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(Ciência dos Alimentos)

Lactobacillus acidophilus CRL 1014 e extrato aquoso de yacon na evolução do processo de carcinogênese química de cólon

ANA PAULA DA SILVA ALMEIDA

ARARAQUARA - SP

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Tese apresentada ao Programa de Pós-Graduação em Alimentos e Nutrição, Área de Ciência dos Alimentos, da Faculdade de Ciências Farmacêuticas da Universidade Estadual Paulista "Júlio Mesquita Filho", para obtenção do título de Doutor em Alimentos e Nutrição, área de Ciência dos Alimentos.

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“Tudo que é seu encontrará uma maneira de chegar até você”

- Chico Xavier -

RESUMO

O câncer de cólon e reto representa a terceira causa mais comum de câncer no mundo, sendo que pesquisas apontam ação benéfica dos probióticos, prebióticos e simbióticos no câncer de cólon. O objetivo desta pesquisa foi definir as etapas da carcinogênese de cólon que são mais afetadas pelo consumo de extrato aquoso de yacon, *Lactobacillus acidophilus* CRL 1014 e extrato aquoso de yacon + *Lactobacillus acidophilus* CRL 1014 em ratos induzidos quimicamente ao câncer de cólon com 1,2-dimetilhidrazina (DMH). Cem ratos Wistar machos foram divididos aleatoriamente em cinco tratamentos: G1: ratos não tratados; G2: DMH; G3 (probiótico): DMH + *L. acidophilus* CRL 1014; G4 (prebiótico): DMH + extrato aquoso de yacon; G5 (simbiótico): DMH + *L. acidophilus* CRL 1014 + extrato aquoso de yacon. Todos os tratamentos foram administrados via oral durante 8 meses, sendo que o *L. acidophilus* CRL 1014 foi administrado na quantidade de 3 mL/ kg de peso/ dia na concentração de 10^9 UFC/ mL e o extrato aquoso de yacon administrado foi calculado em relação ao seu conteúdo de frutooligossacarídeos (FOS). Os animais ingeriram 10% de FOS na dieta. A concentração de FOS no extrato aquoso de yacon foi previamente determinada utilizando kit enzimático (Megazyme®). Cinco animais pertencentes a cada grupo experimental foram sacrificados após a primeira administração de DMH, no terceiro, quinto e oitavo meses. Os tecidos foram submetidos à análise macroscópica, verificando-se o número e volume de massa tumorais, análise de focos de criptas aberrantes (FCA), análise histológica do tecido, ensaio cometa em amostras de sangue periférico, índice de apoptose, imunohistoquímica para p53 e Ki-67. Houve redução no dano no DNA nos tratamentos G3, G4 e G5. Os grupos G5 e G4 apresentaram aumento no índice de apoptose e diminuição da proliferação celular em relação ao G2 ($p<0.05$). Não houve diferença na marcação de p53 nos grupos G3, G4 e G5 em relação ao G2. O grupo G5 apresentou menor número de FCA/ cólon no terceiro mês, sendo que no quinto mês os grupos G3, G4 e G5 apresentaram redução no número de FCA/ cólon em relação ao G2 ($p<0.05$). No último mês, os grupos G3, G4 e G5 não apresentaram efeito no número de FCA/ cólon em comparação com o G2. Todos os tumores nos grupos G3 e G2 eram adenocarcinomas no início da etapa de progressão da carcinogênese, sendo que apenas 50 e 67% dos tumores nos grupos G4 e G5 eram adenocarcinomas. Já na fase mais tardia da progressão da carcinogênese, aos 8 meses de protocolo, os tratamentos G3, G4 e G5 não apresentaram efeito. Em conclusão, os resultados indicam um efeito quimiopreventivo do extrato aquoso de yacon associado ou não ao *Lactobacillus acidophilus* CRL 1014 nas fases iniciais do câncer de cólon.

Palavras-chave: câncer, frutooligossacarídeos, intestino, prebiótico, probiótico.

ABSTRACT

Colorectal cancer is the third commonest type of cancer in both sexes in the world. Research suggests beneficial effects of consuming probiotics and prebiotics on colon cancer. The aim of this project was to define the phases of the development of colon cancer that are more affected by aqueous yacon extract, *Lactobacillus acidophilus* CRL 1014 and aqueous yacon extract + *Lactobacillus acidophilus* CRL 1014 ingested by rats chemically treated with 1,2 dimethylhydrazine (DMH) to induce colon cancer. Hundred male Wistar rats were randomly divided into five treatments: G1: untreated rats; G2: DMH; G3 (probiotic): DMH + *L. acidophilus* CRL 1014; G4 (prebiotic): DMH + aqueous yacon extract; G5 (synbiotic): DMH + *L. acidophilus* CRL 1014 + aqueous yacon extract. All treatments were administered orally for 8 months, *L. acidophilus* CRL 1014 was administered at 10^9 CFU/ mL in 3 mL/ kg body weight and amount of aqueous yacon extract administered was calculated in relation of its fructooligosaccharides (FOS) content using fructan assay kit (Megazyme®). Animals ingested 10% of FOS in diet. Five animals from each treatment group were sacrificed after the first DMH injection and in the third, fifth and eighth months. Tissues were subjected to macroscopic analysis, measuring polyp number, volume and thickness, aberrant crypt foci (ACF) analysis, histological tissue analysis, comet assay in peripheral blood, apoptosis index, and colon samples from the first sacrifice were assessed for K-67 and p53 by immunohistochemical procedures. G3, G4 and G5 showed DNA damage reduction. G5 and G3 groups showed higher apoptosis index and decrease of Ki-67, compared to G2 ($p<0.05$). p53 staining did not differ among G2, G3, G4, G5. G5 showed fewer ACF number per colon in third month and in the fifth month G3, G4 and G5 showed a reduction of ACF number per colon relative to G2 ($p<0.05$). In the last month, G3, G4 and G5 showed no effect on ACF number per colon relative to G2. All tumors in G2 and G3 were adenocarcinomas in the early stage of carcinogenesis progression and 50 and 67% of detectable tumors in G4 and G5 were adenocarcinomas. In the later stage of carcinogenesis progression, at 8 months of protocol, G3, G4 and G5 showed no effect. In conclusion, results indicated a chemoprotective effect of aqueous yacon extract associated or not with *Lactobacillus acidophilus* CRL 1014 in the early phases of colorectal cancer.

Keywords: cancer, fructooligosacchacarides, gut, prebiotic, probiotic.

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ABREVIATURAS

- **AOM:** azoximetano

- **APC:** adenomatous polyposis coli (gene relacionado com a polipose adenomatosa familiar)

- **COX-2:** ciclooxigenase-2

- **DMH:** dimetilhidrazina

- **FCA:** focos de criptas aberrantes

- **FOS:** frutooligossacarídeo

- **HDL**: lipoproteína de alta densidade

- **LDL**: lipoproteína de baixa densidade

- **MAM:** metilazoximetanol

- **MGMT:** O^6-metilguanina DNA metil transferase

- **N^7-mG:** N^7 metilguanina

- **O^6-mG:** O^6 metilguanina

- **PCNA:** antígeno nuclear de proliferação celular

- **PGE-2:** prostaglandina E-2

- **p53:** antígeno de supressão tumoral

- **Ki-67:** antígeno nuclear não-histona

CAPÍTULO I

1. INTRODUÇÃO

O câncer é uma doença que ocorre em diferentes faixas etárias, podendo acometer tanto crianças quanto jovens, adultos e idosos, com número crescente de casos em todo o mundo e apresenta prognóstico relacionado com o estágio evolutivo da enfermidade, presença e localização de mestástases e tipo de massa tumoral caracterizada por análises histológicas do tecido envolvido (INCA, 2009).

O câncer de cólon, pulmão, fígado, estômago e mama estão entre os principais tipos de câncer relacionados com a maioria dos casos de morte a cada ano, sendo que cerca de 30% das causas de morte por câncer são devido a alto índice de massa corpórea, fumo, consumo de álcool, baixo consumo de frutas e vegetais e sedentarismo (WHO, 2014). Segundo a World Health Organization (2014) e o International Agency for Research on Cancer (2012), espera-se que o número de casos anuais de câncer aumentem de 14 milhões em 2012 para 22 milhões até 2014.

A prevenção e o controle do câncer estão entre os mais importantes desafios científicos e de saúde pública. Assim, com o objetivo de reduzir a incidência e a mortalidade, foi criada a política nacional de atenção oncológica, incorporada pela Portaria nº 2.048, de 3 de setembro de 2009, definindo abrangente controle do câncer com a realização de ações preventivas e assistência de alta complexidade. As políticas de prevenção associadas ao amplo acesso à informação sobre prevenção de doenças e a importância da adoção de hábitos mais saudáveis, aumentou a busca da população por uma alimentação mais saudável. Neste contexto, os alimentos funcionais com probióticos, prebióticos ou simbióticos ganharam importância e maior visibilidade.

Desta forma, os alimentos funcionais tornaram-se foco de diversas pesquisas e muitos trabalhos apontam ação preventiva de alimentos contendo probióticos, prebióticos e simbióticos. Vários mecanismos de ação dos probióticos, prebióticos e simbióticos já foram postulados, tais como: aumento da resposta imune, degradação de carcinógenos, produção de compostos antimutagênicos, inibição da formação de células tumorais, dentre outros (DENIPOTE et al., 2010; KUMAR et al., 2010).

O objetivo deste trabalho se fundamenta na grande quantidade de relatos científicos que apontam para a capacidade das bactérias láticas e ingredientes prebióticos em reduzirem ou retardarem o desenvolvimento do câncer de cólon. Entretanto, há poucos relatos na literatura sobre quais etapas da carcinogênese são mais afetadas pelo consumo de prebióticos, probióticos e simbióticos. Da mesma forma, o potencial do yacon foi pouco explorado quando às suas ações anti-carcinogênicas. Frente ao exposto, o estudo da administração diária de *L. acidophilus* CRL 1014, extrato aquoso de yacon e/ou associação de ambos, em animais quimicamente induzido ao câncer de cólon, torna-se interessante, de modo que os resultados possam elucidar os possíveis efeitos e mecanismos de proteção ao desenvolvimento deste tipo de neoplasia.

2. REVISÃO BIBLIOGRÁFICA

2.1. Câncer de cólon: Incidência e fatores de risco

Observa-se pelas estimativas, que desde 2009 o número de casos de câncer de cólon tem aumentado progressivamente (INCA 2009, 2011, 2014), sendo que em 2009 já se configurava como a terceira causa mais comum de câncer no mundo em ambos os sexos e a segunda causa de morte por câncer em países desenvolvidos (INCA, 2009).

Jemal et al. (2011) relataram que o câncer de cólon é o terceiro tipo mais comum de câncer diagnosticado em homens e o segundo em mulheres, sendo que em países desenvolvido as taxas de câncer cólon são de 2 a 5 vezes maiores do que nos países em desenvolvimento, com as maiores incidências encontradas na Austrália, Nova Zelândia, Europa e América do Norte e as menores na África e sudeste da Ásia central.

O câncer é uma importante causa de doença e morte no Brasil, sendo que as estimativas para o ano de 2014 apontam para a ocorrência de aproximadamente 32.600 casos novos de câncer de cólon e reto, sendo 15.070 e 17.530 em homens e mulheres, respectivamente (INCA, 2014) e segundo a World Health Organization (2014) e o International Agency for Research on Cancer (2012) o número de casos de câncer deve atingir até 22 milhões em 2014, sendo que em 2012 o câncer de cólon foi responsável por 694.000 mortes.

Nos Estados Unidos, estima-se que 24.040 mulheres e 26.270 homens virão à óbito em 2014, sendo que cerca de 65.000 mulheres e 71.830 homens serão diagnosticados com câncer de cólon em 2014 (AMERICAN CANCER SOCIETY, 2014).

As estimativas para 2015 apontam a ocorrência de 576 mil novos casos de câncer, sendo 33 mil casos relacionados com câncer de cólon e reto no Brasil, sendo que em 5

anos a sobrevida média global está em torno de 55% nos países desenvolvidos e 40% nos países em desenvolvimento (INCA, 2014).

Esta doença ocorre devido a mutações ou ativação anômala de genes que controlam a proliferação, morte ou diferenciação celulares, sendo que a probabilidade de ocorrer uma alteração genética é aumentada quando a pessoa é exposta a alguns fatores químicos, físicos ou biológicos (GUYTON e HALL, 1996).

Fatores biológicos ou hereditários de risco incluem polipose familiar, síndrome de Lynch e colite ulcerativa (BRADY et al., 2000; INCA, 2009), pois há uma relação entre o número de pólipos, adenomas e o risco de câncer de cólon (adenocarcinoma) (LIU, 2006).

Já os fatores ambientais envolvem sedentarismo, consumo de dietas ricas em gordura e com baixos teores de fibras (BRADY et al., 2000; INCA, 2009), tabagismo e consumo de álcool (DENIPOTE et al., 2010), pois a dieta desbalanceada com excesso energético, carboidratos refinados e carnes vermelhas aumenta a exposição da mucosa a ácidos biliares e ação bacteriana (LIU, 2006; TURNER, 2012).

Entretanto, diversos trabalhos científicos apontam o efeito preventivo dos alimentos contendo microrganismos probióticos e ingredientes prebióticos (SAIKALI et al., 2004; FOTIADIS et al., 2008; DENIPOTE et al., 2010; KUMAR et al., 2010). Da mesma forma, a atividade física, o consumo de alho, vegetais amiláceos, frutas e alimentos contendo folato ou peixes e alimentos contendo vitamina D e selênio podem ter um efeito ou proteção contra o câncer de cólon (WORLD CANCER RESEARCH FUND/ AMERICAN INSTITUTE FOR CANCER RESEARCH, 2007).

Os fatores de risco para o câncer de cólon incluem a dieta, a idade, predisposição genética, obesidade ou o sobrepeso, entretanto, os fatores mais relevantes são a história familiar e a predisposição genética (INCA, 2011), pois populações distintas podem

possuir genes de susceptilibidade ao câncer em diferentes proporções (WEINBERG, 2007).

2.2. Evolução do câncer de cólon

No adenocarcinoma de cólon pode-se esquematizar a formação de áreas displásicas epiteliais, formação de pólipos adenomatosos e progressão para adenocarcinoma com a ativação de determinados oncogenes e desativação de genes supressores de tumor, evasão da vigilância imunológica, ação de fatores de crescimento e angiogênese (DOUGLAS, 2000a).

A progressão de um adenoma para um carcinoma envolve alterações genéticas, podendo-se citar: defeitos no reparo de emparelhamento errôneo de DNA, perda de grupamentos metila no DNA, mutações no gene p53 e mutações em vias de sinalização para fator de crescimento, sendo que não há uma sequência de eventos pré-determinada e única, podendo ocorrer alterações na proliferação celular, processo de apoptose e no processo de supressão tumoral (LIU, 2006; TURNER, 2012).

O crescimento displásico muito espesso é definido como adenoma ou pólipo (WEINBERG, 2007). Entretanto, os pólipos são massas tumorais projetadas no lúmen intestinal que podem ser ou não neoplásicos. Já os adenomas são pólipos neoplásicos provenientes do epitélio displásico, os quais podem evoluir para um adenocarcinoma (Figura 2) (LIU, 2006; TURNER, 2012).

Os adenomas podem apresentar alterações hiperplásicas (células normais, aumento localizado e limitado do número de células), metaplásicas (formação tecidual

diferente do tecido original) e displásicas (lesão com perda de polaridade, citologia anormal, mitoses freqüentes) (WEINBERG, 2007; INCA, 2008).

O adenocarcinoma é uma neoplasia maligna, possui células com perda da capacidade de diferenciação celular, tende a crescer aceleradamente e originar metástases (DOUGLAS, 2000b). Já os adenomas são crescimentos benignos locais e não invadem os tecidos adjacentes. (WEINBERG, 2007) (Tabela 1).

Histologicamente, os adenomas e os adenocarcinomas podem ser denominados como tubulares, quando apresentam mais de 75% de estruturas tubulares ramificadas. Já quando os tumores apresentam mais de 75% de estruturas vilosas são denominados vilosos, sendo considerados tubulovilosos quando 25 a 75% das suas estruturas são tubulares e vilosas. Além disso, os adenocarcinomas podem ser classificados como mucinosos com aspecto de anel de sinete quando apresentam mais de 50% da sua estrutura composta com mucina (BOIVIN et al., 2003).

Com relação à graduação histológica, os tumores são divididos em pouco, moderado e bem diferenciados, conforme a diferenciação citológica das células tumorais e número de mitoses (INCA, 2008).

Tabela 1. Características de tumores benignos e malignos

Critérios	**Benignos**	**Malignos**
Crescimento	Lento, delimitado	Rápido, infiltrativo
Morfologia	Semelhante ao tecido original	Diferente do tecido original
Metástases	Ausentes	Freqüentes
Origem		
Glandular	Adenomas	Adenocarcinomas

(INCA, 2008)

Desta forma, a evolução da carcinogênese de cólon envolve várias alterações genéticas seguida de modificação tecidual com o aparecimento de áreas displásicas, formação de pólipos ou adenomas, os quais podem evoluir para um adenocarcinoma de cólon (LIU, 2006; TURNER, 2012).

2.3. Biomarcadores de câncer de cólon

Vários biomarcadores são pesquisados nos protocolos experimentais visando compreender as vias relacionadas com o desenvolvimento e progressão tumoral, tais como Ki-67 (antígeno nuclear não histona), PCNA (antígeno nuclear de proliferação celular), p53 (antígeno de supressão tumoral), COX-2 (Ciclooxigenase) e apoptose (TAKANO et al., 1996; SAKUMA et al., 1999; GUPTA e DUBOIS, 2001; LIU et al., 2003; COLNOT et al., 2004; CECCARELLI et al., 2005; FIRESTEIN et al., 2008). Entretanto, torna-se necessário conhecer os caminhos genéticos-moleculares da patogênese do câncer para determinar os biomarcadores mais adequados, já que a apoptose, angiogênese, crescimento e proliferação celulares estão correlacionadas com a progressão da carcinogênese (BADREDDINE et al., 2008; GOMES et al, 2008).

A apoptose está relacionada com a função das caspases (GOMES et al., 2008; GARAY et al., 2003), assim, a determinação dessas proteínas, em especial a caspase-3, representa uma importante ferramenta para avaliar o índice de apoptose em tecidos cancerosos. Gomes et al. (2008) verificaram correlação entre o índice de marcagem (porcentagem de área tecidual marcada na prova de imunohistoquímica) e a densidade óptica média (intensidade de coloração do tecido medida por pixels positivos) da caspase-3 em análise de tecido com adenocarcinoma de cólon. Da mesma forma, Moura et al. (2012) relataram maior imunoreatividade da caspase-3 clivada em células de adenocarcinoma de cólon induzido em ratos Wistar.

Além disso, Sugamura et al. (1998) observaram uma diminuição da apoptose em tumores mais diferenciados, malignos, com invasão venosa e metástase em linfonodos.

Com relação a proliferação celular, as células proliferativas presentes em um tumor são detectadas facilmente e rapidamente com a determinação de Ki-67 (GERDES et al., 1983). Colnot et al. (2004) observaram aumento da proliferação celular diretamente relacionada com a expressão de K-67. Johnston et al. (1989) demonstraram que o Ki-67 apresenta marcação no terço médio e final das criptas normais e nos adenomas uma distribuição uniforme da marcação de Ki-67 na cripta. Entretanto, deve-se considerar que o Ki-67 apenas indica um potencial de divisão célular (SCHOLZEN e GERDES, 2000).

Conforme Sugamura et al. (1998), a malignidade e o prognóstico de um câncer pode estar relacionado com aumento de Ki-67. Nabi et al. (2008) também demonstraram que o Ki-67 está relacionado com o grau, estágio e histologia do tumor.

Com relação ao biomarcador p53, Kikuchi et al. (1997) relataram que este marcador apresentou-se positivo em 3% dos adenomas e 77% dos adenocarcinomas, sendo ausente nos pólipos hiperplásicos e na mucosa saudável. Já Baretton et al. (1996) demonstraram um acúmulo de p53 em 16% dos adenomas e 43% dos carcinomas.

Outros biomarcadores também são pesquisados na evolução da carcinogênese, tais como a COX-2, pois há uma estreita evidência da relação da COX-2 com o processo de carcinogênese (GUPTA e DUBOIS, 2001; SHENG et al., 2001; CECCARELLI et al., 2005; FÜRSTENBERGER et al., 2006). Conforme Ceccarelli et al. (2005), a expressão de COX-2 está associada com o espalhamento tumoral. Sakuma et al. (1999) observaram que em câncer avançado há expressão difusa de COX-2.

A COX-2 está relacionada com a produção de várias prostaglandinas, as quais estão relacionadas com alguns processos fisiológicos (GUPTA e DUBOIS, 2001).

Entretanto, nem todas as prostaglandinas apresentam efeito benéfico. Conforme Sheng et al., (2001), a PGE-2 promove alterações fenotípicas, aumento de motilidade e estimulação do crescimento de células cancerosas no cólon.

Assim, a determinação de biomarcadores é uma importante ferramenta para avaliar os mecanismos envolvidos na evolução e formação de metástases, bem como as vias que podem ser inibidas ou estimuladas com determinados protocolos experimentais (BARETTON et al., 1996; ILYIN et al., 2004; CECCARELLI et al., 2005; BABREDDINE et al., 2008; SAKUMA et al., 2009).

2.4. Etapas da carcinogênese química experimental

O desenvolvimento do câncer de cólon representa uma seqüência de eventos com as etapas de iniciação, na qual ocorre alteração no DNA pela ação de algum carcinógeno, seguida pela promoção, onde há uma mudança na expressão do genoma e expansão clonal de células iniciadas formando focos de criptas aberrantes (FCA) e, posteriormente, ocorrem às etapas de progressão, na qual ocorrem múltiplas alterações genéticas e o processo proliferativo torna-se independente, podendo ocorrer a progressão de alguns FCA para adenomas e adenocarcinomas (GOMES-CARNEIRO et al., 1997; BRADY et al., 2000).

As etapas relacionadas com o desenvolvimento do câncer de cólon podem ser evidenciadas e determinadas experimentalmente com a utilização de técnicas laboratoriais, tais como o ensaio cometa (FAIRBAIRN et al., 1995; COLLINS, 2004), contagem de FCA (BIRD, 1995) análises macroscópicas e histológicas (MOURA et al., 2012).

De acordo com Bird (1987), as criptas aberrantes que são observadas em cólon corado com azul de metileno, apresentam "espessamento da parede das criptas colônicas e são mais largas em relação as criptas normais, sendo que após 3-4 semanas de indução os animais apresentam criptas únicas e a exposição repetida ao carcinógeno azoximetano (AOM) acima de 12 semanas levam a elevação no número e tamanho dos focos displásicos". Além disso, os "FCA se coram mais intensamente, geralmente apresentam o lúmen em forma de fenda e apresentam-se elevados do plano na visualização no microscópio" (BOIVIN et al., 2003).

O dano ao DNA, relacionado com a etapa de iniciação do câncer, pode ser avaliado pelo ensaio cometa, pois as células lesadas são identificadas visualmente por uma espécie de cauda formada pelos fragmentos e quebras da fita de DNA (FAIRBAIRN et al., 1995). O ensaio cometa baseia-se na lise celular e denaturação do DNA presente no sangue e avaliação do comprimento da cauda entre outros parâmetros da imagem do cometa formada pelo DNA danificado e visualizado em microscopia de fluorescência após a passagem das lâminas em cuba de eletroforese (Figura 1) (SINGH et al., 1988).

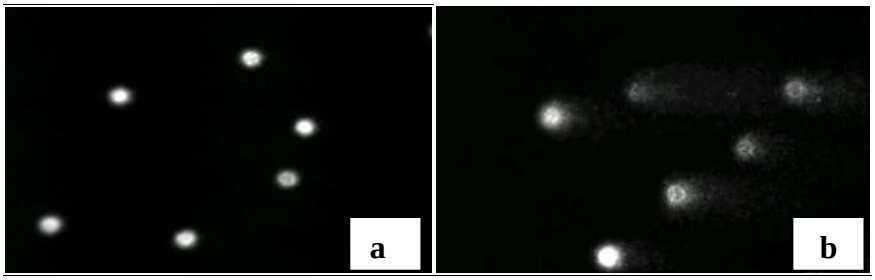


Figura 1. a. Imagem de nucleóides sem dano no DNA observados em microscopia de fluorescência e coloração de brometo de etídeo em amostra de sangue coletada do plexo retro-orbital em rato Wistar. b. Imagem de nucleóides com dano no DNA observados

em microscopia de fluorescência e coloração de brometo de etídeo em amostra de sangue coletada do plexo retro-orbital em rato Wistar (Dados da pesquisa).

As etapas de promoção e progressão podem ser identificadas por análises histológicas pela visualização da formação de FCA (etapa de promoção) e progressão destas lesões para adenomas/ adenocarcinomas, os quais podem originar metástases (etapa de progressão). A presença de FCA evidenciam lesões pré-neoplásicas (BIRD, 1995), sendo que com a evolução da doença, pode-se observar graus de anormalidade tecidual que variam desde o leve, com células pouco diferenciadas da mucosa normal à um amontoado desordenado de células, formando um tecido maligno (Figura 2) (WEINBERG, 2007).

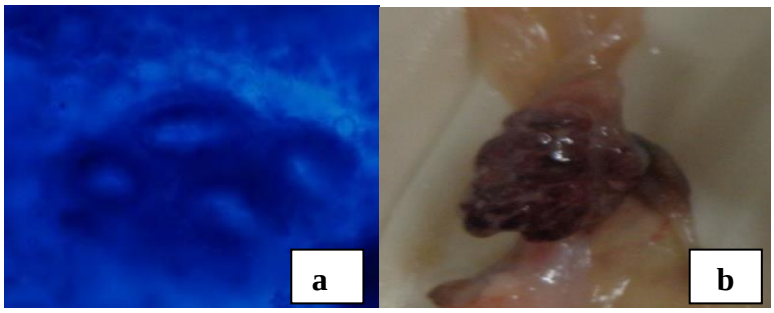


Figura 2. a. Imagem de foco de cripta aberrante com 4 focos observada em microscópio óptico em amostra de cólon de rato Wistar induzido quimicamente ao câncer com 1,2 dimetilhidrazina e corado com azul de metileno. b. Lesão macroscópica em cólon de rato Wistar induzido quimicamente ao câncer com 1,2 dimetilhidrazina (Dados da pesquisa).

Desta forma, pode-se dividir a evolução da carcinogênese química experimental nas etapas de iniciação, promoção e progressão (GOMES-CARNEIRO et al., 1997; BRADY et al., 2000), permitindo a avaliação de intervenções experimentais em modelo animal induzido quimicamente à carcinogênese de cólon.

2.4.1. Indução da carcinogênese química experimental

O monitoramento das etapas de carcinogênese no cólon pode ser realizado em modelo animal induzido quimicamente ao câncer com o composto denominado 1,2 dimetilhidrazina (DMH) (Figura 3) (RONCUCCI et al., 1991; RAVNIK-GLAVAC et al., 2000; PERSE e CERAR, 2005; PERSE e CERAR, 2010; MOURA et al., 2012), sendo que em 1975, Reddy e colaboradores já demonstravam ser possível estudar o efeito da dieta em ratos induzidos quimicamente ao câncer de cólon com DMH.

H3C- NH- NH - CH3 ($C_2H_8N_2$)

Figura 3. Fórmula estrutural e molecular do composto 1,2 dimetilhidrazina (IARC, 1987)

"O DMH é um reagente químico que, quando administrado por via subcutânea na dosagem adequada produz alta incidência de adenomas e adenocarcinomas de cólon, induzindo mutações genéticas no DNA" (IARC, 1999; 1987), sendo que após a administração "o composto é oxidado, desidrogenado a azoximetano (AOM), sofre uma N-oxidação e uma co-oxidação a metilazoximetanol (MAM), o qual é transformado no íon metildiazônio ativo, um composto carcinogênico" (FIALA, 1975, 1977; IARC

1987). Além disso, com a metabolização do DMH, há a formação de O^6-mG (O^6-metilguanina) e N^7-mG (N^7-metilguanina) (MEGARAJ et al., 2014) a partir do AOM formado (NYSKOHUS, et al. 2013).

O azoximetano, subproduto do DMH (FIALA, 1975, 1977; IARC 1987), também é considerado um indutor de carcinogênese (IARC 1974, 1987; LASKO e BIRD, 1995; BURLAMAQUI et al., 2012). Entretanto, Jucá et al. (2014) compararam a administração de DMH na dose de 65mg/ kg/ semana por 5 semanas e AOM na dose de 20mg/ kg/ semana por 2 semanas e concluíram que o DMH é um indutor 50 vezes mais barato e mais eficiente na indução química ao câncer que o AOM, embora ambos apresentem potencial carcinogênico.

Os metabólitos reativos do AOM que induzem a formação de O^6-mG e N^7-mG são detectados no fígado, seguido do colón (proximal e distal), com níveis similares no duodeno, jejuno e íleo com 6 h de administração do indutor (MEGARAJ et al., 2014).

A remoção do O^6meG no organismo ocorre pela ação de uma proteína reparadora de DNA, a O^6-metilguanina DNA metil transferase (MGMT) e pelo processo de apoptose. O MGMT é rapidamente utilizado pelo organismo com a formação de O^6meG dentro de 2h, sendo substituído por uma resposta aguda da apoptose com maiores índices apoptóticos 6-8h após a administração de AOM (NYSKOHUS et al., 2013).

Os roedores são as espécies de laboratório mais utilizadas para protocolos experimentais, pois apresentam tamanho reduzido, ciclo reprodutivo curto, prole numerosa, nutrição variada, docilidade, adaptação e sociabilidade (ANDRADE et al., 2002). A carcinogenicidade do DMH foi testada em camundongos, ratos e hamsters com a administração oral, subcutânea e intramuscular induzindo a formação de tumores em vários locais (IARC, 1974, 1987).

Além disso, entre os roedores, diversos trabalhos demonstram que os ratos são um modelo animal que permite uma boa avaliação da evolução do processo de carcinogênese de cólon e o composto 1,2 dimetilhidrazina (DMH) induz alteração no cólon de ratos com formação de FCA e lesões tumorais, sendo portanto considerado um indutor adequado de carcinogênese de cólon em diferentes protocolos experimentais em ratos (Quadro 1) (JACKSON et al., 1999; LARANJEIRA et al., 1999; JIA e HAN, 2000; RODRIGUES et al., 2002; SIVIERI et al., 2008; MOURA et al., 2012).

Modelo Animal	**Dose de DMH**	**Formação de FCA**	**Pólipos, adenomas e/ou adenocarcinomas**	**Autores**
Ratas SRW	6,8mg/kg/ semanal/ 1, 5, 10 e 20 semanas	não avaliado	acima de 20 semanas, ausência com dose total de 6,8mg/kg	Jackson et al. (1999)
Ratos Wistar	20mg/kg/ semanal/ 10 semanas	16 semanas	32 semanas em todos animais	Jia e Han (2000)
Ratos Wistar	20mg/kg/ semanal/ 14 semanas	42 semanas	42 semanas	Sivieri et al. (2008)
Ratos Wistar	40mg/kg/ 2 vezes por semana/ 2 semanas	4 semanas	30 semanas	Rodrigues et al. (2002)
Ratos Wistar	40mg/kg/ 2 vezes por semana/ 2 semanas	20 semanas	20 semanas	Moura et al. (2012)
Ratas EPM-1 Wistar	65mg/kg/ semanal/ 5 semanas	não avaliado	16 semanas	Laranjeira et al. (1998)

Quadro 1. Protocolos experimentais de carcinogênese química utilizando 1,2 dimetilhidrazina (DMH)

Assim, pode-se considerar que o DMH é um bom indutor de carcinogênese química em ratos, permitindo avaliar com clareza a evolução do processo adenoma-adenocarcinoma de cólon, identificando as etapas de iniciação, promoção e progressão tumoral.

2.5. Probióticos, prebióticos e simbióticos

Os probióticos são microrganismos vivos, que administrados em quantidades adequadas, conferem benefícios à saúde do hospedeiro (FAO/WHO, 2001; BRASIL, 2002), sendo que o microrganismo probiótico deve resistir ao pH gástrico e sais biliares e não apresentar patogenicidade (LEE et al., 2005; SIVIERI et al., 2008; THYRABUNYANON et al., 2009; GIANOTTI et al., 2010). Diversas bactérias dos gêneros *Lactobacillus, Bifidobacterium, Enterococcus,* são utilizadas como probióticos (LAW, 1997; ORDÓÑEZ-PEREDA, 2005; STEFE et al., 2008; KADDOKA et al., 2010),

Dentre os efeitos benéficos relacionados com a ingestão de produtos elaborados com bactérias probióticas, pode-se ressaltar a diminuição do risco e prevenção do câncer de cólon (LAN et al., 2007; LEE et al., 2008; SIVIERI et al., 2008; THYRABUNYANON et al., 2009; BALDWIN et al., 2010; DENIPOTE et al., 2010; KIM et al., 2010; KUMAR et al., 2010).

Outros efeitos benéficos atribuídos aos probióticos incluem: alívio dos sintomas de intolerância à lactose, redução do colesterol sérico, alívio de constipação, prevenção de colite medicamentosa, ulcerativa ou induzida por radiação e casos diarréicos (FOTIADIS et al., 2008). Além disso, promovem aumento do HDL, redução no nível pós-prandial de LDL oxidado e triglicerídeos, (KADDOKA et al., 2010), ação anti-hipertensiva pela melhora no perfil lipídico, diminuição da resistência à insulina (LYE et al., 2009), bem como modulação da resposta imune (MARTIN et al., 2006).

Os mecanismos de ação dos probióticos envolvidos com a prevenção ao câncer de cólon relatados na literatura são diversos, tais como: alteração do metabolismo no cólon, aumento da resposta imune, indução da apoptose, diminuição de resposta

inflamatória, degradação de carcinógenos, produção de compostos antimutagênicos, inibição da formação de células tumorais e da conversão de substâncias pré-carcinogênicas em carcinogênicas, redução da incidência de FCA e formação de tumores, suprimindo a atividade de enzimas bacterianas e diminuindo o dano ao DNA (DENIPOTE et al., 2010; KUMAR et al., 2010).

Já os prebióticos são componentes alimentares não digeríveis que ajudam a microbiota do cólon, estimulando seletivamente o crescimento de bactérias comensais (FORSYTHE, 2002; ROBERFROID, 2003; SANGEETHA et al., 2005; RAFTER et al., 2007). Além disso, alguns estudos indicam a ação preventiva no câncer de cólon dos prebióticos pela diminuição da capacidade metastática, inibição do crescimento, modulação da expressão gênica, melhora na barreira epitelial do cólon, diminuição da exposição ou redução do impacto de agentes genotóxicos a mucosa intestinal, modulação da resposta imune, diminuição da proliferação celular no cólon e da capacidade da água fecal de induzir necrose e danos ao DNA na mucosa (POOL-ZOBEL, 2005; RAFTER et al., 2007; DENIPOTE et al., 2010).

Dentre os prebióticos, pode-se citar os frutooligossacarídeos, os quais são encontrados em uma variedade de plantas, tais como no alho, cebola, aspargos, alcachofra, banana, trigo (ROBERFROID, 2003) e yacon (Figura 4) (SANTANA e CARDOSO, 2008).

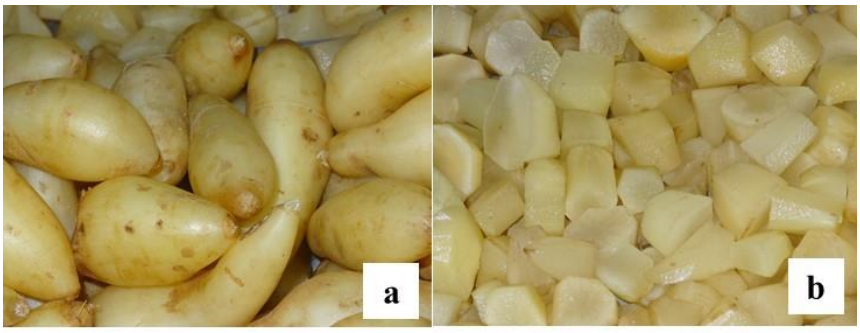


Figura 4. a. Imagem da raiz tuberosa de yacon (*Smallanthus sonchifolius*) "in natura". b. Imagem da raiz tuberosa yacon (*Smallanthus sonchifolius*) após remoção da casca, lavagem e corte (*Smallanthus sonchifolius*) (Dados da pesquisa).

O termo frutooligossacarídeo (FOS) é um nome genérico de todo oligossacarídeo que escapa da digestão no trato intestinal humano, sendo composto principalmente de frutose. Esse prebiótico alcança o cólon para ser fermentado por bactérias benéficas, originando compostos como lactato e ácidos graxos de cadeia curta (butirato, propionato, acetato), favorecendo bactérias dos gêneros *Bifidobacterium* spp e *Lactobacillus* spp em detrimento de microorganismos patogênicos, tais como *Clostridium sp* e *E. coli.* Além disso, diminuem o risco de câncer de cólon pela produção de butirato e aumento de resposta imune local, bem como diminuem o pH no cólon e facilitam a excreção de amônia e aminas na urina (ROBERFROID, 2003; IANNITI e PALMIERI, 2010).

Desta forma, alimentos com probióticos e prebióticos (SAIKALI et al., 2004; FOTIADIS et al., 2008; DENIPOTE et al., 2010; KUMAR et al., 2010) e simbióticos (associação do probiótico com prebiótico) (MOURA et al., 2012) são apontados com potencial de prevenção ao câncer de cólon, sendo que há uma forte evidência de relação entre câncer, dieta e microbiota intestinal (IANNITTI e PALMIERI, 2010).

3.1.1. *Lactobacillus acidophilus*

Dentre as bactérias probióticas pesquisadas quanto ao efeito preventivo sobre o câncer de cólon, pode-se citar o *Lactobacillus acidophilus* (PERDIGON et al., 1987; HOSODA et al., 1996; LANKAPITHRA e SHAH, 1998; RAO et al., 1999; LEE e LEE, 2000; URBANSKA et al., 2009; BALDWIN et al., 2010; KIM et al., 2010). O *Lactobacillus acidophilus* pertence ao gênero *Lactobacilli,* o qual engloba várias espécies com potencial à saúde (LAW, 1997). As bactérias ácido láticas são as maiores representantes de probióticos, podendo ser associadas a vários habitats, tais como: plantas, alimentos, água e silagem, bem como no trato intestinal, vagina e cavidade oral humanos, podendo influenciar beneficamente o hospedeiro (HOLZAPFEL e SCHILLINGER, 2002)

Baldwin et al. (2010) verificaram que na presença de 10^8 UFC/ mL de bactérias ácido láticas (*L. acidophilus* e *L. casei*) há um aumento em 40% da apoptose em células de carcinoma de cólon isoladas de biópsias (CRL-2134) na presença do 5-fluouracil e o fenômeno foi dose dependente em relação ao número de UFC/ mL.

Kim et al. (2010) observaram que o exopolissacarídeo cb-EPS isolado de *L. acidophilus* 606 inibiu a proliferação da linhagem de células tumorais do cólon (HT-29), afetando a morfologia celular e induzindo morte celular.

Hosoda et al. (1996) avaliaram leites fermentados com *L. acidophilus* LA-2. Os pesquisadores observaram diminuição da mutagenicidade da água fecal, bem como alteração benéfica da população microbiana nas fezes, o que pode ter influenciado nesse resultado. Lankapithra e Shah (1998) verificaram que o *Lactobacillus acidophilus* inibiram oito potentes agentes mutagênicos e pró-mutagênicos, entre eles a aflatoxina-B, sendo que dentre os ácidos produzidos, o butirato apresentou maior efeito, seguido

pelo acetato. Além disso, Perdigon et al. (1987) verificaram que o *Lactobacillus acidophilus* aumentou a atividade enzimática e fagocítica dos macrófagos peritoneais e atividade dos linfócitos.

Rao et al. (1999) verificaram que a dieta com *Lactobacillus acidophilus* NCFMTM inibiu a formação de FCA em ratos induzidos quimicamente ao câncer de cólon. Lee e Lee (2000) administraram diariamente *Lactobacillus acidophilus* HY2104 durante 10 semanas a ratos Sprague-Dawley induzidos ao câncer com AOM (15mg/ kg) na segunda e terceira semana de experimento e observaram que o total de FCA sete semanas após a indução foram menores nos grupos que receberam *Bifidobacterium longum* HY800, *Lactobacillus acidophilus* HY210 e ambas cepas probióticas em relação ao controle induzido, sendo que a porcentagem de redução de número total de FCA com *Lactobacillus acidophilus* foi de 38.6%. Bolognani et al. (2001) observaram que a dieta com *L. acidophilus* durante 4 semanas após indução química da carcinogênese de cólon com AOM (duas doses de 12,5mg/ kg/ via subcutânea) diminui o número de FCA em ratos Sprague Dawley alimentados com dieta rica em gordura, com 25% de óleo de milho.

Goldin e Gorbach (1977) verificaram que a atividade das enzimas 8-glucuronidase, azorredutase e nitrorredutase aumenta com a dieta com carne em relação a dieta com fibras, sendo que a administração de *L. acidophilus* diminuiu a atividade das enzimas fecais nitrorredutase e azorredutase em animais alimentados com carne.

Urbanska et al. (2009) verificaram supressão da incidência, multiplicidade e tamanho dos tumores com a administração de *L. acidophilus* microencapsulado e diminuição da liberação de ácidos biliares, sendo que os camundongos com mutação no gene APC apresentaram menos pólipos e adenomas mais avançados com baixo grau de displasia em relação ao controle que não recebeu tratamento.

Desta forma, sugere-se que o *Lactobacillus acidophilus* apresenta diversos efeitos com potencial de prevenção ao câncer de cólon, tais como: alteração da mutagenicidade da água fecal (HOSODA et al, 1996), redução da incidência, volume e multiplicidade tumoral (URBANSKA, et al. 2009), diminuição de FCA (LEE e LEE, 2000; BOLOGNANI et al., 2001), apoptose de células cancerosas (BALDWIN et al, 2010), inibição de agentes carcinogênicos e pró-carcinogênicos (LANKAPITHRA e SHAH, 1998) e aumento da resposta imune (PERDIGON et al., 1987).

2.5.2. Yacon (*Smallanthus sonchifolius)*

O yacon é uma raiz tuberosa originária dos Andes (LACHMAN et al., 2003), maior fonte de frutooligossacarídeos (FOS) encontrado na natureza (SANTANA e CARDOSO, 2008). Entretanto, conforme a origem do yacon analisado, há variabilidade no conteúdo de FOS, compostos fenólicos ou atividade antioxidante, sendo que o conteúdo de FOS pode variar entre 0.7 à 12.3 g/ 100g de peso fresco (CAMPOS et al., 2012).

O yacon apresenta diversos compostos tanto presentes em folhas como na própria raiz, podendo-se citar: os frutooligossacarídeos (CAMPOS et al., 2012), melampolides (LIN et al., 2003), sesquiterpenos lactonas (CHOI et al., 2010), compostos fenólicos (SIMONOSVKA et al. 2003), flavonóides (CHIRINOS et al., 2013) e o ácido ent.-kaurenóico (PADLA, et al. 2012).

Essa raiz tuberosa fornece energia na faixa de 148-224 kcal/ kg de matéria seca (HERMMAN et al. 1997-98) e alto teor de fibras e minerais (VANINI et al., 2009). Além disso, conforme Valentová et al. (2006), o yacon também apresenta variabilidade

em relação a produtividade, formato, peso, isoenzimas, oligofrutanas, compostos fenólicos e conteúdo de DNA nas folhas.

Com relação ao conteúdo de compostos fenólicos, Park et al. (2009), determinaram que o componente fenólico mais ativo no yacon é o ácido clorogênico e o ácido caféico. Entretanto, o ácido clorogênico e o triptofano são os principais antioxidantes no tubérculo (YAN et al., 1999).

Diversos estudos apontam o potencial benéfico do yacon, tais como: ação hipoglicemiante (AYBAR et al., 2001), hepatoprotetora (VALENTOVA e ULRICHOVÁ, 2003; OLIVEIRA et al. 2013); redução de triglicerídeos (GENTA et al., 2005), retenção de cálcio e aumento da dureza óssea (LOBO et al., 2007), aumento no trânsito intestinal (GEYER et al. 2008), redução da insulina e LDL (GENTA, 2009), atividade antioxidante (PARK et al., 2009), redução da gordura visceral abdominal (HABIB et al., 2011), ação imunomoduladora (DELGADO et al., 2012), modificação benéfica da microbiota intestinal e produção de ácidos graxos de cadeia curta (CAMPOS 2012), melhora no perfil lipídico, concentração de creatitina plasmática, albumina sérica e ação em nefropatias (HONORÉ et al., 2012), ação benéfica na espermatogênese (PARK e HAN, 2013). Entretanto há poucos relatos na literatura em relação ao potencial anti-carcinogênico do yacon (MOURA et al., 2012).

Desta forma, torna-se relevante investigar a ação do extrato aquoso de yacon em animais induzidos quimicamente ao câncer de cólon, considerando os compostos bioativos presentes.

Assim, a hipótese testada nessa pesquisa foi verificar o potencial quimioprotetivo do extrato aquoso de yacon, associado ou não ao *L. acidophilus* CRL 1014 em ratos Wistars induzidos quimicamente ao câncer de cólon.

3. OBJETIVOS

3.1. Objetivo geral

- Definir se o consumo do extrato aquoso de yacon associado ou não ao *Lactobacillus acidophilus* CRL 1014 apresenta potencial quimiopreventivo na carcinogênese química de cólon induzida em ratos Wistar.

3.2. Objetivos específicos

- Avaliar a ação do extrato aquoso de yacon na evolução do processo de carcinogênese química de cólon (etapas de iniciação, promoção e progressão da carcinogênese).
- Verificar se o extrato aquoso de yacon apresenta efeito anti-genotóxico frente à agressão química induzida por carcinógeno.
- Verificar o potencial quimioprotetor do extrato aquoso de yacon no desenvolvimento de alterações macroscópicas e microscópicas no cólon.
- Avaliar a influência do extrato aquoso de yacon no comportamento de biomarcadores de risco de câncer de cólon.

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CAPÍTULO II

ARTIGO 1.

Yacon (*Smallanthus sonchifolius)*: Physicochemical Characteristics and Functional Properties

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ARTIGO 1.

Yacon (*Smallanthus sonchifolius)*: Physicochemical Characteristics and Functional Properties

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ABSTRACT

Yacon (*Smallanthus sonchifolius)*, a tuberous root native to the Andes, is found around the world because of it's high adaptability, palatability, safety and beneficial health effects of fructooligosaccharides (FOS) and phenolic compounds, such as chlorogenic acid. Physicochemical characteristics and bioactive compounds of yacon leaves and roots are linked to several beneficial health effects, including lipid profile and calcium absorption, and reduction of visceral fat and blood glucose levels. They also have anti-microbial, anti-carcinogenic and hepatoprotective effects, and positively affect spermatogenesis and gut microbiota. This review covers aspects related to yacon cultivation, physicochemical characteristics and the mechanism of action of bioactive compounds.

keywords*: · health· planting· prebiotic· safety· fructooligosaccharides*

Higlights

-Yacon has several health benefits.

-Different yacon bioactive compounds, such FOS and phenolics were related.

-Yacon has a highest fructooligosaccharide (FOS) content found in nature.

-Chlorogenic acid is also the major yacon compound.

1. Introduction

Yacon [*Smallanthus sonchifolius* (Poeppig et Endlicher) H. Robinson, *Asteraceae*] is a tuberous root native to the Andes (Lachman, Fernández & Orsák, 2003) with the highest fructooligosaccharide (FOS) content found in nature (Santana & Cardoso, 2008). FOS are prebiotics that help gut microbiota stimulate the growth of beneficial bacteria in detriment of pathogenic microorganisms (Roberfroid, 2003).

Yacon cultivation (Fernandes et al., 2006), safety and functional properties make it the subject of many research projects and clinical trials (Aybar et al., 2001; Campos 2012; Delgado et al., 2012; de Moura et al., 2012; Genta, 2009; Geyer et al., 2008; Lobo et al., 2007; Oliveira, Braga & Fernandes, 2013).

Several beneficial properties of yacon root and leaf extract have been studied; however, some mechanisms of action of yacon compounds need to be further investigated. Therefore, the objective of this review was to describe yacon cultivation, physicochemical characteristics, as well the mode of action of bioactive compounds.

2. Methods

Data collected from the web search engines PubMed, Science Direct and Google Scholar were used to carry out this review. Yacon and *Smallanthus sonchifolius* were used as keywords to search for articles published on PubMed and Science Direct (2004-2014) and on Google Scholar (1991 to 2014). A total of 32 articles were found on PubMed and 25 on Science Direct, but one article published on PubMed and four from Science Direct were not included in the present review. The search on Google Scholar resulted in 62 articles, but nine of them were excluded. Considering that some articles were found in more than one web search engine, a total of 81 articles were utilized on this review.

3. Yacon classification and cultivation

Yacon [*Smallanthus sonchifolius* (Poeppig et Endlicher) H. Robinson, *Asteraceae*] (Fig. 1) is a tuberous root native to the Andes, rich in fructooligosaccharides (FOS) and pest resistent due di and sesquiterpenes contents and can used as sweetner and medicinal infusion (Lachman, Fernández & Orsák, 2003).



Fig. 1. Tuberous root- yacon (*Smallanthus sonchifolius)*

According to the botanic classification, this plant belongs to the *Asteraceae* family. Yacon was originally classified within the genus *Polymnia* even though *Smallanthus* has been suggested before. Currently, *Smallanthus sonchifolius* is the most used name, but yacon can also be found in the literature as *Polymnia sonchifolia* or *Polymnia edulis Wedd* (Zardini, 1991).

Yacon receives different common names around the world, including: *aricoma* and *aricuma* in Bolivia, *jicama, chicama* and *shicama* in Ecuador and *arboloco* in Colombia. However, yacon (from the indigenous language Quéchua *yaku,* which means water) is the name more frequently used in most countries. It is note worthy that water is the most abundant compound in the yacon root (Zardini, 1991).

Yacon is a perennial herbaceous plant with vegetative spread and a branching system that gives rise to aerial stems about 2 to 2.5m high. This plant shows a good adaption in different climatic regions, altitudes and soils, which explains its expansion out of the Andean region.Yacon harvest can occur one year after planting. The leaves have a defense system against insects, thus the use of pesticides is not required. Yacon has high productivity, which can exceed 70 t ha^{-1} (Grau & Rea, 1997).

Nowadays, yacon is cultivated in several places, including Europa (Valentová & Ulrichová, 2003), Argentina (Aybar et al., 2001; Genta et al., 2005), Peru (Campos et al., 2012, Genta et al., 2009), Korea (Park et al., 2009), Ecuador (Fernandes et al., 2006; Valentová et al., 2008), Japan (Lin, Hasegawa & Kodama, 2003; Takenaka, 2003), Bolivia (Castro et al., 2013; Fernandes et al., 2006), New Zealand (Fernandes et al., 2006), showing good adaptability in different climatic regions.

Fernandez et al. (2006) reported yacon's adaptability in different climatic conditions as well as how physiological plant status and soil texture can affect root shape.

4. Physicochemical characteristics of yacon

Campos et al. (2012) reported a large variability in FOS content, phenolic compounds and antioxidant activity when analyzing different yacon accessions. Depending on yacon accession the antioxidant capacity is between 23 and 136 μmol TE (trolox equivalent) g dry $matter^{-1}$ and total phenolic compounds range between 7.9 and 30.8 mg CAE (chlorogenic acid) g dry $matter^{-1}$. Accession is "a collection of plant material from a particular location, assigned an identification number" (Aubry, Shoal & Erickson, 2005).

Valentová et al. (2006) showed that yacon variability is related to productivity, yacon shape and weight, isoenzymes, oligofrutans, phenolic compounds and DNA content in leaves.

Yacon has a cellular structure containing cellulose, hemicellulose and pectin compounds. It is an important source of inulin-type fructan (35 g 100 g dry matter^{-1}) with total dietetic fiber content of 45g 100g dry matter^{-1} (Castro et al., 2013). Lachman et al. (2004) reported that the upper part of yacon roots is higher in inulin, whereas the bottom part is rich in fructose.

Furthermore, FOS content ranged from 6.4 to 65.0g 100g dry matter^{-1} (0.7 to 12.3g 100g fresh weight^{-1}) depending on yacon accession and is inversely correlated with reducing sugars, sucrose, total phenolic compounds and positively correlated with dry matter (Campos et al., 2012).

Fernandez et al. (2013) evaluated the contents of short-chain FOS GF3-GF10 and long-chain FOS>GF10 in octoploid and dodecaploid landraces, and suggesting a positive effect of higher ploidy on the FOS content. In addition, Narai-Kanayama, Tokita, and Aso (2007) suggested that fructan 1-exohydrolase is related to the continuous decline in FOS of low degree of polymerizatio) during storage.

Freeze-dried yacon flour contains carbohydrates (86.13%), low protein (2.45% ± 0.09%), lipids (0.87% ± 0.10%), ashes (2.53% ± 0.14%), glucose (7.3%) and fructose (14.1%) (Delgado et al., 2012). Total dietetic fiber is 10.4g 100g dry matter^{-1} (8.7g insoluble fraction and 1.7g soluble fraction) (Castro et al., 2013). Moreover, yacon root contains 93.2% moisture (Chirinos et al. 2013) and yacon has low caloric values and high fiber and mineral contents (Vanini et al., 2009), providing 148-224kcal kg dry matter^{-1} and a good source of potassium (1.8-2.9g kg dry matter^{-1}) (Hermman et al., 1997-98).

Furthermore, depending on yacon accession, reducing sugars ranged from 19.7 to 75.9 g/ 100g dry matter and sucrose ranged from 2 to 16.8g/ 100g dry matter (Campos et al., 2012).

The fresh matter has pH between 6 and 6.5 and uronic acid content between 1.4 and 2.4% of dry matter (Castro et al., 2013). Specific heat is 1304 to 3919 J/ kg^{o}C, heat conductivity varies from 0.21 to 0.52 W/ m^{o}C (Perusselo et al., 2013).

Park & Han (2013) reported that 50% ethanol extract of yacon tubers and leaves contains phenolic acids, such as chlorogenic, ferulic and caffeic acids. Takenaka et al. (2003), using high performance liquid chromatography (HPLC) and NMR spectroscopy, detected five phenolic compounds derivative of caffeic acid in yacon root: chlorogenic acid, 3,5-dicaffeoylquinic acid, 2,4- or 3,5-dicaffeoylaltraric acid, 2,5-dicaffeoylaltraric acid and 2,3,5- or 2,4,5- tricaffeoylaltraric acid. Moreover, Park et al. (2009) found that chlorogenic and caffeic acids are the phenolic compounds more active in yacon, through HPLC. Chlorogenic acid is the major compound found in yacon, with 2.83µg/ 1mg of extract. Park and Han (2013) reported that yacon extract has DPPH scavenging activity in proportion to the total phenolic compounds presents on it.

Yan et al. (1999) established chlorogenic acid content of 48.5 +/- 12.9 *ug*/g and tryptophan content of 14.6 +/- 7.1 *u*g/g (fresh weight) in six different yacon roots. Campos et al. (2012) detected low concentration of carotenoids in some accessions and absence of anthocyanins.

Simonovska et al. (2003) reported that yacon leaves have dicaffeoilquinic acid isomers and an unknown chlorogenic acid, whereas roots contain quercitin flavonoid. Compounds found in yacon leaf extract and their actions are summarized on Table 1.

Table 1. Isolated Compounds of Yacon Leaves

Class	Substance	Biological activity	Reference
Sesquiterpenes lactones,	Enhydrin	Hypoglycemic, antimicrobial activity	Genta et al, 2010; Choi et al. 2010
	Uvedalin	antimicrobial	Lin, Hasegawa and Kodama, 2003
	Sonchifolin	antifungal	Inoue et al, 1995
	Fluctuanin	antimicrobial	Lin, Hasegawa and Kodama, 2003
		nitric oxide inhibition	Hong et al, 2008
Phenolic Acids	Cafeic Acid	antioxidant	Valentová, Sersen and Ulrichová 2005
		inhibit lipoperoxidation	Valentová, Sersen and Ulrichová 2005
	Chlorogenic Acid	hypoglycemic	Valentová, 2004
		inhibit lipoperoxidation	Valentová, Sersen and Ulrichová 2005
		antioxidant	Yan et al. 1999
	Ferulic Acid	antioxidant	Valentová, Sersen and Ulrichová 2005
Flavonoids	Quercetin	anti-inflammatory, and anti-platelet properties	Oh, Endale and Park, 2012
	Rutin	nephropathy preventive	Hao et al, 2012

Neves and Silva (2007) showed that polifenol oxidase from yacon roots (has maximum activity with chlorogenic acid, catechol, 4-methylcatechol, and caffeic acid.

Furthermore, Dou et al. (2010) isolated two diterpenes (ent-kaurane-3β,16 β,17,19-tetrol and ent-kaurane-16 β,17,18,19-tetrol) from yacon leaf extract. Aráoz et al. (2010) analyzed metabolites produced by the secretory canals of yacon root cortex and identified ent-kaurane-type diterpenes. The main component was ent-16-kauren-19-oic

acid (kaurenoic acid) methyl ester and one of the minor components (18-isobutyroyloxy-ent-kaur-16-en-19-oic acid) is a new kaurenoic acid derivative.

Other compounds are found in the yacon leaf. Zheng et al. (2009) isolated octadecatrienoic acid and 13(R)-hydroxy-octadeca-(9Z,11E,15Z)-trienoic acid benzyl glycoside from yacon leaves. Zheng et al. (2010) also found hexenol glycoside, ent-15β-hydroxy-kaur-16-en-19-oic acid and ent-18-hydroxy-kaur-16-en-19-oic acid in yacon leaves.

5. Yacon bioactive compounds

5.1. Fructooligosaccharides (FOS)

Fructooligosaccharides (FOS) are carbohydrates with fructose molecules named fructans, which are synthesized from sucrose and can be inulin, levan or mixed levan types (Benkeblia, 2013). Inulin-type fructans include inulin, oligofructose (product of inulin hydrolysis) and fructooligosaccharides (FOS) which differ in polymerization degree (PD). Inulin PD ranged from 2 to 70 monosaccharide units, while oligofructose and FOS are fructans with a PD lower than 10 monosaccharide units (Apolinário et al., 2014; Kelly, 2008).

FOS are found in several plants, including garlic, onion, asparagus, artichoke, banana, wheat (Roberfroid, 2003) and yacon. However, yacon is the food with the highest FOS content found in nature (Santana & Cardoso, 2008).

5.1.1. FOS activity on gut microbiota and short-chain fatty acid production

FOS shows prebiotic activity like fibres, especially soluble fibres. FOS scape absorption in small intestine and are metabolized by species of colonic bacteria. During FOS fermentation, colonic bacteria transform FOS into compounds, such lactate and short chain fatty acids (SCFA) (butyrate, propionic and acetate acids). This process favors the growth of probiotic bacteria, such as *Bifidobacterium* spp., and *Lactobacillus* spp. in detriment of pathogenic microorganisms, such as *Clostridium* spp. and *E. coli*. (Gibson 1998; Gibson & Roberfroid, 1995; Whelan, 2013).

Our research group has investigated the influence of the prebiotics, especially FOS, in the gut microbiota using the SHIME® reactor (Simulator of Human Intestinal Microbial Ecosystem) (Sivieri et al., 2014). In this study, we observed that the gut microbiota was improved during FOS treatment. To date, few studies on yacon consumption and gut microbiota have been published in the literature. Our research group has also been investigating aqueous yacon extract (100mL daily) in the SHIME®reactor. In accordance with FOS results, a significant increase ($p<0.01$) in the *Lactobacillus* spp. and *Bifidobacterium* spp. populations was observed during yacon treatment. The PCR-DGEE obtained showed that the overall microbial community was reached in the ascending colon of the SHIME® reactor. The SCFA concentration increased ($p<0.05$) during the treatment period, due mainly to significant increased levels of acetic and butyric acids (Sivieri et al., 2014). Such results are expected, considering the high FOS content found in yacon. However, the ability of probiotic microorganisms, such as *Lactobacillus acidophilus* NRRL-1910, *Lactobacillus plantarum* NRRL B-4496 and *Bifidobacterium bifidum* ATCC 15696, to ferment FOS in yacon roots depends of the FOS polymerization degree (Pedreschi et al., 2003).

In agreement with *in vitro* studies, Campos et al. (2012) reported that the administration of yacon flour in an animal model increases *Bifidobacterium* spp., *Lactobacillus* spp. counting and SCFA production (acetate, propionate, butyrate and total SCFA), compared with the control group.

Weaver et al. (2010) also reported that treatments of inulin and inulin blended with FOS affect SCFA production with increase of propionate, butyrate and acetate production and total SCFA. Additionally, these treatments increased total acetate and cecal contents.

According to Campell et al. (1996), fermentable and indigestible oligosaccharides help gut health by lowering pH, increasing SCFA production and *Bifidobacterium* spp. counts. In addition, FOS increase cecal butyrate fatty acid concentration comparing with xylooligosaccharide, cellulose and control treatments in an animal trial.

Regarding SCFA absorption, Levrat, Remése and Demigné (1991) suggested that propionate is more effectively absorbed than acetate fatty acid, because acetate content is higher in the cecum and absorption is similar to both fatty acids. Furthermore, butyrate absorption is proportionally lower than propionate fatty acid absorption.

Bird et al. (2010) suggested that the perspective of prebiotics should be broaden, without changing the definition, by considering changes in the colonic fermentation products, such as butyrate, as key indicators of bowel health. Therefore, yacon can be considered as a good prebiotic ingredient.

5.1.2. FOS and colon cancer

FOS reduce colon cancer risk with butyrate acid production, increase of immune local response, decrease of colon pH and promote ammonia and amines removal in urine (Ianniti & Palmieri, 2010; Roberfroid, 2003).

Butyrate short-chain fatty acid production in the colon by beneficial bacteria decreases cellular proliferation and induces apoptosis, especially in colon tumor cells (Hamer et al., 2008). Increase of butyrate production in the colon causes decrease of aberrant crypt foci and delay of tumor progression in rats (Pool-Zobel, 2005).

According to Delgado et al. (2012), FOS consumption improved mucous membrane immunity. The consumption of 5% commercial FOS, 3 or 5% yacon freeze-dried (34.31% FOS) during 30 days did not affect negatively the immune system in BALB/c rats. However, pro-inflammatory cytokine IL-1β in macrophage culture decreased and IgA fecal levels increased, improving mucous membrane immunity.Vaz-Tostes et al. (2014) also reported the beneficial effect of yacon on immunity. The consumption of yacon flour (0.14g FOS kg body weight^{-1}) during 18 weeks increased IL-4 (interleukin-4) on serum and fecal sIgA (secretory IgA) in overweight preschool children with an inadequate dietary intake of zinc and fiber.

Additionally, yacon promotes the growth of probiotic bacteria, which produce less pro-carcinogenic enzyme β-glucuronidase, in detriment of pathogenic microorganisms (de Preter, 2011).

De Moura et al. (2012) reported that yacon have a beneficial health effect because they can lower colon cancer risk. They reported reduction in number and multiplicity of aberrant crypt foci, invasive adenocarcinomas, cellular proliferation and tumor formation in rats with chemically induced to colon cancer and treated with rat chow added of 1% yacon.

5.1.3. FOS action in diabetes and lipid profile

Sheid et al. (2014) suggesting FOS present on yacon can reduce glucose blood with short-chain fatty acids (SCFA) production or promote glucose absorption in peripheral tissues. Furthermore, Kuryl et al. (2006) suggested that fructans have beneficial effect on glucose metabolism, because a high-fructan diet (10%) increased the glucose transmembrane transport in red blood cells, when compared with inulin and a low fructan diet (5%).

Aybar et al. (2001) reported that diabetic rats taking 2% yacon tea *ad libitum* during 30 days had lower blood glucose levels. The animals also showed improvement in insulin profile, creatinine *clearance*, renal hypertrophy and albumin excretion comparing with diabetic rats in the positive control group.

In addition, Habib et al. (2011) showed that yacon flour (340 or 6800mg FOS/ kg body weight) administered during 90 days to diabetic Wistar rats (Streptozotocin-induced) was linked to beneficial effects on increase in insulin-positive cells, pancreas weight and glucagon-like peptide. The animals had also a reduction in visceral abdominal fat and a slight increase in the plasmatic levels of fasting insulin with smaller FOS doses yielding better results.

Regarding lipid profile, Roselino et al. (2012) reported a positive effect on triglyceride and HDL cholesterol levels in diabetic rats that received yacon extract and soybean treatment and it's association with *Enterococcus faecium* CRL 183 and *Lactobacillus helveticus* ssp *jugurti* 416. According to Genta et al. (2009), premenopausal, obese and slightly dyslipidemic women taking yacon syrup (0.29g and 0.14g FOS/ kg body weight/ day) during 120 days showed improvement in fasting LDL and visceral fat. Genta et al. (2005) also reported a reduction in post-prandial

triglycerides serum in healthy Wistar rats fed with yacon flour (340 mg and 6800mg FOS/ kg body weight). Kuryl et al. (2006) suggested that fructans have beneficial effect on lipid metabolism with β-oxidation of fatty acids in white blood cells. Furthermore, Levrat, Remése and Demigné (1991) showed that inulin treatment (5-20%) increases bile acids pool having a slight hypocholesterolemic effect.

5.1.4. FOS action in bones

The beneficial effect of yacon on bones has also been reported (Lobo et al., 2007; Rodrigues et al., 2012). Lobo et al. (2007) studied growing rats fed with yacon flour (7.5% FOS) during 27 days and reported an increase in calcium levels in the femur and tibia bones. Also, an increase in bone hardness and femur bone weight was observed, comparing with the control groups. According to Rodrigues et al. (2012), yacon flour associated with *Bifidobacterium longum* showed a positive effect on calcium, phosphorous and magnesium concentrations in the tibia bone. Besides, fracture force increased 8.1% with yacon treatment and 14.6% with yacon associated with *Bifidobacterium longum* comparing with the control group. Levrat, Remése and Demigné (1991) showed that inulin treatment (5-20%) causes enlargement of cecal pool of calcium, phosphate and magnesium and an enhanced absorption of theses minerals.

Campos et al. (2012) reported that yacon flour administration in an animal model increases depth and number of bifurcated crypts in the cecum. Similarly, Lobo et al. (2007) reported an increase of bifurcated crypt number in the cecum with yacon treatment (5 or 7.5% FOS). The treatment with yacon (7.5% FOS) showed increased of number and depth of crypts comparing with control group. According to Rodrigues et al. (2012), yacon flour treatment causes an increase in cecum weight. Therefore, these

studies suggest that FOS administration increases cecal calcium absorption and, consequently, calcium levels in bones.

According to Campbell et al. (1997), FOS treatment increases cecal weight compared with the control and cellulose treatments. The authors suggesting that FOS increase crypt depth and cell density by providing butyrate as an energy source, resulting in higher wall weight, compared with the control.

Ohta et al. (1994) suggested that FOS (50g kg^{-1}) increased calcium and magnesium absorption in the colon and rectum, and indigestible and fermentable carbohydrates facilitate the colorectal absorption of these minerals because there is a linear disappearance of calcium and magnesium during colorectal passage. Furthermore, SCFA production can be related to some stimulative effect of mineral absorption. Levrat, Remése and Demigné (1991) also reported an enlargement of the cecum with inulin treatment and an increase in the cecal wall weight and blood flow in the cecal vein. Besides, there was an increase in cecal calcium and magnesium with inulin treatment.

In addition, Weaver et al. (2010) suggested that SCFA production can be related to calcium absorption and cecal content. Moreover, femoral calcium content was related to cecal weight and bone zinc content was increased with treatment of the inulin blended with FOS.

5.2. Phenolic compounds

Takenaka et al. (2003) detected five phenolic compounds (caffeic acid derivatives) in yacon root. According to Park et al. (2009), chlorogenic and caffeic acids are the most active phenolic compounds in yacon.

5.2.1. Antioxidant effects of phenolic compounds

Phenolic compounds have antioxidant effect (Campos et al., 2012), as well as beneficial effects on diabetes (Valentová et al., 2004). According to Campos et al. (2012), there is a hight correlation between antioxidant capacity and total phenolic compounds (Table 1). However, Yan et al. (1999) reported that chlorogenic acid and tryptophan are the major antioxidants in yacon root and chlorogenic acid mechanism of action is related to hydroxyl radical production mediated by iron and tryptophan with hydroxyl radical scavenging activity.

It seems that the antioxidant activity of phenolic compounds have an effect on hepatic damage. Valentová et al. (2004) showed cytoprotective effect of aqueous and organic extract of yacon against hepatic oxidative damage induced by tert-butyl-hydroperoxide and allyl alcohol in a dose-dependent way. Better results were observed with yacon leaf organic extract (1000µg mL^{-1}), which is high in phenolic compounds.

The hepatoprotective effect of yacon was also reported by Oliveira, Braga and Fernandes (2013), who observed alanine-aminotransferase (ALT) reduction in rats with Streptozotocin-induced diabetes and treated with aqueous extract of yacon tuberous roots.

5.2.2. Anti-diabetic effect of phenolic compounds

Hypoglycemic effects of yacon can be associated with the high content of phenolic compounds in leaves and roots (Table 1). However, enhydrin, a sesquiterpene lactone compound, also has hypoglycemic activity. Phenolic compounds show a reduction in glycogenolysis and cytochrome P450 2b and 2e expression compared to the effect of insulin in the organism (Valentová et al., 2004). Satoh et al. (2013) demonstrated the beneficial effect of yacon treatment on hepatic insulin sensitivity in

the insulin-resistant state. The tribbles 3 (Trb3) expression (negative regulator of Akt phosphorylation activity) was reduced in the liver, with yacon treatment. A reduction in gluconeogenic genes was also observed.

In addition, recent studies on the metabolic and molecular basis of chlorogenic acid showed that this compound is capable of regulating glucose and lipid metabolism through AMPK (5' adenosine monophosphate-activated protein kinase) via activation. AMPK via activation leads translocation of GLUT4 transporter (glucose transporter type 4) through intracellular membranes to plasmatic membranes, thus increasing glucose carriage (Ong, Hsu & Tan, 2013).

Park et al. (2009) studied rats with Streptozotocin-induced diabetes taking yacon (200mg/ kg/ p.o.) and chlorogenic acid (10mg/kg) during six weeks. They observed hypoglycemic effect and decrease in plasmatic glucose, total cholesterol, triglycerides, creatinine contents.

Honoré et al. (2012) also reported the beneficial effect of yacon (leaves) when administered daily during four weeks (70mg dry extract/ kg body weight/ p.o.) to rats with Streptozotocin-induced diabetes: decrease in blood glucose levels, improved insulin production and immunoreactivity in Langerhans islets. Improved plasmatic creatinine, total cholesterol and triglycerides were also observed. Moreover, yacon decoction also prevented the glomerular increase and thickening of basal membranes and nephropathy molecular markers.

5.2.3. Spermatogenic effect of phenolic compounds

Park and Han (2013) observed yacon extract increased sperm counts in rat testis, serum testosterone level, seminiferous tubule numbers and diameters, with some tubules

showing signs of spermatogenesis. An increase in sperm numbers was observed with yacon extract, chlorogenic and ferulic acids. In addition, yacon extract and chlorogenic acid inhibited enzymatic testosterone degradation in rat liver homogenate. Therefore, the authors suggest that the effects of yacon tuber extract can be related to phenolic compounds content.

5.3. Melampolides, sesquiterpenes lactones and flavonoids

Lin, Hasegawa and Kodama (2003) found by chromatography six melampolides on yacon leaves and 8-β methacryloyloxy melampolide-14-oic acid methyl ester, showing strong microbiological activity against *B. subtilis* and *Pyricularia oryzae*. According to the authors, sesquiterpene lactones (uvedalin, flutuanin) showed antimicrobial activity (Lin, Hasegawa & Kodama, 2003), whereas sonchifolin presented antifungal activity (Inoue et al., 1995). Further, Choi et al. (2010) reported that enhydrin has antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA).

According to Simonovska et al. (2003), the crude extract of yacon root contains quercetin flavonoid. Quercetin has anti-inflammatory and anti-platelet properties (Oh et al., 2012) and the flavonoid rutin (Simonovska et al., 2003) can prevent nephropathy (Hao et al., 2012). It seems that the reduction in nephropathy molecular markers, such as collagen IV and laminin-1, reported by Honoré et al. (2012) in rats with Streptozotocin- induced diabetes could be linked to this rutin.

5.4. Ent-kaurenoic acid

Antimicrobial activity of yacon may also be related with *ent*-kaurenoic acid. Padla, Solis and Ragasa (2012) showed that *ent*-kaurenoic acid from the dichloromethane extract of yacon leaves has moderate activity against *S. aureus* and *S. epidermidis* and weak activity against *B. subtilis,* in disk diffusion method.

Joung et al. (2010) reported that n-hexane extract of yacon leaves exposed to 4000 lux showed antimicrobial activity against six MRSA strains.

According to Velez et al. (2013), the protective effect of yacon could be mediated by non-specific immunity, such as total S-IgA, improving the immunological intestinal barrier.

Furthermore, Raga et al. (2010) reported hypoglycemic activity after the consumption of yacon leaf aqueous extract in normoglycemic and diabetic mice treated orally with *ent*-kaurenoic acid and normoglycemic mice that received *ent*-kaurenoic acid by intraperitoneal injection.

6. Toxic effects of yacon

Most studies found in the literature reported beneficial effects of yacon (Aybar et al., 2001; Campos et al., 2012; Delgado et al., 2012; Genta et al., 2005). However, we found two articles showing toxic effects of yacon consumption (leaves and roots). Yun et al. (2010) reported anaphylaxis after yacon consumption in a 55-year-old woman. This patient had positive skin prick and intradermal tests to yacon extract, and the food challenge test also confirmed food anaphylaxis. According to de Oliveira et al. (2011), renal toxicity after extended consumption of yacon leaf aqueous extract and renal damage was associated with an increase in blood glucose levels.

7. Conclusions

Several studies are found in the literature reporting yacon health benefits. However, research on the biochemical steps of each compound involved and their mechanisms of action is scarce. Therefore, further studies should be carried out to verify the mechanisms of action of yacon compounds on different pathologies, how they act in synergy, as well also investigate new compounds found in this species.

Conflict of interest "None declared"

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ARTIGO 2.

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ARTIGO 2.

Protective effect of Yacon (*Smallanthus sonchifolius*) and *Lactobacillus acidophilus* CRL 1014 intake in the early phases of colorectal cancer

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Abstract

The aim this study was to investigate the beneficial effect of aqueous yacon extract (AYE) alone or in combination with *L. acidophilus* CRL 1014 against colon carcinogenesis induced by 1,2 dimethyhydrazine (DMH) in male Wistar rats. Animals were randomly allocated into five groups (n=20 each): G1: untreated group; G2: DMH-treated group; G3 (probiotic): DMH + *L. acidophilus* CRL 1014- treated group; G4 (prebiotic): DMH + AYE- treated group; G5 (synbiotic): DMH + *L. acidophilus* CRL 1014 + AYE- treated group. Five animals of each group were sacrificed after the first administration of DMH, and apoptosis and cell proliferation analysis in the colon and comet assay in peripheral blood samples were evaluated. At third, fifth and eighth months after DMH administrations, the colons were submitted to aberrant crypt foci (ACF) count and macroscopical and histological analysis. A significant reduction ($p<0.05$) in leukocyte DNA damage and colonic cell proliferation but a significant increase ($p<0.05$) in colonic apoptosis was observed in G3, G4 and G5 groups and in G3 and G5 groups, respectively. Specially, in the fifth month after DMH administrations, a significant reduction ($p< 0.05$) in ACF development was observed in G3, G4 and G5 groups in relation to the G2 group. The findings suggest a protective effect of aqueous yacon extract, with or without probiotic, at the early steps of colon carcinogenesis.

Keywords: chemical carcinogenesis, colon, prebiotic, probiotic, synbiotic.

Introduction

Colorectal cancer is a disease that affects people of all ages around the world [1] and estimates 22 millions of colorectal cancer cases in the world in 2014 [2,3]. Increased intake of red meat, alcohol and animal fat has been correlated with colorectal cancer risk [4]. Conversely, food with probiotics, prebiotics and synbiotics has been shown to exert a protective effect against colorectal cancer [5,6].

Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host [7]. Prebiotics are non-digestible food components that affect the colonic microbiota, stimulating the growth of beneficial bacteria [8]. Fructooligosaccharides (FOS) are prebiotics found in several plants [8], including yacon root [9]. Yacon (*Smallanthus sonchifolius*) is an Andean tuberous root with the highest known content of FOS in nature [9] and it provides several benefits, such as hypoglycemic [10], hypocholesterolemic [11], antioxidant [12] and antitumor actions [13].

Rodent colorectal cancer can be chemically induced by 1,2-dimethylhydrazine (DMH), or their metabolite azoxymethane, showing similarities with human colorectal cancer in humans, so that the DMH model is an excellent tool to study molecular events, risk factors, and prevention strategies for this malignant disease [14,15]. Colorectal cancer development includes three stages: initiation, promotion and progression [16]. DNA damage can lead to the initiation stage and promotion stage can be monitored by counting the aberrant crypt foci (ACF) [17] while progression stage can be assessed by colonic adenoma and adenocarcinoma development [18]. Several biomarkers have been used in rodent colon carcinogenesis bioassays, such as

preneoplastic lesions (ACF) [17], cell proliferation [19], apoptosis [20] and DNA damage analysis [21].

Several reports have indicated different biological actions of dietary yacon [22-26], but there are few studies on its anti-carcinogenic effect on colon carcinogenesis [13]. Therefore, the aim of this research was to assess an aqueous yacon extract alone, or in association with *Lactobacillus acidophilus* CRL 1014 (probiotic), in male rats submitted to the colorectal carcinogenesis induced by DMH model.

Materials and Methods

Animals and treatment

The animals used in this study were handled in accordance with Brazilian Animal Experimentation College, and approved by the Ethics Committee of the Faculty of Pharmaceutical Science of Araraquara-SP, UNESP (Protocol 16/2010). Hundred 4-weeks old male Wistar rats were obtained from the UNESP animal facility and maintained in opaque propylene boxes, in a room with a controlled photoperiod (12 hours of darkness) under air filtration and controlled temperature (22ºC ± 2ºC) with free access to water and commercial rat chow (23% protein, 49% carbohydrate, 4% fat, 5% fiber, 7% ashes and 6% vitamin C). After a 2-week acclimation period to the housing environment, the animals were randomly allocated into five groups (n=20 each): G1 (untreated animals): animals that received commercial chow; G2 (DMH): animals induced by DMH and that consumed commercial chow; G3 (probiotic): animals induced by DMH and that consumed commercial chow + *L. acidophilus* CRL 1014; G4 (prebiotic): animals induced by DMH and that consumed commercial chow + aqueous yacon extract; G5 (synbiotic): animals induced by DMH and that consumed commercial

chow + *L. acidophilus* CRL 1014 + aqueous yacon extract (Fig. 1). G2 to G5 groups received four subcutaneous injections of 40 mg kg^{-1} of DMH (Sigma-Aldrich, CO, USA, pH 6.5 in NaCl solution) [27,28], two weeks after the beginning of the treatments with probiotic, prebiotic and synbiotic. G1 group received similar injections of 0.9% NaCl solution (DMH vehicle). All treatments were administered orally for 8 months, *L. acidophilus* CRL 1014 was administered at 10^9 CFU/ mL in 3 mL/ kg body weight [27] and amount of aqueous yacon extract administered was calculated in relation of its FOS content. Animals ingested 10% of FOS in diet [29]. FOS concentration of aqueous yacon extract was previously determined using fructan assay kit (Megazyme, Ireland).

Five animals of each group were sacrificed in a CO_2 chamber, 24 hours after the first DMH administration and at the third, fifth and eighth months of the experimental period (Fig. 1). The body weights of animals in all groups were recorded once a week throughout the experiment.

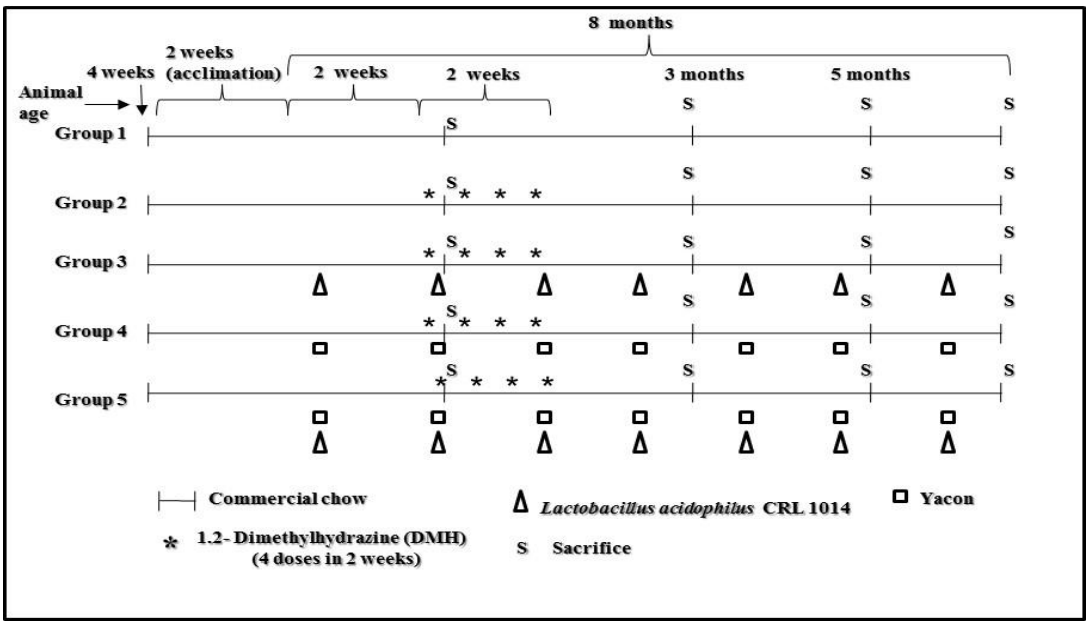


Fig. 1 Experimental design for evaluation of chemopreventive efficacy of aqueous yacon extract and *L. acidophilus* CRL 1014 against experimental colon carcinogenesis. G1 (untreated animals): animals that received commercial chow; G2 (DMH): animals induced by DMH and that consumed commercial chow; G3 (probiotic): animals induced by DMH and that consumed commercial chow + *L. acidophilus* CRL 1014; G4

(prebiotic): animals induced by DMH and that consumed commercial chow + aqueous yacon extract; G5 (synbiotic): animals induced by DMH and that consumed commercial chow + *L. acidophilus* CRL 1014 + aqueous yacon extract. Groups G2 to G5 received 4 subcutaneous injections of 40mg kg^{-1} of DMH, 2 weeks after the begining of the treatments with probiotic, prebiotic and synbiotic.

Probiotic and prebiotic preparation

Lactobacillus acidophilus CRL 1014 was administered to probiotic (G3) and synbiotic groups (G5) at a concentration of 10^9CFU mL^{-1} of sterile peptone water by adding 3mL kg^{-1}bw. The *L. acidophilus* CRL 1014 cells used were collected by centrifugation (5000*g*, 10 min, 4°C) and washed with sterile peptone water after growth in Man Rogosa and Sharpe (MRS) Broth medium (MERCK®, USA) and a bacterial cell count [30]. Aqueous yacon extract was administered to prebiotic (G4) and synbiotic (G5) groups. Yacon root (purchased in a local marketing) were pasteurized at 95ºC/15min immediately after yacon cutting, and yacon was ground to obtain aqueous extract [31,32]. FOS content on aqueous yacon extract was determined with fructan assay kit (Megazyme, Ireland) [33].

Colon processing and histopathological analysis

The colon was removed, opened longitudinally, residual bowel contents was removed with 0.9% NaCl and colon fixed flat in 10% buffered formalin (pH 6.9-7.1) for 48 hours, then maintained in 70% ethanol solution [28]. The localization and volume of each colonic tumor were registered using a calipers of 0.01 mm precision. Measured width (W) and length (L) were used to calculate tumor volume (cm^3) (V) according mathematical equation $V= (L \times W^2)/2$ [34]. Tumors were classed as small, medium or large [13].

The number of aberrant crypt foci (ACF) were identified according Bird`s criteria [17]. The colons were stained with Leishman (Merck®, USA) for 2 min to evaluate ACF count, using an equipped microscope with image capture, at 10 and 40X magnifications. Then, colon samples were embedded in paraffin, cut in 5μm sections and stained with hematoxylin–eosin (HE) for histological analysis [28]. Colon tumors were classified as adenomas or adenocarcinomas using well-established criteria [35].

Immunohistochemical analysis

Ki-67 and p53 labeling indexes was determinated on slides from animals sacrified 24 hours after first DMH administration. Using a labeled polymer system (Universal Dako LSAB®+kit Peroxidase, Denmark A/S), immunoreactivity of Ki-67 (Rabbit monoclonal antibody [SP6], Abcam, USA) and p53 (anti-p53, rabbit polyclonal antibody Abcam, USA) in the colon sections was detected. Briefly, colon sections were dewaxed in xylol and hydrated in absolute alcohol. Slides were treated with 0.01 M citrate buffer (pH 6.0) at 120^0C for 5 min in a Pascal pressure chamber (Dako Cytomation Denmark A/S); 3% H_2O_2 in PBS for 10 min; 1% non fat milk in PBS for 60 min; anti-Ki-67 (1:100 dilution in BSA- bovine serum albumin) or p53 (1:100 in BSA) antibodies overnight at 4^0C; Slides were incubated with Dako Biotinylated Universal link and Streptavidin separately for 15 minutes, each one at room temperature.

Chromogen color was developed accomplished with 3.3-diaminobenzidine tetrahydrochoride (Sigma–Aldrich, Co, USA). The slides were counter-stained with Harris`s hematoxylin. Twenty crypts were analysed in longitudinal section of each slide. Ki-67 index (%): (number of labeled cells in half of crypt/number of cells in half of

crypt) x 100 [13]. p53 staining was regarded as positive when more than 5% of the nuclei showed intense staining [28].

Apoptosis analysis

Apoptosis index was performed on slides from animals sacrified 24 hours after first DMH administration. Slides stained with HE were used to determined apoptosis index and twenty crypts were analysed in longitudinal section of each slide. Apoptosis index (%): (number of cells in apoptosis in half of crypt/ number of cells in half of crypt) x 100 [36].

Comet assay

This technique was performed as described by Singh et al. (1988) [37], with modifications. Briefly, four hours after the last injection of DMH or vehicles, slides were prepared by depositing 5µL of blood of retro orbital plexus mixed with 100µL of low-melting-point agarose (1.5%) on a glass slide coated with normal agar, at 37^0C. Slides (covered with glass coverslip) were chilled at 10^0C for 5min. Slides (after removal of the coverlips) were immersed in freshly prepared lysis solution, composed of 100mL lysis stock solution (2.5 M NaCl, 100.0 mM EDTA, 10.0 mMTris, pH adjusted to 10.0 with solid NaOH, 1000mL of distilled water and 1% N- laurosylsarcosine sodium), 1.0mL Triton X-100 and 10.0mL DMSO (dimethyl sulfoxide). Protected from light, lysis proceed for at least 24h at 4^0C. Slides (washed with phosphate buffered saline (PBS)) were placed in an electrophoresis chamber (25.0 V and 300.0 mA (1.25 V cm^{-1})), and using alkaline electrophoresis buffer (0.3M NaOH, 1mM Na_2EDTA, pH>13) at 4 ^{0}C for

20 min. Slides were neutralized with 0.4 M Tris–HCl buffer, pH 7.5, fixed in absolute ethanol for 10 min and saved for later analysis. For staining, the slides were covered with 50μL of ethidium bromide (20μg/mL) and a coverslip. The material was examined with an epi-illumination fluorescence microscope at 400x magnification connected to an image analysis system (Comet Assay II, Perspective Instruments, Suffolk, UK). A total of 100 nucleoids (two slides per animal) per treatment were analyzed and software used to calculate comet tail length.

Statistical analysis

Quantitative data were compared among experimental groups using ANOVA or Kruskal-Wallis tests followed by Turkey's contrast test. Incidence of tumor volume size was subjected to chi-square test. The statistical difference among the parameters was considered when $p<0.05$.

Results and Discussion

General findings

The oral treatments with prebiotic (AYE), probiotic *(L. acidophilus* CRL 1014) and snybiotic (*L. acidophilus* CRL 1014+AYE) did not significantly modify the body weight gain or food consumption during the experimental period (data not shown). In fact, various experimental evidences have demonstrated that prebiotcs and probiotics are safety for human use [10,36,38,39].

Comet assay

The comet assay has been shown to be sensitive in detecting DNA damage at various levels in different tissues samples [40]. The amount of DNA damage induced by DMH can be verified by the length of tails of the nucleoids produced on leukocytes after electrophoresis (Fig.2). Results showed a significant increase ($p<0.05$) in the length of the comet tail in all groups exposure to DMH (G2-G5 group) when compared to the G1 group (untreated animals) (Fig. 2a and b). On the other hand, a significant reduction ($p<0.05$) in the DNA damage in leukocytes from the G3 (probiotic), G4 (prebiotic) and G5 (snybiotic) groups was observed in comparison to the G2 group (DMH alone) (Fig. 2c). This finding indicate that these oral treatments, before and during DMH exposure, reduced the extent DNA damage in leukocytes and, probably, in epithelial colonic cells target susceptibly to colon carcinogenesis. In corroboration, Pool-Zobel (1996) [41] demonstrated that treatment of the animals with lactic-acid bacteria (*L. acidophilus*, *L. confuses*, *L. gasseri*, *B. longum* and *B. breve*), before DMH administration, inhibits the genotoxic effect this colon carcinogen. However, little has been published on the protective effect of aqueous yacon extract against cancer induced in rats with DMH.

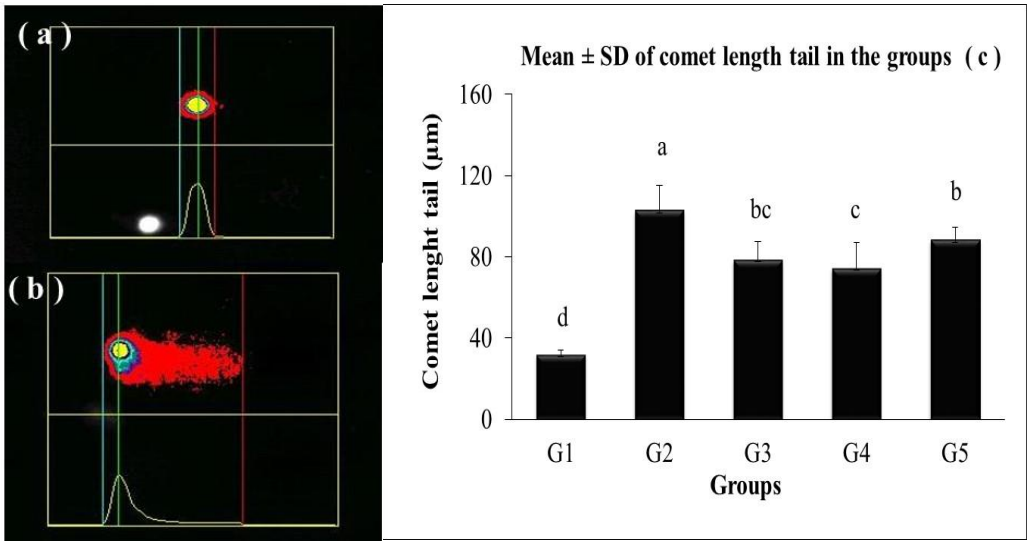


Fig. 2 Comet assay graphic originated with software (Comet Assay II, Perspective Instruments, Suffolk, UK) and results of treatments. a. Comet assay graphic of cell with no DNA damage in an untreated animal. b. Comet assay graphic with DNA damage in DMH animal. c. Mean ± SD (Standard Desviation) of comet tail length in the groups. G1 (untreated animals): animals that received commercial chow; G2 (DMH): animals induced by DMH and that consumed commercial chow; G3 (probiotic): animals induced by DMH and that consumed commercial chow + *L. acidophilus* CRL 1014; G4 (prebiotic): animals induced by DMH and that consumed commercial chow + aqueous yacon extract; G5 (synbiotic): animals induced by DMH and that consumed commercial chow + *L. acidophilus* CRL 1014 + aqueous yacon extract. Different letters indicate significantly different results ($p<0.05$).

Apoptosis Index

Apoptosis index were analysed in the crypts in colon. The results showed that animals treated with aqueous yacon extract associated with *L. acidophilus* CRL 1014 (G5) and *L. acidophilus* CRL 1014 (G3) showed higher apoptotic indexes (22.79% and 20.41%, respectively) when compared with DMH group (G2) ($p<0.05$) (Fig. 3c). Conversely, de Moura et al. (2012) [13] observed no effect on the apoptotic index when animals were fed yacon and *L. casei*. On the other hand, it has to be taken into account that, between 6 and 24 h after chemical induction of cancer, there is a temporary rise in the apoptotic index, which later (at 72 h) falls to a lower value [42]. The damage suffered by the DNA and, therefore, changes in the process of apoptosis, have their

origin in the phase of initiation of carcinogenesis. Now, the transformation of an adenoma into an adenocarcinoma is related to genetic changes and, while there is no exclusive and predetermined sequence of such changes, among those linked to tumor development is a reduction in the rate of apoptosis [43,44].

Ki-67 and p53 Immunohistochemical analysis

Ki-67 and p53 were analysed in the crypts. The staining proportion of Ki-67 in treated groups (G3, G4 and G5) were similar to untreated group (G1) ($p<0.05$). The cell proliferation was 33.79, 33.66, 37.76, 32.61% for groups G1 (untreated), G3 (probiotic), G4 (prebiotic) and G5 (synbiotic), respectively (Fig.3d). According Sakuma et al., (1999) [45] is possible to observe Ki-67 proliferation in cancer cells and in normal cells. In addition, the present cell proliferation results were similar to those obtained by Wu et al. (2014) [20] in the period 0-24h after induction of cancer with azoxymethane (AOM) in mice given inulin as a dietary supplement for 3 weeks.

G4 group did not differ from G2 (DMH) ($p<0.01$), which showed a Ki-67 index of 43.77%. It was observed reduction in the cell proliferation indexes ($p< 0.05$) in the groups fed aqueous yacon extract associated with *L. acidophilus* CRL 1014 and *L. acidophilus* CRL 1014.

In addition, in the present study, the Ki-67 index varied inversely with the apoptotic index. Cell proliferation may lead to an increased risk of developing cancer whereas apoptosis is a protective innate mechanism taking to elimination of the epithelial cells with DNA damage or genomic instability [46]. In agreement with ours results, Linsalata et al. (2005) [47] also observed the influence of a probiotic treatment on the proliferation rate and apoptotic index in healthy animals. Every day for 4 weeks,

they gave rats a cocktail of 2.8×10^{8} CFU/ mL, consisting of 9×10^{10} CFU/g bifidobacteria, 8×10^{10}CFU/g lactobacilli and 20×10^{10}CFU/g *Streptococcus thermophilus*, and reported a lower Ki-67 index and higher apoptotic index, relative to the control group of healthy animals.

However, the relationships between Ki-67, the prognosis and clinicopathological variables can vary, because even tumors with a slow cell cycle and rate of proliferation may contain cells that stain heavily for Ki-67. Conversely, Ki-67 staining may be absent in tumors with a short cell cycle and high rate of proliferation, if there are few cells in active cell cycle [48].

There was no nuclear p53 staining in the healthy rat group (data not shown). Similarly, Sakuma et al. (1999) [45] observed an absence of p53 staining in healthy animals. Whereas the synbiotic, prebiotic, probiotic and DMH groups all showed similar, positive staining.

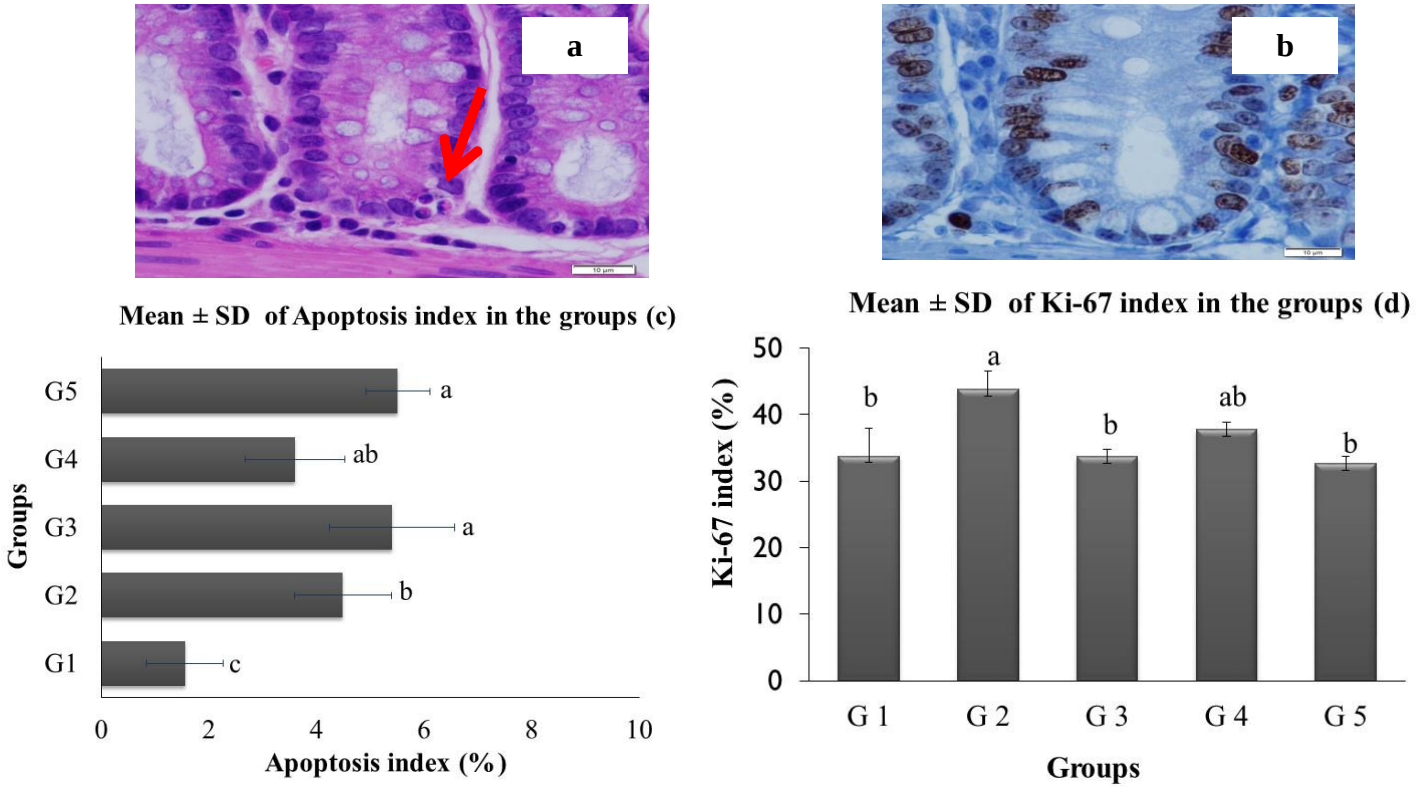


Fig. 3 Representative images (100x objective) and results (Mean ± SD) of apoptosis and Ki-67 immunohistochemical analysis in the different groups 24 hours after induction of colon cancer with DMH in rats. a. Arrow indicate apoptosis on crypt. b. Ki-67 staining on crypt. c. Apoptosis index. d. Ki-67 index. G1 (untreated animals): animals that received commercial chow; G2 (DMH): animals induced by DMH and that consumed commercial chow; G3 (probiotic): animals induced by DMH and that consumed commercial chow + *L. acidophilus* CRL 1014; G4 (prebiotic): animals induced by DMH and that consumed commercial chow + aqueous yacon extract; G5 (synbiotic): animals induced by DMH and that consumed commercial chow + *L. acidophilus* CRL 1014 + aqueous yacon extract. Different letters in the same columns indicate significantly different results ($p<0.05$). Values are mean ± Standard desviation.

Classical ACF analysis

Classical ACF were evaluated in methylene blue-stained whole mount colon at the end of third, fifth and eight months of experimental period (Table 1). There was a significant reduction ($p<0.05$) in mean number of ACF/colon in G5 group (synbiotic) when compared to the G2 group (DMH) in the third month. In the fifth month, a significant reduction ($p<0.05$) in mean number of ACF/colon was detected in G3 (probiotic), G4 (prebiotic) and G5 (synbiotic) groups in relation to the G2 group (DMH). Moreover, no significant difference was observed among groups treated with DMH at late phase of colon carcinogenesis (Table 1). According this results, de Moura et al. (2012) [13], using 1% yacon root, alone or in a synbiotic combination with *Lactobacillus casei* in chow, showed a significant decrease in the number of ACF/colon in both treatments after DMH administrations.

In addition, *L. acidophilus* has demonstrated protective effects in reducing the number of ACF in the colon of animals initiated for colon carcinogenesis [49,50]. However, it should be noted that these studies differed in the extent of reduction of ACF, indicating that the probiotic action was strain dependent. Furthermore, these studies used distinct experimental protocols, with differing treatment periods, doses of DMH and amounts of prebiotic and probiotic products administered.

As the carcinogenic process proceeded, at eighth month of protocol, the various treatments no longer had any effect on the number of ACFs per colon, relative to the DMH group (Table 1). Takayama et al. (2013) [51] agreed with this and demonstrated that the number of ACFs increased with age.

Table 1 Effects of aqueous yacon extract and in association with *L. acidophilus* CRL 1014 or *L. acidophilus* CRL 1014 on development of colonic aberrant crypt foci (ACF) during the 8 months of experimental protocol

Month	Groups			
	G2	G3	G4	G5
Third	12.00±2.68[a]	5.00±1.96[ab]	4.50±2.84[ab]	0.75±0.25[b]
Fifth	36.00±4.00[a]	10.00±1.53[b]	7.67±1.45[b]	18.75±2.53[b]
Eighth	15.25±6.17[a]	23.00±5.53[a]	12.00±4.16[a]	15.00±5.84[a]

G1 (untreated animals): animals that received commercial chow; G2 (DMH): animals induced by DMH and that consumed commercial chow; G3 (probiotic): animals induced by DMH and that consumed commercial chow + *L. acidophilus* CRL 1014; G4 (prebiotic): animals induced by DMH and that consumed commercial chow + aqueous yacon extract; G5 (synbiotic): animals induced by DMH and that consumed commercial chow + *L. acidophilus* CRL 1014 + aqueous yacon extract. Different letters in the same line indicate significantly different results ($p<0.05$). Values are mean ± Standard error.

Histopathological analysis

At sacrifices, there were more tumors on transverse and descending colons, representing 44% and 45% of total tumors observed, respectively. All macroscopic tumors were measured and classified into three categories: small (<0.22cm^3), medium (0.22-0.84cm^3) and large (>0.85cm^3) (Fig. 4d and 4e.). In the initial phase of colon carcinogenesis progression, the animals that ingested aqueous yacon extract (G4) and its associated with *L. acidophilus* CRL 1014 (G5) showed better, compared to DMH group (G2) and probiotic (G3), because only 50 and 66.67% of macroscopic tumors in G4 and G5 were adenocarcinomas (Fig. 4d). Likewise, de Moura et al. (2012) [13] reported that a lower proportion of adenocarcinomas were induced with DMH in animals that ingested a synbiotic mix of yacon and *Lactobacillus casei*.

However, in the later phase, at 8 months of protocol, all treatments (G3, G4, G5) had no effect (Fig. 4d). This corroborates the work of Capurso et al. (2006) [52] , who describe animal models to assess colorectal cancer risk and probiotic treatments, with

results suggesting that these treatments have beneficial effects during initiation of the cancer, which become weaker in the subsequent phases of promotion and progression. Pool-Zobel (2005) [29] reported that prebiotic (with inulin-type fructan) and synbiotic treatments are more effective and the beneficial action more pronounced when they are applied constantly throughout the carcinogenic process. In any case, it must be borne in mind that DMH is a very potent carcinogen whose use will inevitably lead to the development of tumors.

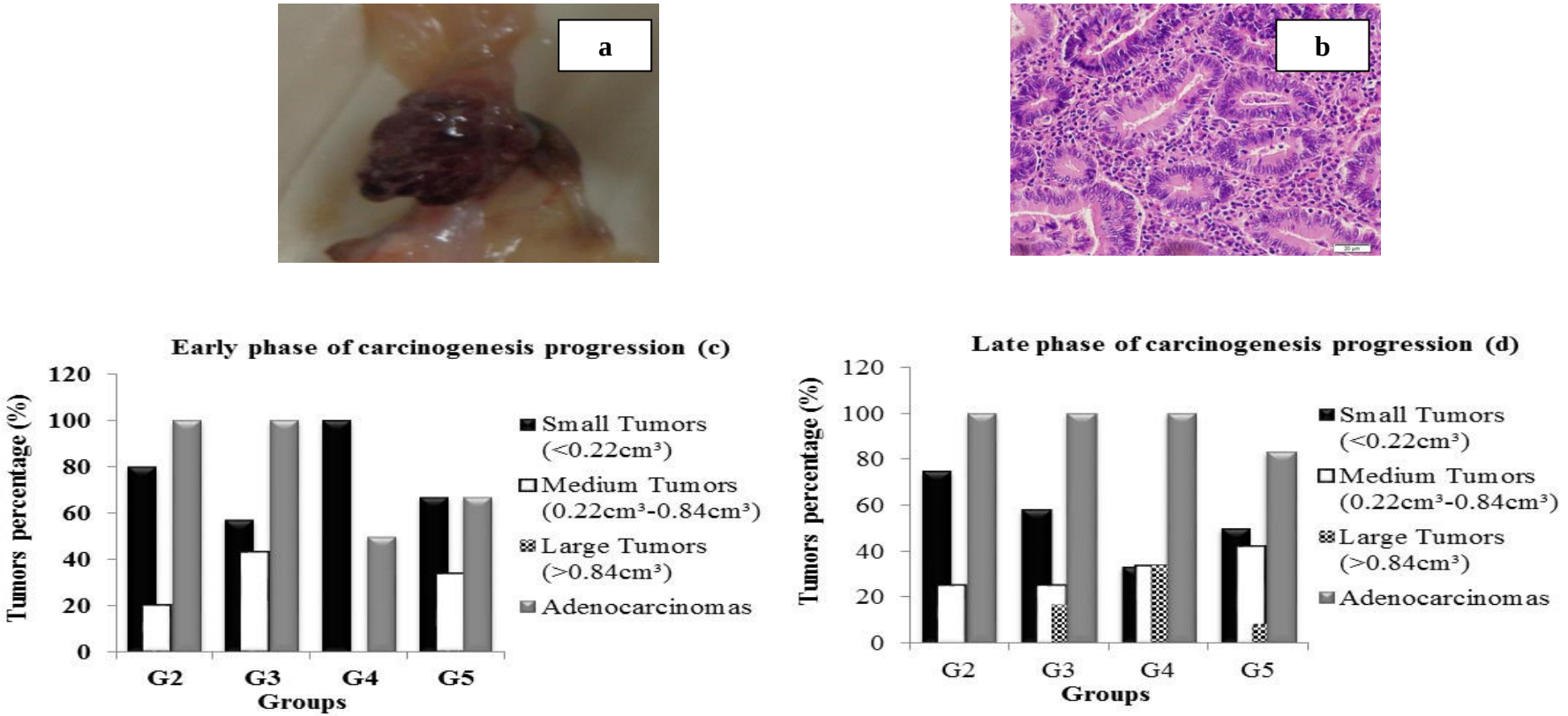


Fig. 4 Representative images and results (Mean ± SD) of tumor percentage in the different groups. a. Macroscopic Tumor. b. HE slide of tumor. c. Tumor size distribution in the different groups in early phase of carcinogenesis progression. d. Tumor size distribution in the different groups in late phase of carcinogenesis progression G1 (untreated animals): animals that received commercial chow; G2 (DMH): animals induced by DMH and that consumed commercial chow; G3 (probiotic): animals induced by DMH and that consumed commercial chow + *L. acidophilus* CRL 1014; G4 (prebiotic): animals induced by DMH and that consumed commercial chow + aqueous yacon extract; G5 (synbiotic): animals induced by DMH and that consumed commercial chow + *L. acidophilus* CRL 1014 + aqueous yacon extract (Chi-Square Test).

Conclusion

The findings this present study suggest that ingestion of *L. acidophilus* CRL 1014, alone (G3) or combined with aqueous yacon extract (G5), had beneficial effects on the early phases of carcinogenesis (lower number of ACF, genotoxicity and Ki-67 index and higher apoptotic index). Therefore, the results indicate aqueous yacon extract, combined or not with *L. acidophilus* CRL 1014 may have potential as a chemopreventive agent against on early phases of colorectal cancer.

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Conflict of interest: None.

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CAPÍTULO III

CONSIDERAÇÕES FINAIS

- Observou-se um efeito benéfico do *L. acidophilus* CRL 1014 e este associado ao extrato aquoso de yacon nos estágios iniciais da carcinogênese (diminuição de FCA, genotoxidade e proliferação celular e aumento de índice de apoptose).

- Na fase inicial da progressão observou-se uma diminuição da porcentagem de adenocarcinomas nos animais que ingeriram o extrato aquoso de yacon e este associado *ao L. acidophilus* CRL 1014.

- Desta forma, os resultados apontam uma ação quimiopreventiva do extrato aquoso de yacon associado ou não ao *Lactobacillus acidophilus* CRL 1014 nas fases iniciais da carcinogênese.

* Como propostas futuras:

- Poderia ser realizado um estudo com ácidos clorogênico, cafeíco e frutooligossacarídeos, os quais são principais compostos presentes no yacon, durante a carcinogênese química de cólon induzida com DMH com o objetivo de verificar se os principais compostos presentes no yacon estão relacionados com os efeitos benéficos do yacon no processo de carcinogênese.

- Determinar os biomarcadores: Ki-67, índice de apoptose, p53, blc-2, ciclooxigenase-2 e prostaglandina (PG-E2) nas fases de iniciação, promoção e progressão da carcinogênese para avaliar se há uma correlação e modificação da proporção desses biomarcadores durante a evolução do processo canceroso.

- Realizar uma avaliação do protocolo experimental utilizando o yacon e o *Lactobacillus acidophillus* CRL1014 em cada etapa da carcinogênese, a fim de verificar se houve efeito somatório dos tratamentos na presente pesquisa.