RITA DE CÁSSIA STAMPINI OLIVEIRA LOPES

EFEITO DO CONSUMO DE SORGO EXTRUSADO (*Sorghum bicolor* L.) ASSOCIADO AO PROBIÓTICO (*Bifidobacterium longum*) NO CONTROLE METABÓLICO, INFLAMATÓRIO E DE TOXINAS URÊMICAS EM INDIVÍDUOS EM HEMODIÁLISE

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

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Ao meu esposo Marco Antônio,

Pelo amor, carinho e companheirismo em todos os momentos de nossas vidas

Aos meus Filhos Felipe e Jéssica,

Fontes de inúmeras surpresas e alegrias infinitas

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Pelo incentivo e apoio

"A persistência é o menor caminho do êxito" (Charles Chaplin)

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BIOGRAFIA

Rita de Cássia Stampini Oliveira Lopes, filha de Adão Gomes de Oliveira e Maria Aparecida Stampini, nascida em 10 de janeiro de 1975 na cidade de Visconde do Rio Branco, Minas Gerais.

Em fevereiro de 1990 iniciou o segundo grau no Colégio Universitário de Viçosa (COLUNI), concluindo os estudos em dezembro de 1992.

Em março de 1995 iniciou o curso de graduação em Nutrição, na Universidade Federal de Viçosa, Minas Gerais, colando grau em agosto de 1999.

Em 2002 recebeu o título de especialista em Nutrição e Saúde, pela Universidade Federal de Viçosa.

Em agosto de 2007 iniciou o curso de mestrado em Bioquímica Agrícola, na Universidade Federal de Viçosa, obtendo o título em outubro de 2009.

Em março de 2014, ingressou no curso de doutorado em Ciência da Nutrição, na Universidade Federal de Viçosa e pleiteou o título de doutora em março de 2018.

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LISTA DE ABREVIATURAS

%	Percentual
µg	Microgramas
µL	Microlitros
µmol	Micromol
3-DXA	3-Deoxiantocianidina
5-MeO-LUT	5-Metoxiluteolinidina
7-MeO-API	7-Metoxiapigeninidina
AGCC/SCFA	Ácido Graxo de Cadeia Curta
AOAC	Associação Oficial de Química Analítica
API	Apigeninidina
BMC/IMC	Índice de massa corporal
IL6	Interleucina 6
IL 8	Interleucina 8
IL10	Interleucina 10
CAT	Catalase
CFU/UFC	Unidade Formadora de Colônica
cm	Centímetros
CRP/PCR	Proteína C reativa
DRC/CKD	Doença renal crônica
g	Grama
G1	Grupo 1
G2	Grupo 2
G3	Grupo 3
GAE/EAG	Equivalente de Ácido Gálico
GC/CG	Grupo Controle
GPx	Glutationa Peroxidase
GS/SG	Grupo Simbiótico
GT	Grupo Teste
HD	Hemodiálise
HPLC	Cromatografia líquida de alta eficiência
IAA	Àcido indol acetico
IC 95%	Intervalo de confiança de 95%

iNOS	Óxido Nítrico sintase
IS	Indoxil Sulfato
Kcal	kilocalorias
Kg	Kilogramas
LUT	Luteolinidina
MDA	Malondialdeído
Mg	Miligramas
min	Minutos
mL	Mililitros
Mmol	Milimol
n	Tamanho amostral
NF κβ	Fator nuclear κβ
ng	Nanograma
nm	Nanômetro
ºC	Graus Celsius
P	Nível de significância (probabilidade)
P	Fósforo
p-CS	P Cresyl sulfato
PD	Bebida probiótica
pH	Potencial de hidrogênio
PM	Leite Pasteurizado
RAA	Renina-Angiotensina-Aldosterona
ROS	Espécie Reativa de Oxigênio
RPM	Rotação por Minuto
s	segundos
SD/DP	Desvio-padrão
SOD	Superóxido Dismutase
TC	Triacilglicerídeos
TAC	Capacidade Antioxidante Total
TGF β	Fator de Transformação do Crescimento
TNF	Fator de necrose tumoral
WHO	Organização Mundial da Saúde

RESUMO

LOPES, Rita de Cássia Stampini Oliveira, D.Sc., Universidade Federal de Viçosa, março de 2018**. Efeito do consumo de sorgo extrusado (*Sorghum bicolor* L.*)* associado ao probiótico (*Bifidobacterium longum*) no controle metabólico, inflamatório e de toxinas urêmicas em indivíduos em hemodiálise.** Orientadora: Hércia Stampini Duarte Martino. Coorientadoras: Andréia Queiroz Ribeiro, Maria Eliza de Castro Moreira eValéria Aparecida Vieira Queiroz

A Doença Renal Crônica (DRC) vem se tornando um grande problema de saúde pública em todo o mundo. Por isso, torna-se necessário investigar novos tipos de alimentos como coadjuvantes no controle metabólico e na redução do risco de comorbidades em portadores de DRC em tratamento hemodialítico. Desse modo, o objetivo deste estudo foi investigar o efeito do consumo de sorgo extrusado (*Sorghum bicolor L*.) associado ao probiótico (*Bifidobacterium longum*) no controle metabólico, inflamatório e de toxinas urêmicas em indivíduos em hemodiálise. Tratou-se de um ensaio clínico controlado, randomizado, simples cego com duração de sete semanas, que foi realizado no setor de nefrologia de um hospital público. Os voluntários, 58 indivíduos com DRC em hemodiálise, foram aleatoriamente divididos em dois grupos: o grupo simbiótico (GS) que recebeu a 100 mL do leite probiótico não fermentado com a cepa *Bifidobacterium longum* e 40 g de flocos de sorgo extrusado; e o grupo controle (GC) que recebeu 100 mL de leite pasteurizado e 40 g de flocos de milho extrusado. Antes da intervenção os participantes foram caracterizados quanto aos aspectos socio-demográficos e clínicos por meio de prontuários médicos e entrevistas e, avaliados em relação peso, altura e IMC. Os cereais extrusados foram caracterizados quanto a composição centesimal, mineral, de compostos bioativos, como taninos condensados, compostos fenólicos totais, flavonoides e a capacidade antioxidante. Durante a intervenção foi avaliado a viabilidade das células no leite com probiótico. As amostras de marcadores metabólicos, sanguíneas e fecais foram coletadas ao ínicio e ao final da intervenção. As amostras de sangue foram centrifugadas, as de fezes foram divididas em alíquotas e ambas foram imediatamente armazenadas a -80 ° C. A partir do soro sanguíneo foram avaliados os marcadores séricos inflamatórios (IL-6, IL-10, TNFα e proteína C-reativa), de estresse oxidativo (capacidade antioxidante total, superóxido dismutase e malondialdeído) e urêmicos (indoxil sulfato, p-cresyl sulfato). Nas amostras fecais foi feito a determinação do pH e da concentração de ácidos graxos de cadeia curta. O sorgo extrusado apresentou maior percentual de carboidratos (aproximadamente 71%), seguido de proteína (aproximadamente 11%) e lipídios (aproximadamente 0,4%). Quando comparado ao milho extrusado, o sorgo apresentou maior porcentagem de fibra alimentar ($p < 0,05$) e maior conteúdo de compostos fenólicos e taninos, consequentemente maior atividade antioxidante ($p < 0,05$). O

número de células viáveis de *Bifidobacterium longum* BL-G301 no leite foi de 9.06 x 10^8 ± 5.4 x 10^8 CFU/100 mL. O consumo do sorgo extrusado associado ao leite probiótico por pacientes em hemodiálise diminuiu os níveis séricos de uréia, p-cresil sulfato, indoxil sulfato, proteína C reativa e malondialdeído ($p < 0,05$), apresentou maior adequação dos valores de albumina sérica (82.8%; n = 24) ($p<0.05$) e aumentou a capacidade antioxidante total e a superóxido dismutase ($p < 0,05$) quando comparado ao grupo controle. Além disso, nas amostras fecais, os indivíduos do GS apresentaram pH menor em relação ao GC ($p < 0,05$). Apesar da concentração de ácidos graxos de cadeia curta, não ter apresentado diferença significativa entre os grupos após a intervenção ($p > 0,05$), a comparação intragrupo mostrou que os indivíduos do GS apresentaram maior concentração de ácidos acético, butírico e propiônico no final da intervenção ($p < 0,05$), enquanto o GC apresentou maior concentração de ácido acético e propiônico ($p < 0,05$). Assim, conclui-se que o sorgo extrusado do cultivar BRS 305 apresentarou maior conteúdo de fibra alimentar, compostos fenólicos e atividade antioxidante quando comparado com o milho extrusado. As características químicas do sorgo extrusado permitiu a sua inclusão na alimentação dos indivíduos com DRC. Além disso, a ingestão do sorgo extrusado associado ao leite probiótico com *Bifidobacterium longum* BL-G301 melhorou a inflamação, o estresse oxidativo e os marcadores urêmicos em indivíduos com doença renal crônica em hemodiálise.

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ABSTRACT

LOPES, Rita de Cássia Stampini Oliveira, D.Sc., Universidade Federal de Viçosa, March, 2018. **Effect of the intake sorghum extruded (*Sorghum bicolor L.*) associated to the probiotic (*Bifidobacterium longum)* in metabolic, inflammatory and uremic toxin control in hemodialysis individuals.** Advisor: Hércia Stampini Duarte. Co-Advisors: Andréia Queiroz Ribeiro, Maria Aliza de Castro Moreira and Valéria Aparecida Vieira Queiroz

Chronic kidney disease (CKD) has become a major public health problem worldwide. Therefore, it is necessary to investigate new types of foods as a coadjuvant in metabolic control and in reducing the risk of comorbidities in individuals with CKD undergoing hemodialysis (HD). Thus, the aim of this study was to investigate the effect of the intake of extruded sorghum (Sorghum bicolor L.) associated with probiotic (Bifidobacterium longum) on metabolic, inflammatory and uremic toxin control in HD subjects. This was a randomized, controlled, single-blind, seven-week clinical trial conducted in the nephrology sector of a public hospital. The volunteers, 58 subjects with CKD on hemodialysis, were randomly divided into two groups: the symbiotic group (SG), which received 100 mL of unfermented probiotic milk with *Bifidobacterium longum* strain and 40 g of extruded sorghum; and the control group (CG) which received 100 mL of pasteurized milk and 40 g of extruded corn. Before the intervention, the participants were characterized in terms of socio-demographic and clinical aspects through medical records and interviews and evaluated in relation to weight, height and BMI. The extruded cereals were characterized as the mineral, centesimal composition of bioactive compounds, such as condensed tannins, total phenolic compounds, flavonoids and antioxidant capacity. During the intervention, the viability of the cells in the probiotic milk was evaluated. Metabolic markers, blood and fecal samples were collected. at the beginning and at the end of the intervention. Blood samples were centrifuged, stool samples were divided into aliquots and both were immediately stored at -80 ° C. Inflammatory serum markers (IL-6, IL-10, and TNFα), oxidative stress (total antioxidant capacity, superoxide dismutase, and malondialdehyde), and uremic (indoxyl sulfate, p-cresyl sulfate) were evaluated from blood serum. pH and the concentration of short chain fatty acids were evaluated in fecal samples. Extruded sorghum presented higher carbohydrate concentration (approximately 71%), followed by protein (approximately 11%) and lipid (approximately 0.4%). When compared to the extruded corn, it presented a higher ($p<0.05$) percentage of dietary fiber, and higher ($p<0.05$) content of phenolic compounds and tannin, consequently higher antioxidant activity. The number of viable *Bifidobacterium longum* BL-G301 cells in milk was $9.06 \times 10^8 \pm 5.4 \times 10^8$ CFU/100 mL. Extruded sorghum associated with unfermented probiotic milk decreased ($p<0.05$) the C-reactive protein, malondialdehyde, p-cresyl sulfate, indoxyl sulfate and urea serum levels, with

a higher adequacy of serum albumin values (82.8%; $p<0.05$) and increased total antioxidant capacity and superoxide dismutase ($p <0.05$) when compared to the control group. In addition, in the fecal samples, GS individuals presented lower pH levels in comparison with GC group ($p <0.05$). Although of the concentration of organic acids presented no significant difference between the groups after the intervention ($p> 0.05$), the intragroup comparison showed that GS individuals showed a higher concentration of acetic, butyric and propionic acids at the endpoint ($p <0.05$), while the GC presented higher concentration of acetic and propionic acids ($p <0.05$). Thus, it is concluded that the extruded sorghum of the BRS 305 cultivar presented higher dietary fiber content, phenolic compounds and antioxidant activity when compared to the extruded corn. The chemical characteristics of extruded sorghum allowed its inclusion in the diet of individuals with CKD. In addition, the ingestion of extruded sorghum associated with probiotic milk with *Bifidobacterium longum* BL-G301 improved inflammation, oxidative stress and uremic markers in subjects with chronic kidney disease on hemodialysis.

1. INTRODUÇÃO

A doença renal crônica (DCR) vem se tornando um grande problema de saúde pública em todo o mundo. Nos últimos anos as taxas mundiais de incidência e prevalência da doença, cresceram em torno de 5% (FILHO; RODRIGUES, 2013; PEREIRA et al., 2012; SESSO et al., 2017). Atualmente, a média global de prevalência da doença é de 13,4%, com maior concentração nas regiões dos Estados Unidos, Canadá, Europa e Austrália (HILL et al., 2016). Em 2016, o Brasil apresentou um número estimado de 122.825 indivíduos com a DRC, o que representa um aumento anual médio de 6,3% nos últimos cinco anos (SESSO et al., 2017).

O seu desenvolvimento está relacionado à diversos fatores de risco, como doenças pré-existentes e fatores genéticos. Uma vez instalada, a perda progressiva da filtração glomerular é associada a um conjunto complexo de alterações fisiológicas, como o estresse oxidativo e a inflamação, resultando em um grande número de complicações e morbidades (BASTOS; BREGMAN; KIRSZTAJN, 2010).

A DRC se divide em diferentes fases, conforme seu desenvolvimento, que exigem tratamentos distintos. O tratamento pré-dialítico ou tratamento conservador é composto por intervenções precoces, mudanças no estilo de vida, dietas hipoproteicas e, consequentemente, restritas em fósforo, além da preparação do indivíduo para o posterior tratamento dialítico (BASTOS; BREGMAN; KIRSZTAJN, 2010; EVANS; TAAL, 2011; GONÇALVES; BARRETO; CANZIANI, 2007). O tratamento dialítico corresponde à hemodiálise (HD), diálise peritoneal automatizada (DPA) e diálise peritoneal ambulatorial contínua (CAPD). Estes tratamentos substituem parcialmente a função renal, minimizando os sinais e sintomas da doença e preservando a vida do paciente (RIELLA; MARTINS, 2001).

No Brasil, o número de pacientes em tratamento dialítico em 2016 foi de 50.807, sendo que 92,1% dos pacientes em diálise crônica faziam tratamento por hemodiálise (SESSO et al., 2017). Apesar dos benefícios da diálise em prolongar a sobrevida dos indivíduos com DRC as condições impostas pela doença e pelo tratamento dialítico resultam em uma série de alterações

sistêmicas, metabólicas e hormonais como fadiga generalizada, anorexia, acidose metabólica e anemia ferropriva (FOUQUE et al., 2008). Essas modificações afetam negativamente a microbiota intestinal e induzem a produção de toxinas urêmicas e marcadores inflamatórios, que são agravadas por intervenções terapêuticas de rotina, como a restrição alimentar de frutas, vegetais, leguminosas e carnes, fonte de fibras, ferro, potássio e fośforo, utilizadas com intuito de controlar o desenvolvimento da doença (RIELLA; MARTINS, 2001). As principais causas de morbidade, hospitalização e mortalidade nestes pacientes são aterosclerose, doença cardiovascular e infecções, que estão relacionadas com o processo de estresse oxidativo, de inflamação e do acúmulo de toxinas urêmica (SHEMA-DIDI et al., 2012).

O estresse oxidativo, que em indivíduos com a DRC está relacionado com o grau da disfunção renal, é evidenciado por baixos valores dos biomarcadores antioxidantes como a superóxido dismutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx) e pelo aumento de agentes oxidantes, como enzimas oxidases, lipoxigenases e cicloxigenases (CACHOFEIRO et al., 2008; CELIK et al., 2013). A presença de outros agravos como o diabetes mellitus, a dislipemia, a hipertensão arterial e o envelhecimento, comumente presentes na população com DRC, ou mesmo a administração de ferro para corrigir a anemia, podem agravar ainda mais o estado de estresse oxidativo associado à uremia (CACHOFEIRO et al., 2008). Estudos clínicos e experimentais têm demonstrado um importante papel do estresse oxidativo na patogênese e na progressão da DRC e suas complicações (IKIZLER et al., 2002; OBERG et al., 2004; TERAWAKI et al., 2004; ZHOU et al., 2012).

O processo inflamatório crônico participa de forma ativa nos mecanismos de progressão da lesão renal, exacerbando o acometimento de outras doenças, como o diabetes, a hipertensão arterial, entre outras (VIANNA et al., 2011). Ele é evidenciado pela elevação de marcadores inflamatórios, como a proteína-C reativa (PCR), citocina pró-inflamatória interleucina 6 (IL-6), e fator de necrose tumoral - alfa (TNF-α). O aumento da concentração sérica destes

marcadores séricos está associado com a instalação de comorbidades e desregulação imune (CHEUNG; PAIK; MAK, 2010).

Nos últimos anos, a progressão da DRC também está sendo associada à alteração na microbiota intestinal. Na DRC, o influxo de ureia para o trato gastrointestinal é grande, aumentando a produção de amônia pelas ureases microbianas e consequentemente reduzindo o pH intestinal. A alteração do pH modifica a composição da microbiota intestinal e da barreira epitelial intestinal e permite a entrada das endotoxinas e outros conteúdos luminais nocivos para os tecidos subjacentes e para a circulação sistêmica (KOTANKO; CARTER; LEVIN, 2006; WONG et al., 2014). Estas alterações podem aumentar a susceptibilidade do indivíduo à infecções, desordens imunes, inflamação, estresse oxidativo, desnutrição e resistência à insulina (MAFRA et al., 2014; VAZIRI et al., 2013).

Nesse sentido, a redução das endotoxinas por meio do equilíbrio da microbiota intestinal e do tempo de trânsito colônico representa uma estratégia terapêutica promissora para os portadores de DRC (ROSSI et al., 2014a), por contribuir para a recuperação do controle metabólico (MEYER; HOSTETTER, 2012). Novas abordagens dietéticas, como a inclusão do sorgo extrusado e probióticos, podem favorecer a alteração no ambiente colônico, aumentar a produção de ácidos graxos de cadeia curta e diminuir o pH colônico, levando a uma redução da produção de endotoxinas e da uremia (ROSSI et al., 2012a).

Os probióticos são definidos pela FAO/WHO como microrganismos vivos, administrados em quantidades adequadas, que conferem benefícios à saúde do hospedeiro (ASHRAF; SHAH, 2011). A sua ação está associada à redução do pH colônico e à modulação da microbiota intestinal, o que contribui para a redução de endotoxinas, da uremia e do estresse oxidativo (ROSSI et al., 2012a). Alguns estudos com DRC tem destacado a utilização de probióticos com a *c*epa *Bifidobacterium longum* para o controle metabólico e inflamatório destes pacientes (OGAWA et al., 2012a; TAKAYAMA; TAKI; NIWA, 2003; TAKI; TAKAIAMA; NIWA, 2005).

O sorgo (*Sorghum bicolor* L.) é um cereal da família *Poaceae*, nativo das regiões tropicais da África, com composição centesimal semelhante à do milho. Possui grande potencial para alimentação humana por ser fonte de fibra alimentar e de compostos fenólicos como taninos e antocianinas (CARDOSO et al., 2017; VÁZQUEZ-ARAÚJO; CHAMBERS; CHERDCHU, 2012). Os cultivares que possuem testa pigmentada, como o BRS 305, apresentam elevada concentração de taninos condensados, podendo contribuir para um maior conteúdo de compostos fenólicos e elevar em até 19% a capacidade antioxidante da dieta, promovendo benefícios para a saúde (CARDOSO et al., 2017; MORAES et al., 2012). No entanto, ainda não existem estudos comprovando a funcionalidade do sorgo como fonte prebiótica. A concentração de fibra alimentar e compostos fenólicos no sorgo podem auxiliar na modulação da microbiota intestinal e no controle do estresse oxidativo em portadores de DRC. Alimentos ricos em taninos e antocianinas podem modificar a composição da microbiota intestinal aumentando a quantidade de bactérias benéficas como a *Bifidobacterium spp* e *Lactobacillus spp* e reduzindo as bactérias patogênicas como *Clostridium spp e Escherichia Coli* (CARDOSO; MARTINO; PINHEIRO-SANTANA, 2014).

Diante do exposto, a hipótese deste estudo é que o consumo de sorgo extrusado associado ao leite probiótico não fermentado durante sete semanas exercerá efeito benéfico no controle urêmico, no estado inflamatório e no estresse oxidativo em indivíduos com DRC em hemodiálise.

1.1 Referências Bibiográficas

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2. OBJETIVOS

2.1 Objetivo geral

Investigar o efeito do consumo do sorgo extrusado (Sorghum bicolor L.) associado ao leite probiótico (Bifidobacterium longum) sobre o controle metabólico, inflamatório e de toxinas urêmcias em indivídos em hemodiálise.

2.2 Objetivos específicos

- Revisar sistematicamente sobre os efeitos da ingestão de prebióticos e / ou probióticos na modulação da microbiota intestinal, no controle de produtos nitrogenados, do estresse oxidativo e da inflamação em indivíduos com doença renal crônica;
- Avaliaçar a composição em nutrientes e compostos bioativos de flocos de sorgo extrusado e o efeito deste cereal consumido com leite probiótico não fermentado na inflamação e no estresse oxidativo em indivíduos com doença renal crônica em hemodiálise;
- Avaliaçar o efeito de flocos de sorgo extrusado e de leite probiótico não fermentado no controle metabólico, nos marcadores urêmicos, no pH fecal e na produção de ácidos graxos de cadeia curta em indivíduos com DRC em hemodiálise.

3. REVIEW ARTICLE: MODULATION OF INTESTINAL MICROBIOTA, CONTROL OF NITROGEN PRODUCTS AND INFLAMMATION BY PRE/PROBIOTICS IN CHRONIC KIDNEY DISEASE: A SYSTEMATIC REVIEW

Artigo aceito para publicação pela revista Nutrición Hospitalaria

3.1 Abstract

Dysbiosis may favor the occurrence of inflammation and oxidative stress in chronic kidney disease (CKD). It has been suggested that the intake of pre/probiotics may control the progression of chronic kidney disease. Thus, the objective of this study was to systematically review the literature on the effects of pre/probiotic intake on the intestinal microbiota, control of nitrogen products, oxidative stress, and inflammation in the CKD patients. The literature search was conducted on MEDLINE, LILACS, Cochrane Library of Clinical Trials, and Science Direct. After careful evaluation by the reviewers, ten potentially relevant articles were selected for this study. Based on previous studies, intake of prebiotics appears to have the following effects: increased Bifidobacteria and Lactobacillus counts; reduced formation of uremic toxin, p-cresol, and its serum concentrations; improved lipid profiles; reduced systemic inflammatory state and concentrations of oxidative stress markers. Similarly, consumption of probiotics can reduce blood urea and serum phosphate concentrations. Furthermore, an increase in fecal volume and intestinal *Bifidobacterium*, and a reduction in p-cresol serum and blood urea concentrations were observed in response to symbiotic intake. These results suggest that consumption of pre/ probiotics may modulate the intestinal microbiota, and promote the growth and metabolism of anaerobic bacteria by decreasing the production of uremic solutes, further causing oxidative stress and systemic inflammation in CKD patients.

3.2 Introduction

Chronic kidney disease (CKD) is a global public health problem. In countries like the United States of America and Australia, it affects 10–13% of the population (1,2). Over the last decades, there has been an increase in the incidence and prevalence of this disease, with a significant increase in the number of patients requiring dialysis therapy (3). In Brazil, statistics show a gradual increase in the prevalence of CKD, with a high proportion of patients receiving dialysis treatment. In 2016, 122.825 CKD patients were recorded to be on dialysis; 92.3% were on hemodialysis (HD) (4,5).

Uremia is a serious complication of CKD and it is associated with the degree of renal injury, food restriction, and hydration status. Besides activating the renin–angiotensin–aldosterone system and favoring the occurrence of vascular calcifications, uremia also changes the microbiota (known as dysbiosis) and increases intestinal permeability (6–8). It results in increased flow of urea into the intestinal lumen, which is then hydrolyzed by microbial urease. The ammonium hydroxide produced increases the local pH, facilitating the growth of pathogenic bacteria and promoting mucosal irritation and damage (9,10). These changes allow entry of endotoxins and other harmful luminal contents into the underlying tissues and systemic circulation, favoring the manifestation of other diseases (6,7,11,12)

Patients with CKD demonstrate significant quantitative and qualitative changes in intestinal microbiota, related to the overgrowth of aerobic and anaerobic pathogenic bacterial species (7,11). These are able to use nitrogen products, increasing the production of uremic toxins, such as indoxyl sulfate and p-cresyl sulfate. These toxins induce inflammation, oxidative stress and cause a pathophysiological impact, which result in structural and functional changes that indirectly influence patient morbidity and mortality (13,14).

In uremic patients, oxidative stress and inflammation, causes dysregulation of the immune system. This is evidenced by the presence of elevated oxidative biomarkers, such as lipid

oxidation and protein oxidation product (15,16). A cross-sectional study involving patients with chronic renal failure demonstrated that the presence of indoxyl sulphate and p-cresyl sulphate, was also associated with elevated concentrations of inflammatory markers and with increased arterial stiffness, which is a characteristic finding in CKD (17). These toxins, in the past decade, have been associated with the progression, the cardiovascular morbidity and the mortality of CKD patients (18). In addition to uremia, the metabolic acidosis, the routine therapeutic interventions used in the treatment of CKD, such as dietary restriction of fruits, vegetables, and foods with high fiber content; the iron intake, the use of phosphate binders; and the use of antibiotics modify the colonic environment. These modifications negatively affect the intestinal microbiota and induce the production of uremic toxins and the occurrence of an inflammatory process (19).

Some interventions are being used to modulate the intestinal microbiota block LPS or attenuate inflammation, or target adsorption of uremic toxin end products of microbial fermentation (20). Review articles describe some of these interventions that the administration of an inhibitor of small intestinal alpha-glycosidase that increases the fermentation of carbohydrates in the colon can reduce the colonic generation of p-cresol; the utilization of essential oils as agents to treat dysbiosis; the oral adsorbents has been used to restore the epithelial tight-junction proteins and reduced plasma endotoxin and markers of oxidative stress and inflammation; the administration of synthetic TLR4 antagonists to inhibit LPS signaling; and the ingestion of prebiotics and/or probiotics that play an important role in the progression of the CKD (12,20).

The results of a recent study suggest that the ingestion of prebiotics and probiotics can increase the production of short chain fatty acids, modulating the intestinal microbiota (17). They can also reduce intestinal permeability, reducing the entry of endotoxins into the bloodstream, and the occurrence of inflammation (21), which may, in turn, play an important role in the progression of the CKD. Therefore, the objective of this study was to systematically review the

literature on the effect of prebiotics and/or probiotics product intake on the intestinal microbiota, oxidative stress, and inflammation in patients with CKD.

3.3 Methods

This systematic review was conducted according to a prespecified protocol and is described according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement (22). This article is based on previously conducted studies and it does not include studies conducted by any of the authors.

3.3.1 Search Strategy

A literature review was conducted in computerized databases MEDLINE (PubMed), Latin American and Caribbean Health Sciences (LILACS), Cochrane Library of Clinical Trials and Science Direct. Databases were searched using the key words: "chronic kidney disease," "hemodialysis," "intestinal permeability," "intestinal/gut microbiota," "inflammation," "oxidative stress," "uremic toxins," "supplementation," "probiotic," "prebiotic," and "symbiotic." These terms were searched alone or in combinations with each other. The search was limited to articles published between 2005-2016.

First, we conducted a manual search on references of all selected articles. To ensure that no relevant data was missed, a survey of the gray literature was conducted using the databases of theses and dissertations from the following sources: the Coordination of Improvement of Higher Education Personnel (CAPES), the Digital Library of theses and dissertations of the Federal University of Viçosa, and Brazilian digital library of theses and dissertations to ensure that we did not miss out important studies. Next, the abstracts of these articles were analyzed

to verify compliance with the inclusion criteria, and full-text articles were subsequently examined to confirm their eligibility.

3.3.2 Selection Criteria

In this review, we included randomized clinical studies that examined the efficacy of prebiotic, probiotic, or symbiotic supplementation in modulating intestinal microbiota and regulating nitrogen products and inflammation in CKD patients (both sexes) who had received the intervention for at least one day. Review articles, animal studies, articles not written in English, and those not related to the topic of interest were excluded.

3.3.3 Data Extraction and Synthesis

All relevant studies identified in the electronic databases were consolidated in a single database to remove all duplicates. After exclusion of the duplicates, two independent reviewers selected the references in three phases: analyses of titles, abstracts, and full texts. Any disagreements related to conflicting data or study eligibility were resolved by a third reviewer. Data including methodological quality, participant information, duration of intervention, and outcome type (changes in intestinal microbiota, nitrogen products, uremic toxins, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, and inflammatory markers) were extracted and collected in duplicates in a Microsoft Excel worksheet. The quality of the included trials was measured using the Cochrane Collaboration's tool for assessing the risk of bias in randomized trials (23). Quality assessment was conducted by 3 independent reviewers, with disagreements resolved by consensus.

3.4 Results

3.4.1 Search Results

During the initial selection process, we identified 1.679 articles. Later, we excluded 781 duplicate articles, and 890 articles were removed after reading their titles and/or abstracts. Eight other articles were identified by reverse search and were considered eligible. However, after reading their abstracts, six did not meet the inclusion criteria and were thus excluded from this study. Finally, we included and critically analyzed a total of ten articles (Figure 1).

According to the Cochrane Collaboration's risk of bias tool for randomized trials, ten selected studies presented varying degrees of bias. All trials had a low or an unclear risk of bias for sequence generation, allocation concealment, blinding, selective outcome reporting, and other sources of bias.

3.4.2 Study Characteristics

The selected articles described studies involving CKD outpatients who were either in stages 3–4 of the disease (5 studies), stages 4-5 without dialysis (1 study), or were receiving HD (4 studies). These studies were further grouped based on the type of supplement that was used for treating the participating patients (Table 1). While two studies tested the effectiveness of prebiotic products in patients with CKD, the remaining 8 studies investigated the efficacy of either probiotics (4 studies) or symbiotics (4 studies) in these patients.

3.4.3 Participant Characteristics

A total of 423 subjects participated in the clinical trials, and the sample size ranged from 12 to 125. Their age ranged from 26 to 82 years and 64.18% of the participants were male. The sample size of three studies were similar, two of which involved patients with stages 3-4 of the disease and one with indviduals HD (28,31,32) (Table 1).

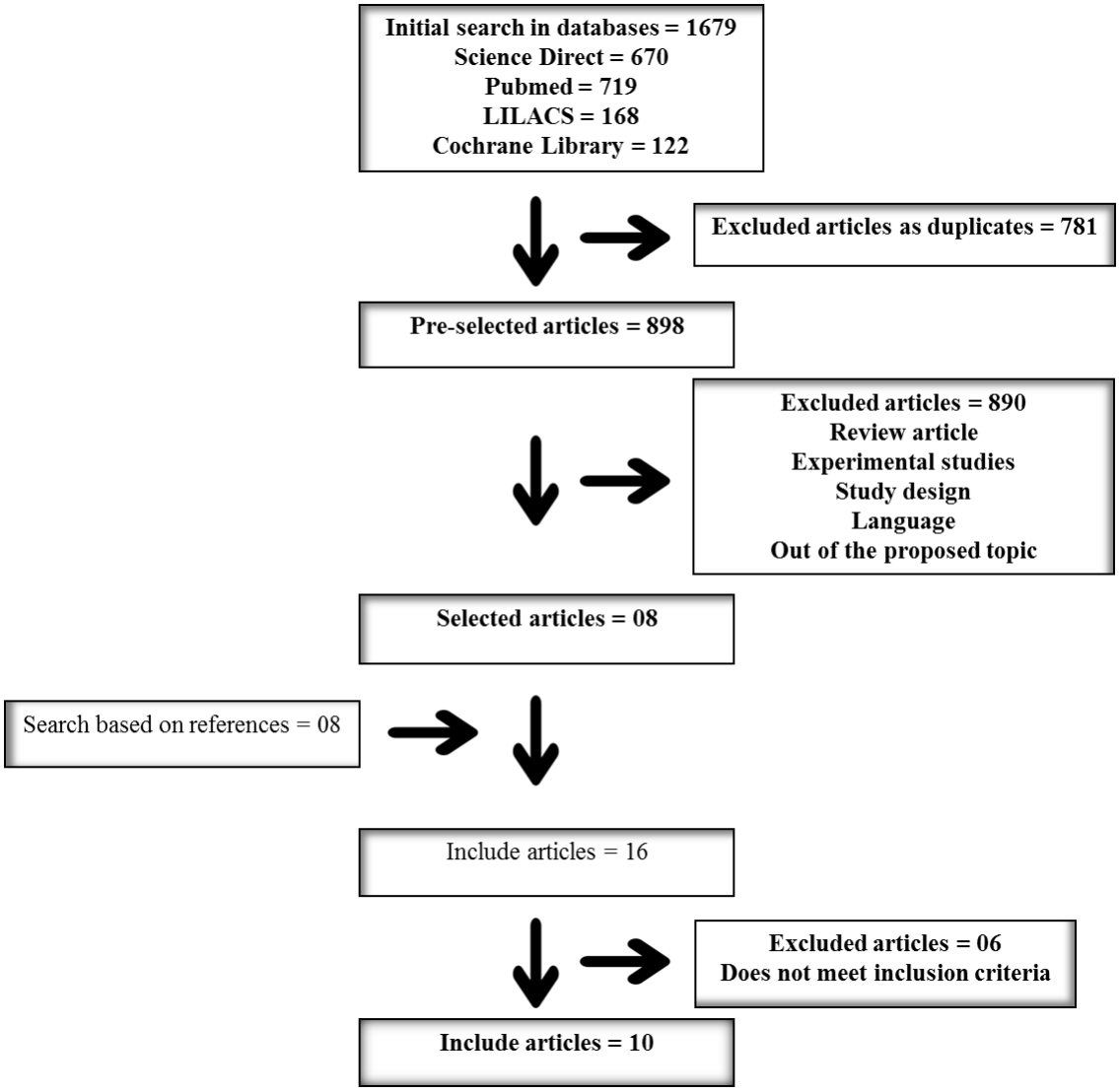

Figure 1: Flowchart of the steps for obtaining the articles selected for this systematic review.

3.4.4 Interventions

Interventions with prebiotics in CKD patients showed that the consumption of dietary fermentable fiber (10 g and 20 g for 6 weeks) and the consumption of lactulose (30 mL three times a day for 8 weeks) improved lipid profiles (total cholesterol and LDL) and oxidative status (increased total antioxidant capacity and decreased malondialdehyde), suppressed the systemic inflammatory responses (TNF-α, IL-6, IL-8, and CRP), and increased *Bifidobacteria* and *Lactobacillus* counts (24,25).

Probiotic supplementation used in outpatient treatment (16×10^9 CFU/day of *Lactobacillus casei shirota* for two months; and 9×10^9 CFU/day of *Lactobacillus acidophilus* (*L. acidophilus*), *Bifidobacterium longum* (*B. longum*), and *Streptococcus thermophilus* (*S. thermophilus*) for three months) decreased the concentrations of blood urea and uremic toxins in treated patients. In addition, HD patients who received 2×10^9 CFU/day of *B. longum* demonstrated a reduction in their serum phosphate concentrations (26–28). However, probiotics had no effect on uremic toxin levels or inflammatory markers in HD patients who used 1.8×10^{11} CFU/day of *S. thermophilus*, *L. acidophilus*, *and B. longum* for two months (29).

Table 1: Characteristics of selected studies and their major results

Author Year	Study Design	Patients n	Intervention	Duration	Major results	Conclusion
Xie et al. (2015) (24)	Randomized, placebo control	HD G1 n=42 G2 n=39 GC n=44	Prebiotic - Soluble fiber with more than 75% of fermentability G1- 10 g G2 - 20g GC - without fibers	6 Week	⬇TC, LDL and TC/LDL relationship; ⬇MDA; ⬇TNF-α, IL-6, IL-8 e CRP; ⬆TAC	In patients on hemodialysis, fermentable fiber in the diet supplementation: Improves the lipid profile and oxidative stress Decreases the systemic inflammatory state.
Tayebi-Khosroshahi et al. (2016) (25)	Randomized, placebo control	Outpatient on Stage 3 and 4* GC n=16 GT n=16	Lactulose 30 mL syrup three times a day	8week	⬆*Bifidobacteria* and *Lactobacillus*	The prebiotic increased *Bifidobacteria* and *Lactobacillus* counts in patients with CKD
Ranghanathan et al. (2009) (27)	Randomized, placebo control, double-blind, crossover	Outpatient on Stage 3 and 4* GC = 13 GT=13	Probiotics *L. acidophilus* *B. longum* *S. thermophiles* $9x10^{10}$ CFU/day	3 months	⬇urea nitrogen and uric acid	The probiotic intake was well tolerated, with a reduction in the concentrations of urea nitrogen and uric acid, being able to contribute to a better quality of life.
Ogawa et al. (2012)(28)	Placebo control	HD GC = 15 GT=15	Probiotics: *B. longum* in tablets 01/day $2x10^{9}$ CFU/day	4 Week	⬇phosphate	*B. longum* can be used to treat hyperphosphatemia in patients on hemodialysis.

Continuation Table 1: Characteristics of selected studies and their major results

Author Year	Study Design	Patients n	Intervention	Duration	Major results	Conclusion
Alatriste et al. (2014) (26)	Randomized	Outpatient on stages 3 and 4 n = 15	Probiotic: *L. casei shirota* G1 - $8x10^9$ CFU/day G2 - $16x10^9$ CFU/day	2 months	G2: larger reduction urea	Dose of $16x10^9$ CFU resulted in a greater reduction in blood urea levels.
Natarajan et al. (2014) (29)	Randomized, placebo control, double-blind, crossover	HD GC = 22 GT=22	Probiotics: *S. thermophilus* L. *acidophilus* *B. longum* $1.8x10^{11}$CFU/day	2 months	Trend to reduce CRP, total indoxilglucuronil and white blood cells	The use of probiotics seems to be safe and well tolerated.
Cruz-Mora et al. (2014) (30)	Randomized, placebo control, double-blind	HD GT n = 8 GC n = 10	Synbiotic: *L. acidophilus* *B. bifidum* ($2x10^{12}$CFU/day) Inulin (2.31 g)	2 months	↑ Bifidobacterium	Synbiotic can increase the bifidobacterium population maintaining the balance of intestinal microflora.
Guida et al. (2014) (31)	Randomized, placebo control double-blind	Outpatient on stages 3 and 4 GT n = 18 GC n = 12	Synbiotic $5x10^9$ CFU/day Probinil neutro®	4 Week	↓ p-cresol	Synbiotic lowers serum concentration of p-cresol in patients with CKD not subject to dialysis.

Continuation Table 1: Characteristics of selected studies and their major results

Author Year	Study Design	Patients n	Intervention	Duration	Major results	Conclusion
Dehghani et al 2016 (32)	Randomized, placebo control double-blind	Outpatient on stages 3 and 4 GT n = 18 GC n = 12	Synbiotic: *L. casei, L. acidophilus L. bulgarigus L. rhamnosus B. breve B. longum, S. thermophilus* Fructo-oligosaccharides (1000 mg/day)	6 weeks	⬇blood urea nitrogen	The intake of synbiotic supplement could reduce blood urea nitrogen in patients with CKD in stages 3 and 4
Rossi et al 2016 (33)	Randomized, placebo control double-blind crossover	Outpatient on stages 4 and 5 n=31	Synbiotic *Lactobacillus, Bifidobacteria, Streptococcus* (9×10^{10}) inulin fructo-oligosaccharides, galacto-oligosaccharides (15 g)	6 weeks (4-week washout)	⬇p-cresol ⬇*Ruminococcaceae* ⬆*Bifidobacteria*	In patients with CKD, synbiotics did decrease serum p-cresol and favorably modified the stool microbiome.

G1, group 1; G2, group 2; G3, group 3; GC, group control; GT, group test; IS, indoxyl sulfate; NF-kB, nuclear factor kappa beta; TC, triglycerides; CRP, C-reactive protein; TAC, total antioxidant capacity; MDA, malondialdehyde; TNF-α, tumor necrosis factor α; IL 6, interleukin 6; IL 8, interleukin 8

*phases 3 and 4 – phase of the chronic kidney disease preceding the hemodialysis phase

Effects of symbiotics were studied in patients receiving outpatient treatment and those receiving HD. An increase in the *Bifidobacterium* population was observed in subjects who consumed a symbiotic containing *L. acidophilus*, *Bifidobacterium bifidum* (2×10^{12} CFU/day), and inulin (2.31 g) for two months. A reduction in serum p-cresol concentration and an increase in stool volume were observed in patients who received 5×10^{9} CFU/day Probinul neutro® for four weeks. An intake of symbiotic supplement with 7 strains of probiotics and fructooligosaccharides (500 mg twice a day for 6 weeks) reduced blood urea nitrogen in patients with CKD stages 3 and 4. The symbiotic intervention that used 3 different types of fibers (15 g) and a combination of probiotics (*Lactobacillus, Bifidobacterium, and Streptococcus genus* (9×10^{10} CFU)) for six weeks (4-week washout) also showed an increase in the *Bifidobacterium* population and a reduction in serum p-cresol concentration (30–33).

3.5 Discussion

In CKD, oxidative stress and inflammation occurs at the onset of the disease and increases with its progression. HD individuals experience increased oxidative stress possibly due to loss of antioxidants during dialysis, interactions between blood and dialysis membrane, and malnutrition (34). However, despite the fact that HD patients present greater oxidative stress and inflammation than other outpatient-treated patients, the results of selected studies indicate that the consumption of probiotics (24,25), prebiotics (26–29) and symbiotic (30–33) supplementation caused positive changes in HD and CKD in HD and CKD patients.

Prebiotics are non-digestible food compounds that stimulate the beneficial growth of microbiota conferring health benefits to the host. They decrease oxidative stress, systemic inflammation, and the production of uremic solutes in patients (35,36). In a randomized placebo-controlled trial, prebiotic consumption for six weeks by individuals receiving HD was associated with the following changes: reduced levels of total cholesterol and LDL, improved

oxidative stress through reduction of malondialdehyde and increase of total antioxidant capacity, and improvement in systemic inflammation (TNF-α, IL-6, IL-8 and CRP) (24). These effects may be due to the ability of dietary fibers to hinder the process of dietary fat digestion and absorption, and favor the production of short chain fatty acids. During micelle formation phase of the digestion process, dietary fibers bind to cholesterol or bile acids forming a gel. This binding reaction delays gastric emptying, decreases dietary fat absorption and bile acid reabsorption by the enterohepatic circulation, and drives the use of hepatic cholesterol for bile acid production (37). Dietary fibers are not digested by humans. They are rather fermented by the intestinal bacteria that release short chain fatty acids. These fatty acids are responsible for modulating the intestinal microbiota and exerting immunomodulatory effects (31,37). Such changes in the intestinal microbiota were observed in a randomized controlled clinical trial. In this study, 30 mL of lactulose supplement three times a day for eight weeks increased *Bifidobacteria and Lactobacillus* counts in HD patients (25)

Probiotics are live microorganisms, when administered in appropriate amounts, may confer benefits to the host´s health (27). Their effect on the immune system is evidenced by an increased expression of anti-inflammatory cytokines (IL-10 and Nrf 2), and a decrease in proinflammatory cytokines (Il-6 and TNFα) and levels of systemic inflammation (38,39).

In a randomized clinical trial, the consumption of probiotics containing 16×10^9 CFU/day of *L. casei shirota* for two months reduced the levels of serum urea in uremic patients with moderate to severe CKD (stages 3–4) (26). Similar results were observed in a pilot study that tested the effects of a probiotic containing different bacterial strains (*L. acidophilus*, *B. longum*, and *S. thermophilus*) at a dose of 9×10^9 CFU/day. After three months, the majority of the participants who received probiotic had reduced concentrations of serum urea nitrogen and uric acid, contributing to a better quality of life for those individuals (27). Oral administration of *B. longum* capsules in a placebo-controlled clinical trial was shown to reduce serum phosphorus concentrations in HD patients (28). This suggests that the reduction of these metabolites in

CKD patients may be associated with the ability of the microbiota to use the metabolic waste as a substrate. A possible explanation for the observed reduction in uremia is related to the ability of certain anaerobic bacteria to degrade urea and uric acid through the production of enzymes, such as uricase, allantoinase, and urease. In an *in vitro* study, *Lactobacillus* exposure to an urea-enriched environment induced the production of enzymes responsible for urea reduction (40). Following the intake of probiotics, an increase in *Lactobacillus* and *Streptococcus* populations in the feces can be explained by the conversion of urea to ammonia, a source of nitrogen for metabolic purposes (27). Probiotics can increase dietary fiber fermentation, reduce the intestinal pH, modulate the intestinal microbiota, and increase calcium ionization. Calcium, in turn, binds to phosphorus, reducing its absorption and leading to a reduction in serum phosphorus concentration (28). On the other hand, serum levels of uremic toxins and inflammatory markers were not affected in HD individuals who received a daily dose of 1.8×10^{11} CFU/day of a probiotic (containing *S. thermophilus*, *L. acidophilus*, and *B. longum*) for two months. However, the results showed a trend for the reduction of C-reactive protein ($p = 0.071$), indoxyl glucuronyl ($p = 0.058$), and white blood cell counts ($p = 0.057$) (29). We believe that if the probiotics were administered in combination with soluble fibers, the results would have been different; this is based on our knowledge that soluble fibers can potentiate the effects of probiotics, resulting in a reduction of these markers. A decrease in uremic toxin concentration may be associated with fermentation of soluble fiber by intestinal anaerobic bacteria. This process of fermentation increases the production of short chain fatty acids and reduces colonic pH. The modified environment favors the growth of beneficial bacteria, inhibits the enzymes involved in generation of p-cresyl sulfate and indoxyl sulfate, improves epithelial barrier function (via induction of mucin production, blocking epithelial binding receptors, and strengthening epithelial tight junctions), and reduces the influx of uremic toxins (Figure 2) (41).

Supplementation of a symbiotic compound containing *L. acidophilus* and *B. bifidum* (2×10^{12} CFU/day) and inulin (2.31 g) for two months improved intestinal dysbiosis in individuals receiving HD, increasing the population of *Bifidobacterium* and preserving the numbers of *Lactobacillus* in the gut (30). Symbiotic intake in different stages of CKD decreased p-cresol serum concentration and normalized bowel habits (31,33). The symbiotic supplement (7 strains of probiotics and fructooligosaccharides), when consumed 500 mg twice a day for 6 weeks can reduce blood urea nitrogen in patients with CKD at stages 3 and 4. One of the mechanisms by which this supplementation can potentially benefit the kidneys is by stimulating growth of gut microbial biomass by increased consumption of dietary fibers; this subsequently decreases ammonia production, increases the ratio of ionized ammonia, and facilitates the use of nitrogenous wastes by bacterial cells. Thus, more ammonia is excreted through the feces, and there is a low level production of potentially damaging forms of nitrogen, such as urea, uric acid, and creatinin (32). These results indicate the need to conduct a study to assess microbiota composition, uremic parameters, and inflammatory biomarkers in response to consumption of symbiotics.

In summary, the mechanism by which prebiotics and/or probiotics modulate the intestinal microbiota and decrease oxidative stress and inflammation may be due to increased intestinal anaerobic bacteria count and maintenance of intestinal barrier integrity (Figure 2). Intestinal barrier integrity may be improved by the production of mucin, blocking of connection receptors, and strengthening of the epithelial junctions (17).

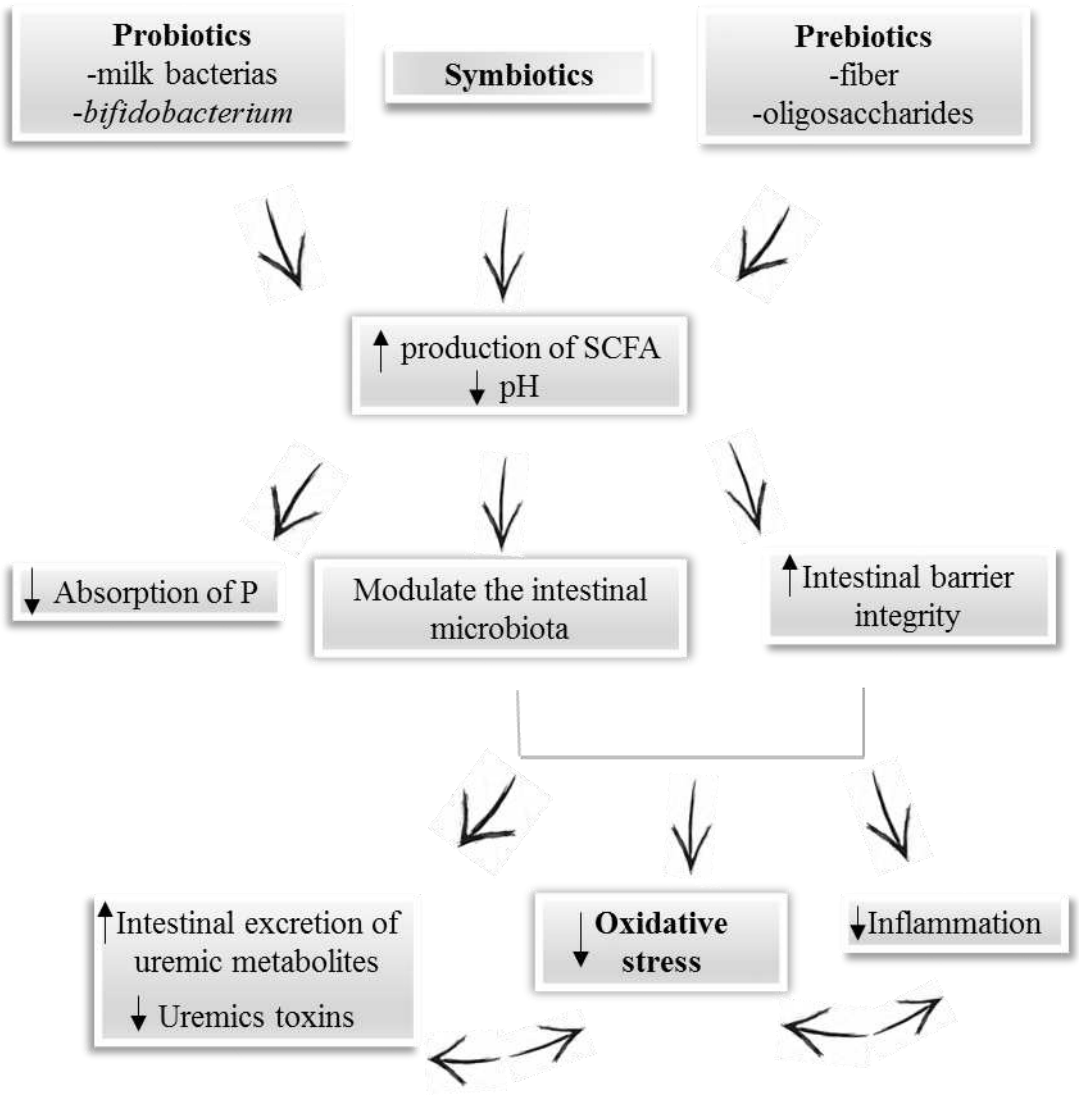

Figure 2: Potential mechanisms involved in the modulation of intestinal microbiota and the reduction of oxidative stress and inflammation by pre- and / or probiotic products in Chronic Kidney Disease. SCFA - short-chain fatty acids; P - phosphorous.

Due to the inclusion and exclusion criteria adopted in this study, we could include only ten studies in our review, and this limitation did not allow us to carry out a meta-analysis study. Our search for relevant studies was confined to the main databases. Thus, it is possible that we might have limited our numbers by missing out some studies that fulfilled our inclusion criteria. Nonetheless, despite these limitations, the present study allowed us to identify "the gaps" in the literature in relation to the topic of interest, allowing us to propose future studies to unravel the mechanisms by which the prebiotics and/or probiotics can control progression of CKD.

3.6 Conclusions

Prebiotics and/or probiotics can modulate the intestinal microbiota by promoting the growth and metabolism of anaerobic bacteria, decreasing the production of uremic solutes, the oxidative stress and the systemic inflammation. There is scarcity of studies that address the effects of prebiotics and/or probiotics on the intestinal microbiota, oxidative stress, and inflammation in CKD patients. Therefore, future studies are needed to provide clarification on topics, such as mechanisms that link regulation of dysbiosis and manifestation of diseases associated with CKD or its precursor diseases, such as diabetes mellitus, hypertension, and atherosclerosis.

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4. ORIGINAL ARTICLE 1: EVALUATION OF THE HEALTH BENEFITS OF CONSUMPTION OF EXTRUDED TANNIN SORGHUM WITH UNFERMENTED PROBIOTIC MILK IN INDIVIDUALS WITH CHRONIC KIDNEY DISEASE

Artigo aceito para publicação pela revista Food Research International

4.1 Abstract

This study investigated the chemical and nutritional composition of breakfast cereal based on whole sorghum, and the effect of its association with unfermented probiotic milk on the inflammation and oxidative stress of individuals with chronic kidney disease. Extruded sorghum breakfast meal presented higher carbohydrate concentration (approximately 71%), followed by protein (approximately 11%) and lipid (approximately 0.4%). When compared to extruded maize breakfast meal, it presented higher percentage of dietary fiber ($p<0.05$), and higher content of phenolic compounds and tannin, consequently higher antioxidant activity ($p<0.05$). Extruded sorghum breakfast cereal combined with unfermented probiotic milk decreased the C-reactive protein ($p<0.05$) and malondialdehyde ($p<0.05$) serum levels and increased the total antioxidant capacity and superoxide dismutase ($p<0.05$) in patients with chronic kidney disease. Therefore, the extruded sorghum ,source of tannin, anthocyanin, and dietary fiber, when consumed with unfermented probiotic milk alleviates the inflammation and oxidative stress in patients with chronic kidney disease .

Keywords: Antioxidant capacity; chronic kidney disease; dietary fiber; phenolic compounds; probiotic; *Sorghum bicolor* L.

4.2 Introduction

Sorghum (*Sorghum bicolor* L.) is a cereal from the *Poaceae* family and has a chemical composition similar to that of corn. Sorghum is native to the tropical regions of Africa and presents great potential for human consumption as a source of dietary fiber and phenolic compounds such as tannins and anthocyanins (Cardoso, Pinheiro, Martino, & Pinheiro-Sant'Ana, 2017; Gomes et al., 2017). Sorghum is consumed in the form of whole grain, as flour in recipes or as a brakfast cereal after subjection to an extrusion process (Anunciação et al., 2016; Khan, Yousif, Johnson, & Gamlath, 2015). Extrusion is a process that combines high pressure, heat and mechanical force in a short time, causing physical and chemical changes to the food matrix. Although the extrusion process influences the bioactive compounds concentrations, it has been used for the products formulation, such as breakfast cereal, popcorn, breads, pastas, and snacks. Thus, this process may be an effective approach to increase the consumption of sorghum by the population (Chávez, Ascheri, Carvalho, Godoy, & Pacheco, 2017).

Sorghum cultivars such as BRS 305, with pigmented seed, heads comprise condensed tannins at high concentrations, which may contribute to a higher phenolic content and increased antioxidant capacity of the diet by 19%, thereby promoting health benefits (Moraes et al., 2017; Moraes et al., 2012). Varieties of sorghum with tannin as well as varieties of sorghum without tannin (such as black sorghum) contain 3-deoxyanthocyanidins, which promote beneficial effects on the body, as an anti-inflammatory and antioxidant activity (Burdette et al., 2010; Awika et al., 2009). However, the tannin sorghum extruded was used because this type has a high molecular weight and degree of polymerization. So, when associated with the extrusion process, the tannin sorghum structure changes by the breakdown of polymers into monomers and dimers of proanthocyanidins, contributing to the antioxidant effect and improving the bioavailability of nutrients (Cardoso et al., 2017; Trompette et al., 2014). In addition, the

fermentation of the fibers and resistant starch present in sorghum, by anaerobic bacteria increases the concentration of short chain fatty acids in the colonic environment favoring intestinal microbiota balance and intestinal mucosal integrity (Rossi et al., 2016). The modulation of intestinal microbiota can contribute to the reduction in inflammation and oxidative stress of patients with some chronic non-transmissible diseases such as chronic kidney disease (CKD) (Rossi, Klein, Johnson, & Campbell, 2012).

CKD has become a major public health problem worldwide based on incidence and prevalence rates, which in the past 4 years have grown by around 5% (Sesso, Lopes, Thomé, Lugon, & Martins, 2016). The overall mean prevalence of the disease is 13.4%, and is highest in the United States, Canada, Europe and Australia in comparison to countries like India and Africa (Hill et al., 2016). CKD promotes the accumulation of organic residues in the body that interact negatively with various biological functions, accentuating the inflammatory state, oxidative stress, cardiovascular dysfunction and risk of death (Barreto et al., 2014; Lemos, Alencastro, Konrath, Cargnin, & Manfro, 2012). To control this accumulation, individuals with CKD need to restrict the intake of some food groups, such as fruits, vegetables and grains, that are sources of micronutrients, dietary fibers, and phytochemicals with antioxidant and anti-inflammatory activity (Riella & Martins, 2001). Hence, the sorghum can be added to the diet of individuals with CKD to help maintain a balanced nutritional composition, providing fibers, antioxidants, and bioactive compounds. Thus, the consumption of extruded sorghum and probiotic milk may exert a beneficial effect on metabolic control, oxidative stress and inflammation in CKD patients on hemodialysis. Foods with probiotics, which are generally manufactured by the fermentation process, such as fermented dairy drinks, have been used to help control of the oxidative stress in CKD patients (Borges et al., 2017; Odamaki et al., 2012; Ogawa et al., 2012; Taki, Takaiama, & Niwa, 2005). Another way of producing dairy drinks with probiotics is by directly adding the microorganisms into the food matrix, without the fermentation stage, to preserve milk flavor (Oliveira et al., 2017). However, there is still no

scientific evidence that milk and extruded sorghum help in the metabolic control of patients with CKD.

In the present study, we aimed to verify that extruded tannin sorghum breakfast meal is a good source of nutrients for individuals with CKD and that cereal improves inflammation and oxidative stress in these patients when consumed with unfermented probiotic milk.

4.3 Material and methods

4.3.1 Cultivating and harvesting the grains

The grains were cultivated in the experimental field of Embrapa Milho e Sorgo, located in Sete Lagoas, Brazil. The BRS 305 hybrid sorghum with a brown pericarp and tannin was cultivated from April to July 2014, and corn grains with a yellow pericarp were cultivated in the 2013/2014 cropping season. After harvesting, the grains were packed in plastic bags and sent to Embrapa Agroindustry of Foods (Rio de Janeiro, Brazil) for processing.

4.3.2 Preparation of breakfast cereals

Sorghum and corn grains were milled in a combined knife-hammer mill TREU (Rio de Janeiro, Brazil) equipped with a sieve size of 1.0 mm producing fine whole meal flour. Either sorghum or corn flour pre-conditioned at moisture of 14.0% was added of 10.0% refined white sugar (Companhia União de Açúcar, São Paulo, Brazil) and 0.5% refined NaCl salt (Ita Serv Sal, Mossoró, Brazil) and processed in a co-rotating twin extruder Evolum HT25 (Clextral Inc., Firminy, France) equipped with a die of four holes of 3,8 mm each, running at a constant screw speed of 350 rpm, feed rate of 10 kg/h and temperature profile of 10 heating zones (from feeding

to die), as following: 30, 60, 80, 100, 100, 100, 110, 110, 120, 120°C. Low temperature of the feeding region was important to avoid water vapor formation hence allowing a positive flow along the barrel. The combination of temperature of 120°C close to the die and screw speed allowed the production of puffed extrudates. A cutter was also installed at the die to produce round shape extrudates. The extrudates were oven-dried at 60°C for 2 h, packed in polyethylene bags, and transported by land to the Federal University of Viçosa, where they were stored at 5 ± 2°C until analysis (Figure S1).

Figure S1: Whole grain sorghum breakfast cereal (A) and whole grain maize breakfast cereal (B)

4.3.3 Macronutrients, moisture, ash, dietary fiber, resistant starch, and minerals analysis

For the determination of chemical composition, 15 grams of sorghum and corn extruded flour were used. The determination of ash, protein, lipids, moisture, total dietary fiber and RS was performed according to the methodology proposed by AOAC (2012). The concentration of carbohydrates was calculated by difference. The total energy value of flours was estimated considering the conversion factors of 4 kcal.g^{-1} for protein or carbohydrate and 9 kcal.g^{-1} per lipid (Frary & Johnson, 2005). Calcium, magnesium, copper, iron, zinc, phosphorus, potassium and manganese levels were quantified by atomic absorption spectrophotometry (Gomes, 1996).

4.3.4 Total phenolic compounds

The total phenolic compounds content in sorghum and corn extruded were determined using the Folin - Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventós, 1999). The extracts were obtained by the addition of 10 mL of methanol:water (60:40/v:v) solution in 1 g of the sample. 0.5 mL of extract were added to 0.5 mL of Folin - Ciocalteu reagent (20%). After homogenization, 0.5 mL of sodium carbonate (7.5%) was added. The reaction mixture was homogenized by vortex (2865 g, 10 s) and incubated at room temperature (30 min). The reading of absorbance was performed in spectrophotometer (Thermo scientific, Evolution 606, USA) at 765 nm. Analytical curve of gallic acid (0,005–0,10 mg/mL) was used to quantify the compounds ($y = 18.66x + 0.084$; $R2 = 0.995$). The results were expressed in mg of gallic acid equivalents/g of flour (mg GAE/g).

4.3.5 Condensed tannins

Condensed tannin (proanthocyanidins) concentration was determined by the vanillin reaction method (Burns, 1971), with slight modifications (Maxon & Rooney, 1972; Prince, Scoyoc, & Butler, 1978). In brief, the tannins were extracted by adding 1% HCl in methanol in 200 mg of the sample, which was agitated at 0.71 g for 20 min at 30 °C. After extraction, the solution was centrifuged at 2790 *g* for 20 min, and 1 mL of the supernatant was mixed with 2.5 mL of the 1% vanillin solution in methanol and 2.5 mL of 8% HCl in methanol. After 20 min, absorbance was measured at 500 nm on a spectrophotometer (Thermo Scientific®, model Multiskan Spectrum 1500). The result was obtained by drawing a standard curve for different concentrations of catechin in methanol ($y = 0.4343x + 0.0161$; $R2 = 0.9984$) and is expressed in mg of catechin equivalent per 100 g of the sample used.

4.3.6 Antioxidant capacity

The antioxidant capacity of the cereals was determined by performing ELISA. For obtaining the extract, 2 g of the samples was diluted in 60% methanol plus 0.1 μM methanolic solution of DPPH (1,1-diphenyl-2-picrylhydrazyl) (Blois, 1958). The absorbance was measured at 517 nm (Thermo Scientific®, model Multiskan Spectrum 1500). The antiradical capacity was expressed in a μmol trolox equivalent/g of the sample (μmol trolox/g).

4.3.7 Flavonoids (3-deoxyanthiocyanidins, flavones, and flavanones*)*

The content of flavonoids (3-deoxyanthiocyanidins, flavones, and flavanones) from sorghum was determined in five replicates according to the method described by Yang et al. (2012). For extraction, 2 g of the sample was added to 20 mL of methanol:HCl (99:1) and stirred for 120 min. Then, the suspension was centrifuged at 2790 ×*g* for 5 min, the supernatant was collected, packed in an amber bottle, and stored in a freezer (−18 ± 1 °C) (Dykes, Seitz, Rooney, & Rooney, 2009). The flavonoids were determined by HPLC, and their identification was performed by comparing the retention time and the absorption spectrum of the peaks of the standards and the samples analyzed under the same conditions. For the quantification, analytical curves constructed by injecting standard solutions with six different concentrations in duplicates were used. The R^2 of the standard curve ranged from 0.9939 to 0.9999, detection limits from 18.98 to 35.12 ng/mL, and quantification limits from 94.90 to 175.60 ng/mL. Compounds are expressed as mg/g sample, as single compounds, and as the sum of 3-deoxyanthocyanins (3-DXA), flavones, flavanones, and flavonoids.

4.3.8 Preparation and viability of milk products

The dairy drinks used in the study were pasteurized milk, used in the control group (CG) and pasteurized milk with addition of the probiotic bacterium *Bifidobacterium longum* BL-G301 (Granotec do Brasil S.A.), used in the symbiotic group (SG). The production of the probiotic dairy beverage was carried out weekly in the Federal University of Viçosa, according to the procedures described by Oliveira et al (2017). The beverages (100 mL) were packed in a high-density polyethylene plastic bottle with aluminum seal, labeled with date of manufacture, validity, instructions for storage and consumption, and stored under refrigeration at 4 ± 2ºC for up to seven days. During the seven weeks of probiotic milk production, samples were collected immediately after preparation and stored under refrigeration at 4°C ± 2°C for seven days to verify the viability of the cells in the final product shelf life. Enumerations of viable *Bifidobacterium longum* cells were performed by plating on Man Rogosa Sharpe (MRS) agar and incubating in anaerobic at 37°C for 72 h (Ashraf & Shah, 2011).

4.3.9 Intervention

It is a clinical, randomized, simple blind study, during 7 weeks. The study protocol was approved by the Human Research Ethics Committee of the Federal University of Viçosa, number 27364314.8.00005153 and was registered at www.ensaiosclinicos.gov.br under the number RBR-2d9ny6. Of the 112 individuals with CKD who underwent hemodialysis in the Nephrology Sector of Hospital São João Batista, in the city of Viçosa, Brazil, 107 were eligible for the study. The participants were older than 18 years and had hemodialysis sections three to four times a week for at least three months. We did not include in the study individuals whose records reported auditory deficiency, with autoimmune disease or with hepatitis B and C virus, with newly implanted catheters, hemodynamic instability, lactose intolerance or milk

discomfort. All the selected ones were clarified as to the objectives, methods of the research and the secrecy of the information. Thus, 99 people with CKD accepted to participate in the project and signed the term of free and informed consent. The participants were randomly into two groups: SG (100 mL of dairy drink with probiotic and 40 g of extruded sorghum flakes) and CG (100 mL of pasteurized milk and 40 g of flakes of extruded corn) and at the end of the intervention there were 58 people included in the study. The reasons why the other 41 did not complete the research were: withdrawal even before the intervention commenced; abdominal discomfort, death, did not fit the food, difficulties in consuming the product at home and hospitalization (Figure 1). The calculation of the sample size indicated that each group should be composed of 23 patients, considering a difference of 30% in the concentration of malondialdehyde (MDA), and a statistical power of 90%, after the intervention.

During the hemodialysis, two food kits were given to patients, one which was consumed in the third hour of hemodialysis and another who was consumed on the interdialytic day at home. The patients, who for some reason could not consume the products in the nephrology sector were instructed to take them home and consume them together the same day. Participants during the hemodialysis days were asked about the consumption of the kit and the presence of some adverse effect to verify adherence or not to the study

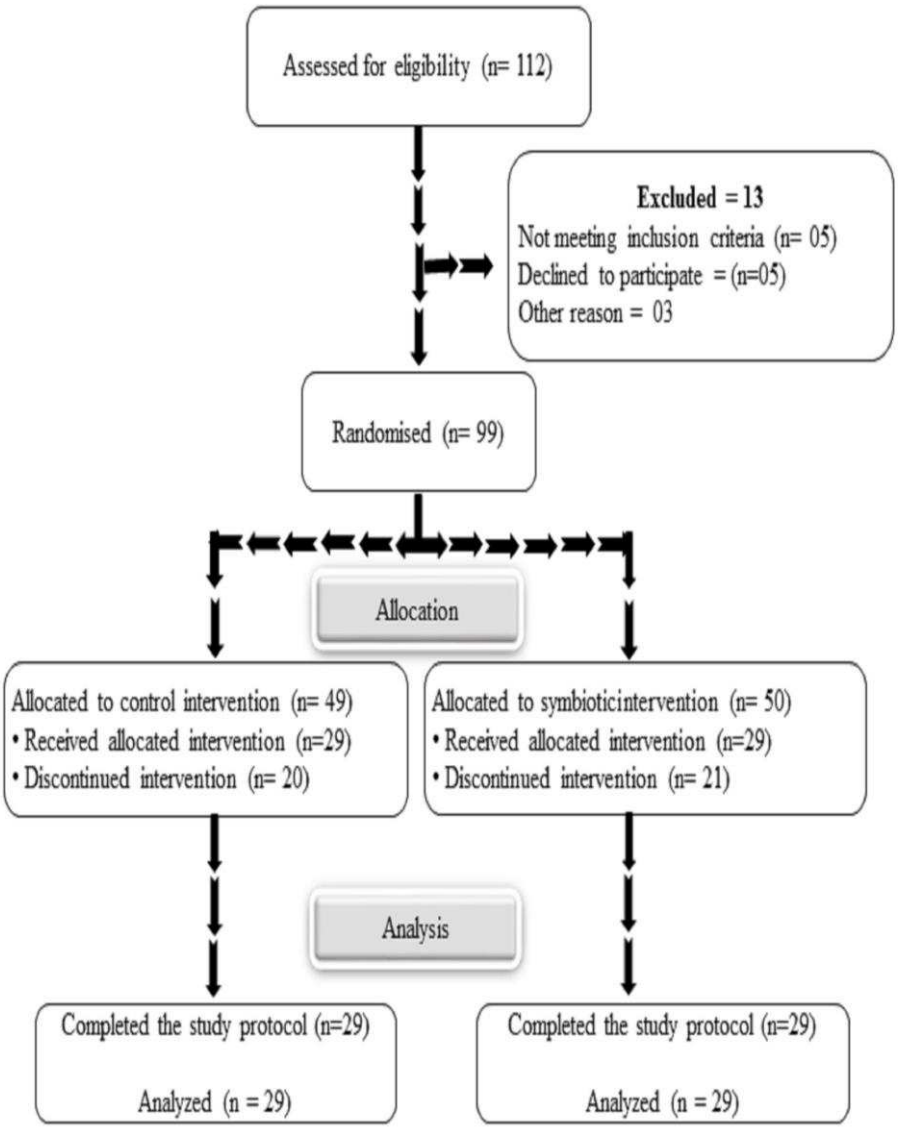


Figure 1: CONSORT diagram showing the flow of participants through each stage of the trial. CONSORT Consolidated Standards of Reporting Trials

4.3.10 Data collection

Socio-demographic and clinical data were obtained before the intervention period, through the collection of information in medical records and through questioners for direct interviews. At the beginning and at the end of the intervention the blood samples were collected by a

professional in the nephrology sector and packed in vacuum tubes. The blood was centrifuged and immediately packed in a freezer at -80^{o}C.

All anthropometric measurements were performed at the end of the hemodialysis session, after 30 minutes of hemodynamic balance. The anthropometric measures included in the study were weight (kg) and height (cm), which were performed according to previously standardized procedures (Jellife, 1968; Lipschitz, 1994; WHO.World Health Organization, 1997).

For the determination of the enzymatic activity of superoxide dismutase (SOD), 30 μL of blood serum was added in duplicate in microplates. One hundred eighty-nine μl of phosphate buffer (50 mM), 6 μl of 3- [4,5-dimethyl-thiazol-2-yl] -2,5-diphenyltetrazolium bromide (MTT) and 15 μl of pyrogallol were added. The plate was incubated for 5 minutes in an oven at 37°C. After the incubation period, the reaction was stopped by the addition of 150 μL of dimethylsulfoxide (DMSO). The plate reading was performed on Elisa reader (Multiskan GO, Thermo Scientific) at 570 nm and values expressed as U SOD/mg protein.

Dosing of blood MDA was performed in duplicate using the thiobarbituric acid reactive substances (TBARS) method (Buege & Aust, 1978). The readings were performed in an ELISA apparatus (Multiskan GO, Thermo Scientific) at 535 nm and the concentration of MDA was determined from the standard curve using standard 1,1,3,3-tetramethoxypropane (TMPO) as standard ($y = 4.2904 - 0.0934$; $R^2 = 0.9945$ - initial reading; $y = 3339 - 1.6362$, $R^2 = 0.9985$ – final reading). The values were expressed as μmol/mg. The protein concentration used in the calculation of SOD and MDA activity was measured by the method of Bradford et al. (1976).

Total antioxidant capacity (TAC) was measured by colorimetric assay using the antioxidant assay kit (CS0790, Sigma Aldrich) according to the protocol provided by the manufacturer. The serum antioxidant concentration was measured by spectrophotometry at 750 nm and was expressed in mM trolox equivalent from the standard curve ($y = -1.82 + 1.3213$, $R^2 = 0.9976$ - initial reading; $y = -1.7808 + 1.3178$; $R^2 = 0.9942$ - final reading).

Serum concentrations of IL-6, IL-10 and TNF-α were detected by means of the multiple base sandwich immunoassay kit (HCYTOMAG-60K-03, Millipore, Billerica, MA, United States) according to manufacturer instruction. In 96-well plates we added 25 µl of serum, which was incubated with antibodies specific for IL-6, IL-10 and TNF-α. The fluorescent signal from the beads was detected in the Luminex 200 equipment, xponent/analyst software, version 4.2 and the levels were calculated according to the standard curves that were established by 5 different concentrations (CV= 0%, R^2 = 1). The C-reactive protein concentration (CRP) was quantified by ultra-sensitive immunoturbidimetry (COBAS-Mira Plus, Roche Diagnostic Systems) using a commercial kit (K079, Bioclin®, Minas Gerais, Brazil).

4.3.11 Statistical analysis

The recorded data were reviewed in order to detect missing information and inconsistencies. The chemical analyses of the extruded cereal were performed in three replicates, except for the flavonoids (five replicates). The normality of the data was evaluated by the Shapiro–Wilk test. The difference between the extruded cereals was evaluated by Student's t-test. The descriptive analysis of the general characteristics of the participants per group was performed. Quantitative variables with normal distribution (according to graphical analysis, asymmetry and kurtosis coefficients and Shapiro-Wilk test) were expressed as mean and standard deviation (SD) and those that did not present normal distribution were expressed in median and minimum and maximum values. Differences between groups were assessed by chi-square test (categorical variables), Student's t-test or Mann-Whitney test (numerical variables). Fisher exact test (categorical variables), paired Student t test or the Wilcoxon test were used to assess differences within the groups. Statistical analysis was performed using the SPSS 20.0 program (SPSS, Inc., Chicago, IL, USA) and the significance level (α) was considered equal to 5%.

4.4 Results

4.4.1 Chemical composition of extruded cereals

4.4.1.1 Macronutrients, moisture, ash, dietary fiber, resistant starch, and minerals analysis

The extruded sorghum breakfast cereal showed higher content of dietary fiber (8.84%), highlights the insoluble fiber, than that showed by extruded corn cereal (7.28%) ($p<0.05$). A similar content of carbohydrate (71.04%) and protein (11.26%) was observed in both the extruded cereal. However, extruded corn showed 49% more lipids ($p<0.05$) and a higher caloric value ($p<0.05$) than that showed by extruded sorghum. The resistant starch content of extruded sorghum (1.03 ± 0.00) was 85% higher than that found in extruded corn (0.15 ± 0.02) ($p<0.05$) (Table 1).

In this study, we observed that the extruded sorghum cereal showed lower phosphorus content (340.33 ± 2.51 $mg.100^{-1}g$) and higher copper (0.33 ± 0.01 $mg.100^{-1}g$), zinc (1.93 ± 0.02 $mg.100^{-1}g$), magnesium (1.45 ± 0.02 $mg.100^{-1}g$), calcium (102.00 ± 1.00 $mg.100^{-1}g$), manganese (1.45 ± 0.02 $mg.100^{-1}g$) and iron (5.59 ± 0.25 $mg.100^{-1}g$) contents than those showed by extruded corn ($p<0.05$). The potassium content in extruded sorghum cereal (353.00 ± 3.02 $mg \cdot 100^{-1}g$) and extruded corn cereal (353.00 ± 1.01 $mg \cdot 100^{-1}g$) was similar.

Table 1. Nutritional composition of extruded sorghum and extruded corn (g.100g^{-1})

Variables*	**Mean** ± SD***	**Mean** ± SD***
	Extruded Sorghum	Extruded Corn
Moisture	6.57 ± 0.28^{a}	6.29 ± 0.31^{a}
Ash	1.87 ± 0.40^{a}	1.66 ± 0.26^{b}
Lipids	0.41 ± 0.13^{b}	0.81 ± 0.91^{a}
Protein	11.26 ± 1.04^{a}	12.66 ± 0.81^{a}
Total dietary fiber	8.84 ± 0.12^{a}	7.28 ± 0.87^{b}
Soluble fiber	0.07 ± 0.55^{b}	0.87 ± 0.35^{a}
Insoluble fiber	8.78 ± 1.70^{a}	6.41 ± 0.90^{b}
Carbohydrates	71.04 ± 0.74^{a}	71.30 ± 0.39^{a}
Resistant starch	1.03 ± 0.00^{a}	0.15 ± 0.02^{b}
Total energy value (kcal· 100 g^{-1})	332.91 ± 1.67^{b}	343.13 ± 5.16^{a}

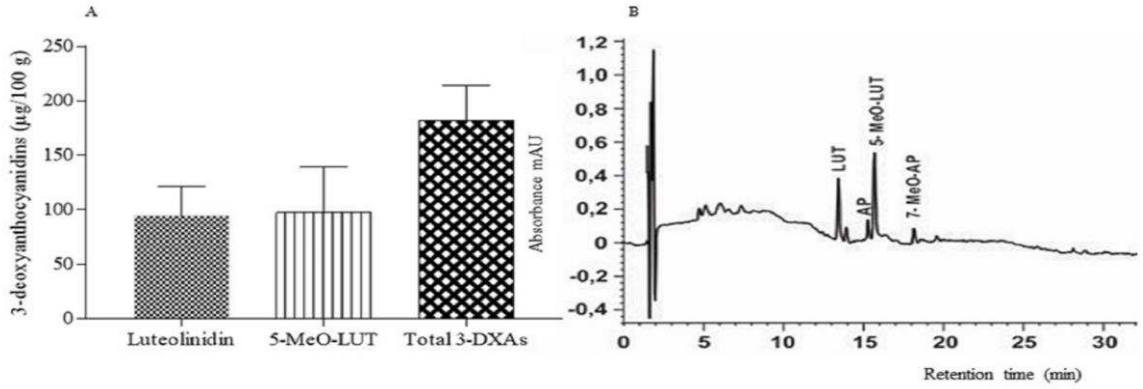
* Values expressed in dry matter. ** Mean of three replicates. *** Standard deviation; same letters on the line do not differ by t test for independent samples at 5% probability,

4.4.1.2 Phenolic compounds and antioxidants

The extruded sorghum breakfast cereal showed higher content ($p<0.05$) of phenolic compounds (1.10 ± 0.02 mg galic acid equivalent/g sample) and condensed tannins (proanthocyanidins) (0.71± 0.08 catechin equivalent/g sample) than that showed by extruded corn cereal (0.81 ± 0.01 mg galic acid equivalent/g sample; 0.00± 0.00 catechin equivalent/g sample, respectively). In addition, the antioxidant activity observed in extruded sorghum cereal (4.68 ± 0.01 µmol trolox/g) was higher than that of the extruded corn cereal (0.68 ± 0.09 µmol trolox/g) ($p<0.05$).

4.4.1.3 Flavonoids

We found two compounds of the 3-DXA group, luteolinidin and 5-methoxyluteolinidin, to be present at similar concentrations and detected traces of apigeninidin and 7-methoxyapigeninidin in the extruded sorghum. None of these compounds were found in the extruded corn cereal (Figure 2A and 2B). The wavelength and retention time of the 3-DXA group in this study are presented in Figure 2C. We did not detect flavones and flavonones in either of the cereals.



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Composto	λmax (nm)	Tempo de retenção (min)
Luteolinidin	239	13.3
Apigeninidin	239	15.1
5-methoxyluteolinidin	239	15.5
7-methoxyapigeninidin	239	17.9

Figure 2. 3-deoxyanthiocyanidins in sorghum extruded cereal. (A) Concentration (µg/100g). (B) HPLC analysis of the extruded BRS 305 sorghum cereal genotype with a brown pericarp. (C) Maximum wavelength and retention time of 3-deoxyanthiocyanidins in the extruded sorghum cereal. The concentration results are expressed as the mean of five replicates ± standard deviation

4.4.2 Viability of probiotic milk

The number of viable *Bifidobacterium longum* BL-G301 cells concentration in a milk was $9.06 \times 10^8 \pm 5.4 \times 10^8$ CFU/100 mL, with a minimum concentration of 2.5×10^8 CFU/100 mL and a maximum of 1.5×10^9 CFU/100 mL, indicating the viability of the product during the intervention period.

4.4.3 Intervention

Of the 58 participants who completed the study protocol, the majority were male (51.7%). The mean age was 63.1± 10.9 years and the time of hemodialysis ranged from 3 to 245 months (55.5 ± 59.1). The level of schooling varied, with the majority having incomplete fundamental education (56.9%) and none with higher education. The patients presented diabetes mellitus (44.8%) and hypertensive nephrosclerosis (22.4%) as the main etiologies associated with CKD. Regarding nutritional status before intervention, 19.61% of CKD patients presented low weight, 52.9% were eutrophic, and 27.4% were overweight/obese. The randomization of the groups was adequate, since they did not present differences in age, BMI, sex, presence of diabetes, and inflammatory or oxidative stress markers ($p>0.05$) (Table 2).

Table 2: Baseline characteristics of subjects with chronic kidney disease on hemodialysis according to experimental groups.

Variables	Control group (n=29)	Symbiotic group (n=29)
Age (years)*	63.03 ± 10.77	63.17 ± 11.16
BMI (kg/m2)*	23.26 ± 9.60	24.74 ± 4.24
Sex †		
Male	21.00 (72.40)	17.00 (58.60)
Female	8.00 (27.60)	12.00 (41.40)
Diabetes†		
Yes	13.00 (44.80)	13.00 (44.80)
No	16.00 (55.20)	16.00 (55.20)
Inflammatory markers		
CRP (mg/dL)•	0.80 (0.04; 2.86)	0.85 (0.01;2.62)
IL10 (pg/mL)•	0.48 (0.12; 2.44)	0.44 (0.10;3.01)
IL6 (pg/mL)•	6.09 (1.12; 15.43)	4.21 (0.98;15.41)
TNFα (pg/mL)*	28.14 ± 12.45	30.27 ± 12.53
Oxidative stress markers		
SOD (U SOD/mg ptn) •	2.21 (0.87; 7.40)	2.32 (1.00; 7.77)
MDA (MDA nmol/g ptn) •	1.03 (0.55; 4.82)	1.01 (0.37;3.28)
TAC (Mm trolox)*	0.49 ± 0.10	0.52 ± 0.09

*Mean ± Std. Deviation, Independent Samples t-test; • median (minimum;maximum), Mann-Whitney test;† n (%), Pearson Chi-Square Tests. BMI - body mass index; CRP - C-reactive protein; IL10 – Interleukin 10; IL6 – Interleukin 6; TNFα - Tumor necrosis fator α; SOD - Superoxide dismutase; MDA – malondialdehyde; TAC - Total antioxidant capacity.

In relation to oxidative stress, we observed that after the intervention, serum MDA decreased ($p<0.05$), and SOD and TAC increased ($p<0.05$) compared to that in CG (Figure 3A). In addition, we observed that delta values for SOD and TAC in the SG group were higher than those in the CG group (Figure 3B).

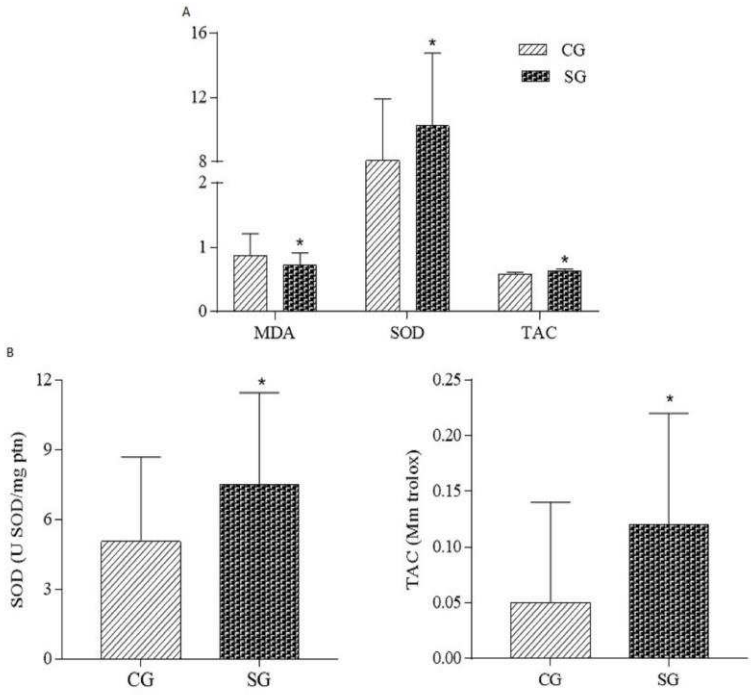

Figure 3: Evaluation of oxidative stress markers in subjects with chronic kidney disease on hemodialysis after intervention. (A)- Comparison between the symbiotic group and the control group. (B)- Comparison of the delta values (end values-baseline values); * Means followed by asterisks differed between groups at 5% probability (Student's t-test or Mann-Whitney).

The SG group presented a decreased in serum CRP concentration ($p<0.05$) in comparison to the intergroups (Figure 4A) and showed a decreased in TNF-α concentration ($p<0.05$) in relation to delta values (final value - initial value) (Figure 4B). This group also presented a reduction in IL-10 concentration (Figure 4C), however, there was no change ($p>0.05$) among IL-6, IL-10 and TNF-α cytokines intergroups (Figure 4a). The SG presented a moderate negative correlation of SOD values with CRP values ($r = -0.373$, $p<0.05$) and IL-10 ($r = -0.387$, $p>0.05$).

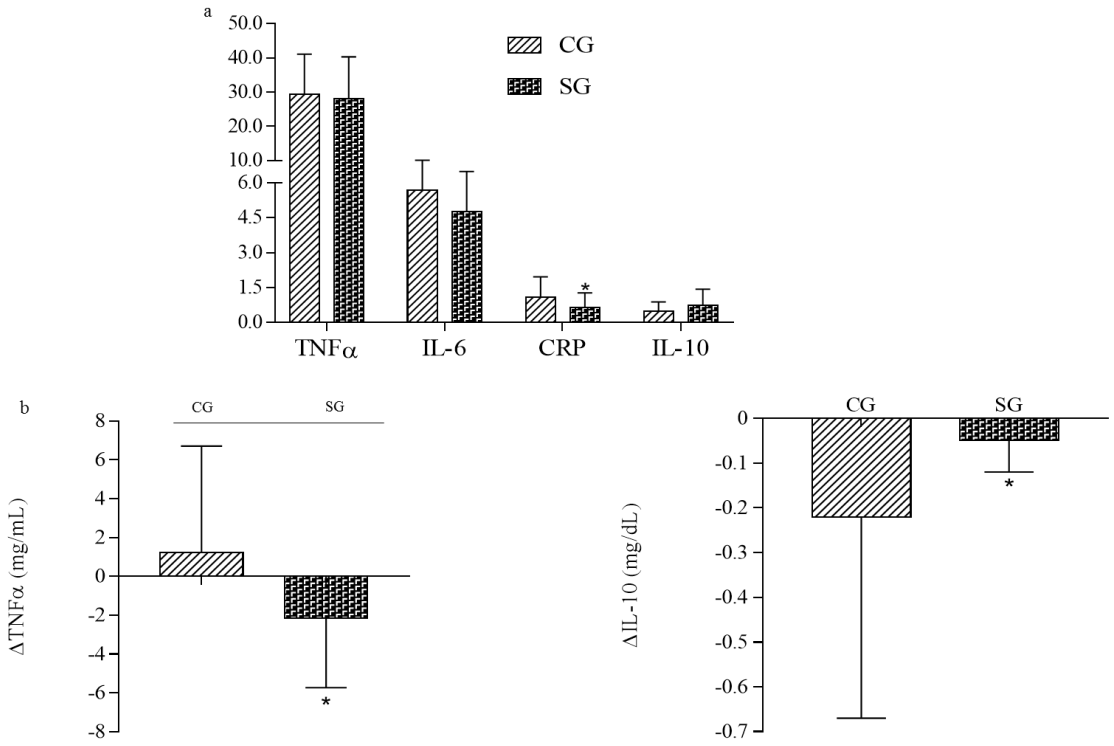

Figure 4: Evaluation of inflammatory markers in subjects with chronic kidney disease on hemodialysis after intervention. A- Comparison between the symbiotic group and the control group. B- Comparison of the delta values (end values-baseline values); * Means followed by asterisks differed between groups at 5% probability (Student's t-test or Mann-Whitney)

4.5 Discussion

This research is the first to investigate the effects of consumption of extruded whole sorghum breakfast cereal and unfermented probiotic dairy beverage on the inflammation and oxidative stress markers in individuals with CKD in hemodialysis. In relation to the chemical composition, the extrusion process promotes the transformation of food and changes its chemical, physical, and nutritional characteristics, as it involves high temperatures, pressures, humidity, and mechanical stress (Cardoso et al., 2017). In our study, the extruded sorghum cereal presented a higher carbohydrate content (approximately 71%), followed by protein

(approximately 11%) and lipid (approximately 0.4%) contents. The extruded sorghum cereal also presented higher content of dietary fiber, phenolic compounds, antioxidant capacity, and tannin in relation to extruded corn cereal. When comparing our result with the centesimal composition of whole sorghum flour of the same genotype, we observed a lower carbohydrate concentration (62.09%) and a higher concentration of lipids (2.60%) and dietary fiber (11.43%) (Martino et al., 2012). Liopart et al. (2013) analyzed the effects of extrusion temperature (164, 182, and 200°C) on the physicochemical properties of extruded red sorghum, and found a high concentration of dietary fiber (9%) and a similar percentage of protein (11%) and ashes (1.5%). Despite the reduction in fiber content promoted by grain processing, extruded sorghum morning cereal continues to be classified as a source of fiber when consumed at a minimum portion of 40 g (3.6 g of total fiber), in accordance with Brazilian legislation which states that for a food to obtain this denomination, it must provide at least 2.5 g of dietary fiber per portion consumed (BRASIL, 2016). In addition, this 40-g portion should provide approximately 12% of the daily recommended requirement of this nutrient, according to the recommendations of the Institute of Medicine (2002).

In our study, we observed that the extruded sorghum cereal showed lower phosphorus content, similar amounts of potassium, and higher, zinc, magnesium, calcium, manganese, and iron content than those showed by extruded corn. High phosphorus and potassium contents could be a limiting factor for considering extruded sorghum as an ideal meal for patients with CKD. The nutritional recommendations advocate that the foods indicated for patients with CKD should have a potassium content of less than 5 mEq or 195 mg per serving (Riella & Martins, 2001). A portion of 40 g of extruded sorghum breakfast cereal (measured as percentage of dietary fiber) has a potassium content of 141 mg, and can be indicated for patients with CKD. With respect to the content of zinc (1.93 mg ± 0.25) found in extruded sorghum cereal, we propose that it can be used as a zinc food source since zinc-rich foods such as meats and legumes have around 0.99 to 4.0 mg of the mineral per 100 g of the product (Andrade, Barros, Mello, &

Takase, 2004). The inclusion of a food source of zinc in the diet could assist in improving the oxidative stress in CKD patient. Zinc can stabilize the structure of SOD, and may be a proton donor during the oxidative cycle of the enzyme (Li, Jiao, Chen, & Liang, 2010).

The content of phenolic compounds, condensed tannin, and antioxidant activity were higher in the extruded sorghum cereal in comparison with extruded corn cereal. However, when comparing our results with the whole sorghum flour, we observed that the whole flour had a higher content of phenolic compounds and tannins than the extruded sorghum cereal, but a similar antioxidant capacity (Moraes et al., 2012). The decrease in the contents of phenolics and tannins may be due to the extrusion of sorghum grains. The condensed tannins (proanthocyanidins) present in sorghum have a high molecular weight and a high degree of polymerization, and the extrusion process can alter this structure by the breakdown of these polymers into monomers and dimers of proanthocyanidins, which have greater bioavailability and a higher antioxidant effect (Cardoso et al., 2017; Trompette et al., 2014). The presence of the antioxidant effect observed in our study after the extrusion process was also observed in a study comparing the extrusion process of three different sorghum cultivars (Cardoso et al., 2014).

We found all compounds of the 3-DXA group in the extruded sorghum cereal, different from that observed by Anunciação et al. (2017), who found four compounds. The difference between the results can be accounted for by the growth process and the difference in extrusion process conditions. A study on extruded sorghum (SC 319 brown pericarp genotype) showed that extrusion leads to a reduction in the content of these flavonoids, with a mean retention of 29.3% (Cardoso et al., 2015).

The absence of flavones and flavonones cannot be attributed to the extrusion process because no study describes the stability of these flavonoids in cereals. Moreover, the results of our study are similar to those described by Cardoso et al. (2015) and Anunciação et al.(2016). The authors not detected these compounds in extruded sorghum of SC391 and SC319

genotypes, both with brown pericarp, indicating the sensitivity of these compounds to heat processing. Although our study has shown a loss in the content of anthocyanins probably due to the extrusion process, sorghum cereal can provide health benefits to patients with CKD because the presence of anthocyanins may activate and enhance the antioxidant defense system of the individual (Shih, Yeh, & Yen, 2007).

The viability of probiotic milk indicated that the concentration of viable *Bifidobacterium longum* BL-G301 cells ($9.06 \times 10^8 \pm 5.4 \times 10^8$ CFU/100 mL) was adequate during the intervention period. Some studies have already been published demonstrating the effect of high (Gionchetti et al., 2007) or low probiotic concentrations on human health (Whorwell et al., 2006), although there is no recognized level of cellular concentration that guarantees an effect on health (Reid, 2008).

Oxidative stress in uremia may be associated with an increase in the expression or activity of NAD(P)H oxidase and with the reduction in natural antioxidant factors, leading to the accumulation of reactive oxygen species (Cachofeiro et al., 2008). Patients on hemodialysis presented low blood antioxidant capacity and high oxidative stress degree due to antioxidant losses during dialysis interactions between the blood and the dialysis membrane and malnutrition that decreases food absorption (Cachofeiro et al., 2008; Coombes & Fassett, 2012).

The symbiotic investigation (40 g of the extruded sorghum cereal and 100 mL of a dairy beverage with probiotic) of seven weeks of intervention, was able to decrease CRP and MDA levels as well as increase TAC and SOD when compared with that of the control product (40 g of extruded corn flakes and 100 mL of milk). These results may be associated with zinc, dietary fiber, tannins, phenolic compounds, and flavonoids contents in the extruded sorghum cereal and the concentration of *Bifidobacterium longum* present in probiotic milk. The intake of the probiotic milk associated with extruded sorghum that presented a high concentration of dietary fiber and polyphenolics compounds may contribute to changes in the colonic environment and the reduction of oxidative stress in CKD patients. The fiber fermentation by the probiotic can

increase the production of short chain fatty acids and decrease the colonic pH, favoring the growth of gram positive bacteria and reducing the presence of lipopolysaccharide, one of the factors responsible for the increase in oxidative stress and inflammation (Kallapura, Pumford, Hernandez-velasco, & Hargis, 2014).

In our study, the oxidative stress was attenuated after the intake of the extruded sorghum cereal with the probiotic dairy drink, since there was an increase in SOD and TAC in the SG. The high intake of polyphenols compounds from BRS 305 extruded sorghum (condensed tannins: 13.06 ± 1.52 mg catechin eq/portion; flavonoids: 76.78 ± 12.92 μg total 3DXAs/portion; phenolic compounds: 44.20 mg of gallic acid eq/portion) can act directly on the inflammatory process owing to their antioxidant and anti-inflammatory properties, avoiding or decreasing the formation of reactive oxygen species (Cardoso et al., 2017; Castilla et al., 2008). Moreover, the structures of these compounds, which are composed of phenolic rings and hydroxyl groups, act directly on the formation of reactive oxygen species by donating hydrogen or electrons and inhibit the oxidative reactions of biological molecules (Vattem & Shetty, 2005). Furthermore, they can also regulate the biosynthesis of glutathione and enzyme expression which modulate the defense system against oxidative stress by continuously converting highly reactive electrophilic species into non-toxic and excretable metabolites, that can modulate the transcription of factor Nrf2 and prevent the activation of nuclear factor NF-κB (Cardoso et al., 2017; Kim, Cha, & Surh, 2010; Saldanha et al., 2016) (Figure 5).

CRP levels has been shown to be a good marker to define this inflammation and the risk of cardiosvascular disease in CKD patients. Its reduction in patients on hemodialysis has been associated with the intake of polyphenols, which have high antioxidant and anti-inflammatory capacity (Seifried, Anderson, Fisher, & Milner, 2007). In fact, our study demonstrated that supplementation with sorghum breakfast cereal (source of phenolic compounds) with unfermented probiotic milk drink can prevent the progression of inflammation, since the CRP levels in the test group decreased after the intervention. García-Mediavilla et al. (2007) showed

that flavonoids can modulate CRP expression and induce changes in nuclear factor kappa B (via NF-κB), contributing to the anti-inflammatory effects through mechanisms that may involve blocking the activation of NF-κB and the resulting increase in the regulation of pro-inflammatory genes (Figure 5).

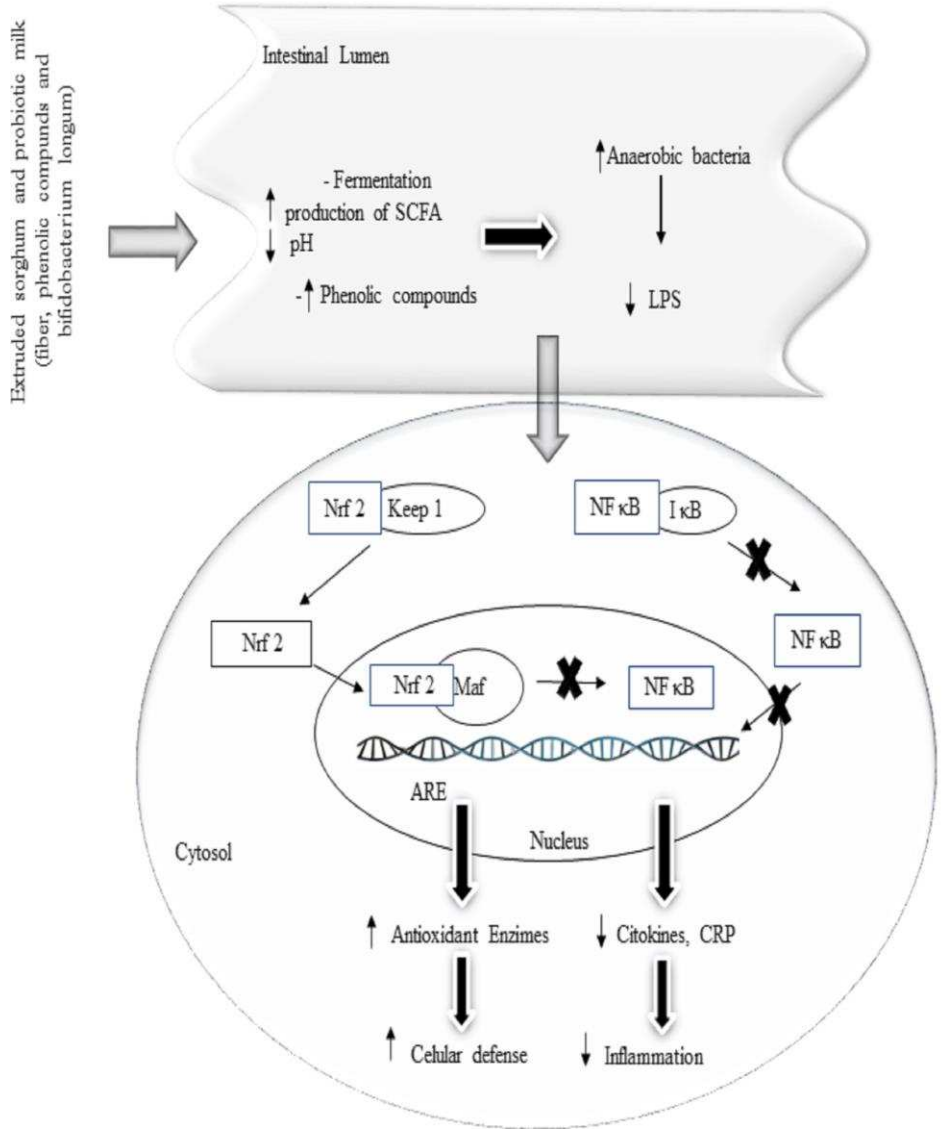

Figure 5: Possible mechanisms that explain the effects of extruded sorghum and probiotic drink consumption on metabolic, inflammatory markers and oxidative stress in CKD, based on our results. Nrf 2, Nuclear factor erythroid-2-related factor-2; Keep 1, repressor molecule, Maf, transcription factor; ARE, antioxidant responsive elements; NF κB; Nuclear factor-kappa B; I κB, repressor molecule; CRP, C-Reactive Protein.

In our study, the results can be attributed to the symbiotic effect of sorghum bioactive compounds and probiotics, and some studies with CKD patients have highlighted the use of probiotic products with the strain *Bifidobacterium longum* for alleviation of inflammation and oxidative stress (Ogawa et al., 2012; Taki et al., 2005). The antioxidant effect has been demonstrated in healthy adults who consumed pasta (7 to 14 days) containing 30% of sorghum flour showed an increase in SOD activity and TAC (Khan et al., 2015).

Thus, the extruded sorghum cereal intake consumed with unfermented probiotic milk drink improved the oxidative stress and inflammation in patients with CKD.

4.6 Conclusion

The extruded sorghum from cultivar BRS 305 presented higher dietary fiber content, phenolic compounds and antioxidant activity than that showed by extruded maize breakfast cereal. These chemical characteristics are suitable to do this food part of the diet of patients with CKD since the intake of this cereal combined with probiotic milk with *Bifidobacterium longum* BL-G301 improved the inflammation and the oxidative stress in individuals with CKD on hemodialysis.

4.7 Conflict of interest

The authors declare that they have no conflict of interest

4.8 Acknowledgements

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5. ORIGINAL ARTICLE 2: SYMBIOTIC MEAL DECREASES UREMIC TOXINS, FECAL PH AND UREA IN HEMODIALYSIS INDIVIDUALS: A PLACEBO-CONTROLLED TRIAL

Artigo submetido à revista Toxins

5.1 Abstract

Generation of uremic toxins p-cresylsulfate (p-CS), indoxyl sulfate (IS) and indole 3-acetic acid (IAA) in hemodialysis (HD) individuals may be associated with the intestinal microbiota and recognized markers of disease progression. This study investigated the effect of symbiotic meal on uremic toxins, in fecal pH and metabolic markers in HD individuals. We conducted randomized single-blind and placebo-controlled intervention study with 58 HD subjects (20 F/38M, 63.1± 10.9-old) who were randomly allocated in symbiotic group (SG, 40 g of extruded sorghum plus 100 mL of unfermented probiotic milk) or control group (CG, 40 g of extruded corn plus 100 mL of pasteurized milk), during 7-wk. Metabolic markers and uremic toxins, fecal concentration of short chain fatty acid and pH value were determined. The SG group had decreased serum p-CS and IS, as well as decreased urea concentration ($p<0.05$) compared to CG. SG showed higher fecal butyric acid and lower pH compared to baseline and CG ($p<0.05$). In addition, serum p-CS and fecal pH were positively correlated to urea concentration in SG participants at the endpoint. The consumption of the symbiotic meal during 7-wk reduced colonic pH, increased the production of short chain fatty acid by intestinal microbiota, and reduced serum uremic toxins and urea in HD subjects.

Key words: *Bifidobacterium longum,* chronic kidney disease, indoxyl sulfate, p-cresylsulfate, *Sorghum bicolor* L., urea.

5.2 Introduction

Chronic kidney disease (CKD) is a worldwide public health problem with estimated prevalence between 10% to 13% [1]. Cardiometabolic risk factors are among several complications of CKD and hemodialysis (HD) treatment and cardiovascular disease is the major cause of morbidity and mortality in HD patients [2]. Atherosclerosis pathogenesis in CKD patients has been associated with traditional risk factors, such as hypertension, diabetes, and age, as well as non-traditional risk factors, such as uremic toxins and anemia [3].

CKD patients have been presenting a progressive retention of uremic toxins, which can negatively impact many body functions and increase cardiovascular mortality [4,5]. Uremic toxins, p-cresylsulfate (p-CS), indoxyl sulfate (IS) and indole 3-acetic acid (IAA) are toxins commonly used as markers of CKD progression [6], because these metabolites accumulate in the blood flow and may contribute to uremic syndrome. They induce renal tubular cells to produce free radical, promote vascular calcification, and aortic wall thickening [7].

Reducing proteolytic bacteria are known to regulate uremic toxin synthesis though probiotics and/or prebiotics use [7,8]; however, this mechanism has been little explored. Probiotic is a live microbial food supplement that beneficially affects the host animal by improving its gut flora balance when administered in adequate quantities [9,10]. Some studies have highlighted the use of probiotics with the strain *Bifidobacterium longum* for metabolic control in CKD patients[11–13].

Sorghum (*Sorghum bicolor* L.) is a cereal from the Poaceae family native to the tropical regions of Africa. Its grains have high levels of fibers and bioactive compounds, mainly in pericarp, which can help in the control of organic residues in the body [14–16]. Cultivars with pigmented pericarp, such BRS 305, have high levels of condensed tannins (proanthocyanidins) that have demonstrated high antioxidant activity and way attenuating uremia, cardiovascular

dysfunction and mortality in CKD patients [14,16–18]. However, no studies were found assessing the functionality of sorghum in CKD patients.

Thus, in this study we hypothesized that consumption of symbiotic meal (symbiotic group - SG) is better than placebo meal (control group – CG) to reduce colonic pH, increase short chain fatty acid production by intestinal microbiota, and subsequently reduce serum uremic toxins and metabolic markers in HD subjects. Our aim was to investigate the effect of the symbiotic meal (extruded sorghum associated unfermented probiotic dairy beverage) on uremic toxins, in fecal pH and metabolic markes in HD individuals during a 7-wk intervention.

5.3 Results

5.3.1 Baseline characteristics of the participants

The 58 participants showed hemodialysis time ranges from 3 to 245 months and 63.1± 10.9 years old. Patients presented diabetes mellitus (44.8%) and hypertensive nephrosclerosis (22.4%) as the main diseases related to CKD. At the baseline, 19.6% of patients under HD were underweight, 52.9% were normal-weight and 27.4% were overweight/obese. There was differences in baseline characteristics between groups ($p > 0.05$) (Table 1).

Table 1 – Baseline characteristics of HD subjects, according to intervention groups.

Variables	All patients (n=58)	Control group (n=29)	Symbiotic group (n=29)
Age (year)*	63.10 ± 10.90	63.03 ± 10.77	63.17 ± 11.16
BMI ($Kg.m^{-2}$)*	23.99 ± 3.65	23.26 ± 2.87	24.74 ± 4.24
Sex †			
Male	38.00 (65.52)	21.00 (72.40)	17 (58.6)
Female	20.00 (34.48)	8.00 (27.60)	12 (41.4)
HD time (months) •	32.00 (14.00;77.00)	31.00 (14.00; 71.50)	34 (15.50; 34.00)
Cause of Kidney Disease †			
Diabetes	26.00 (44.80)	13.00 (44.80)	13.00 (44.80)
Hypertensive nephrosclerosis	13.00 (22.40)	6.00 (20.70)	7.00 (24.30)
Glomerulonephritis	3.00 (5.10)	2.00 (7.00)	10.00 (3.40)
Polycystic kidney disease	2.00 (3.50)	1.00 (3.40)	1.00 (3.40)
Obstructive uropathy	2.00 (3.50)	1.00 (3.40)	1.00 (3.40)
Other causes	12.00 (20.70)	6.00 (20.70)	6.00 (20.70)
Metabolic markers			
Creatinine (mg/dL) *	8.60 ± 3.07	8.71 ± 3.32	8.48 ± 2.84
Potassium (mEq/l) *	5.61 ± 0.86	5.71 ± 0.96	5.51 ± 0.75
Urea (mg/dL) *	148.03 ± 20.54	143.13 ± 19.53	152.93 ± 20.67
Iron (µg/dL) *	58.50 ± 22.33	56.46 ± 25.45	62.03 ± 17.46
Hematocrit (%)*	33.62 ± 8.90	33.25 ± 5.99	34.26 ± 11.29
Red blood cell (millions/mm^3) •	4.00 (3.00;4.00)	4.00 (3.00;4.00)	4.00 (3.00;4.00)
Hemoglobin (g/dL) •	11.05 (9.57;12.55)	11.40 (9.80;12.80)	11.00 (9.45;12.15)
Alkaline Phosphatase (U/I) •	320.50 (251.50;495.00)	322.00 (226.00;520.00)	319.00 (256.00;485.00)
Phosphrus (mg/dL) •	5.50 (4.80;6.50)	5.80 (3.13;8.90)	5.50 (3.60;7.30)
Albumin (g/dL) •	4.00 (3.66;4.10)	4.00 (3.60;4.10)	4.00 (3.71;4.09)
Uremic Toxins (µM)			
IAA •	17.20 (12.82;26.95)	15.41 (3.53;62.28)	20.34 (5.12; 56.86)
IS *	160.59±75.72	167.87±75.88	153.30±76.20
p-CS •	306.01 (240.17;584.64)	288.70 (248.66;578.71)	339.06 (213.27; 593.16)
Short chain fatty acid (mmol/L)			
Acetic acid*	4.33±1.98	4.92±2.15	3.79±1.69
Propionic acid*	2.69±2.44	3.04±2.84	2.39±2.07
Butyric acid*	2.27±1.72	2.44±1.88	2.25±1.62

* Mean ± Std. Deviation, Independent Samples t-test; •median (p25;p75), Mann-Whitney test;† n (%), Chi-Square Tests. Level of significance 5%. BMI - body mass index; IAA - indole 3-acetic acid, IS - indoxyl sulfate, p-CS - p-cresyl sulfate.

5.3.2 Body Mass index and Metabolic markers

BMI values showed no statistical difference in the SG (24.94 ± 4.31) in relation the CG (23.84 ± 2.75) ($p>0.05$), after 7-wks. HD patients intaking the symbiotic meal and CG presented similar ($p>0.05$) nutritional state classification, respectively, underweight (16.0% and 15.4%), normal-weight (52.0% and 65.4%) and overweight/obese (32.0% and 19.2%).

The SG decreased urea concentration and showed a higher percentage (82.8%; n = 24) of serum albumin adequate compared to CG ($p<0.05$). The other metabolic markers showed no significant difference between groups ($p>0.05$). SG and CG groups incresed serum albumin in relation to the baseline ($p<0.05$). However, only SG decreased urea and phosphorus concentration in relation to baseline ($p<0.05$) (Figure 1).

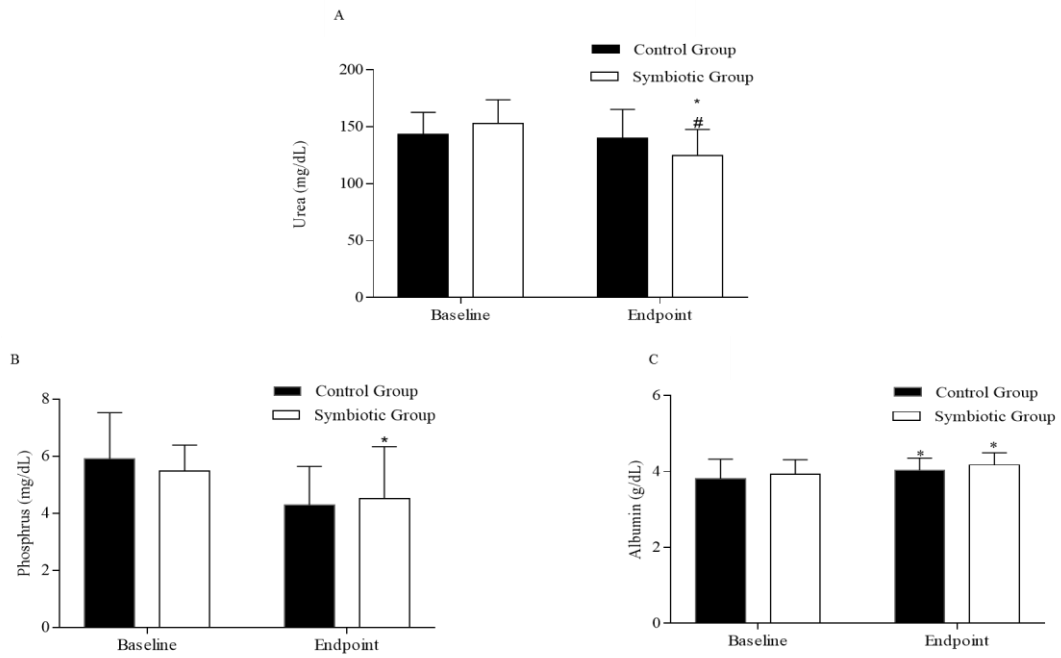

Figure 1 - Metabolic markers of HD individuals before and after 7-wk. A- Urea; B- Phosphorus; C- Albumin. *$p<0.05$ from paired-t test for comparison between baseline and endpoint values for symbiotic group; #$p<0.05$ from Student-t test for comparison between groups (control vs. symbiotic) at the endpoint values.

5.3.3 Short chain fatty acid, fecal pH, uremic toxins and correlations

The SG presented higher concentration of acetic, butyric, propionic acids at endpoint (7-wk), compared to baseline (p<0,05) and CG presented higher concentration of acetic and propionic acid (p<0,05) at endpoint (7-wk), compared to baseline (p<0.05), with no difference between groups (Table 2). In addition to, SG presented lower fecal pH than CG, after 7-wk(p<0.05).

The SG decreased serum p-CS and IS (p<0.05), compared to the CG. In addition, the CG presented a reduction (p<0.05) in p-CS concentration while the SG showed a reduction in IAA, IS and p-CS concentration at endpoint (7-wk), compared to baseline (Table 2). In relation to delta values (delta final – delta initial), the SG presented a larger decreased in serum IAA and IS concentration (p<0.05) than the CG (Figure 2).

Table 2 –Concentration of short chain fatty acid and fecal pH in subjects with chronic kidney disease on hemodialysis, according to intervention groups.

Variables	Control group			Symbiotic group			
	Baseline	Endpoint	P[1]	Baseline	Endpoint	P[1]	P[2]
Uremic toxins (µM)							
IAA	15.41 (11.23;21.08)	14.91 (12.11;19.44)	0.87	20.34 (14.07; 29.91)	14.04 (10.92;20.98)	0.01*	0.97
IS	167.87±75.88	157,18±62,47	0.30	153.30±76.20	123,94±56,71	0.04*	0.04*
p-CS	288.70 (253.82;570.20)	282.19 (231.12;363.64)	0,01*	339.06 (220.04; 583.79)	248.76 (165.51;292.86)	<0.001*	0.05*
SCFA (mmol/l)							
Acetic acid	4.92±2,15	7.90±3.80	0.02*	3.79±1.69	6.51±2.24	0.006*	0.08
Propionic acid	3.04±2,84	6.17±3.66	0.02*	2.39±2.07	6.36±3.70	0.002*	0.93
Butyric acid	2.44±1,88	3.64±2.94	0.18	2.25±1.62	3.91±2.41	0.004*	0.69
Fecal pH	6.16±1.25	5.75±1.03	0.03*	6.39±0.99	5.12±0.90	0.000*	0,04*

Mean ± Std. Deviation, Independent Samples t-test or paired-t test; Level of significance 5%.

Median (p25; p75) Mann-Whitney or Wilcoxon test; Level of significance 5%.

[1] statistical intragroup (baseline vs. endpoint).

[2] statistical between groups.

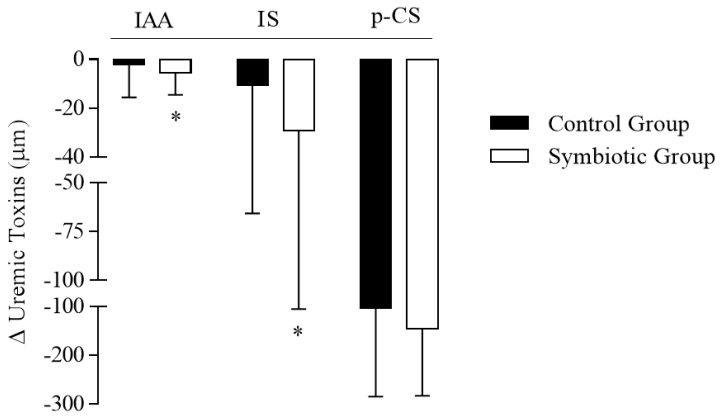

Figure 2: Delta values (delta final – delta initial) of uremic toxins of control group and symbiotic group. IAA - indole 3-acetic acid, IS - indoxyl sulfate, p-CS - p-cresyl sulfate.

The correlation between serum urea and p-CS and urea with pH fecal was positive in SG at the endpoint (Figure 3 A-B). No correlation was observed in control group (p>0.05).

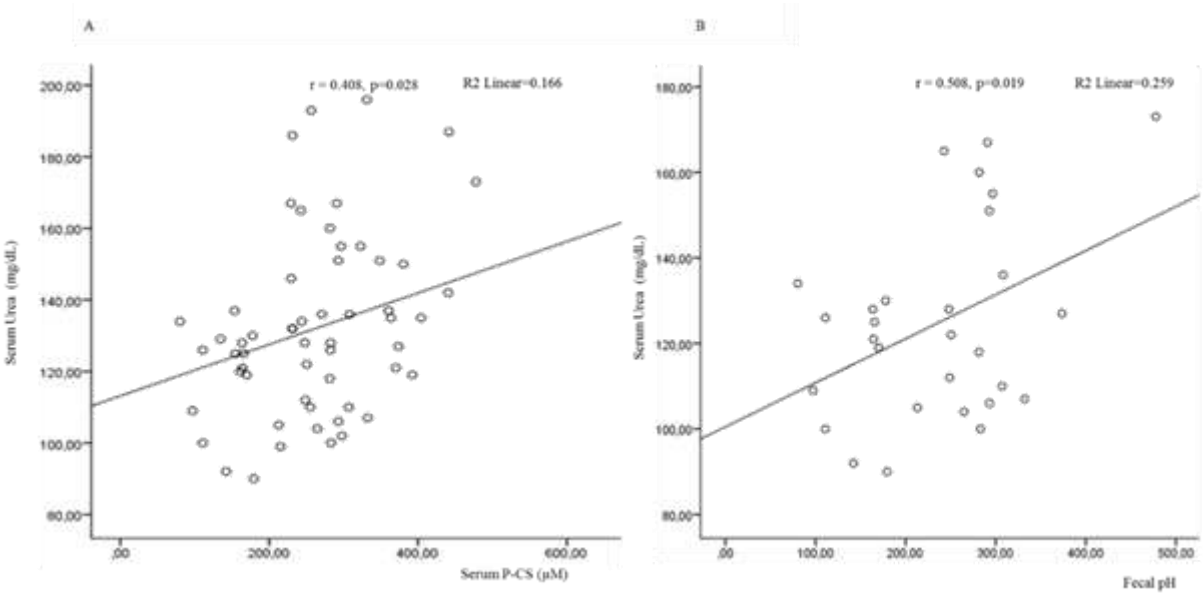

Figure 3- Spearman correlation in SG individuals at the endpoint (n=29). A-Correlation between serum urea and p-CS. B-Correlation between serum urea and fecal pH. p-CS: p-cresylsulfate

5.4 Discussion

This research is the first to investigate the effects of symbiotic meal (extruded sorghum breakfast meal plus unfermented probiotic dairy beverage) on metabolic and uremic toxins control in HD individuals.

The main result of this study was that symbiotic meal decreased serum uremic toxins in HD subject after 7-wk of intervention. This result has protected effect, since CKD patients present a progressive retention of uremic toxins, with a negative impact on many body functions and increase in cardiovascular mortality. The increase of the toxins in the bloodstream can increase intestinal permeability caused by influx of urea and expansion of urease-possessing bacteria [4,5]. Probably, symbiotic meal improves the composition of intestinal microbiota, favoring the growth of anaerobic bacteria and reducing the permeability of the intestinal barrier, decreasing production and diffusion of uremic toxins from the intestinal lumen into the bloodstream. These effect may decrease a renal fibrosis, delay the progression of CKD, and improve azotemia [5]. Our results differ of some studies with symbiotic therapy in CKD patients [19–21] because it presented a reduction in the serum concentrations of both toxins, IS and p-CS.

The second point is that SG group decreased serum urea after 7-wk of intervention. The symbiotic meal may decrease production of urea from ammonia in the liver by decreasing ammonia production by urease and its influx into the gastrointestinal tract. The concentration of dietary fibers (3,6 g/portion), and bioactive compounds (condensed tannins: 13.06 ± 1.52 mg catechin eq/portion; flavonoids: 76.78 ± 12.92 μg total 3DXAs/portion; phenolic compounds: 44.20 mg of gallic acid eq/portion) in extruded sorghum and the *Bifidobacterium longum* ($7.4x10^8 \pm 5.4x10^8$ CFU/100 mL portion) in the probiotic milk [16] can decrease ammonium hydroxide production from ammonia, which decrease the gut's luminal pH, preventing dysbiosis and intestinal mucosa alterations and, subsequently, decreasing uremic toxins

production and systemic inflammation [5,22,23]. The polymeric tannins from sorghum can interact with starch production resistant starch. Then, the dietary fiber and resistant starch fermented by intestinal microbiota can acidify the colonic environment, which inhibit the growth of bacteria acid, such as species producers of urease enzyme[5,14,23,24]. Similar results was observed by Dehghani et al. [23] in subjects with CKD who received two capsules of 500 mg of a symbiotic for six weeks and by Alatriste et al. [25] in individuals with CKD who received a dose of $16x10^9$ CFU of *Lactobacillus casei shirota* (LcS) for eight weeks. However, in our study we used a food instead capsule. Another important factor is that the decrease of serum uremic toxins values may be related to the lower production of ammonia by the intestinal microbiota, since this is directly related to intestinal permeability [5].

Other discussion point is related to the acidification of colonic environment confirmed in our study with reduction of fecal pH and increase concentration of acetic, butyric, propionic acids in the HD subjects who received symbiotic meal. This acidification may be related to ammonia rate, as well as to improve the intestinal microbiota composition and the serum uremic toxins concentration [4]. The urea concentration in our study was correlated to fecal pH, suggesting the increase of anaerobic bacteria and reduction of toxins uremics. As a consequence, the reduction of urea concentration leads to a reduction in the production of p-CS. Some studies with probiotic have documented the reduction pH colonic in CKD individuals [4,27]. The reduction of serum p-CS and IS concentration found in our study may be related to the inhibition of the enzymes involved in generation of p-CS and IS and by increase of fecal butyric acid concentration at the endpoint in the SG. Both cereals are classified as fiber sources because they have a value of this nutrient greater than 2.5 g per serving[28]. It is known that fibers, when fermented, produce short chain fatty acids, among them acetate, butyrate and propionate. However, sorghum present higher concentration of fiber than corn. This fact was responsible for causing an increase in the concentration of butyric acid and reducing in fecal pH in the symbiotic group (sorghum), which was not observed in the control group (corn).

Butyric acid exerts a variety of effects on intestinal function as reinforce the colonic defense barrier through of mucins production and antimicrobial peptides, and decrease intestinal epithelial permeability by increasing the expression of tight junction proteins reducing the influx of uremic toxins [5,27,28].

This study also showed that o SG presented a higher percentage of serum albumin adequate compared to CG, and a reduction in serum phosphorus concentration in SG at endpoint compared to baseline. In addition, the use of symbiotic meal was able to alter the serum phosphorus and potassium values within the normality values. This result can also be associated with intestinal acidification which can increase the calcium ionization and, consequently, its connection with phosphorus, preventing its absorption [11]. In addition, the albumin production is related to inflammation, then the adequacy of albumin found in our study may be related to the intake of the symbiotic product, which was previously observed in our study decreasing the inflammation in HD subjects [16].

Although our study was a small single-center study and the number of participants limited us to non-stratification of the products tested, the sample size was adequate to examine the hypotheses. However, further studies should evaluate the isolated effects of extruded sorghum breakfast meal and unfermented probiotic milk on uremic markers.

5.5 Conclusion

The intake of the symbiotic meal (sorghum extruded breakfast meal plus unfermented probiotic milk) for 7-wk reduced the serum uremic toxins and metabolic markers in HD subjects; and the reduction of fecal pH and the increase of SCFA production by intestinal microbiota can be the explanation for these results.

5.6 Materials and Methods

5.6.1 Subjects

One hundred and twelve HD subjects in a single dialysis center (Brazil) were evaluated for eligibility. Of these, one hundred and seven met the inclusion criteria and were selected for the study. Inclusion criteria were: patients older than 18 years old that had been having hemodialysis sessions three times a week for at least the past three months. In this study, we did not include individuals whose records reported auditory deficiency, autoimmune disease, hepatitis B and C virus infection, newly implanted catheters, hemodynamic instability, lactose intolerance or milk discomfort.

Thus, ninety-nine selected participants were randomly distributed into two groups, placebo (control) and symbiotic, and evaluated during 7-wk. At the end of the intervention, 58 volunteers had completed the study (Figure 4) and were included in the analyses of this work.

Sample size was calculated considering: (1) difference in the IS concentration as main outcome (12); (2) published SD (± 15.5) for IS concentration; (3) statistical power of 90%; and (4) significance level of 5%. For all these criteria, the sample size required was a minimum of 22 volunteers per group [31].

The study protocol was approved by the Human Research Ethics Committee of the *Universidade Federal de Viçosa* (protocol number 701.796/2014) and was registered at www.ensaiosclinicos.gov.br, with ID number RBR-2d9ny6. All participants read and signed the written informed consent.

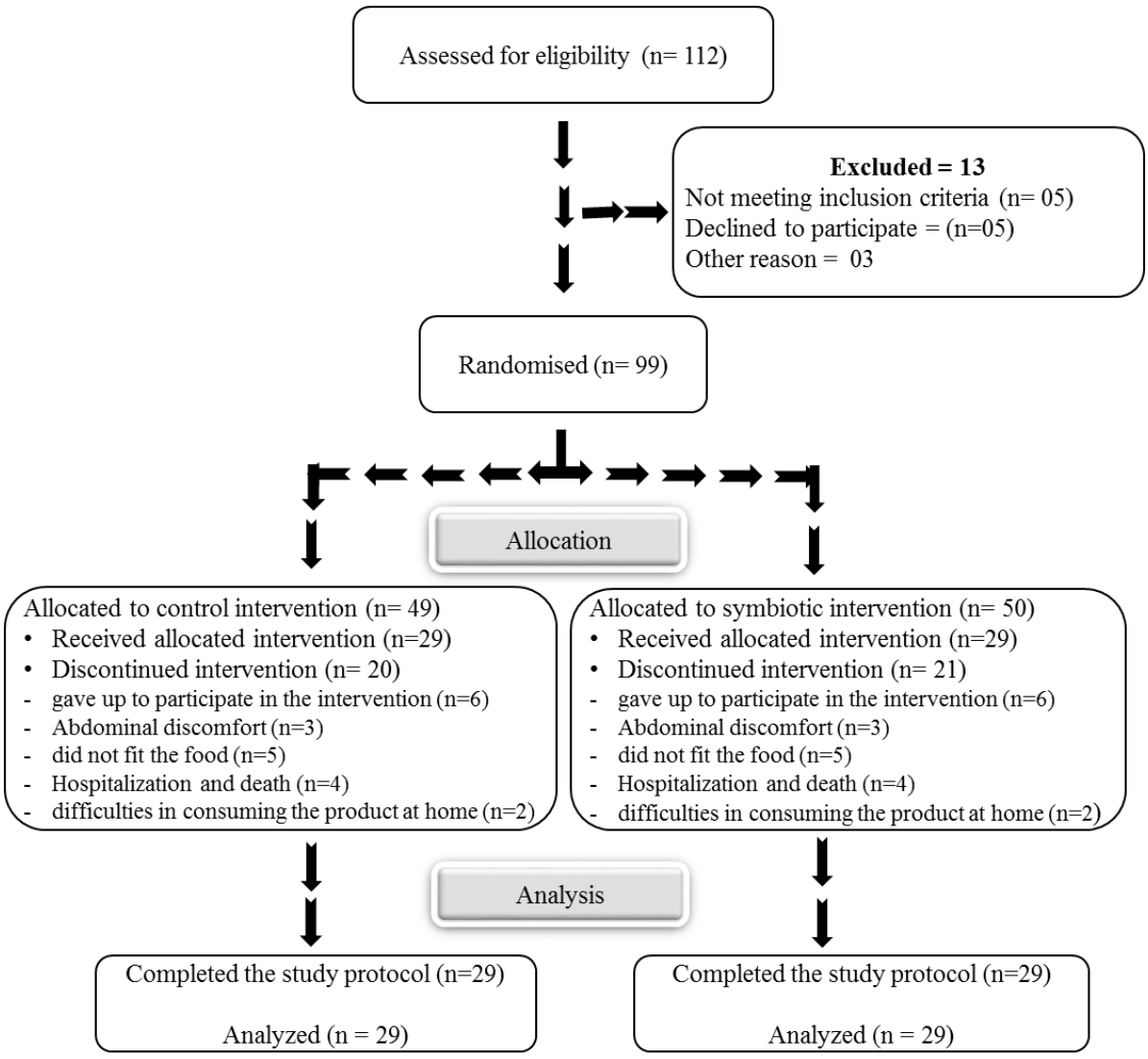

Figure 4: CONSORT diagram showing the flow of participants through each stage of the trial. CONSORT Consolidated Standards of Reporting Trials.

5.6.2 Study design

This is a controlled, randomized, simple blind study, which took place for 7 weeks and respected blood collection routine of the chosen nephrology sector. Socio-demographic, clinical and anthropometric data were obtained before the intervention period by medical records and interviews. Participants were randomly assigned to one of the groups: symbiotic group – SG (100 mL of probiotic dairy drink and 40 g of extruded sorghum flakes) or control group – n CG (100 mL of pasteurized milk and 40 g of extruded corn flakes) (Figure 5). During the HD

sessions, patients received two food kits, one was consumed on the third hour of hemodialysis and the other was consumed on the interdialytic day at home. Patients who could not consume the products in the dialysis center were instructed to take them home and consume them together the same day. Participants were asked about the consumption and if there were any adverse effect during HD days (two d/wk) to assess intervention compliance.

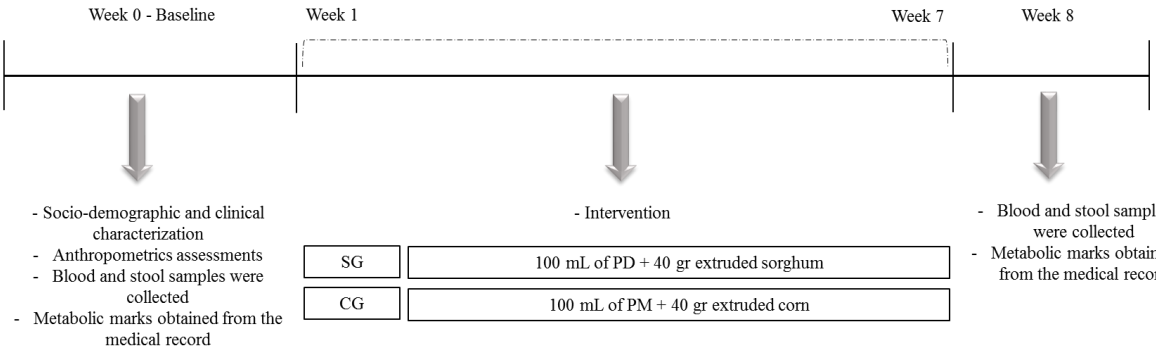

Figure 5: Schematic representation of study protocol (control group: *n* = 29; symbiotic group: *n* = 29). CG – control group; SG – symbiotic group; PM – pasteurized milk; PD – probiotic drink

At the beginning and at the end of the intervention, blood samples were collected by a qualified professional in the chosen nephrology sector; blood samples were centrifuged and immediately stored at -80ºC. Participants collected stool samples in sterile bottles, kept at -18ºC until the hemodialysis procedure. Participants used Styrofoam packaging with ice cubes to transport samples to the nephrology sector. Samples were collected during HD sessions, aliquoted and stored at -80 ° C.

5.6.3 Products preparation

Sorghum grains were grown in the *Embrapa Milho e Sorgo* experimental field, located in Sete Lagoas, Brazil. The BRS 305 sorghum hybrid, with brown pericarp containing tannins, was cultivated from April to July 2014, and corn grains were cultivated in the 2013/2014 crop.

After harvest, the grains were packed in plastic bags and sent for processing to *Embrapa Agroindústria de Alimentos*, Rio de Janeiro, Brazil.

Sorghum whole grains were milled into flour using a 3600-disk mill (Perten Instruments, Huddinge, Sweden), with opening size 2, and the corn kernels milled in a TREU hammer mill (Rio de Janeiro, Brazil) equipped with a sieve of 1.6 mm opening. The flour mixture received 10% refined sugar (União de Refindores, São Paulo, Brazil) and 0.5% NaCl (Serv Sal, Mossoró, RN), and was processed in a Clextral Evolum HT25 double co-rotating extruder (Clextral Inc., Firminy, France) at a constant screw speed of 350 rpm and profile temperatures: 30, 60, 80, 100, 100, 100, 110, 110, 120, 120 °C, from the feed zone. We injected distilled water between the first and second feed zones by means of a J-X 8/1 piston metering pump (AILIPU Pump Co. Ltd., China), adapted to provide a moisture content of 13%. The shape and size of the extrudates were obtained by a cutter knife (Clextral Inc., Firminy, France) equipped with a 4-blade knife installed at the 2 mm die exit with four holes in rotation at 400 rpm to obtain spherical extrudates. These were then oven dried at 60°C for 2 h, packed in polyethylene bags and transported by land to the *Universidade Federal de Viçosa*, where they were sampled (40 g, proportion stipulated according to the percentage of food fiber present in extruded sorghum; Lopes et al, 2018 – in press) in plastic packaging, sealed by means of a sealing machine, labeled and stored at 5 ± 2ºC.

The dairy drinks used in the study were pasteurized milk for the control group (CG), and pasteurized milk with added probiotic bacterium *Bifidobacterium longum* BL-G301 (Granotec do Brasil S.A.) for the symbiotic group (SG). Probiotic dairy beverage production was carried out weekly at the *Universidade Federal de Viçosa* according to procedures described by Oliveira et al [32]. The beverages (100 mL) were packed in a high-density polyethylene plastic bottle with aluminum seal, labeled with date of manufacture, validity, instructions for storage and consumption, and stored under refrigeration at 4 ± 2ºC for up to seven days. The viability of the probiotic product was performed weekly and the concentration of viable *Bifidobacterium*

longum BL-G301 cells averaged $7.4x10^8 \pm 5.4x10^8$ CFU/100 mL, with minimum concentration of $2.5x10^8$ and maximum of $1.5x10^9$ CFU/100 ml.

The chemical composition of the products used in this study is described in Table 3.

Tabel 3: Chemical composition of the products used in the intervention/portion*

	Symbiotic Product	Control Product	p
Energy (Kcal)	196.16 ± 0.67	200.25 ± 2.06	0.031
Carbohydrate (g)	33.42 ± 0.29	33.52 ± 0.15	0.617
Protein (g)	7.90 ± 0.42	8.56 ± 0.33	0.140
Lipids (g)	3.36 ± 0.05	3.53 ± 0.04	0.012
Total Fiber (g)	3.54 ± 0.45	2.91 ± 0.35	0.037
Phosphorus (mg)	136.00 ± .00	143.20 ± 0.40	<0.001
Potassium (mg)	141.20 ± 1.20	140.80 ± 0.40	0.613
Iron (mg)	2.23 ± 0.10	1.45 ± 0.01	<0.001
Calcium (mg)	160.80 ± 0.40	140.93 ± 1.01	<0.001
Tannin (mg catechin eq.)	28.40 ± 0.07	-----	
Phenolic compounds (mg gallic ac. eq.)	44.20 ± 0,84	32.60 ± 0.36	<0.001
Total 3 – DXAs (mg)	72.78 ± 12.92	-----	

*Calculation based on the chemical composition of the extruded cereals [16] and the chemical composition of pasteurized milk

5.6.4 Data collection

All anthropometric measurements were performed at the end of the hemodialysis session, after 30 minutes of hemodynamic balance. Anthropometric measures included in the study were dried weight (kg) and height (cm), performed according to previously standardized procedures [33-35]. Body mass index (BMI) was calculated by weight/height^2 ratio, and the weight-status of each individual was calculated according to the World Health Organization cut-off points for adults [35] and to the Lipschitz cut-off points for elderly people [34].

The metabolic markers analyzed assessed were: albumin, urea, creatinine, potassium, phosphorus, calcium, hemoglobin, hematocrit, iron and alkaline phosphatase. Data for these metabolites were obtained from medical records collected before and after the intervention.

Uremic toxins (IS, p-CS, and IAA) were quantified by high-performance liquid chromatography (HPLC) with fluorescent detection. Plasma samples were processed as described in Meert et al [36]. The ultra-filtered serum was injected into an HPLC system (Shimadzu Prominence) consisted of a Rheodyne injector (model 7125), a quaternary pump (Shimadzu LC-20AD) controlled by the LC Solution software, and a fluorescence detector (Shimadzu RF-20A). Serum separation was achieved with C8 Luna column (Phenomenex, Luna 5µm, 100Å, 150x4,6mm), eluted with 50mM ammonium formate pH 3.0, alongside methanol proportions increasing from 35% to 70% along the run at a flow rate of 0.7 mL/min. During the run, the fluorescence wavelengths varied λexc = 280 nm and λemi = 383 nm to IS and λexc = 265 nm and λemi = 290 to p-CS and IAA.

Fecal hydrogen potential (pH) was measured in digital pHmeter T-1000 (Tekna, São Paulo, Brazil). We filled one gram of feces in falcon-type tube (15 mL capacity) and added 10 mL of ultrapure water. After vortex homogenization, the pH was read.

For the extraction of organic acids in fecal contents, around 500 mg of faeces were homogenized with the addition of 1 mL of ultrapure water. The samples were centrifuged (Himac CT 15RE, Hitachi) at 12000 g for 10 min at 4°C. Then, supernatants were treated as described by Siegfried et al [37]. Organic acids (acetic, propionic and butyric acid) were determined by high performance liquid chromatography (HPLC) in a Dionex Ultimate 3000 Dual detector HPLC (Dionex Corporation, Sunnyvale, CA, USA) coupled to a refractive index (RI) Shodex RI-101. The following chromatographic conditions were used: Bio-Rad HPX-87H column (300 mm x 4.6 mm) equipped with a Bio-Rad Cation H guard column, column temperature 45°C, injection volume of 20 µL. The mobile phase was composed by concentrated sulfuric acid, EDTA and ultrapure water; flow rate of 0.7mL/min. The following organic acids

were used for the calibration of the standard curve: acetic, succinic, formic, propionic, valeric, isovaleric, isobutyric and butyric acid. The standard solutions of the acids were prepared with a final concentration of 10 mmol/L, except acetic acid (20 mmol/L). Standards and a quantitative curve was used to determine all solute levels.

5.6.5 Statistical analysis

Recorded data were reviewed to detect missing information and inconsistencies. Subsequently, a descriptive analysis of the general characteristics from participants was performed per group. Quantitative variables with normal distribution (according to graphical analysis, asymmetry and kurtosis coefficients and Shapiro-Wilk test) were expressed as mean and standard deviation (SD) and those that did not present normal distribution were expressed in median and p25-p75. Differences between groups were assessed by the chi-square test (categorical variables), Student's t-test or Mann-Whitney test (numerical variables). Paired-t test or the Wilcoxon test was used to assess differences within the groups. Spearman's rank-correlation coefficient was used to assess the correlation of serum urea with p-CS and fecal pH for each tested group. Statistical analysis was performed using the Statistical Package for Social Sciences 20.0 program (SPSS, Inc., Chicago, IL, USA) and significance level (α) was considered equal to 5%.

5.7 Acknowledgements

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5.8 Author Contributions

All authors significantly contributed to this paper: data collection (R.C.S.O.L. and J.M.V.T.), sample analysis (R.C.S.O.L and M.E.C.M), data analysis (R.C.S.O.L and M.E.C.M.), data interpretation (R.C.S.O.L, M.E.C.M and H.S.D.M), writing of the paper (R.C.S.O.L) and review of the paper (J.M.V.T, M.E.C.M, V.A.V.Q, H.H.M.H and H.S.D.M.).

5.9 Conflicts of Interest

The authors declare that they have no conflict of interest.

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6. Conclusão Geral

- O sorgo extrusado do cultivar BRS 305 demonstrou ser fonte de fibra alimentar, compostos fenólicos e atividade antioxidante. Embora este cereal tenha apresentdo alta concentração de minerais, como fósforo e potássio, uma porção de 40 g pode ser incluída na alimenação dos indivíduos com DRC em hemodiálise.
- A ingestão do sorgo extrusado do cultivar BRS 305 com leite probiótico não fermentado com *Bifidobacterium longum* BL-G301 reduziu os níveis séricos de malondialdeído e proteína C reativa, além de aumentar os níveis séricos de superóxido dismutase e a capacidade antioxidante total do soro em indivíduos em hemodiálise. O grupo que recebeu a intervenção simbiótica apresentou também uma correlação negativa entre proteína C reativa com superóxido dismutase.
- A ingestão do sorgo extrusado do cultivar BRS 305 e leite probiótico não fermentado com *Bifidobacterium longum* BL-G301 reduziu as concentrações séricas de indoxil sulfato e p-cresil sulfato, bem como a concentração sérica de ureia e pH fecal quando comparado ao grupo controle. Também promoveu maior produção de ácido acético, butírico e propiônico ao final da intervenção quando comparado aos valores iniciais. Além disso, no final da intervenção, o grupo simbiótico apresentou uma correlação positiva entre p-cresil sulfato e pH com a concentração da ureia.
- A ingestão do sorgo extrusado do cultivar BRS 305 associado ao leite probiótico não fermentado com *Bifidobacterium longum* BL-G301 melhorou a inflamação, o estresse oxidativo e os marcadores urêmicos em indivíduos com doença renal crônica em hemodiálise.

7. CONSIDERAÇÕES FINAIS

Este estudo avaliou a ingestão do sorgo extrusado associado ao leite probiótico não fermentado por indivíduos em hemodiálise. A partir dos resultados encontrados podemos dizer que este produto simbiótico atenuou a inflamação, o estresse oxidativo e os níveis séricos de marcadores urêmicos nestes pacientes, provavelmente pela modificação da microbiota intestinal. Assim, estudos futuros são necessários para avaliar o efeito deste produto na composição da microbiota intestinal.

Embora este estudo tenha sido de centro único o tamanho da amostra foi adequado para examinar as hipóteses. No entanto, o número de participantes nos limitou a não-estratificação dos produtos testados. Por isso estudos adicionais devem avaliar se estes produtos oferecidos isoladamente teriam o mesmo efeito na inflamação, no estresse oxidativo e nos marcadores urêmicos nesta população.

APÊNDICES

Apêndice A: Aprovação do Comitê de Ética

UNIVERSIDADE FEDERAL DE VIÇOSA - UFV

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: PREVALÊNCIA DE DOENÇA CELÍACA ENTRE PACIENTES COM DOENÇAS RENAIS CRONICAS E IMPLEMENTAÇÃO DE ESTRATÉGIAS DIETÉTICAS

Pesquisador: Sônia Machado Rocha Ribeiro

Área Temática:

Versão: 2

CAAE: 27364314.8.0000.5153

Instituição Proponente: Departamento de Nutrição e Saúde

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 701.796

Data da Relatoria: 04/07/2014

Apresentação do Projeto:

Trata-se de um estudo prospectivo para avaliar a prevalência de doença celíaca entre pacientes com doença renal crônica e implementar estratégias nutricionais e dietéticas com intuito de corroborar para qualidade de vida e diminuir possíveis complicações clínicas associáveis entre ambas patologias.Espera-se conhecer a problemática relacionada entre a doença celíaca e a doença renal em tratamento hemodialítico para a implementação de protocolos e rotinas de atendimento nutricional, visando a melhoria da condição clínico-nutricional dos pacientes atendidos na referida unidade hospitalar.

Objetivo da Pesquisa:

Investigar a associação entre doença celíaca e a doença renal em pacientes submetidos ao tratamento hemodialítico e implementar estratégias dietéticas.

Objetivos Secundários:

Avaliar a presença das principais manifestações clínicas relacionadas à DC em pacientes portadores de DRC em tratamento hemodialítico;

Investigar parâmetros bioquímicos séricos e biópsia de mucosa intestinal sugestivos de intolerância à gliadina;

Calcular a prevalência de portadores de exames positivos para DC;

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Correlacionar os resultados encontrados para Anti-endomísio IgA anticorpos EMA com os exames bioquímicos de: PCR, IL-6, Potássio, Fósforo, Cálcio, Albumina, Glicemia de jejum, Hemoglobina glicada,Hematócrito, Hemoblobina, Ktv.
Avaliar o estresse oxidativo dos participantes.
Avaliar o estado nutricional e a ingestão alimentar dos participantes.
Realizar a intervenção dietética com o uso de probiótico.
Analisar os exames marcadores de DC e de controle metabólico da DRC após a intervenção dietética.
Realizar avaliação antropométrica dos participantes antes e após a intervenção dietética para a retirada de glúten.
Oferecer acompanhamento nutricional no programa pró-celíaco (atividade de extensão da UFV) para os participantes com exames positivos para DC.
Elaborar receitas de baixo custo adaptadas para fins especiais da doença renal crônica e doença celíaca.

Avaliação dos Riscos e Benefícios:

Descritos de forma adequada.

Comentários e Considerações sobre a Pesquisa:

Todas as alterações solicitadas foram atendidas.

Considerações sobre os Termos de apresentação obrigatória:

Apresentados de forma adequada. Foi anexado aos documentos um parecer técnico do serviço de Nefrologia e um parecer técnico do serviço de Gastroenterologia do Hospital São João Batista esclarecendo todas as pendências identificadas anteriormente.

Recomendações:

Quando da coleta de dados, o TCLE deve ser elaborado em duas vidas, rubricadas em todas as suas páginas e assinadas, ao seu término, pelo convidado a participar da pesquisa, ou por ser representante legal, assim como pelo pesquisador responsável, ou pela(s) pessoa(s) por ele delegada(s), devendo as páginas de assinaturas estar na mesma folha. Para a submissão, não é necessária a assinatura do TCLE.

Conclusões ou Pendências e Lista de Inadequações:

Não há.

Situação do Parecer:

Aprovado

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Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Ao término da pesquisa é necessária a apresentação do Relatório Final e após a aprovação desse, deve ser encaminhado o Comunicado de Término dos Estudos.

Projeto analisado durante a 3ª reunião de 2014.

VICOSA, 27 de Junho de 2014

Assinado por:
Patrícia Aurélia Del Nero
(Coordenador)

Apêndice B: Termo de adesão do serviço de nefrologia

SERVIÇO DE NEFROLOGIA

HSJB

Termo de Adesão

O serviço de Nefrologia do Hospital São João Batista, (CNPJ: 021356080001/59), declara ter interesse em participar do projeto ESTUDO CLÍNICO PARA SUBSIDIAR NOVAS ESTRATÉGIAS DE TERAPIA NUTRICINAL NA DOENÇA RENAL CRÔNICA EM TERAPIA RENAL SUBSTITUTIVA como parceira da Universidade Federal de Viçosa, que submeterá a proposta de projeto visando o apoio do CNPq, por meio da chamada 36/2014- Pesquisa sobre doenças renais.

Viçosa, 04 de novembro de 2014.

Márcia Garcia Gouvea
NEFROLOGISTA
CRM: 16 379

Drª Márcia Garcia Gouvea

Responsável Técnica do Serviço de Nefrologia

Apêndice C: Termo de consentimento livre e esclarecido

UNIVERSIDADE FEDERAL DE VIÇOSA
DEPARTAMENTO DE NUTRIÇÃO E SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA DA NUTRIÇÃO
Av. P.H. Rolfs, s/n - Campus Universitário - 36570-000 – Viçosa, MG – Brasil

FONE: (31) 3899-2899 Fax: (31) 3899-3176 E-mail: ppgcnut@mail.ufv.br

Termo de Consentimento Livre Esclarecido

Eu_____estou sendo convidada(o) a participar da pesquisa intitulada: **Prevalência de Doença Celíaca entre Pacientes com Doenças Renais Crônicas e Implementação de Estratégias Dietéticas** do Departamento de Nutrição e Saúde da UFV. A pesquisa será realizada por Andreza de Paula Santos (andrezauba@hotmail.com), Karla Pereira Balbino (karla.balbino@ufv.br), Rita de Cássia Stampini Oliveira Lopes (rita.lopes@ufv.br)sob a orientação das professoras Ana Vládia Bandeira Moreira, Sônia Machado Rocha Ribeiro e Hércia Stampini Duarte Martino (telefones para contato: 31 3899- 3730; e 31 9389 0514). Fui informado sobre o significado e importância do projeto e os objetivos do estudo. Fui informado que ao participar da pesquisa terei que responder questionários sobre sintomas relacionados à doença celíaca e ingestão alimentar; terei que realizar exames bioquímicos, cujo sangue será obtido no mesmo momento em que realizo os exames de rotina na nefrologia; minha altura e peso corporal serão avaliados e receberei intervenção dietética, atendimento nutricional e orientação dietética por profissionais nutricionistas qualificados. Ao participar da pesquisa autorizo que as informações registradas em meu prontuário sejam disponibilizadas para as pesquisadoras. Também fui informado que a pesquisa não acarretará nenhum ônus financeiro para mim e para minha família. Estou esclarecido que as informações coletadas serão mantidas em sigilo e que dados obtidos poderão ser divulgados em trabalhos científicos, sem que haja identificação das pessoas que participaram do estudo. Também fui informado de que posso me recusar a participar do estudo, ou retirar meu consentimento a qualquer momento, sem precisar justificar, e se eu sair da pesquisa, não sofrerei qualquer prejuízo ou penalidade. Estou ciente de que, este termo foi redigido conforme determina a Resolução CNS 466/2012 e caso eu tenha dúvida ou sinta prejudicado, poderei, imediatamente, recusar-me a participar ou a continuar fazendo parte da pesquisa. Receberei assistência durante toda pesquisa bem como me é garantido o livre acesso a todas as informações e esclarecimentos sobre o estudo, antes, durante e depois da minha participação. Fui informado de que não há nenhum valor econômico a receber ou pagar por minha participação.

As pesquisadoras do estudo me ofertaram uma cópia deste Termo de Consentimento Livre Esclarecido, conforme recomendações da Comissão Nacional de Ética em Pesquisa (CONEP).

Andreza de Paula Santos (Doutoranda, Pesquisadora)	Karla Pereira Balbino (Mestranda, Pesquisadora)
Rita de Cássia Stampini O. Lopes (Doutoranda, Pesquisadora)	Hércia Stampini Duarte Martino (Docente, Pesquisadora, Orientadora)
Sônia Machado Rocha Ribeiro (Docente, Pesquisadora, Orientadora)	Ana Vládia Bandeira Moreira (Docente, Pesquisadora, Co-Orientadora)

_____

Assinatura do Participante

Viçosa, ---/-----/----------