaired Student's t-test or Mann Whitney, (*) $p < 0.05$, (**) $p < 0.001$. DC - Animals exposed to DMH and receiving diet Control; DY - Animals exposed to DMH and receiving PBY diet.



Group	Less or Equal to 3 ACF			More than 3 ACF			AC Total		
	Proximal colon	Middle colon	Distal colon	Proximal colon	Middle colon	Distal colon	Proximal colon	Middle colon	Distal colon
DC	7,29 ± 1,92	5,86 ± 0,83	4,29 ± 1,73	0,57 ± 0,43	1,0 ± 0,31	0,43 ± 0,20	12,86 ± 4,82	12,86 ± 2,41	8,14 ± 3,03
DY	9,33 ± 3,07	4,83 ± 1,83	3,17 ± 0,73	0,50 ± 0,22	0,50 ± 0,22	0,17 ± 0,17	15,50 ± 4,51	10,33 ± 3,86	4,83 ± 1,51

Figure 7. Aberrant crypt foci (ACF) in BALB/c mice-colon induced to pre-neoplastic colorectal lesions and submitted to 8 weeks of dietary treatment. (A) proximal colon; (B) middle colon; (C) distal colon; (D) total aberrant crypt. The data are expressed as mean ± SEM (n = 7 (DC); n = 6 (DY)). Statistical difference between groups were analyzed by the unpaired Student's t-test or Mann Whitney, (*) $p < 0.05$, (**) $p < 0.001$. (E) Photomicrographs representative of colon luminal surface: DC - Animals exposed to DMH and receiving diet Control; DY - Animals exposed to DMH and receiving PBY diet; The black arrows indicate ACF containing three aberrant crypts (DC) and two aberrant crypts (DY). 100x magnification. (F) Numerical mean values of ACF per crypt classification per intestinal segment.

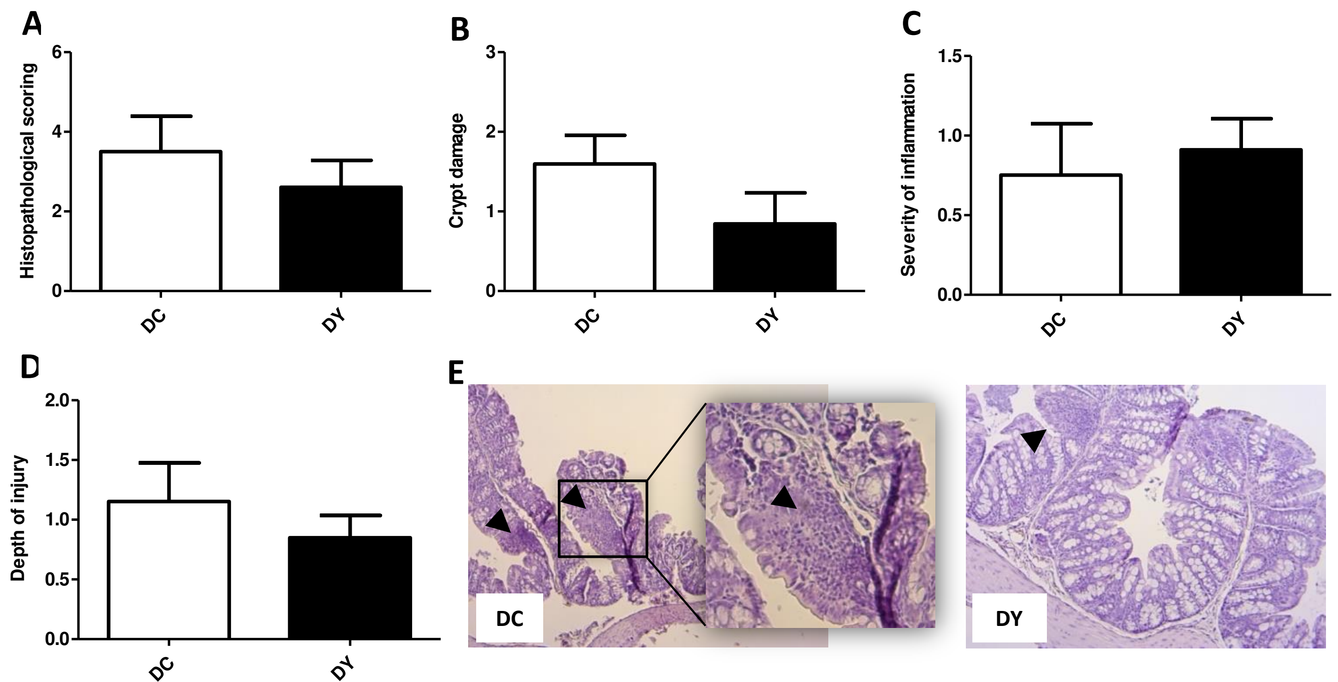


Figure 8. Histopathological score of proximal colon of BALB/c mice induced to pre-neoplastic colorectal lesions and submitted to 8 weeks of dietary treatment. (A) histopathological score; (B) crypt damage; (C); severity of inflammation (D) depth of injury. The data are expressed as mean ± SEM (n = 7 (DC); n = 6 (DY)). Statistical difference between groups were analyzed by the unpaired Student's t-test or Mann Whitney, (*) $p < 0.05$, (**) $p < 0.001$. DC, control diet; DY, PBY diet. (E) Photomicrographs representative of proximal colon mucosa: DC - Animals exposed to DMH and receiving diet Control; DY - Animals exposed to DMH and receiving PBY diet; The black arrows indicate inflammatory infiltrates. 100x magnification; Highlight: 200x magnification.

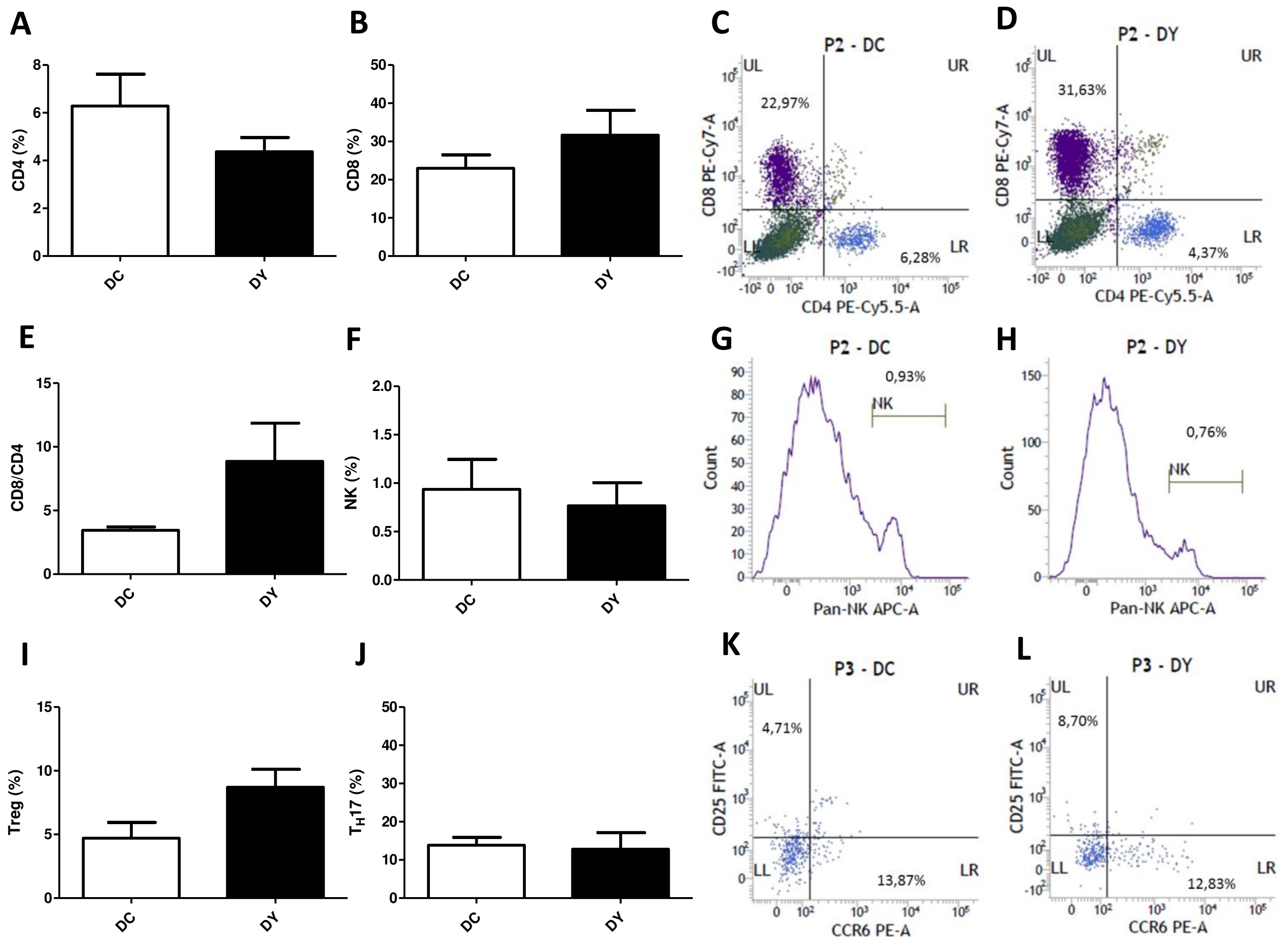


Figure 9. Phenotypic profile of lymphocytes in BALB/c mice-colon induced to pre-neoplastic colorectal lesions and submitted to 8 weeks of dietary treatment. (A) % $CD4^+$ cells; (B) % $CD8^+$ cells; Flow cytometry plots from forward scatter/side scatter-gated lymphocytes cells marked with anti-CD8 (PE-Cy7) on the upper left side quadrants (% CD8 cells) and anti-CD4 (PE-Cy5) on the lower right side quadrants (% CD4 cells) on DC (C) and DY groups (D); (E) % $CD8^+/CD4^+$ ratio; (F) % NK cells (Pan NK^+ cells); Histogram plots of gated lymphocytes cells marked with anti-Pan-NK (APC) on DC (G) and DY groups (H); (I) % Treg cells ($CD4^+CD25^+$ cells); (J) % Th17 cells ($CD4^+CCR6^+$). Flow cytometry plots from gated $CD4^+$ cells marked with anti-CD25 (FITC) on the upper left side quadrants (% Treg cells), and anti-CCR6 (PE) on the lower right

side quadrants (% Th17 cells) on DC (K) and DY groups (L). The results are represented as mean ± SEM of each experimental group (n = 5 (DC); n = 6 (DY)). Statistical difference between groups were analyzed by the unpaired Student's t-test or Mann Whitney, (*) $p < 0.05$, (**) $p < 0.001$. DC - Animals exposed to DMH and receiving diet Control; DY - Animals exposed to DMH and receiving PBY diet.

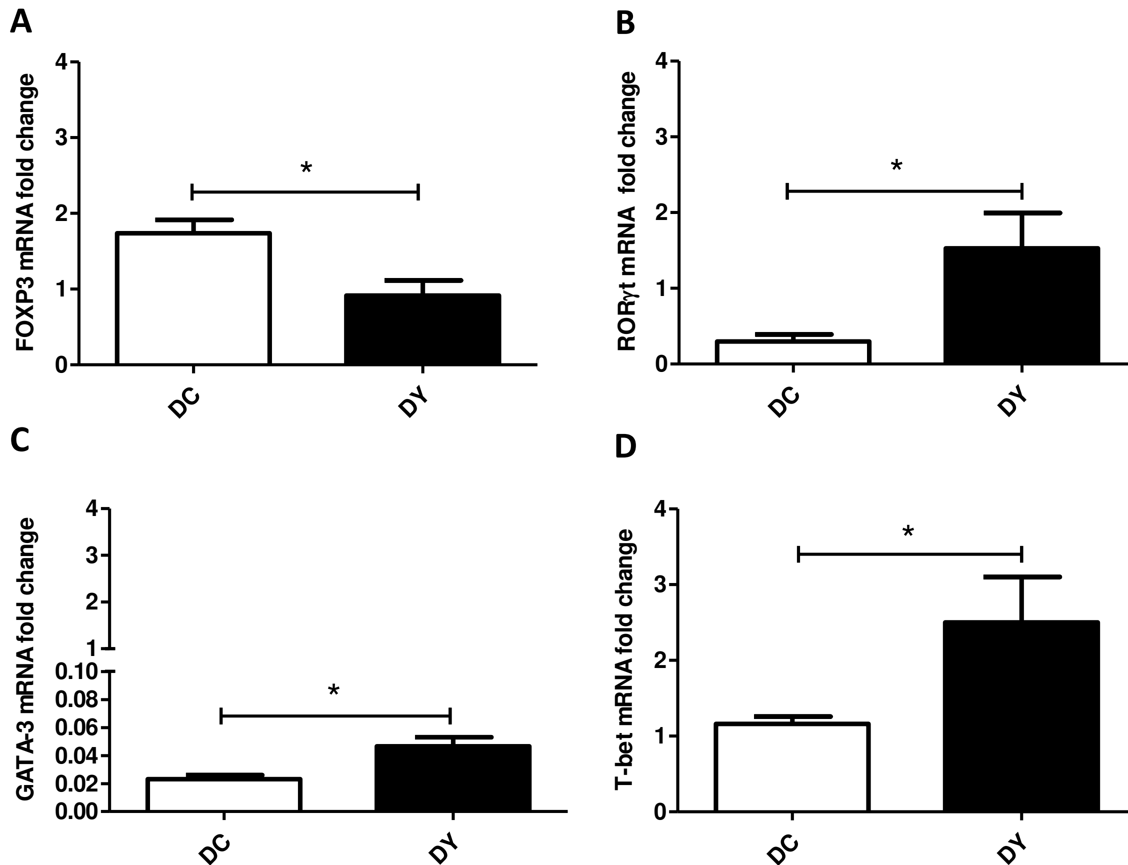


Figure 10. Transcription factor expression of immune response on the colon of BALB/c mice induced to pre-neoplastic colorectal lesions and submitted to 8 weeks of dietary treatment. Real-time PCR analysis of the colonic gene expression of FOXP3 (A), RORγt (B), GATA-3 (C) and T-bet (D). Gene expression was calculated in relation to the constitutive GAPDH gene and presented as a relative variation of the control group. The data are expressed as mean ± SEM (n = 6 (DC); n = 4 (DY)). Statistical difference between groups were analyzed by the unpaired Student's t-test or Mann Whitney, (*) $p < 0.05$, (**) $p < 0.001$. DC - Animals exposed to DMH and receiving diet Control; DY - Animals exposed to DMH and receiving PBY diet.

6 CONCLUSÕES GERAIS

O PBY incluído à dieta de camundongos sadios promoveu a redução da ingestão de alimentar, melhorou a umidade e viscosidade das fezes, intensificou a concentração fecal dos AGCC acético, propiônico, butírico, isovalérico, valérico e capróico, aumentou o número de células Treg e regulou negativamente a expressão do fator de transcrição RORγt no cólon. Em camundongos induzidos às lesões pré-neoplásicas do cólon, a dieta PBY não favoreceu a redução do número de FCA, porém, promoveu redução do pH fecal, aumentou a umidade e a viscosidade das fezes, aumentou a produção dos AGCC fórmico, acético, propiônico, butírico e valérico nas fezes e modulou a expressão de fatores de transcrição no intestino, reduzindo FOXP3 e aumentando RORγt e T-bet, este último ligado ao controle e melhor prognóstico do CCR.

Sugere-se que o PBY, durante os estágios iniciais da carcinogênese colorretal, possa contribuir com a integridade da mucosa intestinal por promover o aumento da concentração de AGCC fecal e modular a resposta imune no cólon para o tipo Th1. Essa modulação poderia auxiliar na redução do risco e agravamento das lesões neoplásicas. Além disso, pode-se inferir que o consumo do PBY por camundongos sadios favoreça a regulação da saciedade, melhore o trânsito e integridade dos tecidos intestinais, module a população de linfócitos intestinais por aumentar a diferenciação das células Treg, e direcione a resposta imune adaptativa anti-inflamatória por regular negativamente a expressão de RORγt. Esses achados nos levam a concluir que o PBY pode ser considerado uma fonte prebiótica promissora capaz de auxiliar na modulação da resposta imune intestinal de maneira benéfica, sendo mais eficaz em situação de saúde, de modo a contribuir com a redução do risco de CCR. A avaliação do perfil das interleucinas intestinais, da produção de sIgA, e do perfil microbiota intestinal poderia contribuir na compreensão do direcionamento da resposta imune gerada em decorrência do consumo deste prebiótico, tanto na saúde quanto no CCR.

ANEXO 1

Cálculo amostral

O tamanho amostral foi calculado baseando-se na comparação entre os valores de duas médias de referência. Para isso utilizou-se a fórmula:

$$N = \frac{2 \times (z\alpha/2 + z\beta)^2 \sigma^2}{d^2}$$ (Miot, 2011)

Onde: $z\alpha/2$= nível de confiança; $z\beta$= poder da amostra; σ- Desvio padrão; d = diferença que se deseja obter. Adotou-se o nível de confiança de 95% ($\alpha \leq 0,05$) (Z=1,96) e considerou-se um poder de 90% da amostra (1- α = 90% (logo, β = 10% (0,10) e Z=1,28).

Foi utilizada como variável principal o número de criptas aberrantes. Os focos de criptas aberrantes (FCA) são os primeiros achados microscópicos de alterações na mucosa do cólon em animais quimicamente induzidos ao câncer colorretal (CRC).

Os valores do número de criptas do estudo de de Moura et al (2012) foram utilizados para os cálculos:

	Grupos/tratamento			
	Não tratado	**0,5% yacon**	**1,0% yacon**	**Simbiótico**
Nº de criptas ± EPM	304,26 ± 51.27	153,82 ± 32.44	110,04 ± 19.41	103,04 ± 22.08
Nº animais	5 ou 12	5 ou 12	5 ou 12	5 ou 12

Assim, tem-se que:

- Média do número de animais: 5 + 12 = 8,5 = 9 animais/grupo → 4 grupos x 9 = 36 animais (total)
- Média do erro padrão de todos os grupos: 31,3
- Conversão para Desvio padrão (DP) → DP = EP x √n → DP = 31,3 x √36 → DP = 187,8
- Considerando uma redução do número de criptas de: 304,26 (grupo não tratado) – 110,04 (tratado com 1% yacon) = 194,22

Cálculo do tamanho amostral:

$$N = \frac{2 \times (1{,}96 + 1{,}28)^2 \, 187{,}8^2}{194{,}22^2} = 19{,}63 \text{ animais/grupo}$$

Assim, o tamanho da amostra encontrado foi de 20 animais por grupo. Considerando que o estudo consistirá em 4 grupos experimentais **Grupo DY,** grupo que receberá a injeção de DMH e a dieta suplementada com o concentrado à base de yacon (PBY); **Grupo DC,** grupo que receberá a injeção de DMH e a dieta controle; **Grupo CY**, grupo que não receberá a injeção de DMH e receberá o dieta suplementada com PBY; **Grupo CC**, grupo que não receberá a injeção de DMH e receberá a dieta controle), serão necessários 80 animais para o presente estudo.

Referências Bibliográficas

de Moura NA, Caetano BFR, Sivieri K, Urbano LH, Cabello C, Rodrigues MAM, et al. Protective effects of yacon (Smallanthus sonchifolius) intake on experimental colon carcinogenesis. Food Chem Toxicol 2012;50:2902–10. doi:10.1016/j.fct.2012.05.006.

Miot HA. Tamanho da amostra em estudos clínicos e experimentais. J Vasc Bras 2011;10:275–8. doi:10.1590/S1677-54492011000400001.

ANEXO 2

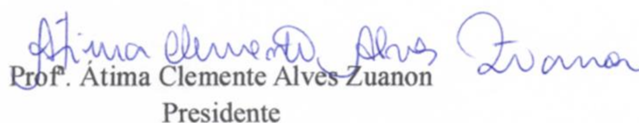
Aprovação pela Comissão de Ética no Uso de Animais (CEUA) da Universidade Federal de Viçosa (UFV)

CERTIFICADO

A Comissão de Ética no Uso dc Animais - CEUA/UFV ccrtifica quc o processo nº 30/2016, intitulado "Respostas imunomodulatória envolvida na terapêutica experimental de lesões pré-neoplásicas utilizando um concentrado à base de yacon *Smallanthus sonchifolius"*, coordenado pela professora Maria do Carmo Gouveia Pelúzio do Departamento de Nutrição e Saúde, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTI, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTI, portanto sendo aprovado por esta Comissão em 25/05/2016, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 30/2016, named "immunomodulatory responses in precancerous lesions experimental therapy using a yacon based concentrate *Smallanthus sonchifolius*", is in agreement with the a actual Brazilian legislation (Lei Nº 11.794, 2008), Normative Resolutions edited by CONCEA/MCTI, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific Purposes and Teaching) and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTI therefore being approved by the Committee on May 25, 2016 valid for 12 months.



Profª. Átima Clemente Alves Zuanon
Presidente
Comissão de Ética no Uso de Animais – CEUA/UFV