UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ENGENHARIA DE ALIMENTOS

ADRIANE CRISTINA GARCIA LEMOS

"AVALIAÇÃO *IN VITRO* E *IN VIVO* DAS PROPRIEDADES FUNCIONAIS E EFEITOS PREBIÓTICOS DOS GALACTO-OLIGOSSACARÍDEOS (GOS)"

"IN VITRO AND IN VIVO EVALUATION OF THE FUNCTIONAL PROPERTIES AND PREBIOTICS EFFECTS OF THE GALACTO-OLIGOSACCHARIDES (GOS)"

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Orientadora: Profa.Dra. Gláucia Maria Pastore

"IN VITRO AND IN VIVO EVALUATION OF THE FUNCTIONAL PROPERTIES AND PREBIOTICS EFFECTS OF THE GALACTO-OLIGOSACCHARIDES (GOS)"

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciência de Alimentos da Faculdade de Engenharia de Alimentos da Universidade Estadual de Campinas para obtenção do Título de Doutora em Ciência de Alimentos.

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“Somente triunfa no mundo quem se levanta e busca as circunstâncias, e as cria se não as encontra”

(Bernard Shaw)

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Dedico às pessoas mais importantes da minha vida

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RESUMO GERAL

Galacto-oligosacarídeos (GOS) são prebióticos obtidos via transgalactosilação enzimática da lactose. Dentre os vários benefícios associados ao consumo de GOS destaca-se a capacidade de estimular o crescimento e atividade de bactérias benéficas no cólon. O objetivo deste estudo foi avaliar as propriedades prebióticas dos GOS sintetizados, a partir da lactose, por β-galactosidase de *Scopulariopsis* sp. A digestibilidade e a fermentabilidade foram avaliadas *in vitro*, enquanto os efeitos prebióticos foram avaliados *in vivo* em um conjunto de experimentos com ratos Wistar. Os resultados observados *in vitro* demonstraram que os GOS produzidos neste estudo são indigeríveis, altamente fermentáveis e convertidos em ácidos graxos de cadeia curta (acetato, propionato e butirato). Estudos *in vivo* demonstraram que o consumo de diferentes doses de GOS por 42 dias não produziu efeitos tóxicos nos animais, evidenciado a partir de avaliações clínicas, exames hematológicos, bioquímicos, necroscópicos e histológicos. Os ratos suplementados com GOS apresentaram maior (p<0.05) população cecal de bifidobactérias ($\log_{10}$ 10,05 ± 0,27 UFC/g) e lactobacilos ($\log_{10}$ 8,92 ± 0,16 UFC/g). Para os ratos não suplementados com GOS estas proporções foram de $\log_{10}$ 8,22 ± 0,33 e 7,2 ± 0,15 UFC/g, para bifidobacterias e lactobacilos, respectivamente. Por outro lado, a população de *Escherichia coli* foi significativamente reduzida (p<0.05), sendo 24,75% menor, quando comparada ao grupo controle sem GOS. Além disso, a fermentação dos GOS pelas bactérias intestinais resultou em um aumento na produção de ácidos graxos de cadeia curta de 2,73 vezes, em relação aos animais sem acréscimo de GOS na dieta.

Observou-se, ainda, que o grupo suplementado com GOS apresentou maiores valores de espessura total da mucosa, altura dos vilos e profundidade das criptas, evidenciado pela maior relação altura de vilosidades:profundidade de cripta em relação ao grupo controle.

Palavras-chave: Galacto-oligossacarídeos, digestibilidade, fermentação, ácidos graxos de cadeia curta, microbiota intestinal, morfometria intestinal.

ABSTRACT

Galacto-oligosaccharides (GOS) are prebiotics obtained via transgalactosylation enzymatic of lactose. Among the many benefits associated with consumption of GOS stands the ability to stimulate growth and activity of beneficial bacteria in the colon. The objective of this study was to evaluate the properties of prebiotic GOS synthesized from lactose by β-galactosidase from *Scopulariopsis* sp*.* The digestibility and fermentability were evaluated *in vitro,* while the prebiotic effects were evaluated *in vivo* in a series of experiments with Wistar rats. The results observed *in vitro* showed that the GOS produced by this study are indigestible highly fermentable and converted into short-chain fatty acids (acetate, propionate and butyrate). *In vivo* studies have showed that consumption of different doses of GOS for 42 days produced no toxic effects in animals, as evidenced from clinical, hematological, biochemical, and histological necropsy. The rats supplemented with GOS had higher (p<0.05) cecal populations of bifidobacteria ($\log_{10}$ 10.05 ± 0.27 UFC/g) and lactobacillus ($\log_{10}$ 8.92 ± 0.16 UFC/g). For rats not supplemented with GOS these proportions were $\log_{10}$ 8.22 ± 0.33 and 7.2 ± 0.15 UFC/g, for bifidobacteria and lactobacillus, respectively. Furthermore, the population of *Escherichia coli* was significantly reduced (p<0.05) and 24.75% less when compared to controls without GOS. Furthermore, the GOS fermentation by intestinal bacteria resulted in an increase in the production of short chain fatty acids from 2.73 times in compared with those without the addition of GOS diet.

It was observed also the supplemented group with GOS showed higher values of total mucosal thickness, villous height and crypt depth, evidenced by the higher ratio of villus height: crypt depth in the control group.

Keywords: Galacto-oligosaccharides, digestibility, fermentation, short chain fatty acids, intestinal microbiota, intestinal morfometric.

SUMÁRIO

LISTA DE ABREVIATURAS E SÍMBOLOS

A

AGCC - Ácidos Graxos de Cadeia Curta

D

DP - Degree Polymerization

E

EPI - exocrine pancreatic insufficiency

F

FCE - Feed efficiency Conversion

FOS - Fructo-oligosaccharides

G

GOS - Galacto-oligosaccharides

GGT - Gamma Glutamyl-transferase

GIT - Gastrointestinal Tract

GC-MS - Gas Chromatography Mass Spectrometry

H

HDL-C - High Density Lipoprotein Cholesterol

HPLC - High-performance Liquid Chromatography

L

LDL - Low Density Lipoprotein

LDL-C - Low Density Lipoprotein Cholesterol

M

MCV - Mean Corpuscular Volume

MCH- Mean Corpuscular Hemoglobin

MCHC - Mean Corpuscular Hemoglobin Concentration

N

NDOs - Non-Digestible Oligosaccharides

P

PT- Prothrombin Time

S

SCFA - Short Chain Fatty Acids

SCCs- Short-chain Carbohydrates

T

TC - Total Cholesterol

TG - Triglycerides

Chapter 1

Chapter 3

Chapter 5

Chapter 7

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

ESTRUTURA DA TESE

Esta tese está estruturada em sete capítulos. Nos Capítulos I e II são apresentados uma revisão da literatura sobre produção, aplicação e efeitos benéficos do prebióticos galacto-oligosacarídeos (GOS) na saúde humana. No Capítulo III são apresentados os resultados obtidos ao testar o potencial prebióticos *in vitro* dos GOS produzidos por *Scopulariopsis* sp. No Capítulo IV apresentam-se os resultados obtidos *in vivo* ao testar a segurança toxicológica dos GOS em ratos. No Capítulo V são apresentados os resultados obtidos ao testar os efeitos prebióticos dos GOS sobre a microbiota cecal, características das fezes e função intestinal em ratos. No Capítulo VI são apresentados os resultados obtidos ao testar os efeitos dos prebióticos GOS e FOS sobre a morfologia intestinal de ratos. O Capítulo VII descreve os resultados obtidos ao testar os efeitos prebióticos de diferentes oligossacarídeos. Os capítulos foram mantidos na forma de artigos e formatados de acordo com as normas das revistas os quais serão submetidos. A tese também fornece um resumo dos principais resultados obtidos nos experimentos.

INTRODUÇÃO GERAL

Consumidores preocupados com a saúde estão buscando cada vez mais alimentos saudáveis que possam contribuir com a saúde e o bem-estar. Este crescente interesse por uma alimentação equilibrada tem incentivado pesquisas de novos componentes naturais biologicamente ativos e tem mudado o entendimento do papel da alimentação sobre a saúde (THAMER & PENNA, 2006; ARAÚJO, 2007). Neste contexto, os alimentos funcionais vêm ganhando posição de destaque na alimentação humana (SAAD, 2006)

A Portaria n.º 398, de 30 de abril de 1999, do Ministério da Saúde, define alimento funcional como sendo "todo aquele alimento ou ingrediente que, além das funções nutricionais básicas, quando consumido como parte da dieta usual, produza efeitos metabólicos e/ou fisiológicos e/ou efeitos benéficos a saúde, devendo ser seguro para consumo sem supervisão médica" (BRASIL, 1999, CRAVEIRO & CRAVEIRO, 2003).

Atualmente, os grupos biologicamente ativos mais conhecidos, que agem como ingredientes funcionais, são: fibras solúveis e insolúveis, flavonóides, carotenóides, fotosteróis, fitostanóis, ácidos graxos (ômega 3 e ômega 6), probióticos e prebióticos (TORRES, 2001; MERINO, 2006; BORTOLOZO & QUADROS, 2007; ABREU et al., 2011).

A literatura científica sobre probióticos e prebióticos apresentou crescimento expressivo nos últimos 10 anos. Seus mecanismos de ação vêm sendo investigados experimentalmente (MORAIS & JACOB, 2006; ROBERFROID, GIBSON, HOYLES, 2010). Os prebióticos têm sido estudados como ingredientes

em vários alimentos, entre eles bebidas lácteas funcionais ou simbióticas (SILVA & STAMFORD, 2000; ABREU et al., 2011).

Os prebióticos são componentes alimentares não-digeríveis que afetam beneficamente o hospedeiro, por estimularem seletivamente a proliferação ou atividade de populações de bactérias desejáveis no cólon. Adicionalmente, os prebióticos podem inibir a multiplicação de patógenos, garantindo benefícios adicionais à saúde do hospedeiro (GIBSON & ROBERFROID, 1995; ROBERFROID, 2007, ROBERFROID, GIBSON, HOYLES, 2010).

Entre as substâncias prebióticas, destacam-se a Inulina, fruto-ologosacarídeos (FOS), galacto-oligosacarídeos (GOS), soja-oligosacarídeos, xilo-oligosacarídeos, isomalto-oligosacarídeos, e lactulose entre outros (SAKI, MATSUMOTO, TANALA, 1999; CHEN & WALKER, 2005).

Porém, segundo ROBERFROID et al. (2010) somente dois tipos de oligossacarídeos são considerados prebióticos, ou seja, frutanos do tipo inulina e os galacto-oligossacarídeos (GOS). Estes têm demonstrado repetidamente a capacidade seletiva de estimular o crescimento de bifidobactérias e, em alguns casos, os lactobacilos levando a uma significativa mudança na composição da microbiota intestinal.

Em relação à estrutura química, os fruto-oligossacarídeos (FOS) são oligômeros de frutose compostos por moléculas de sacarose às quais adicionaram uma, duas ou três unidades de frutose através de ligações glicosídicas β(2,1) à subunidade frutose da sacarose. As estruturas resultantes são denominadas: 1-kestose (GF2), nistose (GF3) e 1F β-frutofuranosil nistose (GF4), e possuem a

seguinte distribuição percentual: 34% de 1-kestose, 53% de nistose e 10% de 1F β-frutofuranosil nistose (FORTES & MINIZ, 2009).

Os FOS podem ser encontrados naturalmente em alguns alimentos de origem vegetal como frutas e hortaliças, também podem ser obtidos enzimaticamente a partir de enzimas vegetais e microbianas. Dependendo da fonte enzimática os FOS podem diferir-se pelo tamanho da cadeia e pela ligação entre as moléculas de açúcares (YUN, 1996; BOSSCHER, LOO, FRANCK, 2006).

Os GOS são uma mistura de oligossacarídeos encontrados naturalmente no leite humano e pode ser produzido através da ação da enzima β-galactosidase utilizando a lactose como substrato (MACFARLENE, STEED, MACFARLENE, 2008; SANTOS, SIMIQUELI, PASTORE, 2009; MARTINS & BURKERT, 2009).

São oligossacarídeos resistentes à ação hidrolítica das enzimas intestinais atingindo o cólon praticamente intacto, onde são seletivamente fermentados pelas bifidobactérias (CHUNG & DAY, 2004, BÚRIGO et al., 2007), permitindo o crescimento da flora bifidogênica, reduzindo a colonização de patógenos (ROBERFROID, 2007).

Os aumentos dos níveis de bactérias benéficas no intestino resultantes do consumo dos GOS são associados a uma serie de efeitos positivos nos sistemas digestório e imunológico. Estes incluem o desenvolvimento da barreira da mucosa, ativação do sistema imunológico, metabolismo dos ácidos biliares, síntese de vitaminas do complexo B, maior absorção de cálcio da dieta (BRUZZESE et al., 2006; DUARTE et al., 2010, SANGWAN et al., 2011).

A fermentação dos GOS através das bactérias resulta em um aumento na produção de ácidos graxos de cadeia curta (AGCC), especialmente acetato,

propionato e butirato (CUMMINGS, MACFARLANE, ENGLYST, 2001; GIBSON et al., 2004; DUARTE et al., 2010, SANGWAN et al., 2011). A taxa e a quantidade da produção dos AGCC depende das espécies e as quantidades da microbiota presente no cólon, a fonte de substrato e do tempo de trânsito do intestino (WONG et al., 2006).

OS AGCC têm importante papel na fisiologia do intestino, é reconhecido como principal fonte de energia para o enterócito; estimula a proliferação celular do epitélio; melhora o fluxo sanguíneo; aumentam a absorção de água e sódio, diminui o pH intraluminal (LAJOLO et al., 2001, ROBERFROID, GIBSON, HOYLES, 2010), favorecendo a absorção de vitamina K, magnésio e de cálcio no colon (DONATTO et al., 2006). Além disso, os AGCC têm sido associados com a redução do risco de desenvolver algumas doenças, como a síndrome do colon irritável, doença inflamatória intestinal, câncer de cólon e redução de colesterol (HIJOVA & CHMELAROVA , 2007; TOPPING & CLIFTON, 2008).

Os GOS também têm sido associados com a melhoria dos hábitos intestinais. Seu efeito na melhoria da obstipação é largamente atribuído ao aumento da biomassa microbiana que resulta de sua fermentação, bem como promovem um aumento na freqüência de evacuações (NIITTYNEN, KAJANDER, KORPELA et al., 2007), efeitos estes que confirmam a sua classificação no conceito atual de fibras da dieta (ROBERFROID, 2002; KAUR & GUPTA, 2002; COSTALOS et al., 2008).

Dentre os prebióticos os GOS tornaram-se foco de atenção na área de compostos bioativos em alimentos, devido aos seus benefícios de saúde e potenciais para melhorar a qualidade dos alimentos (DUARTE et al., 2010).

Em virtude dos efeitos benéficos produzidos pelo GOS, tem havido um considerável interesse por parte das indústrias em desenvolver produtos alimentícios que contenham estes ingredientes funcionais (GIBSON, FULLER, 2000; ROBERFROID, 2000; ROBERFROID, 2007; DUARTE et al., 2010; RAIZEL et al., 2011).

Com isso, se faz necessário a realização de pesquisas, com o intuito de comprovar os reais benefícios do seu consumo. Nesse sentido, este trabalho teve como objetivo avaliar *in vitro* e *in vivo* os possíveis efeitos prebióticos dos GOS produzido pela β-galactosidase por *Scopulariopsis* sp.

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JUSTIFICATIVA

Este trabalho se propôs a investigar as possíveis propriedades funcionais e os efeitos prebióticos relacionados com os galacto-oligossacarídeos (GOS) produzidos pela atividade enzimática da β- galactosidase pelo fungo *Scopulariopsis* sp. Pouca investigação tem sido documentada na literatura científica a respeito da atividade prebióticas frente aos parâmetros fermentativos e ao efeito bifidogênico dos GOS produzido por *Scopulariopsis* sp. Neste contexto, faz-se necessário, investigar seus efeitos prebióticos *in vitro* e *in vivo*.

Evidências científicas têm demonstrado que os GOS são capazes de atuar no organismo humano, produzindo efeitos metabólico e/ou fisiológicos benéfico à saúde humana, mas a contribuição mais importante destes açúcares está associada a uma melhora na composição da microbiota intestinal, devido às características fermentativas especiais que estes compostos apresentam no organismo humano.

Diante dos benefícios da ingestão dos GOS demonstrados por meio de pesquisas e pouco trabalhos que avaliou as propriedades funcionais e os efeitos prebióticos dos GOS produzido por *Scopulariopsis* sp, justifica-se a realização deste estudo.

OBJETIVOS

Objetivos Gerais:

Avaliar *in vitro* a digestibilidade a fermentabilidade de um novo galacto-oligosacarídeos produzido pela atividade enzimática da β-galactosidade derivada do fungo *Scopulariopsis* sp, bem como avaliar *in vivo* os efeitos da suplementação dos GOS sobre os parâmetros histopatológicos, hematológicos, bioquímicos, microbiota intestinal, produção de ácidos graxos de cadeia curta (AGCC) de cadeias curtas. Valores de pH e efeitos tróficos sobre o intestino delgado utilizando ratos Wistar como modelo animal.

Objetivos Específicos:

Capítulo 1 - Realizar levantamento bibliográfico abordando as propriedades físico-químicas e tecnológicas dos GOS;

Capítulo 2- Realizar levantamento bibliográfico com ênfase nos principais efeitos benéficos dos GOS para a saúde humana;

Capítulo 3- Avaliar a digestibilidade e a fermentabilidade *in vitro* dos GOS utilizando enzimas digestivas e inoculo fecal de ratos;

Capítulo 4- Avaliar os efeitos da suplementação dos GOS sobre os parâmetros histopatológicos, hematológicos e bioquímicos em ratos Wistar;

Capítulo 5- Avaliar os efeitos da suplementação dos GOS sobre a microbiota intestinal, produção de ácidos graxos de cadeia curta (AGCC) de cadeias curtas e valores de pH em ratos Wistar;

Capítulo 6- Avaliar os efeitos da suplementação dos GOS sobre a produção de ácidos graxos de cadeia curta (AGCC), valores de pH e os possíveis efeitos tróficos no intestino delgado de ratos Wistar;

Capítulo 7- Avaliar os efeitos da suplementação de diferentes oligossacarídeos sobre a produção de ácidos graxos de cadeia curta (AGCC), valores de pH cecal e fecal, microbiota intestinal e morfologia intestinal utilizando ratos Wistar como modelo animal e comparar os efeitos prebióticos dos GOS com os prebióticos comerciais.

Galacto-oligosaccharides: Definition, Composition, Physicochemical Properties and Technological

CHAPTER 1

Literature review

Galacto-oligosaccharides: Definition, Composition, Physicochemical Properties and Technological

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Abstract

Galacto-oligosaccharides (GOS) are naturally-occurring oligosaccharides in human milk and can be produced through the action of β-galactosidase using lactose as substrate. In recent years, some of the most significant developments in functional food have related to development new product containing prebiotic GOS. The number of total launches in prebiotic products was five times higher in 2011 than in 2005. The major growth was observed in products containing GOS. On average every three days a new product with GOS was launched in the world. Factors driving growth of the GOS in prebiotic products was the growth in awareness of the health benefits of prebiotics. The GOS can be incorporated in to a wide variety of products, ranging from infant milk formula, yogurts, energy drinks, beverages (fruit juices and other acid drinks), fermented milks, flavored milks, and confectionery products. The increased interest of the consumers for these products heightened the need for the development in new biotechnological methods efficient and inexpensive GOS production. This article presents a review of the literature on the production, applications and physiological effects of prebiotics GOS.

Keywords: Galacto-oligosaccharides, β-galactosidase, prebiotics, technological properties, transgalactosylation.

Resumo

Galacto-oligosacarídeos (GOS) são oligossacarídeos de ocorrência natural no leite humano e podem ser produzidos através da ação da β-galactosidase usando lactose como substrato. Nos últimos anos, alguns dos desenvolvimentos mais significativos em alimentos funcionais têm relacionado ao desenvolvimento de novos produtos contendo os prebióticos GOS. O número total de lançamentos de produtos prebióticos foi cinco vezes maior em 2011 do que em 2005. O maior crescimento foi observado em produtos que continham GOS. Em média a cada três dias um novo produto com GOS foi lançado no mundo. Fatores que impulsionam o crescimento dos GOS em produtos prebióticos foi o crescimento da consciência dos benefícios de saúde dos prebióticos. Os GOS podem ser incorporados em uma grande variedade de produtos, que vão desde fórmula de leite infantil, yogurts, bebidas energéticas, bebidas (suco de fruta e bebidas ácidas e outros), leites fermentados, leites aromatizados e produtos de confeitaria. O aumento do interesse dos consumidores para estes produtos aumentaram a necessidade do desenvolvimento de novos métodos biotecnológicos eficientes e de baixo custo para a produção de GOS. Este artigo apresenta uma revisão da literatura sobre a produção, aplicação e efeitos fisiológicos do prebióticos dos GOS.

Palavras-chave: Galacto-oligosacarídeos, β-galactosidase, prebioticos, propriedades tecnológicas, transgalactosilação.

1 – Introduction

Galacto-oligosaccharides (GOS) are oligosaccharides that consist mainly of galactose monomers linked together through several different structural configurations (PLAYNE; CRITTENDEN, 2009). They are classified as prebiotic food because they can selectively stimulate the growth of bifidobacteria and lactobacilli in the lower intestine (SANZ-VALERO, 2009).

GOS have been established as prebiotic ingredients in *vitro* and *in vivo* studies (animal and human). Currently, GOS are produced by *β*-Galactosidase using lactose as substrate (DUARTE et al., 2010). GOS is a promising food additive because it is stable in acidic and high- temperature conditions. Therefore, GOS can be applied in a wide variety of foods such as fermented milk products, breads, jams, confectionaries, beverages, etc. (SAKO et al., 1999).

The production of GOS, like other oligosaccharides, is becoming of increasing interest, as their beneficial effects on human health effects and wide applications as prebiotic food, new biotechnological capabilities promise to expand of the GOS market exponentially. (YANG; BEDNARCIK, 2001; NERI et al., 2011).

The composition of the obtained GOS mixture and, above all, its chemical and structural composition will contribute not only to the development of advanced fractionation/separation processes of the different GOS but will also play an

important role in the understanding of the mechanisms associated with the prebiotic properties of the GOS (NERI et al., 2011).

Recent evidence suggests that the GOS stimulates the growth of bifidobacteria and lactobacilli and reduces the growth of pathogens, modulation of the immune system, relief of constipation, reduction in the risk of colon cancer, increased absorption of minerals, improved synthesis of vitamins of the B-complex, reduction in serum total cholesterol and lipids (SANGWAN et al., 2011).

Despite the increasing number of publications documenting the production and application the GOS, several studies have shown that the main problem for your production is purification. Therefore, there is now considerable interest in new biotechnological methods to enhance production and yield of the GOS. This review focuses on production, applications and physiological effects of the GOS prebiotic are summarized.

2 - Galacto-oligosaccharides: Definition, Composition and Properties

The GOS were recently defined as “a mixture of oligosaccharides occurring naturally in human milk and can be produced from lactose. GOS generally comprise a chain of galactose units that arise through consecutive transgalactosylation reactions, with a terminal glucose unit. The degree of

polymerization of GOS can vary quite markedly, ranging from 2 to 8 monomeric units (SANTOS; SIMIQUELI; PASTORE, 2009).

The GOS are characterized by their indigestible nature, therefore they belong to the group of prebiotics, which are defined as a “selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health”(PETROVA; KUJUMDZIEVA, 2010). For this reason, GOS are also known as ‘Bifidus growth factors’ for their ability to stimulate the growth of *Bifidobacteria* in the human body ” *(*MAHONEY*,* 1998*).*

The GOS composition fraction can varies in chain length and β-glycosides linkages of the interconnection monomeric units, depending on the enzyme source (TOBA; ADACHI,1978). Quantitatively, the amount of the different GOS products apparently follow the order: di-, tri- tetra-saccharides and the linkages synthesized are predominantly β-(1→6) > β-(1→3) and β-(1→2) (MARTÍNEZ-VILLALUENGA et al., 2008).

There has been considerable interest in supplementing foods with prebiotic ingredients because the levels of naturally-occurring oligosaccharides in diets are considered inadequate to produce substantial effects on health (MANNING; GIBSON, 2004). In this context, GOS should be considered as foodstuff valuable additives, as they have numerous dietetic benefits (ANGUS; SMART; SHORTT, 2005).

GOS are stable compounds, and they remain unchanged even after high temperature treatment and are also quite stable during long-term storage at room temperature. It has been suggested that their stability is better than fructo-oligosaccharides (SAKO; MATSUMOTO; TANAKA, 1999; CRITTENDEN; PLAYNE, 1996). This property allows their use in thermally treated foods. Thus, there is no concern for potential decomposition of GOS during typical food processing conditions (PLAYNE; CRITTENDEN, 1996).

GOS is an ideal ingredient to formulate in healthy products targeting specific groups such as infants, children, women, and the elderly (SANGWAN et al., 2011). Because of their stability, in addition to infant foods, GOS can also be incorporated into a wide variety of other foods.

Recently, they have been used in beverages (fruit juices and other acid drinks), meal replacers, fermented milks, flavored milks, and confectionery products (DUARTE et al., 2010). Specialized foods for the elderly and hospitalized people are also promising fields of application of GOS (SAKO; MATSUMOTO; TANAKA, 1999; DUARTE et al., 2010). Besides the food sector, other areas, such as the cosmetic and pharmaceutical industries, can also exploit the physicochemical and physiological properties of GOS (BOCKMUHL et al., 2007; KRUTMANN, 2009).

3 - Production and Structure of GOS

3.1 - Enzymatic Synthesis of GOS

GOS have been produced by β-galactosidases that have transgalactosylation activities (Figure 1), which result in the formation of 4'- or 6'-galactosylactose, longer oligosaccharides, transgalactosylated disaccharides and nonreducing oligosaccharides(ANGUS; SMART; SHORTT, 2005).

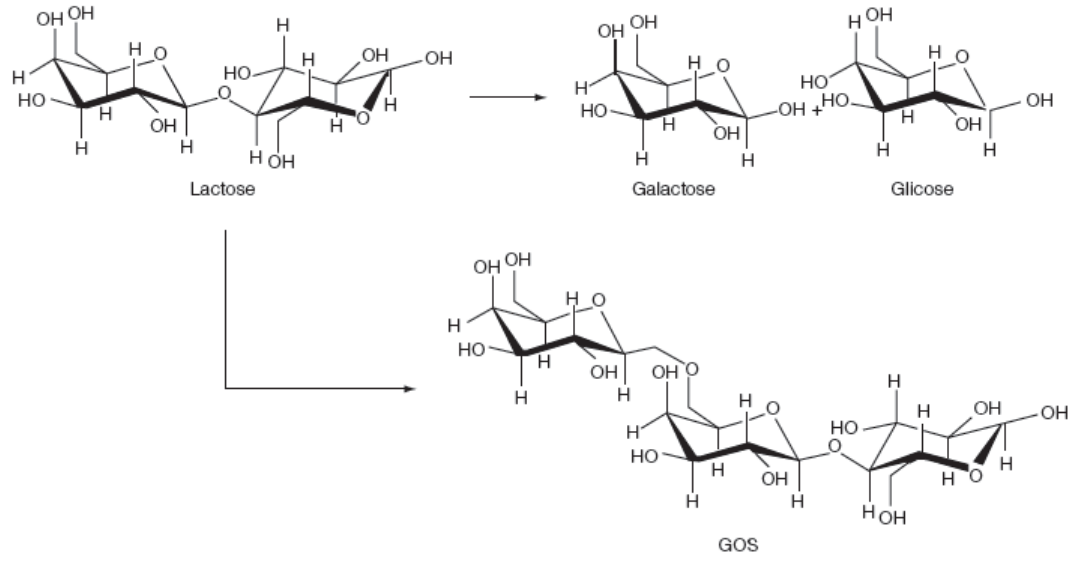

Figure 1- Chemical Structure of GOS produced through the transgalactosylation reaction catalyzed by lactase (MARTINS; BURKERT, 2009)

The β-galactosidase can catalyze the transgalactosylation reaction as well as the hydrolysis of lactose (Figure 2) (ANGUS; SMART; SHORTT, 2005; MANUCCI, 2009). Depending of the lactose concentration, the proportion of transgalactosylation to hydrolysis reaction varies (GOULAS; TZORTZIS; GIBSON, 2007). When water concentration in the system is high, the hydrolysis of lactose occurs predominantly. The transgalactosylation reaction increases with a decrease

in water activity (ZÁRATE; LÓPEZ-LEIVA, 1990). Apart from lactose concentration, other factors influence the reaction, such as: reaction conditions temperature, pH and the presence of specific inhibitors or activators for the enzyme (TZORTZIS; VULEVIC, 2009).

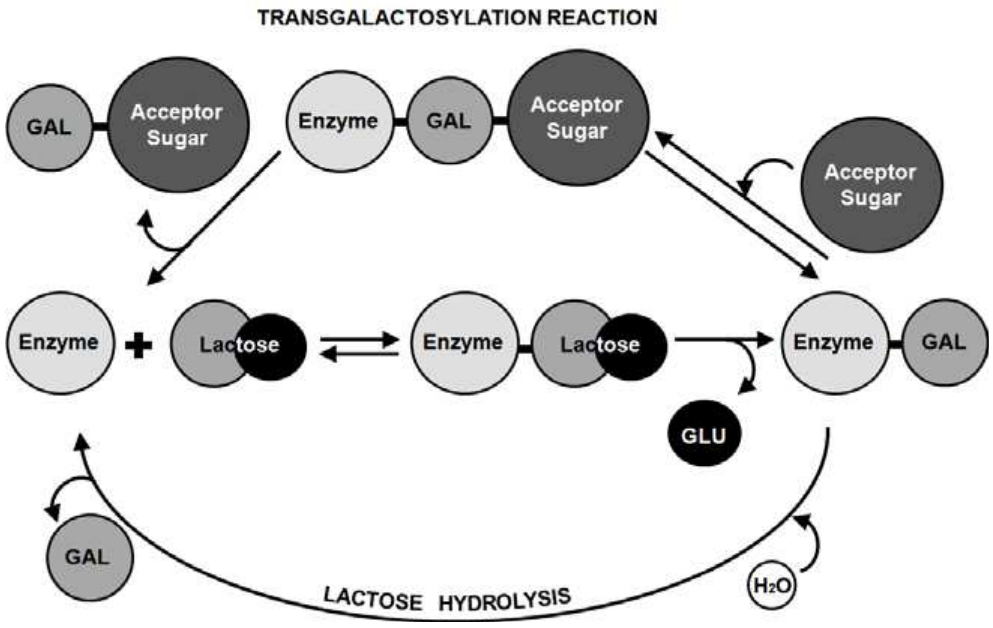

Figure 2 - Trans-galactosylation proposed reaction mechanism by β-galactosidase on lactose (GOULAS et al., 2007).

Converting lactose into GOS by β-galactosidase is a kinetically controlled reaction by means of the competition between hydrolysis and transgalactosylation. Specifically during this conversion is thermodynamically favored lactose hydrolysis which generates D-galactose and D-glucose, competes with the transferase activity that generates a complex mixture of various galactose-based di- and oligosaccharides of different structures (DUARTE et al., 2010).

The knowledge of the reaction time course (or lactose conversion) is required to determine the point of maximum yield of the desired product (DUARTE et al., 2010; PLACIER et al., 2009). The final product obtained during lactose conversion and the glycosidic linkage between the monomers depends on the enzyme source and the physicochemical conditions in the catalytic environment (PLACIER et al., 2009).

Transgalactosylation involves both intramolecular and intermolecular reactions. Intramolecular or direct galactosyl transfer to D-glucose yields regio-isomers of lactose. Intermolecular or indirect transgalactosylation is the route by which disaccharides, trisaccharides, and tetrasaccharides, and eventually longer GOS, are produced from lactose (PLACIER et al., 2009).

The proportion of transgalactosylation to hydrolysis reactions varies depending on enzymes different sources (PLAYNE; CRITTENDEN, 1996). The commercial products are manufactured using β-galactosidase isolated from several sources such as bacteria and fungi (BODUN et al., 2001; CRITTENDEN; PLAYNE, 2001).

Considering that the β-galactosidase enzymes from different microorganisms display different rate constants for hydrolysis for specific glycosidic linkages and that synthesis of GOS is kinetically controlled, synthetic product mixtures made with different enzymes are likely to contain different profiles of glycosidic linkages (CRITTENDEN; PLAYNE, 2001). In this context, these

enzymes fundamentally differ in their ability to catalyze the transgalactosylation reaction relative to hydrolysis, and in their affinity for the GOS synthesized compared to the affinity for lactose (PLACIER et al., 2009).

The main problem for GOS production is purification, since GOS mixtures produced by transgalactosylation always contain considerable amounts of non reacted lactose and monosaccharide (PLAYNE; CRITTENDEN, 1996; MONTAÑÉS et al., 2009). Purified products with more than 90% (w/w) of GOS are available from some manufacturers (PLACIER et al., 2009).

Besides the differences in the purity among commercially available products, there are differences also in the linkages of the oligosaccharide chain due to the different enzymes used in their production. Moreover, depending on their oligosaccharide composition, GOS products vary in terms of their bifidogenic and other protective actions (PLACIER et al., 2009).

GOS have been manufactured and commercialized by very few companies around the world. GOS production was estimated in 1995 in Europe was about 15,000 tones (TANAKA et al., 1983). Examples of companies that are currently involved in GOS production is shown in Table 1. These GOS products usually contain 24–55% oligosaccharides, and smaller amounts of lactose, glucose and galactose (PLAYNE; CRITTENDEN, 1996).

Table 1 - Companies that are currently involved in GOS Production

Manufacturer	Trade name
Friesland Foods Domo (The Netherlands)	Vivinal GOS
Yakult Honsha (Japan)	Oligomate
GTC Nutrition (United States)	Purimune
Dairygold Food Ingredients (Ireland)	Dairygold GOS
First milk ingredients	Promovita
Nissin Sugar Manufacturing Company (Japan)	Cup-Oligo
Snow Brand Milk Products (Japan)	P7L
Clasado Ltd. (UK)	Bimuno

(SANGWAN et al.,2011)

4 - Physicochemical Properties of GOS

The prebiotic properties of the galacto-oligosaccharides have been established in several studies, both *in vitro* (ITO et al., 1990) and *in vivo* (GIBSON; FULLER, 2000). During the last years, galacto-oligosaccharides were reported to be beneficial for human health and they are now recognized as prebióticos (MACFARLANE; STEEDS; MACFARLANE, 2008).

Because of the configuration of their osidic bonds, GOS resist hydrolysis by salivary and intestinal digestive mammals' enzymes. Therefore they reach the colon virtually intact (MACFARLANE; STEEDS; MACFARLANE, 2008), where they are then fermented selectively by beneficial intestinal bacteria (Figure 3) including *Lactobacillus* and *Bifidobacterium* (GOPAL et al., 2001). This fermentation that's

helps to create an environment unfavorable to the growth of some pathogenic bacteria (BRUZZESE et al., 2006).

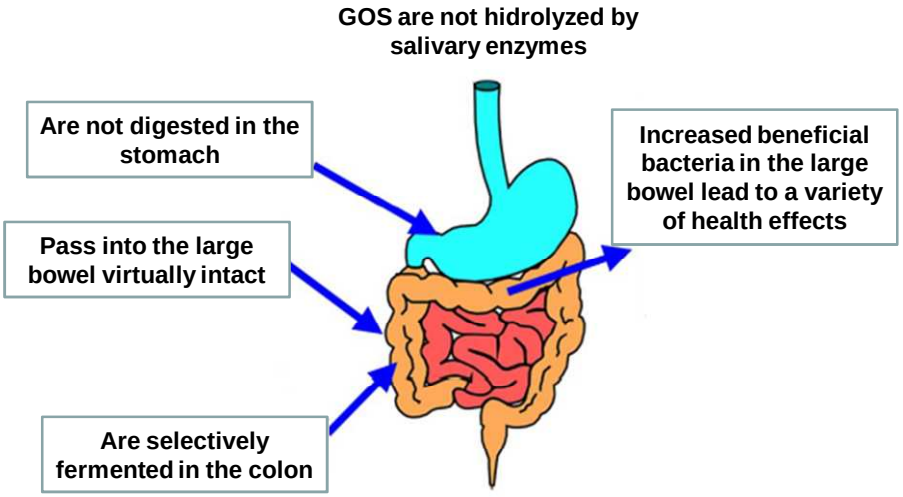

Figure 3 - Behavior of GOS in the human digestive tract (Adapted from TUOHY et al., 2005)

The major products of fermentation includes carbon dioxide, hydrogen, short chain fatty acids (SCFA) and bacterial cell mass (CUMMINGS; MACFARLANE; ENGLYST, 2001).

GOS have been classified as one of the few prebiotics that follow three criteria: (i) resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption; (ii) fermentation by intestinal microflora; and (iii) selective stimulation of the growth and/or activity of intestinal bacteria associated with health and well-being (GIBSON et al., 2004; IQBAL et al., 2011).

Many *in vivo* studies have assessed the GOS effect on intestinal microbiota and they showed significant increases in the bifidobacteria population (MALINEN et al., 2002; DEPEINT et al., 2008; VULEVIC et al., 2008), whereas in other investigation a bifidogenic effect of GOS was not detected (DAVIS et al., 2010).

Differences in the type, purity, and composition of the GOS used in these studies, as well as differences in experimental design and methods of analysis, have likely contributed to these varying outcomes (MACFARLANE; STEED; MACFARLANE, 2008).

However, it has previously been suggested that the daily dose of a prebiotic is not a determinant of the prebiotic effect (DAVIS et al., 2010; ROBERFROID, 2007). According to this argument, the prebiotic effect is influenced by the starting number of bifidobacteria in the subjects prior to administration of the prebiotic, which means that the larger the number of initial fecal bifidobacteria present in an individual, the greater the potential for a bifidogenic effect (DAVIS et al., 2010).

The increased activity of these health-promoting bacteria results in a number of health-related benefits both directly by the bacteria themselves and indirectly by the organic acids that they produce via fermentation. Examples of potential health-promoting benefits are inhibition of the growth of harmful bacteria (MACFARLANE; STEED; MACFARLANE, 2008).

5 - GOS and Health Benefits

Several mechanisms that GOS elicit beneficial effects have been described. Mechanisms related to microflora modification to a more-healthy are linked to selective proliferation of beneficial bacteria especially *Bifidobacterium* and *Lactobacillus* in the gut (Figure 4). The increased levels of beneficial bacteria in the gut resulting from the consumption of GOS are associated with a number of positive effects in the digestive and immune systems. These effects include the development of the mucosal barrier, production of short-chain fatty acids, pH reduction, immune system activation, synthesis of vitamins of the B-complex and enhanced absorption of dietary calcium (HUGHES; HOOVER, 1991; ONISHI; YAMASHIRO; YOKOZEKI, 1995; BRUZZESE et al., 2006).

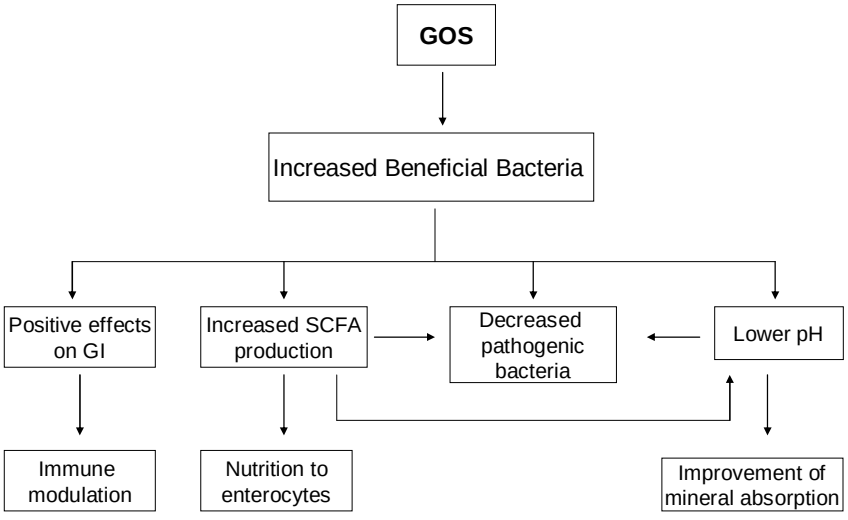

Figure 4 - Different mechanisms that GOS elicit beneficial effects (Adapted from DONOVAN et al., 2009).

Another mechanism promoted by GOS is associated with the production of the short-chain fatty acids (SCFA). GOS are not digested and absorbed in the small intestine, but are available for colonic bacterial fermentation resulting in the production of SCFAs (i.e., acetate, propionate, and butyrate). The SCFAs are produced in varying amounts depending on the diet and the composition of the intestinal microbiota. However, highest concentrations of SCFA are found in the proximal part of the colon (SANGWAN et al., 2011).

Acetate is a fuel for the skeletal and cardiac muscle, the kidneys, and the brain. Butyrate is the preferred fuel of the colonic epithelium, in particular the distal colon and rectum. Propionate is metabolized by the liver and play a role in cholesterol lowering (HIJOVA; CHMELAROVA, 2007). Another benefit of SCFAs is that they increase growth of intestinal epithelial cells and control their proliferation and differentiation (TOPPING; CLIFTON, 2001).

The rate and amount of SCFA production depends on the species and amounts of microflora present in the colon, the substrate source and the gut transit time. SCFAs are readily absorbed and this colon absorption is a very efficient process with only 5–10% being excreted in the feces (GUARNER; MALAGELADA, 2003).

The SCFAs have been associated with reduced risk of some diseases as colon cancer, increased dietary mineral absorption, improvement in bowel habit,

might reduce or lower serum cholesterol levels and serum lipid (HIJOVA; CHMELAROVA, 2007; WONG; JENKINS, 2007).

6 - Applications of Galacto-oligosaccharides

There has been considerable interest in supplementing foods with prebiotic ingredients because the levels of naturally-occurring oligosaccharides in diets are considered inadequate to produce substantial effects on health (MANNING; GIBSON, 2004). In this context, GOS should be considered as foodstuff valuable additives, as they have numerous dietetic benefits (ANGUS; SMART; SHORTT, 2005).

GOS are stable compounds, and they remain unchanged even after high temperature treatment and are also quite stable during long-term storage at room temperature. It has been suggested that their stability is better than Fructo-oligosaccharides (CRITTENDEN; PLAYNE, 1996; SAKO; MATSUMOTO; TANAKA, 1999). This property allows their use in thermally treated foods. Thus, there is no concern for potential decomposition of GOS during typical food processing conditions (MONTAÑÉS et al., 2009).

GOS is an ideal ingredient to formulate in healthy products targeting specific groups such as infants, children, women, and the elderly (SANGWAN et al., 2011). Because of their stability, in addition to infant foods, GOS can also be incorporated into a wide variety of other foods. Recently, they have been used in beverages

(fruit juices and other acid drinks), meal replacers, fermented milks, flavored milks, and confectionery products (DUARTE et al., 2010). Specialized foods for the elderly and hospitalized people are also promising fields of application of GOS (SAKO; MATSUMOTO; TANAKA, 1999; DUARTE et al., 2010).

Besides the food sector, other areas, such as the cosmetic and pharmaceutical industries, can also exploit the physicochemical and physiological properties of GOS (BOCKMUHL et al., 2007; KRUTMANN, 2009).

7 – Conclusions

In conclusion, development of functional food products will continue to grow in the future as consumer demand for healthful products. In this context, the GOS have attracted the interest of pharmaceutical and food industries due to their functional properties. However, the main problem for GOS production is low-yielding. Therefore, are still required the development of cheap and efficient production techniques

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Galacto-oligosaccharides and Heath Benefits Effects

CHAPTER 2

Literature review

Galacto-oligosaccharides and Heath Benefits Effects

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Abstract

Galacto-oligosaccharides (GOS) are prebiotic functional foods that have many health benefits by stimulating the growth and/or activity of bifidobacteria in the colon. The present review summarizes the physiological effects and health benefits of the prebiotics GOS. *In vivo* experiments have demonstrated that the GOS stimulates the growth of bifidobacteria and lactobacillus, reduces the growth of pathogens, modulation of the immune system, relief of constipation, reduction in the risk of colon cancer, increased absorption of minerals, improved synthesis of vitamins of the B-complex, promotes the reduction of total cholesterol and lipids. In conclusion, several experimental and clinical data demonstrate that the GOS are selectively fermented ingredient, modulate the gut microbial ecosystem and confers benefits health in animals and humans.

Keywords: Galacto-oligosaccharides, prebiotic, functional foods, health benefits.

Resumo:

Galacto-oligossacarídeos (GOS) são alimentos funcionais prebióticos que têm muitos benefícios de saúde por estimular o crescimento e/ou atividade de bifidobactérias no cólon. A presente revisão resume os efeitos fisiológicos e os benefícios de saúde do prebióticos GOS. Experiências *in vivo* demonstraram que os GOS estimulam o crescimento de lactobacilos e bifidobactérias, reduz o crescimento de patógenos, modula do sistema imune, alivia a constipação, reduz do risco de câncer do cólon, aumenta a absorção de minerais, melhora à síntese de vitaminas do complexo B, promove a redução do colesterol total e lipídeos. Em conclusão, vários dados experimentais e clínicos demonstraram que os GOS são ingredientes seletivamente fermentados, que modulam o eco-sistema microbiano intestinal e confere benefício de saúde em humanos e animais.

Palavras-chave: galacto-oligosacarídeos, prebiotico, alimentos funcionais, benefícios à saúde.

1 – Introduction

Consumers are increasingly interested in the health benefits of foods and have begun to look beyond the basic nutritional benefits to the potential disease prevention and health enhancing compounds contained in many foods (1). This interest combined with a more widespread understanding of how diet affects disease, rising health-care costs and an aging population are driving a growing and robust market for functional foods and natural health products (2).

“Functional foods” are generally defined as a food that due to their physiologically active substances, benefit health in addition to providing basic nutrition, and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease (3-4-5). The prebiotics have assumed the status of “functional’ foods”, capable of providing additional health benefits like prevention or delaying onset of chronic diseases (6).

A prebiotic was originally defined in 1995 as a “non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health” (7-8). However, a more recent definition described prebiotic as “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host wellbeing and health” (8).

Common prebiotics in use include inulin, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), soya-oligosaccharides, xylo-oligosaccharides,

pyrodextrins, isomalto-oligosaccharides and lactulose. The majority of the studies have so far focused on inulin, FOS and GOS (9-10-11-12-13).

Galacto-oligosaccharides (GOS) have become the focus of a great deal of attention in the field of functional foods, owing to their known health benefits and potential to improve the quality of many foods (14-15). Numerous studies in both animals and humans have demonstrated the health benefits of GOS. This article presents the literature review of the physiological effects and human health benefits of GOS.

2- Galacto-oligosaccharides: Physiological Effects and Health Benefits

GOS resist breakdown by human digestive secretions and arrive relatively unchanged in the lower regions of the intestinal tract where they can be utilized as an energy source by the resident microflora, selectively stimulate the growth and/or metabolic activity of only beneficial microbiota organisms (16).

GOS provide their health benefits by 2 main mechanisms, one is by selective proliferation of beneficial bacteria especially bifidobacteria and lactobacillus in the gut, which provide resistance against colonization of pathogens thereby reducing exogenous and endogenous intestinal infections (17).

These organisms provide specific health benefits for the host that include normalization of colonic transit time, increased production of short-chain fatty acids, improved pathogen resistance, enhanced mineral absorption, favorable modulation of blood lipids, reduce cancer risk improved gut mucosal barrier and immune function (17-18).

2.1 - Bifidogenic Activity in the Colon

The bifidogenic effects of GOS are attributed to non-digestibility and selective fermentation in the colon by beneficial bacteria, particularly bifidobacteria and lactobacillus (19). The bifidogenic effect is often associated with a reduction of the pH and changes in the SCFA pattern (20).

The proposed mechanism: GOS are not digested and absorbed in the small intestine, but are available for colonic bacterial fermentation resulting in the production of SCFAs, as end-products of fermentation (20). These molecules decrease the intraluminal pH, directly inhibiting the growth and activities of harmful microorganism, and contributing to stimulation of the growth of Bifidobacteria (21).

The health promoting attributed to bifidobacteria and other beneficial bacteria is due to the growth inhibition of harmful bacteria, stimulation of immune functions, lowering of gas distention problems, improved digestion/absorption of essential nutrients and synthesis of vitamins (21).

2.2 - Relief of Constipation

Constipation is a frequent problem, particularly among the elderly and pregnant women. For these individuals, consumption of GOS can offer relief to their constipation (19-20). The effect of GOS on fecal characteristics has been evaluated in a number of studies (Table 1). GOS consumption is associated with a softening effect on feces in healthy adults, and may relieve the symptoms of constipation in adults and elderly people (23).

The proposed mechanism: GOS pass undigested into the large intestine and stimulate bacterial fermentation in the colon. The bacterial fermentation of GOS increases bacterial mass, which in turn increases fecal bulk (24). Undigested oligosaccharides and fermentation products may also produce an osmotic effect in the gut, which increases the water content of feces (25). The increased bowel content stimulates peristalsis in the colon (26). However, the laxative effect of GOS varies from patient to patient, as does the effect of any other fiber (27).

2.3 - Maintenance of Normal Intestinal Balance of Microflora

The human digestive tract plays host to some five hundred or more species of bacteria which is made up of both beneficial and harmful bacteria. Intestinal infection may occur by an overgrowth of harmful bacteria in the gut (28).

The prebiotics may be able to alter the composition of the microflora in different segments of the gastrointestinal tract and can also affect other intestinal characteristics influencing intestinal health, such as increased fecal bulk, shortened colonic transit time, changes in the composition of the gut microbiota, lowered intraluminal pH and changed bile acid profiles (29). Another important role of helpful gut flora is that they prevent species that would harm the host from colonizing the gut, an activity termed the "barrier effect" (30).

The proposed mechanism: The process of fermentation of prebiotic GOS since it produces lactic acid and different fatty acids, also serves to lower the pH in the colon, preventing the proliferation of harmful species of bacteria and facilitating that of helpful species (31-32). Metabolism of GOS leads to the production of SCFA, which are important for supporting a healthy intestinal barrier (particularly in the lower GI tract) and also inhibits the growth of harmful bacteria (32).

2.4- Reduction in Serum Cholesterol

Several studies have suggested that ingestion of GOS is effective in improving lipid profiles, including the reduction of serum/plasma total cholesterol, LDL-cholesterol and triglycerides or increment of HDL-cholesterol (33).

The proposed mechanism: prebiotics have been suggested to reduce cholesterol via various mechanisms; one of the purported mechanisms has been mainly attributed to SCFAs. Prebiotics are fermented in the colon by large bowel bacteria, yielding short-chain fatty acids (SCFAs) such as butyrate, acetate and propionate (33).

Butyrate is known to inhibit liver cholesterol synthesis and provide a source of energy for human colon epithelial cells, meanwhile propionate may inhibit the synthesis of fatty acids in the liver, thereby lowering the rates of triacylglycerol secretion (31). Propionate is also involved in the control of hepatic cholesterol synthesis and it reduces the rate of cholesterol synthesis which could lead to the lowering of plasma cholesterol levels (34-35).

Another possible mechanism by which prebiotics could reduce cholesterol level is by selective proliferation of beneficial bacteria especially bifidobacteria and lactobacillus in the gut. The lowering of the cholesterol could be attributed to assimilation of cholesterol in the diet by some strains of Lactobacillus (36).

The animal studies have shown some evidence for the lipid-lowering effects of the GOS however, there are difficulties in demonstrating equivalent effect in humans. The number of human studies is, however, limited and results concerning effects on plasma cholesterol and triacylglycerol are not conclusive (37-38).

2.5 - Increase in Absorption of Different Minerals in the Intestine

There is extensive evidence in experimental animals that prebiotics can increase the absorption of a variety of minerals, including calcium, magnesium, iron, and zinc (39). Several theories have been proposed to explain the stimulatory effect of prebiotic substances on intestinal calcium absorption.

The proposed mechanism: Most of the proposed mechanisms are that prebiotic substances that escape digestion in the small intestine are substrates for the formation of short-chain fatty acids (SCFAs), essentially acetate, propionate, and butyrate). These SCFAs contribute to a reduced luminal pH in the large intestine, increase solubility of calcium in the luminal contents and so increase passive concentration-dependent calcium absorption in the colon (40).

Studies in humans and in animals has demonstrated that administration of GOS resulted in more efficiently absorbed calcium and increased bone density, indicating the prevention of bone losses (17-41-42-43). Although the majority of studies on calcium metabolism have so far been performed on rats, experimental evidence indicates that GOS have a role to play in increasing bioavailability of this mineral (Table 1), but as yet, these results have been translated into only a few human trials.

2.6- Improved Synthesis of B-Complex Vitamins

There is evidence that prebiotics contributes to synthesis of the vitamins of the B-complex and Vitamin K.

The proposed mechanism: The fermentation of prebiotics on the colon leads to the selective stimulation of growth of the bifidobacteria population. The Bifidobacteria, are known to produce B-complex vitamins (B1, B2, B3, B6, B9, B12) and vitamin K, which are important for various metabolic, mucosal and nerve functions within the body (44).

2.7 - Prevent Colon Cancer

Studies *in vitro* and *in vivo*, together with a few human trials, have been conducted to try to establish whether the GOS in general have an anticarcinogenic activity. The anticarcinogenic effect might be exerted by several possible mechanisms:

The proposed mechanism: Most of the proposed mechanisms are that metabolism of GOS leads to the production of SCFA (acetate, propionate and butyrate). The three main acids stimulate colonic sodium and fluid absorption and exert proliferative effects on the colonocyte (45). SCFA contribute to normal large bowel function and prevent pathology through their actions in the lumen and on the colonic musculature and vasculature and through their metabolism by colonocytes (46). Anti-carcinogenic activity has been mainly attributed to SCFAs in particular butyrate (47).

Butyrate is the preferred “fuel” for colonocyte function in the healthy gut epithelium, but is thought to exert certain effects on pre-neoplastic or neoplastic colonocytes. These may include the induction of apoptosis, modulation of oncogene expression (e.g., inhibition of proto-oncogenes), induction of certain differentiation markers and regulation of systems involved in cellular adhesion and/or migration (48).

Total and relative molar concentrations of the main SCFA, acetate, propionate and butyrate produced in the human intestine, depend on the site of fermentation, diet and composition of the intestinal microbiota (46).

The other proposed mechanisms is by selective proliferation of beneficial bacteria especially bifidobacteria and lactobacillus in the gut. Attention has already been directed to the potential of bifidobacteria to influence the bacterial enzymes involved in potential mutagenic and carcinogenic activity (49). More direct evidence for protective properties of prebiotics against cancer has been obtained by assessing the ability of cultures to prevent DNA damage and mutations (50).

2.8 - Effect on the Immune System

Several experimental and clinical data demonstrate that the prebiotics can influence the intestinal microbiota and modulate the immune response. Increased SCFA production, and increase in immunogenic bacteria such as lactobacillus and bifidobacteria are the two main methods by which prebiotics can exert their effects on the immune system (49).

In vitro and animal studies have suggested that the addition of complex fermentable carbohydrates to the diet can modulate the type and function of cells from different regions of the gut-associated lymphoid tissue (51), increase immunoglobulin production in the small intestine and cecal mucosa, and alter the profile of inflamatory cytokines in plasma and intestinal cells (52-53).

The proposed mechanisms: GOS stimulate the growth beneficial bacteria and decreases the population of potentially pathogenic bacteria in the gastrointestinal tract, resulting in reduced quantity of harmful toxins produced by pathogens in the gut, and helps to support the immune system (54). Although the mechanisms underlying the effects of dietary GOS (either from human milk or alternative sources) are not fully understood, the development of a balanced intestinal flora is obviously a key element in this relationship (55).

Another mechanism proposed is by production of SCFAs (acetate, propionate and butyrate) which enhance immune protection by promoting the production of T helper cells, antibodies, leukocytes, and cytokines, stimulate lymph mechanisms (48-49 -56).

Butyrate, which serves as a fuel for colonic epithelial cells, stimulates apoptosis suppress both cytokine-induced and constitutive expression of the transcription factor NF-*κ*B in HT-29 cell lines (57). One of the proposed beneficial effects of butyrate on human intestinal health is the prevention and inhibition of colon carcinogenesis (58-59-60).

Table 1 - Summary of Studies Designed to Determine the Prebiotic Effects of GOS

Prebiotics/ Oligosaccharides	Experimental design	Animals/ Subjects	Dose; duration of the study	Effects	Ref.
GOS	single-blinded study	18 elderly volunteers	0, 2.5, 5.0, and 10.0g. dose for 3 weeks, treatment period	GOS revealed an increase in bifidobacteria populations, dosage to 5 or 10g	**61**
GOS	multicenter, double-blind study,	159 healthy infants, formula supplemented with (GOS) (77 infants), or to a standard follow-on formula (control, 82 infants).	supplemented with 5 g/L (GOS) for 6 weeks, treatment period	addition of GOS (5 g/ L) to a follow-on formula positively influences the bifidobacteria flora and the stool consistency in infants	**55**
GOS	Randomized, Placebo controlled, double-blind	44 elderly volunteers with Rome II positive IBS	7 g/d prebiotic GOS or 7 g/d placebo, for 12-week, treatment Period	GOS stimulating gut bifidobacteria in IBS patients	**56**
GOS	Randomized, Placebo controlled, double-blind, crossover	44 elderly volunteers (16 men & 28 women); 64-79 years old.	5.5 g/d, two 10 weeks treatment period	B-GOS significantly increased the numbers of beneficial bacteria, especially bifidobacteria, at the expense of less beneficial groups compared with the baseline and placebo.	**48**
GOS	double-blind, placebo-controlled,	Rats	4 g kg(-1) day for 10 days before colitis induction. later Assessed 72 h	GOS stimulate the growth of bifidobacteria in rats. No reduction in IBD inflammation.	**57**

GOS	double-blind, randomized crossover study	Huamns (n=12)	20g	Increased Ca absorption in menopausal women	**58**
GOS	double-blind, placebo-controlled,	Rats	5 g/100 g of diet	Increased Ca and Mg absorption	**59**
GOS	double-blind, placebo-controlled	Rats (overiectomized)	5 g/100 g of diet	Stimulated Ca absorption. Reduced loss of bone mass and calcium content	**59**
GOS	double-blind, placebo-controlled,	ovariectomized (OVX) Wistar rats	5g/100g of diet for 20 days	GOS produced a significant hypocholesterolemic effect in the OVX rats	**59**
GOS	Double-blind randomized controlled trial, two-period cross-over study	Humans 14 elderly subjects (mean age 80 years), suffering from constipation	9 g of GOS per day) or a placebo for 6 weeks	The weekly defecation frequency was higher during the GOS period (mean 7.1, range 3–15) than during the control period (mean 5.9, range 1–14)	**62**
GOS	single-blind cross-over study	Human (12 volunteers)	dose 0, 2.5, 5.0 or 10.0 g of GOS, respectively, for 7 d	GOS increase the multiplication of bifidobacteria and lactobacilli in the human intestinal microflora. No significant change on stool weight	**63**
GOS	double-blind, placebo-controlled,	Rats (n**=** 344)	5% or 20% w/w of GOS for 6 weeks	The aberrant crypt multiplicity and the colorectal tumor incidence in rats fed an HGOS (20%, w/w) were significantly lower than those in rats fed an LGOS (5% w/w) diet.	**64**
GOS with 4 probiotic organisms	randomized, double-blind, placebo-controlled trial	Humans (children) GOS (n = 461) or a placebo (n = 464)	GOS + 4 probiotics for 2 to 4 weeks	Significant reduction in IgE-associated diseases, eczema and atopic eczema	**65**
GOS	double-blind, placebo-controlled,	mice	(5% w/w) for 4 weeks	GOS has no detectable effect on the intestine villus height but increased the total protein GOS was also able to increase sucrase activity in cultured Caco-2 cells	**66**

Inulin, FOS and GOS, controls	controlled trial randomized Double-blind	Humans	15 g, Patients treatment periods of 3 weeks	lipids or glucose absorption No significant changes in blood	**67**
GOS	randomized, double-blind, placebo-controlled	Humans (n=18)	GOS (2.5;5.0 or10g/d) For 16 week	GOS (5.0g/d) significant bifidogenic shifts in the fecal microbial community of healthy human adults	**68**

3 - Possible Adverse Effects of Galacto-oligosaccharides

The bacterial fermentation of the GOS increases the production of gases in the colon and may cause adverse gastrointestinal symptoms such as flatulence and transient osmotic diarrhea, which are common side-effects of increasing fiber intake (69).

Usually, tolerance to GOS depends on the amount of the product eaten (23-70). Similarly, the frequency of gastrointestinal symptoms increases when the dose of GOS increases (21- 63). In most of the studies, amounts of 12g of GOS or less daily were well tolerated (25-27), but 15g of GOS per day increased flatulence (22). However, individuals vary considerably in their response to GOS (24), as with their response to any other easily fermented fiber (23-70).

4 – Conclusions

In conclusion, several experimental and clinical data demonstrate that the prebiotic GOS are selectively fermented ingredient, modulate the gut microbial ecosystem and confers benefits health in animals and humans. In addition, GOS can be incorporated in different types of food products.

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In Vitro Digestibility and Fermentation of Galacto-oligosaccharides (GOS) Produced by β-galactosidase from *Scopulariopsis* sp

In Vitro Digestibility and Fermentation of Galacto-oligosaccharides (GOS) Produced by β-galactosidase from *Scopulariopsis* sp

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Significance and Impact of the Study: This is the first study reporting of a potential prebiotic mode of activity for Galacto-oligosaccharides (GOS) synthesized by β-galactosidase for *Scopulariopsis* sp. The GOS produced by *Scopulariopsis* sp. demonstrated prebiotic effects *in vitro,* are not digestible by digestive enzymes, fermented by cecal bacteria and converted to short chain fatty acids in rat large intestine. The fermentation patterns obtained *in vitro* might be used to predict behavior *in vivo*. The type of oligosaccharide fermented influences the nature of the fermentative end products. Short chain fatty acids are considered to be beneficial to health.

ABBREVIATIONS

DP - Degree Polymerization

FOS - Fructo-oligosaccharides

GOS - Galacto-oligosaccharídes

GIT - GastroIntestinal Tract

HPLC - High-performance Liquid Chromatography

SCFA - Short-Chain Fatty Acid

Abstract

Digestibility and fermentation properties of galacto-oligosaccharídes (GOS) by β-galactosidase from *Scopulariopsis* sp were assessed *in vitro*. The GOS digestibility was assessed by using a full model of gastrointestinal digestion, including gastric and small intestinal environments, simulation physiological digestion conditions. Fermentation properties were estimated under anaerobic conditions in mixed fecal bacterial, quantified at 0, 4, 8, 12, 24 and 48 h. GOS were resistant to human salivary enzymes, gastric juice, porcine pancreatic enzymes and rat intestinal mucous enzymes, decreased intestinal pH and resulted in higher (p<0.05) total short-chain fatty acid (SCFA) concentrations. The GOS produced by β--galactosidase from *Scopulariopsis* sp demonstrated prebiotic effects *in vitro,* once that they are non digestible, fermented by cecal bacteria and converted to short chain fatty acids in the large intestine and potential benefit on health. The results from the current study indicate that GOS produced by β-galactosidase from *Scopulariopsis* sp has future perspectives promising for applications in food industry.

Keywords: Galacto-oligosaccharides, prebiotic, digestibility, fermentation, *in vitro*, short chain fatty acid, *Scopulariopsis* sp.

1 - Introduction

The concept of colonic health has become a target for the development of functional foods such as “probiotics, prebiotics and synbiotics” and other dietary components that target the colon and affect its environment, composition of the microflora, as well as the physiology of the colon, and display distinct health benefits (Roy et al., 2006).

“A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host wellbeing and health” (Gibson & Roberfroid, 1995; Gibson et al., 2004; Roberfroid, 2007). The principal concept associated with both of these definitions is that the prebiotic has a selective effect on the microbiota that results in an improvement in the health of the host (Gibson et al., 2004).

For a dietary substrate to be classed as a prebiotic, at least three criteria are required: (1) the substrate must not be hydrolysed or absorbed in the stomach or small intestine, (2) it must be selective for beneficial commensal bacteria in the large intestine such as the bifidobacteria, (3) fermentation of the substrate should induce beneficial luminal/systemic effects within the host. (Gibson and Roberfroid, 1995; Gibson, 1999; Manning and Gibson, 2004).

Based on these criteria listed, only a few groups of food ingredients qualify to be used. A good number of food materials because of their chemical structure are not absorbed in the upper part of the gastrointestinal tract (GIT) or hydrolyzed by the digestive enzymes in humans. Such foods have been called “colonic foods”, foods entering the large intestine which also serve as food for the endogenous micro-organisms (Gibson and Roberfroid, 1995; Gibson et al., 2004).

Intake of prebiotics can significantly modulate the colonic microflora by increasing the number of specific bacteria and thus changing the composition of the microbiota (Gibson & Roberfroid, 1995). Many of these microflora-associated activities have a direct impact on host health. Short chain fatty acids (SCFA) are the major bacterial fermentation products in the large intestine. Up to 95% of the SCFA (acetate, propionate and butyrate) produced during carbohydrate fermentation may be taken up and utilized by the host (Cummings et al., 1987; Tuohy et al., 2005).

Common prebiotics in use include inulin, fructo-oligosaccharides (FOS), galactooligosaccharídes (GOS), soya-oligosaccharides, xylo-oligosaccharides, pyrodextrins, isomalto-oligosaccharides and lactulose. The majority of studies have so far focused on inulin, FOS and GOS (Macfarlane et al., 2008).

Galacto-Oligosaccharides (GOS) are classified as prebiotics, defined as non-digestible food ingredients that beneficially affect the host by stimulating the growth and/or activity of beneficial bacteria in the colon. The increased activity of these

health-promoting bacteria results in a number of health-related benefits both directly by the bacteria themselves or indirectly by the organic acids they produce via fermentation (Gibson, 1998; Roberfoid, 200; Macfarlane,et al., 2008).

GOS naturally occurs in human milk and can be commercially produced from transgalactosylation reaction of B-galactosidase from lactose (Boon et al., 2000; Hung and Lee, 2002). GOS are broadly used in food for both infants and adult (Duarte et al., 2010). Besides the food sector, other areas, such as the cosmetic and pharmaceutical industries, can also exploit the physicochemical and physiological properties of GOS (Bockmuhl et al., 2007; Krutmann, 2009).

Different oligosaccharides with prebiotic properties, such as inulin, fructooligosaccharides (FOS), galacto-oligosaccharides, and lactulose, are commercially available, but currently there is increasing interest in the identification and development of new prebiotic compounds, perhaps with added functionality (Mandalari et al., 2007; Menne et al., 2000; rao, 2001).

However, the wide variety of new candidate prebiotics becoming available for human use requires that a manageable set of in vitro tests be agreed on so that their nondigestibility and fermentability can be established without recourse to human studies in every case (Cummings et al., 2001;)

Many different techniques have been used to evaluate the prebiotic potential of carbohydrates. *In vitro* techniques using fecal inoculum and fermentation systems designed to model the gut are A more useful initial test for evaluate prebiotic potential.

Oligosaccharides are attracting increasing interest as prebiotic functional food ingredients. In this context, GOS produced by β-galactosidase from *Scopulariopsis* sp are novel candidate prebiotics of interest great for the development of functional food. For this reason, we will initially evaluate your prebiotic activity using *in vitro* methods.

This study we investigated the digestibility and fermentation products of GOS produced by β-galactosidase from *Scopulariopsis* sp *in vitro*, using a full model of GastroIntestinal Tract (GIT) digestion, including gastric and small intestinal environments and a colonic model consisting of *in vitro* fermentation systems using fecal inoculum from rats to predict the fate of the GOS in the digestive tract.

2 – Materials and Methods

2.1 - GOS Substrate: The fungi *Scopulariopsis* sp ATCC 44206 isolated previously from soil by Pastore & Park (1979) was used to produce β-galactosidase in this study. *Scopulariopsis* sp fresh spores were inoculated in wheat bran medium (Koji process) and incubated at 30ºC for 5 days. Then the enzyme was extracted by mixing water to culture of *Scopulariopsis* sp on wheat bran and concentrated by precipitation with ethanol (70% of final concentration) at 4ºC. The resulting precipitate was centrifuged, the solids were lyophilized and the dry powder used as crude β-galactosidase enzyme. Activity of β-galactosidase was estimated using *O*-nitrophenyl-β D-galactopyranoside (ONGP) as substrate.

Enzyme activity was defined as the amount of enzyme that liberated 1 µmol of *O*-nitrophenol (ONP) per minute under the standard assay conditions. To synthesis galactooligosaccharides, crude β-galactosidase enzyme at a concentration of 10 $U.mL^{-1}$ were added to a 40% (w/v) lactose solution in a 200mM potassium acetate buffer (pH 5.0) and incubated on a reciprocal shaker at 45℃/12h/150rpm. The reaction was stopped in boiling water bath for 10 minutes to inactivate the enzyme, then diluted, and filtered through a 0.45 µm membrane to remove insoluble particles. Oligosaccharides formed were analyzed by high-performance liquid chromatography (HPLC) (Waters 600E) with a refractive index detector as described previously (Santos et al., 2009). This fraction contained mono-, di-, tri- and tetrasaccharides as follows (%w/w): 50% lactose, 15% glucose, and 7% galactose, in this fraction GOS represent 28%.

2.2 - *In Vitro* Digestion: *In vitro* digestibility of GOS synthesized by β-galactosidase from *Scopulariopsis* sp was investigated according the method described by Asano et al. (2003). These investigators simulated gastric and small intestinal (hydrolytic) digestion. The commercial GOS prebiotic were chosen as a positive control (Oligomate 55 - Yakult Pharmaceutical Ind. Co. Ltd. Tokyo, Oligomate 55, contains 55% GOS) for *in vitro* digestibility studies, while maltose (Wako Pure Chemicals, Osaka), a non prebiotic carbohydrate, was used as a nonselective control (0.2g of substrate was weighed in triplicate and incubated with artificial human saliva - Sigma Chemical, St. Louis, MO), artificial gastric juice prepared according to Lian *et al.* (2003), porcine pancreatic enzymes (Wako Pure

Chemicals) and digestion by rat intestinal mucous enzymes (Sigma Chemical), each substrate solution in a test tube and incubated at 37℃ for 4 h.

Tubes containing reagents, but no substrate, were run as blank. The tubes were analyzed after simulated hydrolytic digestion, at each fermentation sampling time (0 and 4 h).

2.3 - Analysis: Oligosaccharides remaining after digestion were measured by HPLC and the results again shown as the average of three experiments. After deactivation of digestive enzymes, the incubation solutions were diluted 100 fold with water and centrifuged at 5200x*g* for 10 min. The supernatant was used for HPLC analysis after filtering through a 0.45 µm membrane filter (Advantec, Tokyo). HPLC was performed with a Shimadzu LC-10 (Kyoto) device and a pulsed amperometric detector (Dionex, Sunnyvale, USA). A CarboPac PA1

(Dionex) column was used with 100 mM NaOH elution and the flow rate maintained at 1.0 ml/min. The hydrolysis rate was calculated from the following formula: Hydrolysis rate (%) = 100 -100 (Sa/Sb), in which Sa represents samples after treatment and Sb represents samples before treatment (Suzuki, 2004)

2.4- Fermentation by Fecal Flora:

2.4.1- Substrates: The galactooligosaccharídes synthesized by β-galactosidase from *Scopulariopsis* sp were used as substrates. The Commercial GOS Oligomate® was used as positive control and it was purchased from Yakult

Pharmaceutical Ind. Co. Ltd. (Tokyo). Each substrate was dissolved in deaerated 0.1 M phosphate buffer (pH 7.0) at a concentration of 10.0%.

2.4.2 - Fecal incubation*:* Rat cecal contents (n=3) were pooled to serve as the source of inoculum for the *in vitro* fermentation experiment. Male Wistar rats (14 weeks of age) were anesthetized by intraperitoneal injection of a mixture of ketamine (150 mg/kg) and xylazine (10 mg/kg). The gut was removed and the contents were collected in a plastic bag, packed with an Anaero- Pack (Mitsubishi Gas Chemical Inc., Tokyo). Fecal samples were maintained at 37℃ until inoculum was prepared (within 10 min). The experimental protocol was approved by the Animal Experimentation Ethics Committee of University of Campinas.

2.4.3 - *In Vitro* Fermentation Model: The composition and the preparation of the *in vitro* medium have been described in detail Flickinger et al (2000), and the composition are shown in Table 1. All components except vitamin and SCFA mixes were added before autoclave medium sterilization. Filter-sterilized vitamin solution was added just before dispensing the medium that was maintained under anaerobic conditions at all the time after preparation.

Table 1 - Microbiological Medium Composition of Used in Vitro Experiments.

component	concentration in medium, mL/L
solution A[a]	330.0
solution B[b]	330.0
trace mineral solution[c]	10.0
water-soluble vitamin solution[d]	20.0
olate:biotin solution[e]	5.0
riboflavin solution[f]	5.0
hemin solution[g]	2.5
short-chain fatty acid mix[h]	0.4
resazurin[i]	1.0
distilled H2O	296.1
Na2CO3	4.0
cysteine HCl-H2O	0.5
trypticase	0.5
yeast extract	0.5

[a] Composition (g/L): NaCl, 5.4; KH2PO4, 2.7; CaCl2-H2O, 0.16; MgCl2-6H2O, 0.12; MnCl2-4H2O, 0.06; CoCl2-6H2O, 0.06; (NH4)2SO4, 5.4. [b] Composition (g/L): K2HPO4, 2.7. [c] Composition (mg/L): ethylenediaminetetra acetic acid (disodium salt), 500; FeSO4-7H2O, 200; ZnSO4-7H2O, 10; MnCl2-4H2O, 3; H3PO4, 30; CoCl2-6H2O, 20; CuCl2-2H2O, 1; NiCl2-6H2O, 2; Na2MoO4-2H2O, 3. [d] Composition (mg/L): thiamin-HCl, 100; D-pantothenic acid, 100; niacin, 100; pyridoxine, 100; *p*-aminobenzoic acid, 5; vitamin B12, 0.25. [e] Composition (mg/L): folic acid, 10; D-biotin, 2; NH4HCO3, 100. [f] Composition: riboflavin, 10 mg/mL in 5 mmol/L of Hepes. [g] Composition: hemin, 500 mg/mL in 10 mmol/L of NaOH. [h] Composition: 250 mL/L each of *n*-valerate, isovalerate, isobutyrate, and DL-R-methylbutyrate.
[i] Composition: resazurin, 1 g/L in distilled H2O.

An aliquot (16 mL) of the medium was aseptically transferred to tubes containing the substrate and control tubes. Each substrate was dissolved at 10.0% (w/v). All the tubes were stored at 4ºC for approximately 12 h to enable hydration of the substrates before initiation of fermentation. After that, the tubes were placed in a water bath 37ºC approximately 30 min before inoculation (Faber, 2010).

Fecal inoculum (10% w/v) was prepared using fresh feces from three healthy rats. Feces were mixed together and diluted 1:10 (w/vol) in phosphate buffer (pH7.0). These fecal sample dilutions were filtered through four cheesecloth four layers.

Appropriate samples and blank tubes were aseptically inoculated with 1.5 mL of diluted fecal. Tubes were incubated at 37℃ with pe riodic mixing for 4, 8, 12, 24 and 48 h. (Hernot et al., 2009). In a control experiment, the substrate solution was replaced by the bicarbonate buffer. First, the pH of the tubes was measured with a standard pH-meter (Pack pH 21- Hanna Instruments). Then, 2 mL sample was taken from each tube for SCFA analyses. Finally, 1 mL of sample was taken and frozen at -80℃ for carbohydrate analyses of the cu lture medium (Faber et al., 2010).

2.3- Carbohydrate Analysis: carbohydrate contents in the cultures were measured to determine the time that oligosaccharides disappear in the samples and compared GOS with FOS prebiotic. Samples were removed (1 mL) from the batch culture fermentation diluted 100 fold with water, centrifuged at 5200 x *g* for 10 min and they were filtered (0.25 µm) with membrane filter (Advantec, Tokyo). The carbohydrate content in the culture was measured by HPAEC-PAD (Dionex Bio-LC DX 300) with a CarboPac PA-100 carbohydrate column (Dionex). Control experiment was prepared by adding each sugar.

2.4 – Short Chain Fatty Acids Analysis: The fluid (2 mL) removed from the sample tubes for SCFA was immediately added to 0.5 mL of 25% metaphosphoric acid, precipitated for 30 min, and centrifuged at 20000g for 20 min.

The supernatant was decanted and frozen at -20 ℃ i n microfuge tubes. After freezing, the supernatant was thawed and centrifuged in microfuge tubes at 10000g for 10 min. Acetate, propionate, and butyrate concentrations in the

supernatant were determined using a Agilent GC (Model 7890A) equipped with a flame ionization detector (FID) and Nukol columm (Supelco, 30 m x 0.320 mm i.d, x 0.25µm film thickness). The injection was performed in the splitless mode for 1 min at 220℃. Oven temperature was programmed at 100℃ for 5 min, then increase to 200℃ at 8℃.min $^{-1}$ and held for 1 min at the end temperature. Nitrogen was used as the carrier gas with a flow-rate of 60 mL.min^{-1}. Injector and detector were kept at 220℃. Short-chain fatty acid concentration values also were corrected for blank tube production of SCFA. All samples were run in triplicate and an error of 5% was considered acceptable.

2.5 - Statistical Analysis: The statistical analyses were performed using the GRAPHPAD PRISM 5.0 software. All values are given as means ± SEM. The results were analyzed using a paired Student's t-test. Probability value of $p < 0.05$ was considered statistically significant.

3- Results

3.1 - pH Values: Figure 1 presents the pH change after 4, 8, 12, 24 and 48 h in *vitro* fermentation. All substrates had similar pH values (7.0) at 0 h. The GOS synthesized by β-galactosidase from *Scopulariopsis* sp fermentation resulted in the most rapid decline in pH and the lowest pH values (p<0.05) at 4, 8 and 12 h. Conversely, Oligomate GOS had the slowest decline in pH and the highest pH values (p<0.05) at 4, 8, 12 and 24 h of fermentation. The pH decrease was greater at 12 h for these two substrates. However, the GOS synthesized by *Scopulariopsis* sp resulted in the greatest (p<0.05) pH decrease (-1.5) compared with Oligomate GOS (-1.0) after *in vitro* fermentation. The pH values after 24 h and 48h of in vitro fermentation were not significant (p>0.05) from each group.

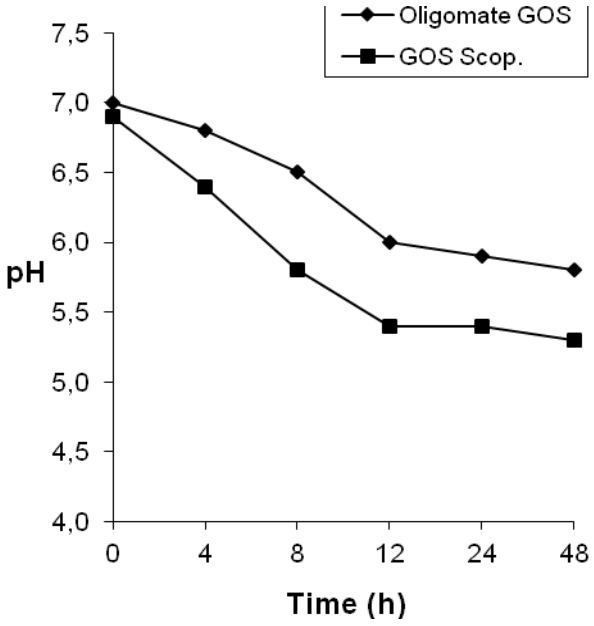

Figure 1- The pH values of GOS synthesized by β-galactosidase from *Scopulariopsis* sp and GOS Commercial after different time of *in vitro* fermentation using rat's cecal microflora. Values are mean (SEM), result of the paired Student's t test compared to pH.

3.2 - *In Vitro* GOS Digestibility: Digestibility of GOS synthesized by β-galactosidase from *Scopulariopsis* sp was carried out *in vitro* digestion experiments and compared with commercial GOS prebiotic; control experiment was carried out using maltose. Initially we examined the decomposition of oligosaccharides by saliva using human salivary α-amylase. GOS synthesized by β-galactosidase from *Scopulariopsis* sp and commercial GOS were not digested by human salivary α -amylase (Figure 2) during 4h incubation. But maltose was decreased by about 32 % after 4h.

Next we examined digestibility of GOS produced by β-galactosidase from *Scopulariopsis* sp and commercial GOS by artificial gastric juice (Figure 3), both showed resistance to this juice after 4h incubation. Similarly, maltose showed resistance to artificial gastric juice after 4h incubation.

GOS produced by β-galactosidase from *Scopulariopsis* sp and commercial GOS were not decomposed during 4h incubation by porcine pancreatic enzymes, but maltose was decreased by about 16% after 4h (Figure 4).

Finally, our examination of the decomposition of GOS produced by β-galactosidase from *Scopulariopsis* sp and commercial GOS by intestinal mucous enzymes using rat intestinal acetone powder (Figure 5) showed that two were not decomposed during 4h incubation. About 92% of maltose was hydrolyzed by rat intestinal mucous enzymes after 3h incubation and about 96% of maltose was hydrolyzed after 4h.

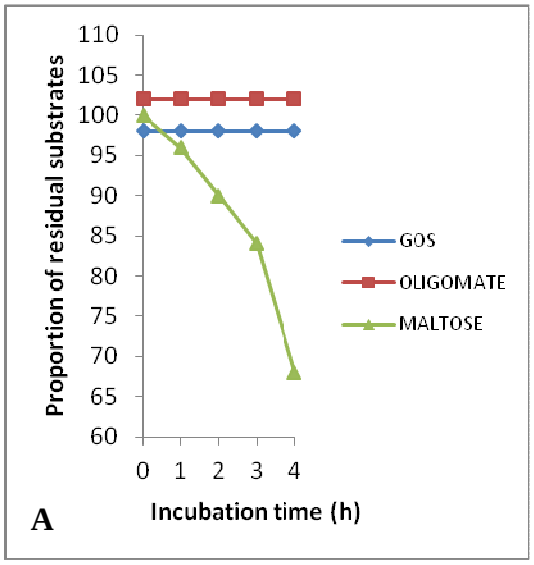

Figure 2- GOS digestion by human salivary α-amylase after 4h incubation.

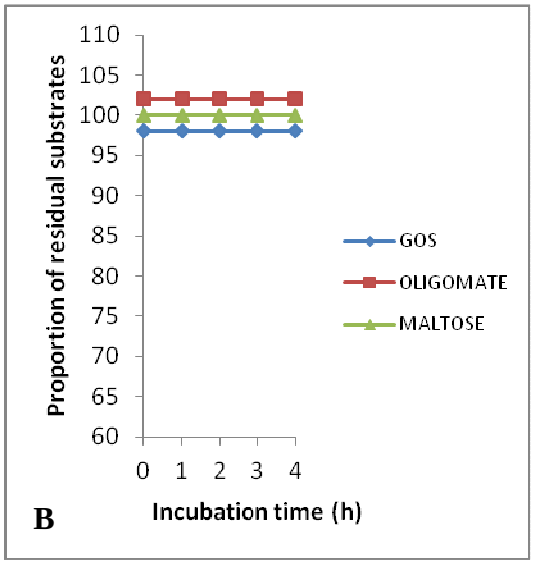

Figure 2- GOS digestion by human salivary α-amylase after 4h incubation.

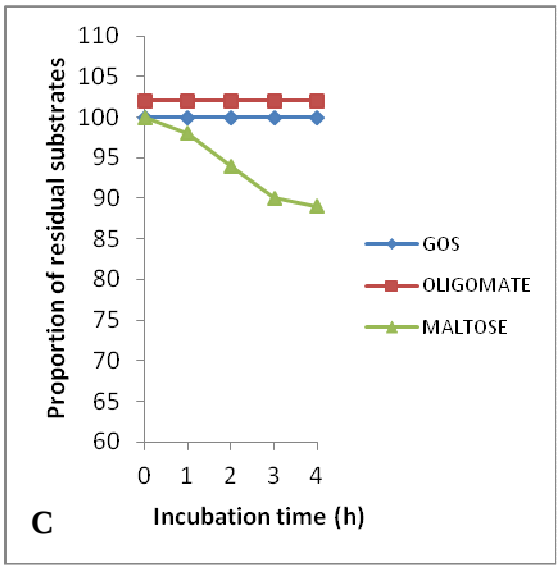

Figure 4 - GOS digestion by porcine pancreatin after 4h incubation.

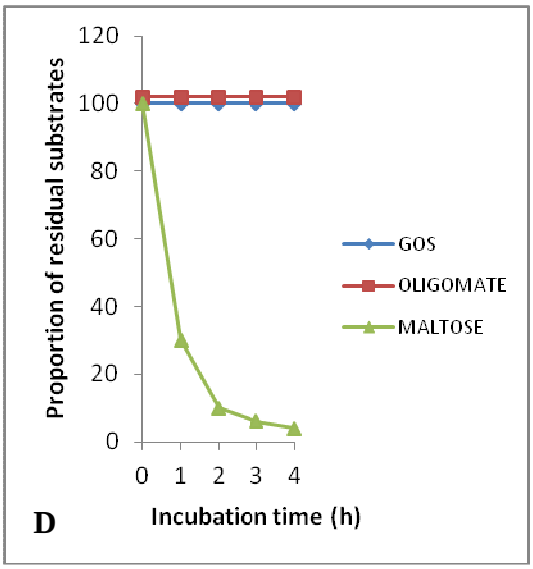

Figure 4 - GOS digestion by porcine pancreatin after 4h incubation.

3.3 - Carbohydrate Analysis: Carbohydrate contents in the cultures were measured after 0, 4, 8, 12 and 24h to determine the time oligosaccharides disappeared (Figure 6). The GOS produced by *Scopulariopsis* sp mixture the carbohydrate disappeared after 12h incubation period. The commercial GOS mixture the carbohydrate disappeared in the cultures after 24h under this experimental condition. When compared the commercial GOS and GOS produced by *Scopulariopsis* sp. The GOS produced by *Scopulariopsis* sp was broken down faster than commercial GOS. The FOS disappeared after 8h incubation period.

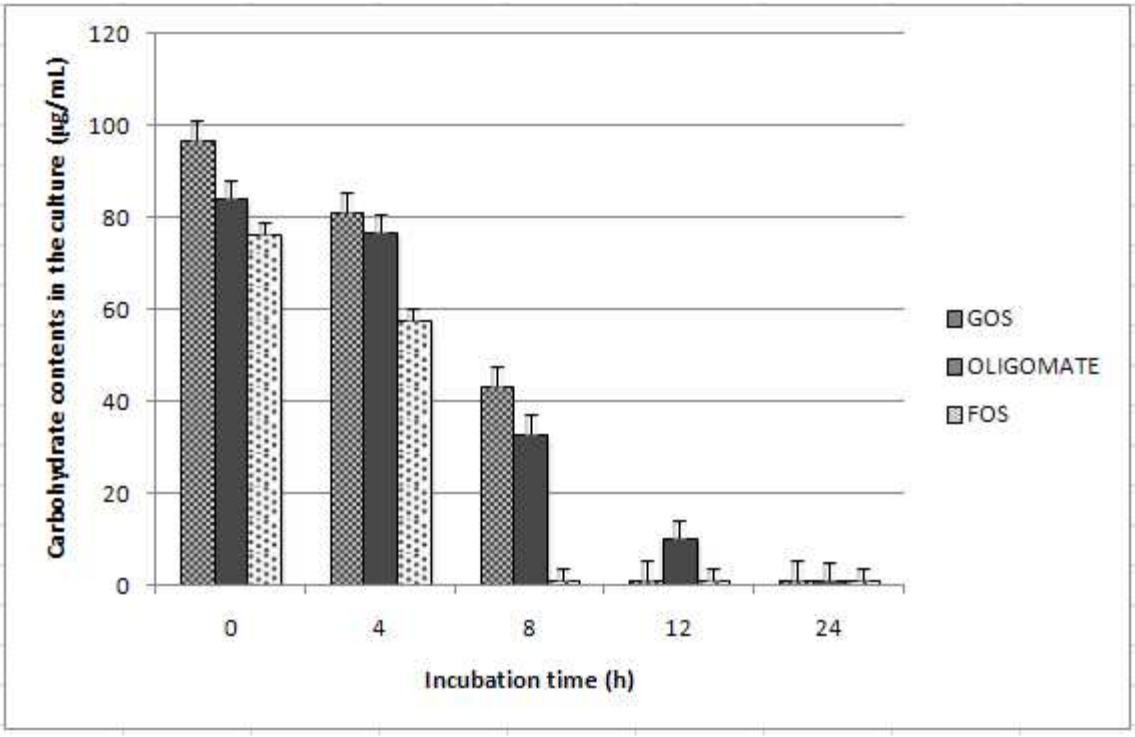

Figure 6 - Disappearance of GOS in the culture during the in vitro cecal incubation. Values are means ± SEM (n=6).

3.4 - Concentration Short Chain Fatty Acids: Determination of short chain fatty acids as the fermentation end products is showed in Table 2. Acetic, propionic and butyric acids were the main fatty acids in the fecal content with GOS produced by β-galactosidase from *Scopulariopsis* sp or commercial GOS. *In vitro* GOS fermentation produced by β-galactosidase from Scopulariopsis sp resulted in the greatest (p<0.05) SCFA concentration including acetate, propionate and butyrate when compared to commercial GOS.

Concentration of acetate, propionate and butyrate (p<0.05) after 48h fermentation were 29.75; 4.16 and 1.49 *µmol*g, respectively for GOS produced by β-galactosidase from *Scopulariopsis* sp and 25.57; 2.85 and 1.19 *µmol*/g for commercial GOS (p<0.05). The molar ratio of acetate:propionate:butyrate was 72:16:12, respectively for GOS synthesized by β-galactosidase from *Scopulariopsis* sp and 67:18:15, respectively, for commercial GOS after 48h fermentation.

Fermentation of GOS produced by β-galactosidase from *Scopulariopsis* sp resulted highest (p<0.05) SCFAs total concentration (155.03 *µmol*/g) after 48h when compared with commercial GOS (101.43 *µmol*/g). GOS produced by β-galactosidase from *Scopulariopsis* sp*,* resulted in the most rapid accumulation of organic acids, achieving 78% of total organic acids production after 12h fermentation. The interaction of the substrate and the time that it was significant (p<0.01) for acetate and attained a maximal rate of SCFA production in time 48h. Propionate and butyrate attained a maximal rate in time 12h. After 12h of

fermentation, propionate and butyrate had lowest rates of production GOS produced by β-galactosidase from *Scopulariopsis* sp and commercial GOS.

Table 2 - Concentrations at 0, 4, 8, 24 and 48h of short-chain fatty acids produced during fecal fermentation

Fatty acid(s)	Incubation time (h)	[1]GOS	[2]GOS
		µmol /g substrate	
Acetic acid	0	N.D	N.D
	4	9.78±1.44a	3.59±1.22b
	8	17.73±1.71a	7.82±0.64b
	12	23.94±1.27a	12.97±1.72b
	24	29.54±1.42a	27.32±1.89b
	48	29.75±2.25a	25.57±2.32b
Propionic acid	0	N.D	N.D
	4	2.83±0.94a	2.06±0.38a
	8	6.35±0.87a	3.65±0.010b
	12	7.35±1.72a	6.31±0.92b
	24	4.49±2.06a	3.68±1.75b
	48	4.16±1.67a	2.85±1.49b
Butyric acid	0	N.D	N.D
	4	2.79±1.53a	2.25±1.74a
	8	5.37±0.56a	3.98±0.72b
	12	5.60±1.71a	4.87±0.58b
	24	3.86±1.19a	3.32±1.51a
	48	1.49±0.45a	1.19±0,89a
Total SCFAs	0	0	0
	4	15.40±4.024a	7.90±0.83b
	8	29.45±6.871a	15.45±1.31b
	12	36.89±10.12a	24.15±2.32b
	24	37.89±14.65a	24.32±4.30b
	48	35.40±15.60a	29.61±7.41b

Values for individual acids are means ± standard deviations of the results.
Results sharing the same superscript in the same row are not significant different (p<0.005).
[1]GOS, produced by *Scopulariopsis sp;* [2]GOS, commercial Oligomate.
Total SCFA = (acetate+propionate+butyrate).
Amounts of generated of SCFA (*µmol*) from 1.0g of each oligosaccharides. N.D. (not detected).

4- Discussion

After 48h of fermentation, GOS produced by β-galactosidase from *Scopulariopsis* sp resulted in the lowest pH values. The change of pH was probably caused by highest total SCFA productions.

These data indicate that bacteria present in the large intestine of rats may ferment these substrates. Similarly, Flickinger et al. (2000) reported 17% decrease in pH values when short-chain fructo-oligosaccharides were fermented for 11h by canine fecal microflora.

The test substrate was not degraded by digestive enzymes after 4h the incubation. These results suggested that GOS produced by β-galactosidase from *Scopulariopsis* sp were confirmed as non-digestible. Therefore, it was expected that GOS reached the large intestine following the digestive tract without digestion and in the colon content fermented by the colonic microbiota.

The non-digestibility of prebiotics can be demonstrated *in vitro* by treatment with pancreatic and other gastrointestinal digestive enzyme. In the GOS, several *in vitro* experiments have shown its non-digestibility and stability to hydrolysis enzyme. Van Loo et al. (1999) studied the effects of various non-digestible oligosaccharides and concluded that more than 90% of GOS arrives into the colon.

Similar finding was observed by Asano et al. (2003) examined by *in vitro* digestion method and observed that mannooligosaccharides were resistant to human salivary α-amylase, artificial gastric juice, porcine pancreatic enzymes and rat intestinal mucous enzymes, but maltose was hydrolyzed after 4h.

The non-digestible oligosaccharides are connected by β-2, 1 bonds and they are not hydrolyzed by digestive enzymes in the upper gastrointestinal tract and, therefore, reach the colon intact. Reach the colon intact, they are fermented by beneficial bacteria commensal to the colon, e.g. bifidobacteria, lactobacillus, which are stimulated to grow and/or are metabolically activated. However, only a few non-digestible oligosaccharides have been identified which can be classified as prebiotics (Gibson and Roberfroid, 1995).

In the cultures with GOS produced by β-galactosidase from *Scopulariopsis* sp the oligosaccharides disappeared after a 12h incubation period and commercial GOS oligosaccharides disappeared in the cultures after 24h. GOS produced by β-galactosidase from Scopulariopsis were more selectively utilized than commercial GOS, This can be attributed to the fact that there are differences also in the composition of the oligosaccharides and differences in the position of the glycosidic linkages occur, due to the different enzymes used in their production (Duarte et al. 2010).

The time of GOS produced by β-galactosidase from *Scopulariopsis* sp and commercial disappearance in the culture was different from that of

fructooligosaccharides. FOS disappeared after 8 h incubation period, it must be inferred that FOS (long-chain), were more rapidly fermented by bacteria of the colonic than GOS (short-chain). Thus, it is possible that the utilization more selective of the FOS by bacteria consist on its structure of the carbohydrate and the bacterial species present in the ecosystem.

Results similar were reported by Asano et al. (2003) that investigated the utilization of fructooligosaccharides by an anaerobic incubation with human feces *in vitro*; these investigators reported that carbohydrate was not detected in the fecal culture with fructo-oligosaccharides after 8h of incubation. Fructooligosaccharides were broken down faster than manno-oligosaccharides by fecal flora. The difference of the time course seemed to be due to differences in species and quantity of bacteria in the feces which utilized each oligosaccharide.

A more meaningful in vitro method for studying prebiotic oligosaccharides is the use of mixed culture (faecal inocula). Study of the changes in populations of selected genera or species can then establish whether the fermentation is selective. The use of faecal inocula probably gives a representation of events in the distal colon (Gibson and Rastall, 2006).

GOS produced by β-galactosidase from *Scopulariopsis* sp resulted in greater fermentation as indicated by greater acetate, propionate, butyrate and total SCFA production compared to substrate evaluated. The GOS commercial produced much less acetate (approximately 77µmol/g) compared to the GOS produced by β-

galactosidase from *Scopulariopsis* sp (approximately 110µmol/g). Differences in composition of oligosaccharides may have been a factor. The commercial GOS contains 55% GOS with glucose, galactose and lactose making up the remaining 45%. GOS produced by β-galactosidase from *Scopulariopsis* sp contains 28% GOS, 50% lactose, 15% glucose, and 7% galactose.

Compositions of short chain fatty acids showed variance in individual composition and it was apparently due to the difference of intestinal microflora. The potential for maximal SCFA production at 12h of fermentation, indicate that fermentation is occurring for simply breakdown of oligosaccharides. However, after 24h of fermentation substrates decreased production of propionate and butyrate for two substrates. The progressive decline in total SCFA concentrations coupled with the rise in pH suggests that the amount of substrate available for fermentation is limiting.

After 48h of fermentation, the molar ratios of acetate, propionate, and butyrate were 67:18:15 for the GOS commercial and 72:16:12 to GOS produced by β-galactosidase from *Scopulariopsis* sp. These variations may be due to disparities in the molecular structure of substrates used.

Any food ingredient that enters the large intestine is a candidate prebiotic. However, to be effective, selective fermentation by the colonic microbiota is required; much evidence supports the belief that the currently identified prebiotics are fermented (Molis et al., 1996; Alles et al., 1996)

Prebiotics have been shown to be a source of SCFAs both *in vitro* and *in vivo*, but *in vivo*, the study of SCFAs is more difficult and relies mostly on determination of the concentrations in feces (Gibson et al., 1995). Study *in vitro* using fecal inoculum from 6 healthy volunteers, compared production of SCFAs from 17 different carbohydrate sources, only FOS and inulin were established as prebiotics (Wang and Gibson ,1993).

Smiricky-Tjardes et al. (2003) evaluated the fermentative characteristics of the galacto-oligosaccharídes *in vitro* and *in vivo* and reported that the GOS increased beneficial bacteria *in vivo* and SCFA concentrations both *in vitro* and *in vivo*. Results similar were reported by Vickers et al. (2001) these investigators described an increased SCFA production as a result of fermentation of short-chain fructo-oligosaccharides and long-chain fructo-oligosaccharides with canine fecal microflora. Therefore, substrates that produce high amounts of SCFA in vitro, namely the fructans, xylo-oligosaccharides, and the galacto-oligosaccharides, may do the same in vivo.

The nature of the carbohydrate determines its fermentability, both the structure of the carbohydrate and the bacterial species present in the ecosystem are probably important factors in controlling the fermentation of short-chain carbohydrates (SCCs) (Van Laere et al., 1997; Cummings et al., 2001).

Van Laere et al. (1997) produced a range of different SCCs with widely different sugar compositions and molecular sizes and tested their breakdown by several

strains of *Bifidobacterium*, *Clostridium*, *Bacteroides,* and *Lactobacillus.* Fermentability differed with oligosaccharide structure. The fructans were extensively fermented, except by clostridia, whereas few species were able to break down arabinoxylan under the conditions of the experiment. Xylooligosaccharides were well fermented. Linear oligosaccharides were catabolized to a greater degree than were those with branched structures. Bifidobacteria utilized low degree of polymerization (DP) carbohydrates first and bacteroides utilized those with a high DP. Metabolic collaboration among species was evident in carbohydrate breakdown.

An acidic pH optimizes the growth conditions for acidophilic bacteria. These include the large group of lactic acid bacteria, e.g. lactobacillus and bifidobacteria. In contrast, an acidic milieu provides only suboptimal conditions for pathogenic microorganisms. Lowering the pH thus reduces colonization by pathogenic microbes and promotes mineral absorption (Rémésy et al., 1993; Chonan & Watanuki, 1995)

The major products of prebiotic metabolism are SCFAs, the gases hydrogen and carbon dioxide, and bacterial cell mass. Much has been written about SCFA production in the hindgut and about the differing metabolic significance of the individual acids (Cummings, 1995).

SCFA produced by bacterial fermentation contribute to normal large bowel function and prevent pathology through their actions in the lumen and on the

colonic musculature and vasculature and through their metabolism by colonocytes. Approximately 95-99% of the short chain fatty acids produced during bacterial fermentation are absorbed rapidly from the intestinal lumen. Butyrate is the preferred energy source for the epithelial cells in the large intestine and supplies up to 70 % of their total energy requirements (Scheppach, 1994). Propionate has been suggested to spare amino acids that would be used in gluconeogensis in the post absorptive state (Demigne and Remesy, 1991).

The GOS produced by β-galactosidase from *Scopulariopsis* sp are indigestible, were fermented by rat cecal bacteria and the products of fermentation were short chain fatty acids. To study these physiological functions of GOS *in vivo*, experiments with animals and human are in progress.

To be classified as a prebiotic, the substrate must "be a selectively fermented ingredient that allows specific changes, both in composition and/or activity, in the gastrointestinal microflora that confers benefits upon host well-being and health". (Roberfroid et al.1998). On the basis of this definition, GOS produced by β-galactosidase from *Scopulariopsis* sp has prebiotic potential based on its indigestibility and SCFA production. GOS was highly fermentable and may positively affect large bowel health.

5- Conclusion

In conclusion, GOS synthesized by β-galactosidase from *Scopulariopsis* sp resisted hydrolytic digestion and they were well fermented as indicated by a decrease in pH, increased SCFA production or concentration. Thus, GOS exhibited prebiotic effects. However, these effects need to be performed using human volunteers.

6- Acknowledgments

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Effects of the Galacto-oligosaccharides (GOS) Produced by β-galactosidase from *Scopulariopsis* sp on Hematological and Biochemical Parameters in Male Wistar Rats

Effects of the Galacto-oligosaccharides (GOS) Produced by β-galactosidase from *Scopulariopsis* sp on Hematological and Biochemical Parameters in Male Wistar Rats

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Abbreviations:

GOS:Galacto-oligosaccharides; FOS: fructo-oligosaccharides; Hb:Haemoglobin; DP: degrees of polymerization; GGT: Gamma glutamyl-transferase; BW: Body weight; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; PT: Prothrombin Time

Abstract

Galacto-oligosaccharides (GOS) are nondigestible oligosaccharides occurring naturally in human milk and can be manufactured by enzymatic transgalactosylation from lactose using β-galactosidase, are of great interest for food and feed applications because of their beneficial effects on health. Some beneficial effects attributed to consumption of GOS include reduced risk of cancer of the colon, increased in mineral absorption, control serum cholesterol levels and immune modulation of the gastrointestinal tract. The present study aims at investigating the effects of oral administration of GOS produced by β-galactosidase from *Scopulariopsis* sp on hematological and biochemical blood parameters in rats. Male albino-Wistar rats weighing between 365 to 375g were supplemented with different doses of GOS in a 42 -days experiment. The result shows that the body weight, feed intake and water consumption was not statistically significant when compared with the control (p<0.05). Hematologic and biochemical variables were not affected by the supplementation with GOS. Histopathologic examination of the organs obtained at autopsy did not reveal any alterations. The data obtained showed that consumption of GOS produced by β-galactosidase derived from *Scopulariopsis* sp for 42 days is safe and does not have of harmful effects variables tested in rats.

Keywords: Galacto-oligosaccharides, haematology, Clinical biochemistry, Pathology**,** rats.

Resumo

Galacto-oligossacáridos (GOS) são oligossacáridos não digeríveis que ocorrem naturalmente no leite humano e podem ser produzidos por transgalactosilação enzimática a partir de lactose utilizando β-galactosidase, são de grande interesse para aplicações na alimentação humana e animal devido aos seus efeitos benéficos para a saúde. Alguns efeitos benéficos atribuídos ao consumo de GOS incluem o risco reduzido de cancer de cólon, aumento na absorção de minerais, controle dos níveis de colesterol sérico e e modulação imunológica do trato gastrointestinal. O presente estudo tem como objetivo investigar os efeitos da administração oral dos GOS produzidos pela β-galactosidase a partir de *Scopulariopsis sp* sobre os parâmetros sanguíneos hematológicos e bioquímicos em ratos. Ratos machos Wistar albinos pesando entre 365 a 375g foram suplementadas com diferentes doses de GOS em um experimento de 42 dias. O resultado mostra que o peso corporal,consumo de ração e água não foi estatisticamente significativo quando comparado com o controle (p <0,05). Variáveis bioquímicas e hematológicas não foram afetados pela suplementação com GOS. O exame histopatológico dos órgãos obtidos na autópsia não revelou quaisquer alterações. Os dados obtidos mostraram que o consumo de GOS produzidos por β-galactosidase derivada de *Scopulariopsis* sp por 42 dias foi seguro e não teve efeitos nocivos nas variáveis testadas em ratos.

Palavras-chave: galacto-oligosacarídeos, hematologia, bioquímica clínica, patologia, ratos

1 - Introduction

Galacto-oligosaccharides (GOS) is a collective term for a group of carbohydrates composed of oligo-galactose with some lactose and glucose. GOS were recently defined as “a mixture of those substances produced from lactose, comprising between 2 and 8 saccharide units, with one of these units being a terminal glucose and the remaining saccharide units being galactose and disaccharides comprising 2 units of galactose” (Tzortzis and Vulevic 2009; Torres et al, 2010).

Galacto-oligosaccharides are naturally occurring in human milk; however, commercial preparations are produced by enzymatic activity of *β*- galactosidase on lactose in a reaction known as transgalactosylation (Sako et al, 1999; Angus et al., 2005 ; Macfarlane et al.2006 ; Maischberger et al., 2008 ; Sangwan et al.2011). Converting lactose into GOS by glycoside hydrolases (GH) results in mixtures containing GOS of different degrees of polymerization (DP), unreacted lactose, and monomeric sugars (glucose and galactose) (Torres et al, 2010).

GOS are non-digestible oligosaccharides and they pass on the colon where they are fermented selectively by beneficial intestinal bacteria, which implicate a balanced and advantageous microbiota (Sako et al., 1999 ; Cummings et al, 2001; Boehm et al. 2007; Mischberger et al., 2008), belong, because of their indigestible nature, to the group of prebiotics (Schaafsma 2008, Sangwan et al. 2011).

Several microbial glycoside hydrolases have been proposed for the synthesis of GOS from lactose. In this context, these enzymes fundamentally differ in their

ability to catalyze the transgalactosylation reaction relative to hydrolysis, and in their affinity for the GOS formed as compared to the affinity for lactose. (Torres et al, 2010). Mixtures of oligossacharides of various chain length and glycosidic bonds are formed during production (Tannock et al., 2004; Neri et al., 2011).

Besides the differences in purity of GOS mixtures, differences in the position of the glycosidic linkages occur, because different enzymes have different regiochemical selectivity. Depending on oligosaccharide composition, GOS products will vary in terms of prebiotic activity, as well as other physiological effects (Torres et al., 2010).

Many beneficial effects on health have been attributed to GOS including: improvement of defecation, stimulation of mineral absorption, elimination of ammonium, colon cancer prevention, as well as protection against certain pathogenic bacteria infections (Hopkins et al. 2003; Shoaf et al., 2006; Macfarlane et al., 2008).

Because of their known health benefits, GOS have become the centre of a great deal of attention in the field of functional foods (Sako et al. 1999, Angus et al., 2005). There is continuous interest in finding microorganisms with adequate properties for industrial uses and able to produce specific GOS mixture with better yields (Torres et al., 2010).

Intake of GOS produced by β-galactosidase from *Scopulariopsis* sp as a dietary supplement requires a safety assessment because its potential use as a prebiotic ingredient is of great interest. Therefore, the purpose of this study is to evaluated the toxic effects resulting from the supplementation of GOS orally in rats, focus on weight parameters, clinical hematological, biochemical and histological necropsy.

2 – Material and Methods

2.1 - Test Substance

GOS syrup used in this study was produced by transgalactosylation from lactose using β-galactosidase derived from *Scopulariopsis* sp produced in the Bioaromas laboratorie (Department of Food Science, School of Food Engineering, University of Campinas, São-Paulo-Brazil). The GOS syrup containing approximately 28% GOS, 50 % lactose, 15% glucose and 7% galactose (Santos et al., 2009).

Reference controls were provided as white powder. The FOS (Raflitose P95) contain 95% oligofructose with degree of polymerization (DP) between 2 and 8 and 5% of glucose, fructose and sucrose were obtained from ORAFTI (Belgium). The FOS was prepared weekly (50%w/v) in suspension in deionized water. This mixture was stored refrigerated and a sufficient amount was removed each day for supplementation of the control animals.

2.2 - Animals and Treatment

Thirty-two (32) male wistar albino rats (*Rattus Wistar*) weighing 365–375 g, were obtained the *Center* of Multidisciplinary investigation in Biology (CEMIB) of the University of Campinas, São-Paulo-Brazil). The rats were given 1 wk to acclimatize during which time they consumed the basal diet ad libitum. After acclimatization, the rats were housed in cages (*n*= 1/cage) kept at 22 ± 3℃, with 40–70% relative humidity and controlled lighting that provided a 12-h light:dark cycle.

2.3 - Acute Toxicity

During the whole experiment, the animals received water and commercial Labina-Purina chow (23.5% protein, 6.5% fat, 70.0% carbohydrate, Purina 5008, St. Louis, Mo.) *ad libitum*. The rats were randomly assigned to four groups (n=8 per group). Group reference control supplemented with FOS (Orafti®P95) 1000mg/kg/bw/day. Groups supplemented with different doses of GOS at 300, 500 and 1000mg/kg/bw/day (OECD Category 4>300-2000mg/kg/bw). Dose volume was calculated based on a density determination performed at the laboratories of Food Engineering (1mL/500mg GOS). Individual doses were based on the most recent body weights.

The GOS syrup was administered orally by intragastric cannula insertion for 42 consecutives days. Oral administration was selected as the route of administration as this is the potential route of exposure to humans; gavage was chosen due to potential palatability issues. All animal manipulations were carried out in the

morning to minimize the effects of circadian rhythm. The protocol was approved by Animal Experimental Ethics Committee of the University of Campinas.

Parameters Evaluated

2.4 - Clinical Observations: The animals were observed daily twice a day for 42 days to assess general health (mortality and morbidity). Detailed clinical observations were performed once weekly prior to dosing and prior to scheduled euthanasia. The visual observations included changes in skin and fur (hair), eyes and mucous membranes and also respiratory, circulatory system, as well as body weight of each animal at the beginning and 42 days of the trial.

2.5 - Body Weights and Food Consumption: Body weight, as well as food and water consumption, was recorded daily. Feed efficiency was calculated as the weekly body Weight gain divided by the food consumption.

2.6 - Hematology and Clinical Chemistry: At the end of the experiment, rats were deprived of food overnight, anesthetized with ether and blood was collected by cardiac puncture (1 mL into EDTA for hematology and 2 mL into sodium heparin for blood clinical chemistry). Blood was centrifuged at 1500 x g (model TJ-6 centrifuge, rotor TH-4 with buckets, Beckman Coulter, Fullerton, CA) for 15 min. and the plasma was immediately removed from the cells. The plasma was stored

in a freezer at -20 ºC for later biochemical analyses. All samples were processed within 4–6h.

Auto haematological analyzer Hemo 960V (Shinova Systems Co., Ltd.) was used to analyse the following blood parameters: erythrocyte count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, total and differential leukocyte count and platelet count. Prothrombin time and fibrinogen were assessed with the Coagulation analyzer (Coagulometro Drake, model Quik Timer SL0055).

The biochemical constituents as glucose, cholesterol, triglycerides, HDL, LDL, total proteins, albumin, amylase and Gamma glutamyl-transferase (GGT), were measured by enzymatic methods using (Labtest®) reagents according to the manufacturer's instructions. All tests were performed at the Laboratory of Analyses Clinical of Medicine Veterinary.

Pathology

2.7 - Gross Necropsy: Detailed gross necropsy, including careful examination of the body external surface, orifices and cranial, thoracic and abdominal cavities and their contents was performed in both groups. The organs (spleen, liver, thymus and pancreas) were evaluated macroscopically according to color, size and texture. After the necropsy the organs were weighed and their wet weights immediately recorded. Results were expressed as organ weight relative to 100g body weight.

2.8 - Histopathology: All tissues examined in the study were fixed in 1.23 mol/L buffered formalin (pH 7.4) and the samples were sent to the Laboratories of Histopathology, University of Adamantina – FAI (Adamantina – SP, Brazil) for histopathologic study. The tissues were then treated with graded alcohol, embedded in paraffin, sectioned in 5 µm slices and stained with hematoxylin and eosin for examination under the light microscope. The tissues were examined microscopically by a board-certified veterinary pathologist.

2.9 - Statistics Methods: Results were expressed as the mean ± SEM. Statistical differences between means were compared using Tukey´s multiple range test. Differences over time were analyzed using one way analysis of variance. All statistical analyses were performed with GraphPad Prism version 5.0 (GraphPad software, Inc.; La Jolla, CA, USA). The $p < 0.05$ level was taken as significant.

3 - Results

3.1 - Survival and Clinical Observations

In study, no mortality was observed in rats after 42 days of daily oral ingestion of different doses of the GOS (300, 500 and 1000mg/kg/bw).During the daily observation of the occurrence of toxic signs on the different systems (autonomic, behavioral, sensory, neuromuscular, cardiovascular, respiratory, ocular, gastrointestinal, genitourinary, skin) did not show toxic signs in the treated groups during the trial period. From the clinical point view the animals maintained a normal appearance corresponding to their species.

3.2 - Body Weight, Food and Water Consumption

No significant differences were found ($p \geq 0.05$) between the initial and final body weight of rats supplemented with GOS and the control rats (Table 1). The cumulative balance in grams of body weight in relation to the initial weight of the animals behaved similarly in groups supplemented with different concentrations of GOS and control group, which is indicative of the not occurrence of changes in this parameter.

Similarly, the groups supplemented with GOS and control did not differ in food or water consumption. Feed efficiency remained constant until the end of the study did not differ between groups GOS and control (Table 1). We considered the gain/feed ratio as the most sensitive measure of growth performance.

Table 1 - Effect of GOS supplementation on body weight, gain, feed and water intakes in rats after 42 days.

Variables	**Treatment**					
	Control	**GOS** 300mg/kg	**GOS** 500mg/kg	**GOS** 1000mg/kg	**SEM**	***p*-Value**
Initial body weight (g)	365.3± 0.26	368.4± 0.27	370.9± 0.34	367.6± 0.29	0.89	0.835
Final body weight (g)	538.8±3.18	539.2±2.80	542.4±2.77	544.5±3.00	0.78	0.875
Total weight gain (g)	192.50± 0.50	189.92± 0.62	190.50± 0.55	192.20± 0.58	0.96	0.653
Total feed intake	767.76±1.24	753.06±1.29	755.16±1.68	768.60±1.48	0.96	0.735
water (ml/d)	47.5±0.36	48.5±0.39	48.7±0.21	47.9±0.39	0.52	0.530
[2]FCE	0.26±0.52	0.25±0.56	0.25±0.44	0.25±0.41	0.63	0.665

[1] Results are expressed as means ± SEM. (n = 8). Groups did not differ, p>0.05.
[2]Feed efficiency conversion – FCE (weight gain/food consumed ratio g/g).

3.3 - Hematological and Biochemical Parameters

The effects of the GOS on the hematological and biochemical parameters of the male Wistar rats were investigated. There were no significant changes in blood parameters between groups supplemented and control group (Table 2). These values are within physiological limits established for the species (Charles River Laboratories; 2006).

There were no changes in blood parameters in the rats supplemented with different doses of GOS (300; 500 and 1000 mg/kg/bw/d). These values are with in limits established for the species (Charles River Laboratories; 2006). No statistically significant differences were noted in blood parameter between rats supplemented with different doses of GOS and control group (Table 2). Therefore, the supplementation with different doses of GOS not was considered toxicological.

The supplementation of different doses of GOS did not affect serum lipids, glucose and enzymes (Table 2). No significant differences were observed in clinical chemistry in the 300, 500 and 1000 mg/kgbw/day groups when compared to the control.

Table 2- The effects of different doses of GOS on the hematological and biochemical parameters in Wistar rats[1]

Hematological variables	Control	GOS 300mg/kg	GOS 500mg/kg	GOS 1000mg/kg
Red blood cells				
Erythrocytes (10^6/ml)	8.34±0.43	8.39±0.36	8.35±0.28	8.27±0.33
Hemoglobin (*g/dL)*	15.80±0.65	15.50±0.34	14.80±0.72	15.60±0.87
Hematocrit (%)	42.80±1.50	43.30±1.60	44.90±1.30	42.60±1.90
MCV, *fL*	55.50±0.90	52.40±1.20	53.90±1.55	55.70±1.67
MCH	18.40±0.92	17.90±0.74	18.20±0.86	18.70±0.98
MCHC (%)	33.80±0.84	35.40±0.92	33.40±0.65	34.80±0.66
PT (s)	13.20±1.25	13.50±0.56	12.80±0.94	12.90±1.32
Fibrinogen (mg/dL)	334±35.26	328±42.46	326±38.92	338±40.84
White blood cells				
Leukocytes (*10^3/mm^3)*	10.97±2.43	10.83±2.64	10.57±3.37	11.34±3.40
Neutrophils %	17.92±5.20	18.36±3.40	17.62±6.20	18.26±8.70
Lymphocytes, *%*	80.39±5.40	84.42±5.70	83.38±6.30	80.73±4.70
Monocytes, *%*	0.92±1.78	0.98±1.58	0.88±1.68	0.94±1.54
Eosinophils, *%*	1.20±1.80	1.30±1.90	1.50±1.20	1.30±1.70
Basophils, *%*	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Platelets				
Platelets (10^3/mL*)*	475±47.30	494±56.39	482±44.56	479±6.85
Mean platelet volume, *fL*	6.65±0.43	5.38±0.57	6.66±0.41	5.91±0.38
Clinical biochemistry				
Glucose mg*l/dL*	135.0±24.0	128.0±18.0	132.0±26.0	123.0±22.0
CT *mgl/dL*	63.0±18.0	62.0±12.0	59.0±10.0	65.0±16.0
TG *mgl/dL*	78.0±33.0	75.0±28.0	82.0±32.0	80.0±26.0
HDL-C *mgl/dL*	52.0±12.0	58.0±18.0	55.0±24.0	59.0±23.0
LDL- C *mgl/dL*	19.3±24.0	18.6±22.0	19.0±19.0	18.9±21.0
Total protein *g/dL*	6.7±1.6	6.5±0.9	6.7±1.4	6.2±0.6
Albumin *g/dL*	3.3±0.6	4.0±0.2	3.6±1.3	3.5±1.5
Globulin g/dL	3.4±0.2	3.6±0.3	3.4±0.5	3.6±1.1
Amylase *U/dL*	228.0±32.0	232.0±24.0	226.0±38.0	230.0±17.0
GGT[2] *U/L*	236.3±0.8	232.1±1.4	230.9±0.6	233.5±0.4

[1]Values are means ± SEM, n = 8. Groups did not differ, p >0.05.
MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; PT: Prothrombin Time.
CT, Cholesterol Total; TG – Triglycerides; GGT, Gamma glutamyl-transferase.

Pathology

3.4 - Gross Necropsy: At the end of the study, rats were subjected to detailed post-mortem examinations of internal organs, which did not show macroscopic differences in size, color or texture. Necropsy did not reflect any damage in the organs examined. The organs (liver, spleen, thymus and pancreas) examined macroscopically showed no pathological changes for any of the animals supplemented orally with GOS, behaving in all cases equal to the control.

3.5 - Relative Organ Weights

Table 4 one can see the quotients of organ weights (spleen, liver, thymus and pancreas)/ body weight. For these variables did not show any significant difference (p>0.05) between the treated and control groups after 42 days of supplementation. The repeated oral administration of different doses of GOS did not affect the mean relative in the organ weights.

Table 3 – Relative organ weights (liver, spleen, thymus and pancreas) of rats after 42 days of daily oral administration of different concentrations of GOS[1]

Parameters	Control	GOS 300mg/kg	GOS 500mg/kg	GOS 1000mg/kg
Liver	0.67±0.70	0.65±0.50	0.68±0.40	0.67±0.80
Spleen	0.27±0.11	0.28±0.17	0.24±0.15	0.24±0.12
Thymus	0.17±0.80	0.16±0.30	0.15±0.80	0.15±0.50
Pancreas	0.07±0.21	0.09±0.32	0.08±0.28	0.09±0.23

[1]Values are means ± SEM, n = 8. Groups did not differ, p>0.05.
Organs weight was expressed in percentage related to animal body weight.
Relative weight (%) = absolute organ weight/body weight X 100%

3.6 - Histopathology Parameters

Histopathology showed no change in cell morphology of the organs examined (spleen, liver, thymus and pancreas) and no evidence of toxicity was observed. These results were positively correlated with the results of the hematological and biochemical parameters.

4 - Discussion

There has been a number of studies that reveal the prebiotic potential of GOS as health promoting in man and animals (Chonan et al., 1995; Teuri et al., 1998; Chonan et al., 2001; Vulevic et al., 2008; Fanaro et. Al, 2009; Silk et al., 2009; Davis et al., 2010). This compound as food ingredients are of great interest as potential ingredients for functional foods. In this study the toxicological effect of GOS produced by β-galactosidase from *Scopulariopsis* sp in experimental animals were analyzed.

The results of individual clinical examinations in both the treated and control groups of the male Wistar rats indicate that GOS was well tolerated during the treatment period (42-days), with no mortality or signs of morbidity observed. In addition, observations were made daily during the study and no differences among the groups supplemented with different doses of GOS and controls rats in changes in skin, eyes, and mucous membranes, as well as in behavioral changes. From the clinical point of view the animals maintained mainteda normal appearance corresponding to their species.

The 42-d oral administration doses of 300, 500 and 1000 mg/kg did not affect the body weight, food and water consumption. These results indicate that GOS showed no toxicity at the doses and routes of administration tested.

There was no significant difference in the organ weights (liver, thymus, spleen, and pancreas) between control and experimental animals supplemented with different doses of GOS.

Histological evaluation of tissues belonging to all structures of the supplemented animals showed normal results (no changes considered to be indicative of pathology), or revealing of important functional and structural damage that could be associated with GOS supplementation at the dosages and periods.

Analysis of blood parameters is a relevant risk assessment; any change in the hematological system has a high predictive value of the toxicity in the humans and in animals. The results of the hematological parameters shows that the rats supplemented with different doses of GOS did not show variations of importance of a biological standpoint. No changes were observed in the red series and also the values of hematocrit and hemoglobin and the counts of white blood cells, since the values are within the normal range for the species (Charles River Laboratories, 2006).

The coagulation time was not changed in groups supplemented with GOS compared with the control. The biochemical analysis showed no statistically significant differences among the groups supplemented with different doses of GOS. Cholesterol and triglycerides were not altered, indicating no role in lipid metabolism of the GOS. Also no changes were observed in blood glucose.

Several studies have showed that the administration of prebiotics are effective in improving lipid profiles, including the reduction of serum/plasma total cholesterol, LDL-cholesterol and triglycerides (Trautwein et al., 1998; Letexier et al., 2003; Hsu et al., 2004; Li et al., 2007). GOS have also been shown to decrease serum cholesterol and triglycerides, respectively, in animal models (Chonan et al., 1995; Beylot, 2005).

In this study, there were no significant differences in ($p \leq 0.05$) serum lipids in groups supplemented with GOS when compared with control group. The GOS supplementation not reduced serum total cholesterol, LDL-cholesterol and triglycerides or increment of HDL-cholesterol. However, studies have shown hypocholesterolemic effects in rats (Chonan, 1995).

The animal studies have shown some evidence for the hypocholesterolemic effects of the GOS however, there are difficulties in demonstrate equivalent effect in humans (Vulevic et al. 2008) The number of human studies is, however, limited and results concerning effects on plasma cholesterol and triacylglycerol are not conclusive (Roberfroid et al., 2010).

Many of the proposed mechanisms and experimental evidence specifically targeting cholesterol-lowering effects remain controversial. Additionally, little information is available on the effective dosage of prebiotics needed to exert hypocholesterolemic effects (Ooi et al., 2010).

Although animal studies have demonstrated significantly lower blood glucose concentrations with prebiotics (Delzenne et al., 2007). In this study, GOS supplementation had no effect on glucose.

However, studies in humans have yielded conflicting data. Consumption of 20 grams/day of inulin-type fructans by healthy volunteers did not modify fasting plasma glucose and insulin concentrations after 4 weeks (Luo et al., 1996). Consumption of 8 grams/day of inulin-type fructans in non-insulin-dependent diabetic reported significantly lower blood glucose levels after 4 weeks. (Yamashita et al., 1994).

Another study examining the effects of chronic consumption of FOS by type 2 diabetics on plasma glucose showed that consumption of 20g FOS/d for 4 weeks had no effect on fasting plasma glucose or insulin levels (Luo et al., 2000).

In relation to the hematological parameters, similar data were observed for the values of the control group. The study results indicate that the GOS syrup produced by β-galactosidase from *Scopulariopsis* sp administered orally at a dose of 1000mg/kg does not produce any signs of toxicity or death in rats. Similar results

were reported by Anthony et al. (2005) when syrup GOS vivinal was administered to rats by gavage at 2500 or 5000mg/kg/bw/day for 90 days no adverse toxicological effects attributable to treatment.

The results of this study have established that the oral administration of GOS produced by β-galactosidase from *Scopulariopsis* sp was safe up to 1000 mg/kg, and the intake of different concentrations 300, 500, 1000 mg/kg of GOS was found to be safe and has no adverse effect on the evaluated parameters. However, the dosage level 1000mg/kg/bw/day not were considered sufficient for conventional acute toxicity test.

On the basis of all these results and under conditions in which the study was conducted was not possible to estimate the LD_{50} in rats after administration GOS oral. Pursuant to the provisions in the OECD, the substances that exhibit an LD_{50} greater than 5000 mg / kg by mouth may be considered practically non-toxic or unclassified.

Therefore, this suggests that further studies should be performed in vivo LD_{50} dose levels; to ensure that oral administration of GOS produced by β-galactosidase from *Scopulariopsis* sp does not induce any toxic effect, which can be the beginning of the safer use of this compound as food ingredient on the future.

5 – Conclusion

Comparing the effects physiological, biochemical analyses and the results of pathological examination, it was concluded that the syrup GOS obtained by the action of β-galactosidase derived from *Scopulariopsis* sp has no significant toxicity even at high doses in the experimental model used. However more studies are needed to confirm the safety of application of the GOS in human food products.

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Effects of Galacto-oligosaccharides (GOS) Supplementation on Intestinal Microbiota, Stool Characteristics and Bowel Function in Wistar Rats

Effects of Galacto-oligosaccharides (GOS) Supplementation on Intestinal Microbiota, Stool Characteristics and Bowel Function in Wistar Rats

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Abstract

Galacto-oligosaccharides (GOS) are non-digestible oligosaccharides that have many health benefits on human health by stimulating the growth and/or activity of *Bifidobacterium* in the colon. This study evaluated the effects of Galacto-oligosaccharides (GOS) produced by β-galactosidase from *Scopulariopsis* sp on the alteration of cecal microbiota, cecal pH, cecal short-chain fatty acids (SCFAs) concentration, stool characteristics, pH fecal and bowel function in Wistar rats. The rats were randomly assigned to 2 groups: control and supplemented with GOS (250 mg/(kg body wt d) for 42 days. After 42 day treatment, the rats supplemented with GOS presented higher humid, dried fecal weight and decreased the fecal pH, the feces contain no excess of undigested food (fat globules, starch granules and muscle fibers), protease activity was observed in the feces.The group that received supplementation of GOS presented a significant increase in the amount of *Bifidobacterium* and *Lactobacillus* ($p<0.05$), inhibited the growth of *E.coli*, and decreased the cecal pH. The SCFAs were significantly increased ($P<0.05$) by intake of GOS, indicating active fermentation. The potential physiological benefits of the GOS appear to relate to colonic health in terms of effects on bowel function, cecal microflora and SCFA metabolism. These results suggest that the GOS produced by β-galactosidase from *Scopulariopsis sp* have a great prebiotic potential and may be beneficial to gastrointestinal health.

Keywords: Galacto-loligosaccharides, Cecal microbiota, Trypsin fecal, Short-chain fatty acids (SCFAs), Rats.

Abbreviations: GI, gastrointestinal tract; FOS, fructooligosaccharides; GOS, galacto-oligosaccharides; SCFAs, Short-chain fatty acids; EPI, exocrine pancreatic insufficiency

1 - Introduction

The mammals animals gut is populated by an array of bacterial species, which develop important metabolic and immune functions, with a marked effect on the nutritional and health status of the host (Ley and others 2008).The metabolic activity developed by the gut microbiota contributes to the digestion of dietary compounds, salvage of energy, supply of micro-nutrients and transformation of xenobiotics (Laparra and others 2010).

A balanced gut microbiota composition confers benefits to the host, while microbial imbalances are associated with metabolic and immune-mediated disorders (Nadal and others 2007; Santacruz and others 2009). The development of the intestinal microflora provides the basis for a barrier that prevents pathogenic bacteria from invading the gastrointestinal tract. The composition of the intestinal microflora together with the gut immune system allows resident bacteria to exert a protective function. In addition gut bacteria are involved in vitamin synthesis (especially vitamins B and K) and in the metabolism of xenobiotics (Salminen and others 1988).

The composition of the gut microbiota is influenced by endogenous and environmental factors (diet, antibiotic intake, xenobiotics etc.). Of these factors, the diet is considered a major driver for changes in gut bacterial diversity that may affect its functional relationships with the host (Ley and others 2008).

Thus, modification of the flora by dietary means offers one of the most effective opportunities for development of functional foods (Salminen and others 1988). In this context, prebiotics has stood out for its beneficial effects on the function and microbiota composition.

Prebiotics are defined as “nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth of one or a limited number of bacterial species in the colon. Modification by prebiotics of the composition of the colonic microflora leads to the predominance of a few of the potentially health-promoting bacteria, especially, but not exclusively, lactobacillus and bifidobacterium (Gibson and Roberfroid 1995).

Researchers have suggested that these bacteria protect the host by competing with bacterial or fungal pathogens for available nutrients and space and modulating the immune system (Guarner and others 2003; Wagner 2008; Gaboriau-Routhiau and others 2009, Roberfroid and others 2010). In addition, it has been reported that some short chain fatty acids (SCFA) including acetic, propionic and butyric acids are released during the fermentation of the prebiotic. As a whole, SCFAs acidify the luminal pH which suppresses the growth of pathogens. They are rapidly absorbed by the colonic mucosa and contribute towards energy requirements of the host (Cummings 1991; Blaut 2002; Vogt and others 2003).

Prebiotics are found naturally in many foods, and can also be isolated from plants (e.g., chicory root) or synthesized (e.g., enzymatically, from sucrose) ” In order for

a food ingredient to be classified as a prebiotic, it has to be demonstrated, that it: (a) is not broken down in the stomach or absorbed in the GI tract, (b) is fermented by the gastrointestinal microflora; and (c) most importantly, selectively stimulates the growth and/or activity of intestinal bacteria associated with health and wellbeing (Roberfroid 2007).

Common prebiotics in use include inulin, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), soya-oligosaccharides, xylo-oligosaccharides, pyrodextrins, isomalto-oligosaccharides and lactulose (Macfarlane and others 2006; Macfarlane and others 2008). There is also a range of new prebiotic compounds emerging, and these include: pecticoligosaccharides, lactosucrose, the sugar alcohols, gluco-oligosaccharides, levans, resistant starch, xylosaccharides and soy-oligosaccharides (Hume and others 2011).

Of the many prebiotics that are available, the only ones for which sufficient data have been generated to allow consideration of their potential for classification as functional food ingredients are the inulin, fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) (Roberfroid 2007).

Galacto-oligosaccharides (GOS) are non-digestible carbohydrates, which are resistant to gastrointestinal digestive enzymes, but are fermented by specific colonic bacteria. They are classified as prebiotic food because they can selectively stimulate the growth of *Bifidobacterium* and *Lactobacillus* in the lower intestine (Sako and others 1999).

The health benefits of Galacto-oligosaccharides are the increased presence of good bacteria (*Bifidobacterium* and *Lactobacillus*, as a fermentative substrate and include protection against enteric infections; increased mineral absorption; immunomodulation for the prevention of allergies and gut inflammatory conditions; trophic effects of SCFAs on the colonic epithelium; preventing of diarrhea and constipation, and reduced toxigenic microbial metabolism that may reduce risk factors for colon cancer (Gibson and others 2004; Macfarlane and others 2008; Torres and others 2010).

GOS produced by transgalactosylation from lactose using β-galactosidase and are of great interest for food and feed applications because of their prebiotic properties (Maischeberger 2007). GOS have been extensively studied for their physiological roles on the intestinal flora and functions (Sako and others 1999).

The prebiotic concept is based on the selective stimulation of the host's own beneficial microbiota by providing specific substrate for their growth and metabolism. Today, the effect is measured by using *Bifidobacterium* or *Lactobacillus* as markers (Roberfroid and others 2010).

Therefore, the present study evaluated the effects of GOS produced by β-galactosidase from *Scopulariopsis* sp on the cecal content microbiota, cecal and fecal pH values, short-chain fatty acids (SCFA) concentrations, stool characteristics and bowel function in Wistar rats.

2 - Material and Methods

2.1 - GOS Production

The GOS mixtures obtained by the action of the β-galactosidase synthesized by *Scopulariopsis* sp were investigated for their effects on the intestine physiology of rats. The GOS were prepared with 40%(w/v) of lactose solution (0,1 M of acetate buffer, pH 5.0), temperatures 45℃, were added the enzyme concentration (10 $U.mL^{-1}$), reaction time was 12 hours of reaction. Under these conditions, the enzyme converted 20% of lactose into oligosaccharide (80.8 $mg.mL^{-1}$ de 4'galactosyl-lactose). The mixture of contain 22% monosaccharides, 50% lactose and 28% GOS (Santos and others 2009).

2.2 - Animals

Twenty-four male Wistar rats weighing approximately 380g±20g were obtained from the Center of Multidisciplinary investigation in Biology (CEMIB) of the University of Campinas (São Paulo, Brazil). Animals were housed individually in plastic boxes with temperatures (21-24ºC), humidity (55%) under a 12h light-dark cycle. All animals were given *ad libitum* to diet and water. The animal use protocol was reviewed and approved by Animal Experimental Ethics Committee of the University of Campinas (protocol n°1707-1).

2.3 - Experimental Design

Rats were assigned randomly in two groups (n = 12 per group), and they were fed with commercial diet (Purina Laboratory Animal Co., Ltd., Shanghai, China).

Control group were given gavage of maltodextrin (250mg/kg BW·d), and GOS groups were given gavage of (250mg/kg BW·d). The duration of the study was 42 d. During the experimental period, rats were weighted every day before the supplementation. At the end of the experimental period, the rats were anesthetized with ketamine/xylazine (100:10 mg/kg) and euthanized by cardiac puncture. After necropsy, cecum were removed and immediately processed for microbial counts and analysis of SCFA.

2.4 - Fecal Weight and Humidity

Feces were collected for 3 days (72-hour) consecutives beginning on 35, 36 and 37 days and stored in a freezer (-20℃). After the third day of collection, we obtained the wet weight of feces in electronic analytical balance (Metla Toledo, model AB204) with a sensitivity of 0.0001g. Next the feces were dried in an oven at 105 ºC. After 22 hours, weighings were begun at 30-minute intervals until two consecutive weights had differences of less than 1.0 mg. The fecal humidity was calculated using the formula [(humid fecal weight - dry fecal weight) / humid fecal weight] x 100 (Freitas and others 2004).

2.5 - Protease Activity in Feces

Two tests were performed with fresh feces (15 minutes of defecation) to assess the presence of pancreatic enzymes in the feces. The x-ray film digestion test and the gelatin digestion test. A fecal sample was obtained from each of the clinically normal rats (n=24) for to evaluate the fecal protease activity.

The x-ray Film Digestion Test: The x-ray film digestion test is performed by mixing one part feces with nine parts 5% sodium bicarbonate solution in a test tube. A strip of x- ray film, were placed on a Kodak X-OMAT scientific imaging film with gelatin coating. Hydrolysis of the gelatin on the X-ray film was determined after four hours of incubation at 37ºC in a humid incubator by washing the film with distilled water. At the end of the incubation period, the film is examined to detect digestion of the film coating. A positive reaction is indicated by a clear zone on the film after it has been rinsed with running water. If the coating has been digested, the feces are considered to be positive for protease activity. The assay was repeated three times.

The Gelatin Digestion Test: The gelatin digestion test is performed by mixing 1ml of the described feces-bicarbonate solution with 2ml of melted 7.5%gelatin and incubating this mixture for 1 hour at 37ºC. After the incubation period, the tube is allowed to cool and then is checked for solidification. If the gelatin remains liquid, the feces are considered to be positive for protease activity (proteases in the feces digested the gelatin).

2.6 - Microscopical Examination of the Feces

Fecal sample is also examined for the presence of undigested food components, fat globules, starch granules and muscle fibers. Test is performed by mixing one part feces with nine parts of water.

Fecal Starch: non-digestible starch in the feces is detected by staining feces with lugol solution. A small amount of feces (1-2 drops) is mixed with one to two drops of lugol solution (2% iodine solution) on the surface of a microscopic slide. A covers lip is placed over the mixture, which then is observed microscopically. Undigested starch appears as dark, blue to black granules. The presence of a 5 or more to blue granules in the feces per x 40 microscopic field is considered to be positive result.

Fecal Fat: This test is performed to detect non-digestible fecal fat. The test is performed by mixing a small amount of feces (1-2 drops) with one to two drops of Sudan III stain on the surface of a microscopic slide. A cover slip is placed over the mixture, and the slide then is examined microscopically. Undigested fat appears as large orange-red droplets. The presence of a 10 or larger, refractive, orange droplets per x 40 microscopic field is considered to be a positive result, thereby indicating a deficiency of fat digestion enzyme (lipase).

Fecal Muscle Fibers: The test is performed by mixing a small amount of feces (1-2 drops) with one to two drops of staining Lugol's iodine on the surface of a microscopic slide. A cover slip is placed over the mixture, and the slide then is examined microscopically. Undigested, striated muscle fibers appear as stain light brown. Undigested fibers have visible striations (40x) running both vertically and horizontally. Partially digested fibers exhibit striations in only one direction, and digested fibers have no visible striations. Only undigested fibers are counted, and

the presence of more than 10 is reported as increased. Five to 6 fields should be evaluated.

2.7 - Microbiological Analysis

The cecal contents were diluted 1:9 with distilled water. In duplicates, 0.05ml of each dilution was plated selective media. The specific agar plates used were as follows: plate count agar – PCA (Merck; total aerobes) Wilkins –chalgren agar (Difico; total anaerobes), Mac-Conkey *agar* (Merck; *Enterobacteriaceae*), eosine methylene blue agar (Oxoid, *Escherichia coli*), de Man, Rogosa and Sharp – MRS agar (Oxoid; *Lactobacillus*), and MRS agar (Oxoid; *Bifidobacterium*)

Enumeration of Bifidobacterium was determined using the method described by Moriya et al (2006). The Commercial MRS medium (Oxoid) was sterilized at 121ºC for 15min and was added the inhibitors and the aminoacid L-cysteine added. The inhibitors concentration in the final medium were: lithium chloride 3 mg/ mL, nalidixic acid 15 µ/mL, neomycin sulphate 100 µg/mL and paramomycin sulphate 200 µg/mL. The L-cysteine concentration was 0.5 g/mL in the final medium.

Plates were incubated at 37ºC aerobically or in anaerobic conditions (Anaerogen® - anaerobiosis generator from Oxoid), for 24 or 72 h as appropriate. After incubation, plates containing 20 to 200 colonies were enumerated, and the counts were expressed as log10cfu/g dry weight. The selectivity of the growth conditions was confirmed by microscopic examination.

2.8 - Determination of SCFAs

Concentrations of short-chain fatty acids in cecal content sample were measured in duplicate as described by Smirick-Tjardes et al. (2003) with modifications. cecal sample were acidified with 250 g/L metaphosphoric acid using a mixture of 0.5 g sample:0.8 mL acid:2.8 mL distilled H2O, precipitated at room temperature for 30 min, and then centrifuged at 25,900 × g for 20 min. The supernatant was decanted and frozen at −20℃ in microfuge tubes. After freez ing, the supernatant was thawed and centrifuged in microfuge tubes at 13,000×g for 10min. The supernatant was used in GC/MS analysis.

The SCFA concentration was analyzed with gas chromatography GC/MS (Agilent 5975C, inert MSD) equipped with a Nukol capillary columm (Supelco, 30m x 0.25 mm x 0.25µm film thickness). The injection was performed in the splitless mode for 1 min at 220℃. Oven temperature was programmed at 100℃ for 5 min, then increase to 200℃ at 8℃ min^{-1} and held for 1 min at the end temperature. Injector and detector temperature was 200℃; the carrier gas was nitrogen at a flow rate of 25 mL/min. Volatile free acid standard mix (Supelco4697-U) was used as internal standard. Short chain fatty acid concentrations were calculated by relative to the internal standard by the peak height ratio method. SCFA concentrations are expressed in units of µmol SCFA/g and all results given as the mean, standard errors (SEM).

2.9 - Fecal and Cecal pH

At the end of the experimental period, 1g of feces were freshly collected from each rat and homogenized with 10 mL of distilled water (Brambillasca and others 2010), using a vortex shaker (Vortex, model QL-901, Biomixer) and pH was immediately determined using a pH meter (Pack pH21). Cecal content pH was all determined in the undiluted cecal contents immediately after killing the animals.

2.10 - Statistical Analyses

Results were expressed as means with their Standard errors of the mean. Results were analysed by an unpaired Student's t test to determine significant differences between control and experimental group. P values of <0·05 were considered significant, using GaphPad Prism 5.0 version (GrapPad software, Inc.; La Jolla, CA, USA). All microbiological concentration was subjected to $\log^{10}$ transformation before analysis.

3 - Results

3.1 - Fecal Weight and Humidity

The total quantities of feces excreted during the three days (72-hour) after supplementation of GOS are summarized in Table 1. When productions of humid feces were compared, significant differences ($p<0.05$) were found between the groups. The group supplemented with GOS had greater production of humid feces than the control group.

Significant differences ($p<0.05$) were also found between groups when dry weight of feces was compared. The GOS group had greater dry weight of feces than the control group. The total quantity of feces (g), humid and dry, was significantly higher ($p<0.05$) in the group supplemented with GOS than the control group.

The feces humidity of the total three days (72-hour) revealed significant differences between groups ($p<0.05$). Rats supplemented with GOS had a significant increase in humidity of feces. Visually, differences in fecal volume are obvious between the groups (Table 1).

Table 1- Effect of GOS supplementation on the production of humid feces (g), dry feces (g) and humidity of feces (%) during the three days collection (72-hour).

Production of feces	Maltodextrin (n=12)	GOS (n=12)	p-value
Humid weight (g)	24.35±0.90[a]	36.57±1.36[b]	0.0042
Dry weight (g)	15.73±0.56[a]	19.49±0.44[b]	0.0039
*Humidity (%)	31.66±3.04[a]	43.29±1.85[b]	0.0038

Values are mean (SEM). Within rows, values with different letters indicates significant differences (p<0.05) between groups.
*The humidity was calculated using the formula [(humid fecal weight - dry fecal weight)/wet fecal weight] X 100.

3.2 - Protease Activity in Feces

Protease activity could be detected using the X-ray film in all rats the control group and supplemented with GOS. Results similar were observed with gelatin digestion test. The feces were considered to be positive in both groups for protease activity. Moreover, not differences were observed in protease activity in the two tests has were performed with fresh feces.

3.3 – Non-digestible/Non-absorbed Food Particles

Starch was usually present in small quantities and often visible macroscopically when stained with Lugol iodine. Undigested Starch ranged from none to five per low powered field. No Statistically significant differences (p<0.05) were found between groups (Table 2).

Table 2- Undigested food components (fat globules, starch granules and muscle fibers) in fecal sample in the rats supplemented with GOS for 42 consecutives day.

Food components	Maltodextrin (n=12)	GOS (n=12)	*p*-value
Starch granules	4.63±0.47	4.52±0.32	0.0034
Fat globules	3.45±1.45	4.69±1.15	0.0062
Muscle fibers	1.58±0.56	1.32±0.62	0.0022

Values are mean (SEM) There were no significant differences (p>0.05) between groups. Maltodextrin, GOS.

The presence of small numbers of neutral fat were observed macroscopically when stained with Sudan III, usually contained at least 8 neutral fat droplets with diameters greater than 20 microns per high powered field. Results were similar in two groups.

Undigested muscle fibers ranged from none to four per low powered field in both groups, generally, microscopic findings for undigested /unabsorbed food particles were similar in control groups and supplemented with GOS. No Statistically significant differences (p<0.05) were found for undigested /unabsorbed food particles between groups.

3.4 - Microbiological Analysis

According to the microbiological analyses statistically significant differences (p<0.05) were found in the bacteria population between groups. Compared with control group, the GOS supplementation significantly increased (p<0.05) the population of *Bifidobacterium* (Table 3). The cecal *Bifidobacterium* populations were inversely associated with cecal pH (p<0.05). The cecal population of

Lactobacillus was higher in the group supplemented with GOS. The concentration of total anaerobes was significantly higher (p<0.05) in animals supplemented with GOS when compared to animals supplemented whit maltodextrin.

However, the number of cecal *Enterobacteriaceae* and *E. coli* were significantly reduced (p<0.05) by oral administration of GOS. The total aerobes in the cecum were not significantly affected (p>0.05) after the supplementation of GOS. The total aerobes were similar in the animals the two groups (Table 3).

Table 3- Microbiota population of the cecum contents of rats in the control and GOS group.

	Maltodextrin (n = 12)	**GOS group (n = 12)**
***Cecal microbiota**		
Total anaerobes	8.1±0.22[a]	10.8±0.26[b]
Bifidobacterium	8.2±0.33[a]	10.0±0.27[b]
Lactobacillus	7.2±0.15[a]	8.9±0.16[b]
Total aerobes	7.6±0.22[a]	7.2±0.25±[a]
Enterobacteriacea	8.9±0.14[a]	7.2±0.16[b]
Escherichia coli	8.9±0.26[a]	6.2±0.34[b]

* Values are mean log colony-forming units (CFU)/g of cecal content, n = 12.
[a,b]Within rows, values with different letters indicates significant differences (p<0.05) between groups.

3.5 - Cecal Concentration of SCFAs

Significant differences were detectable in the cecal concentration of SCFAs between control rats and rats supplemented with GOS. The supplementation of GOS resulted in higher (p<0.05) cecal acetate, propionate and butyrate concentrations compared with group control.

The cecal SCFA pool, expressed as µmol/cecum, was altered by supplementation of GOS. The supplementation of GOS resulted in higher (p< 0.05) cecal acetate, propionate and butyrate concentrations compared with maltodextrin. The maltodextrin substrate produced much less acetate (approximately 12.93 *µmol*) compared to the GOS (30.25*µmol*). GOS resulted in higher (p<0.05) cecal propionate concentrations (16.27*µmol*) compared with control (4.07 *µmol*). Cecal concentrations of butyrate were higher (p< 0.05) for rats supplemented with GOS (8.05*µmol*) compared with maltodextrin (2.97*µmol).* Total SCFA production was greatest (p<0.05) for the GOS compared with control (Table 4).

Table 4 - Means of short-chain fatty acids (SCFA) in cecal contents of rats supplemented with GOS.

Cecal SCFA	**Maltodextrin** (n = 12)	**GOS** (n = 12)	***p*-value**
	Cecum (µmol/g of content)		
Acetate	12.93±4.66[a]	30.25±1.55[b]	$p < 0.001$
Propionate	4.07±0.33[a]	16.27±1.20[b]	$p < 0.022$
Butyrate	2.97±0.57[a]	8.05±1.27[b]	$p < 0.005$
*Total SCFAs	19.97± 1.20[a]	54.57± 1.31[b]	$p < 0.001$

Values are means ± SEM, *n* =12. Results are expressed as µmol SCFA/g
*Total SCFA = acetate + propionate + butyrate
Means within a rows with no common superscript different significantly ($p<0.05$)

3.6 - Fecal and Cecal pH

In the rats supplemented with GOS we observed significantly lower cecal and fecal pH values (Figure1) compared with rats control. However, comparing fecal and cecal pH these differences were not significant for two groups.

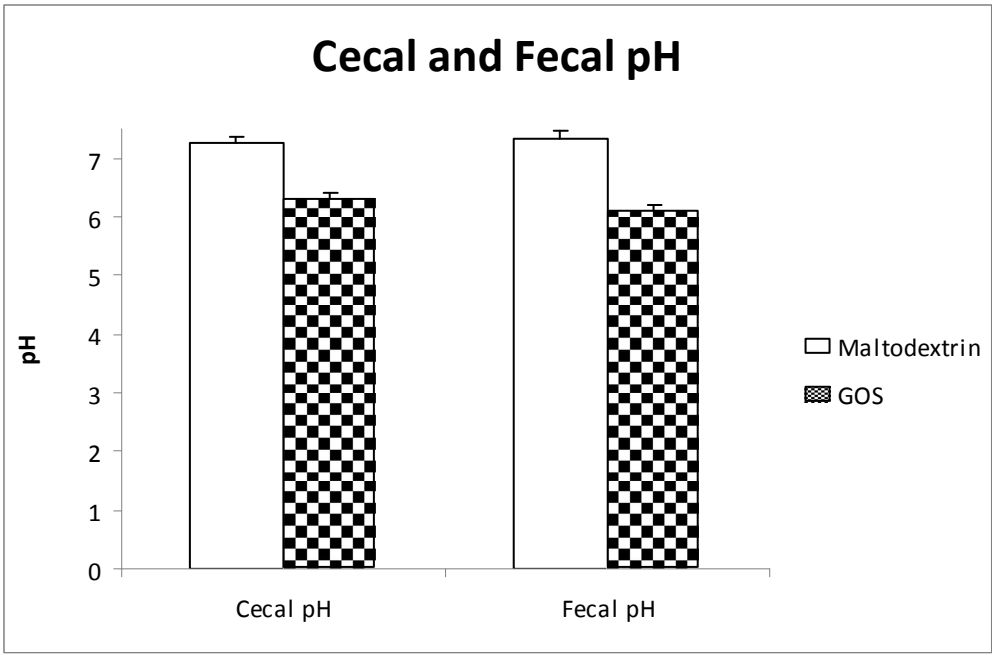

Figure 1 - Fecal and cecal pH of rats supplemented with GOS or maltodextrin Values are means, ± SEM, n = 12. *p< 0.05

4 - Discussion

The supplementation with prebiotic GOS produced by β-galactosidase from *Scopulariopsis* sp may positively modulate gut microbiota composition, resulting in higher humid and dried fecal weight and caused higher fecal humidity than did maltodextrin, probably due to greater fermentation.

The prebiotic reaches the large intestine completely intact, is fermented by the gastrointestinal microflora; selectively stimulating the growth of *Bifidubacterium* and *Lactobacillus* in the lower intestine. (Tuohy and others 2005). This bacterial growth leads directly to an increase in microbial biomass in the colon, resulting in increase in fecal weight (Cummings and others 1997).

Undigested oligosaccharides and fermentation products may also produce an osmotic effect in the gut, which increases the water content of feces (Niittynen and others 2007). This increase in fecal bulk stimulates passage through the colon, shortening transit time. Colonic water resorption is reduced, stool becomes softer and heavier, and stool frequency increases. Together these factors alleviate constipation and improve colon evacuation (Swennen and others 2006; Rose and others 2007).

However, the extent of the effect depends on the chemical and physical nature of the oligosaccharides and the extent to which they are fermented in the colon. Fermentable oligosaccharides stimulate increases in microbial biomass in the colon, resulting in some increase in fecal weight (Cummings and others 1997).

All rats supplemented with GOS, produced feces of similar appearance. The stools were bulky, green-brown, well formed but soft. They contained little starch, occasional undigested muscle fiber and moderate amounts of fat. The feces were considered to be positive in for protease activity. Excess undigested or unreabsorbed material then appear in the feces, may provide indirect evidence of maldigestion and malabsorption (Strasinger and Di Lorenzo 2008).

Malabsorption is defined as impaired absorption of nutrients and maldigestion is a consequence of impaired digestion of nutrients in the intestinal lumen, or at the terminal digestive site of the brush-border membrane of mucosal epithelial cells. It can occur because of congenital or acquired disease in which pancreatic enzyme activity, bile acid concentration, or small intestinal mucosal enzymes are decreased or absent (Dressman and Lennernäs, 2000). Interference with food digestion in small animals is typically due to exocrine pancreatic insufficiency (EPI), whereas most cases of absorption failure are caused by small intestinal disease.

The presence of excess undigested starch is suggestive of a deficiency in starch-digesting enzyme or a increase intestinal transit time. Starch-digesting enzymes (i.e., α-amylase) are produced by the pancreas, and deficiency in these enzymes is suggestive of EPI (Thrall and others 2004).

Excess of undigested fat in feces is suggestive of steatorrhea caused by neutral fats (triglycerides), thereby indicating a deficiency of a fat-digesting enzymes (i.e., lipases). Detection of steatorrhea is useful for the diagnosis of EPI and small bowel

disorders that cause malabsorption. Absence of bile salts that assist pancreatic lipase in the breakdown and subsequent reabsorption of triglycerides produces an increase in stool fat (steatorrhea). Normal feces have few if any neutral fat droplets evident (Thrall and others 2004).

Muscle fibers in the feces are suggestive of defective digestion of these fibers, most likely resulting from EPI (e.g., inadequate fecal protease activity). Muscle fibers also can be present in the feces because of increased intestinal motility and subsequente decrease in intestinal transit time (Thrall and others 2004).

This test is designed to detect proteolytic activity in fresh stool samples, presumably of pancreatic origin. The test evaluates the ability of a fresh fecal sample to digest off the gelatin coating of undeveloped radiographic film or to digest varying dilutions of gelatin in test tubes. Unfortunately, a significant number of both false positive (proteolytic activity is present) and false negative reactions (no proteolytic activity is present) occur (Hardy 2010).

False positive results can occur due to proteolytic activity present in normal fecal bacteria, and false negative secondary to bacterial degradation of pancreatic enzymes and having long delays between collection of the sample and performance of the test. Because of the non-specificity of the stool analysis tests, a number of other tests have been developed to lend better diagnostic sensitivity and specificity to the diagnosis of EPI (Hardy, 2010).

The cecal and fecal pH was significantly reduced with supplementation of GOS when compared to control. The acidic cecal and fecal pH resulting from ingestion of the oligosaccharide diets probably is caused by the greater level of total SCFA production (Campbell and others 1997). A low intestinal pH is good for intestinal health, because the growth of many known pathogens is inhibited under such conditions (Gibson and others 1994; Gibson and others 1995; Asahara and others 2001).

This study shows that supplementation with GOS resulted in significant increases in cecal *Bifidobacterium* and l*actobacillus* concentration, indicating that the microbiota of the large intestine were capable of completely fermenting GOS. *Bifidobacterium*, together with *lactobacillus*, has an important function in the eco-physiology of the colonic microbiota. These organisms have been linked to increased resistance to infection and diarrhoeal disease (Drakoularakou and others 2009).

The bifidogenic properties of GOS were demonstrated in various animal and human study (Rowland and Tanaka, 1993; Moro and others 2002; Drakoularakou and others 2010). Davis and others (2010) studied the effects of GOS in healthy adults reported significant increases in *Bifidobacterium.* Other research has obtained similar results, elderly supplemented with GOS 5.5 g/d of GOS for 10 wk, significantly increased the numbers of beneficial bacteria, especially *Bifidobacterium* (Vulevic and others 2008).

The addition of GOS (5g/L) to a follow-on formula positively influences the *Bifidobacterium* flora and the stool consistency in infants during the supplementation period at weaning (Fanaro and others 2009). Depeint and others (2008) showed that 7g/d of the GOS significantly increased the *Bifidobacterium* populationan in healthy human. Smiricky-Tjardes and others (2003) demonstrated the effect of dietary with GOS on ileal and fecal bacterial communities in growing pigs. The authors found a significant increase in fecal *Bifidobacterium* and *Lactobacillus* for animals fed diets containing GOS.

Rats fed diet containing s (4g kg-1days) increased the colonic levels of *Bifidobacterium (*Holma et al., 2002). Similar results were observed in vitro gut model, the prebiotic capacity of the GOS was further investigated in a pig feeding trial, a significant increased the density of *Bifidobacterium* were observed.(Tzortzis and others 2005). However, recent studies also suggest that the bifidogenic effect of GOS is not only dose dependant, but relies on undefined host factors (Davis and others 2010).

GOS are non-digestible carbohydrates, which are resistant to gastrointestinal digestive enzymes, in the colon; they are fermented by intestinal bacteria. This bacterial fermentation in the colon leads to the production of short chain fatty acids (SCFA), mainly acetate, propionate, butyrate, and lactate (Anderson and others 1984; May and others 1994).

The GOS tested in this experiment increased cecal concentration of SCFA (acetate, propionate and butyrate). Similar results were reported by Pan and others (2009) showed that the after 14 d. treatment, SCFA in mice cecum were significantly increased (p<0.05) by intake oligosaccharides, especially FOS and GOS.

The present study demonstrated the cecal SCFA concentration in the rats supplemented with GOS are in the order from: acetate >propionate> butyrate (30.25; 16.27 and 8.05 *μmol*) and control group: acetate >propionate> butyrate (12.93; 4.07 and 2.97 *μmol*) respectively. Survey data from various populations show that fecal SCFA are in the order predicted from that equation, i.e., acetate > propionate ≥ butyrate (Hoverstad 1984; Cummings 1991).

The SCFAformed at fermentation is quantitatively and qualitatively influenced by the type and amount of carbohydrate substrate. Further, certain combinations of carbohydrates may have synergistic effects on the SCFA pattern and may also shift the site of fermentation (Henningsson and others 2001).

The SCFA contribute to normal large bowel function and prevent pathology through their actions in the lumen and on the colonic musculature and vasculature and through their metabolism by colonocytes (David and others 2001).

Production of SCFAs is one of the most important physiological actions mediated by the microbiota (Cummings 1995). A great proportion (80-85%) of these SCFA

is absorbed by the mucosa of the colon and the other part is either evacuated in the feces or is used for growth and multiplication of bacteria (Guerin-Deremaux and others 2010).

Another benefit of SCFAs is that they increase growth of intestinal epithelial cells and control their proliferation and differentiation (Guarner 2003). Acetate and propionate provide energy for brain, muscle and heart, while butyrate provides about 50% of the daily energy requirements of the gastrointestinal mucosa (Macfarlane and Gibson 1997; Roediger 1989).

The butyrate was produced in a higher concentration when rats were supplemented with GOS (8,05 *µmol)* compared with control (2,97 *µmol).* Butyrate, however, is a fatty acid of particular interest since is known to be the preferred energy substrate for colonocytes. In addition to the trophic effect of butyrate on the mucosa, it is an important source of energy for the colonic epithelium and can regulate both the growth and the differentiation of colonic cells (Williams and others 2003; Rafter and others 2004; Tuohy and others 2005).

Other researchers have demonstrated the trophic effects of SCFA on the intestinal epithelium in rats (Sakata, 19887; Koruda et al. 1988; Kripke et al. 1989). A systemic mediatory mechanism that transmits the stimuli of SCFA to the epithelial cells is proposed for the trophic effect *in vivo* (Sakata 19887).

In humans, the results of the studies examining the effects of GOS on the concentration of SCFAs in the feces are contradictory (van Dokkum and others 1999). It should be noted that the increase in the production of SCFAs in the colon is difficult to determine in humans, because SCFAs are rapidly absorbed in the gut, and thus the amount of SCFAs in the feces does not necessarily correspond to their intracolonic production (Bouhnik and others 1997; Alles and others 1999).

Analysis of intestinal contents and feces for SCFA concentration may not be a good indicator of production since less than 5% of the bacterially derived SCFA appears in feces due to efficient colonic uptake (Smiricky-Tjardes and others 2003).

The data indicate that GOS produced by β-galactosidase from *Scopulariopsis* sp was fermented by intestinal bacteria, significantly increased the *Bifidobacterium* and *Lactobacillus* population, increased SCFA production in a lower pH, produces health benefits related to their interactions with the GI, establishes this prebiotic as an attractive ingredient for foods and dietary supplements.

5 - Conclusion

In conclusion, the GOS produced by β-galactosidase from *Scopulariopsis* sp show a great prebiotic potential as indicated by increased SCFA production and positive microbial modifications in rats. These physiological effects in conjunction with the physicochemical characteristics enable GOS to be promising prebiotic food ingredients. However, further studies of the effects of the GOS on intestinal microflora composition and the concentrations of SCFA are necessary to sustain their beneficial health effects.

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Effects of Galacto-oligosaccharides (GOS) and Fructo-oligosaccharides (FOS) Supplementation on Growth Performance, Cecal Content short-chain fatty acids (SCFAs) Concentration and Gut Morphology in Male Adult Rats

Effects of Galacto-oligosaccharides (GOS) and Fructo-oligosaccharides (FOS) Supplementation on Growth Performance, Cecal Content short-chain fatty acids (SCFAs) Concentration and Gut Morphology in Male Adult Rats

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Abbreviations: SCFA, short-chain fatty acids; FOS, fructooligosaccharides; GOS, galactooligosaccharídes; NDO, non-digestible oligosaccharides BW, body weight; VH, villus height; CD crypt depth, H.E, hematoxiline-eosin.

Abstract

Galacto-oligosaccharides (GOS) and Fructo-oligosacharides are nondigestible oligosaccharides confer beneficial health effects on animals and humans. The purpose of this study was to evaluate the effects of FOS and GOS produced by β-galactosidase from *Scopulariopsis* sp on growth performance, cecal short-chain fatty acids, pH and small intestinal mucosal morphology in Wistar rats. The study was carried out on 36 wistar adult male rats, weighing 380 - 420g, were allocated equally to three groups (n=12). Rats were respectively give gavage of Fructo-oligossacharides (FOS) and galacto-oligosaccharides (GOS) (250mg/Kg/day) for 42 days. Food intake and body weight was determined daily during experimental period. At the end experimental period, cecal contents obtained post-mortem were collected, pH measured and processed for SCFA analysis and small intestine were collected for analysis Morphometric. In the present study, no significant differences (p>0.05) among experimental groups and control group in average weight, weight gain and food intake. The supplementation of GOS and FOS resulted in higher cecal butyrate concentrations compared with the control. The cecal SCFA were higher while pH was lower (p<0.05) in groups supplemented whit oligosaccharide (FOS and GOS). The supplementation with prebiotics increased (p<0.05) villus height, villus height/crypt depth ratio and decreased crypt depth in jejunum and ileum. The Oligossacharides FOS and GOS were fermented, increased concentration of SCFA, low pH and exert trophic effects in the small intestine. The FOS and GOS exert protective effects in the colon.

Keywords: Galacto-oligossacharides, Fructo-oligosaccharides, Short-Chain Fatty Acid, Intestinal Morfometric, Rats.

1 - Introduction

Prebiotic is defined as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" [1]. Modification the composition of the colonic microflora by prebiotics leads to the predominance of a few of the potentially health-promoting bacteria, especially, but not exclusively, lactobacillus and bifidobacteria [1].

The major fermentation products of prebiotic metabolism in large bowel are short-chain fatty acids (SCFA), which had different effects on colon morphology and function such as supply of energy to the intestinal mucosa, lowering of the pH, and stimulation of sodium and water absorption [2,3,4].

Fructooligosaccharides (FOS) and galactooligosaccharides (GOS) are classified as non-digestible oligosaccharides (NDOs), have been demonstrated to possess prebiotic activity in human [5,6,7,8].

FOS is short-chain polymers of b 1–2-linked fructose units, which are produced commercially by hydrolysis of inulin or by enzymatic synthesis from sucrose. They are not hydrolyzed in the human small intestine, but degraded in the colon by the resident microflora [9]. They are mainly known for their ability to increase the endogenous growth of intestinal lactobacilli and bifidobacteria in humans and animals, which has long been regarded as beneficial to health [9, 10].

GOS belong to the group of NDO, containing two to five molecules of galactose and one molecule of glucose connected through glycosidic bonds; they are produced commercially from lactose, which is abundant in cheese whey, by

enzymatic transgalactosylation with ß-galactosidase present in either free or immobilized form [11].

They are classified as prebiotic food because they are completely soluble and are fermented by specific bacteria present in the colon, resulting in the production of SCFA [12].

The modulation of the composition and metabolic activity of the intestinal microbiota through dietary interventions has aroused interest in the use of components that can beneficially on physiology intestinal [13].

Several authors have reported that prebiotics exert important trophic effects on the intestinal epithelium, stimulating the proliferation and differentiation of colonic epithelial cells [14, 15]. These studies opened up a whole new field of investigation in relation to gastrointestinal physiology and prebiotics.

We hypothesized that GOS produced by β-galactosidase from *Scopulariopsis* sp can act beneficially on the intestine physiology. Therefore, an experiment was carried out to investigate the effects of GOS and FOS supplementation on growth performance, cecal short-chain fatty acids, pH and intestinal morfometric in rats.

2 - Materials and Methods

2.1 - Composition of the GOS

The GOS syrup was prepared by reaction a high concentration of lactose (40% wt/vol) with a β-galactosidase enzyme for 24 h at 37℃. The enzyme was produced from *Scopulariopsis* sp isolated by Pastore and Park [16], deposited at the American Type Culture Collection (ATCC), accession number 44206.

The GOS syrup was analyzed by HPLC as described previously [17]. This fraction contained mono-, di-, tri- and tetrasaccharides as follows (%w/w): 50% lactose, 15% glucose, and 7% galactose. In this fraction, GOS per se, i.e., except glucose, galactose and lactose represent 28% by weight.

The commercial FOS used in the experiment was Raftilose® P95 (Orafti-Active Food International, Tienen, Bélgica), a product provided by Clariant S.A., São Paulo, contains 95% oligofructose with a degree of polymerization (DP) 2~7 and 5% of glucose, fructose and sucrose.

2.2 - Animals and Treatments

These experiments were conducted on 36 Wistar adult male rats, weighing 380 - 420g, (3 - 4 mo of age) were obtained from the Vivarium Center- Campinas State University (CEMIB/ UNICAMP). The rats were randomized in 3 groups (n=12 per group) a control group (no oligosaccharide), FOS group and GOS group (250mg/kg/day), respectively. Rats were housed in plastic cages in a temperature

controlled room with a 12h light/dark cycle. They were allowed *ad libitum* to commercial rat diet Labina® (Purina do Brazil Ltda. Ribeirão –Preto) and water.

The supplement was administered orally (oral gavage) for a period of 42 consecutive days. Food intake and body weight was determined daily during experimental period. All procedures involving animals were reviewed and approved by the Animal Experimentation Ethics Committee (CEEA-IB-UNICAMP) at the University of Campinas (Brazil, SP), under the protocol number: 1707-1.

2.3 - Experimental Design

The ration consumption (food intake) and the body weight were recorded daily. The feed conversion ratio was calculated by the correlation between the weight gain and food intake at the end of the experimental period.

At the termination of the experiment, the rats were anaesthetised with subcutaneous injection of ketamine-xylazine-acepromazine cocktail (1.4 ml/kg body wt) and killed by exsanguination through a cardiac puncture. After laparotomy, the cecum and colon with contents was removed. The Cecal contents were collected, pH measured, and a 0.5g aliquot immediately processed for SCFA analysis.

The intestine was removed washed with sterile 0.9% (w/v) NaCl, and immersed in a fixing solution (formol 5%) for 24h for subsequent histological examination. The organs (thymus, spleen and liver) were excised and weighed (Bel Mark 210A Model analytical balance, Capacity: 100 mg to 210g).

2.4 - Cecal SCFAs Analysis

Cecal content SCFA concentrations were determined in duplicate using the method described by of Smirick-Tjardes et al. (2003) with modification. Cecal contents were acidified with 250 g/L metaphosphoric acid using a mixture of 0.4 g sample:0.8 mL acid:2.8 mL distilled H2O and centrifuged at 25,900 1 g for 20 min. 2), precipitated at room temperature for 30 min, and then centrifuged at 25,900 × g for 20 min. The supernatant was decanted and frozen at −20℃ in microfuge tubes. After freezing, the supernatant was thawed and centrifuged in microfuge tubes at 13,000 × g for 10 min. The supernatant was used in GC/MS analysis.

The cecal content digesta was analysed for SCFA concentration with the gas chromatography GC/MS (Agilent 5975C, inert MSD) equipped with a Nukol capillary columm (Supelco, 30m x 0.25mm x 0.25µm film thickness). The injection was performed in the splitless mode for 1 min at 220℃. Oven temperature was programmed at 100℃ for 5 min, then increase to 200 ℃ at 8℃.min $^{-1}$ and held for 1 min at the final temperature. Injector and detector temperature was 200℃; the carrier gas was nitrogen at a flow rate of 25 mL/min. Volatile free acid standard mix (Supelco4697-U) was used as internal standard. Cecal SCFA pool size was calculated as the product of SCFA concentration in cecal digesta mass.

2.5 - Histological Analyses

The histological cuts (duodenum, jejunum and ileum) were dehydrated in a series of crescent concentrations of alcohol, diafinized in xylol, and immersed into paraffin. Cuts of 5 µm thickness were obtained. The slides obtained were stained with hematoxiline-eosin (H.E). Three cross-sections for each intestinal sample

(total of 24 samples for each of the 3 intestinal segments per dietary treatment) were prepared after staining with H.E [19]. A total of 10 intact crypt-villus units were selected in triplicate for each intestinal cross-section (30 measurements for each sample).

2.6 - Morphometric Analyses

The slides were evaluated by light microscopy (Carl Zeiss, Germany). The image of the material analyzed was magnified using 40X objective lens. Images were captured by a camera (Sony) coupled to the microscope and connected to an image analyzer and measured using the ZoomBrowser EX software, ten villus and crypts were scored for each rat, and means calculated were used in the statistical analysis.

After capturing the images, morphological indices were determined using an image Tool Software. The measurements were taken in millimeters (mm). The variables studied in the small intestinal mucosa through the morphometric analysis were: height of the villus (VH), depth of the crypts (DC) and villus/crypt ratio (VH:DC). Villus height was measured from the tip of the villus to the villus-crypt junction; crypt depth was defined as the depth of the invagination between adjacent villi [19]. The results expressed mean ± SD

2.7 - Statistical Analyses

One-way ANOVA was used to study differences in Weight gain, feed intake, organ weight and structure of the small intestine. The differences were compared by Tukey multiple comparison test (parametric data). All data are expressed as means and standart deviation (SD), and the level of statistical significance was 5% (p<0.05). The data were processed and analyzed using the GRAPHPAD PRISM 5.0 software.

3 - Results

3.1 - Growth Performance and Absolute Weight of Organs

Body weight (BW) body weight gain, feed intake and feed conversion ratio of rats the control group and supplemented with prebiotics are presented in Table 1. The initial BW of rats did not differ (p>0.05) between the groups supplemented with prebiotics (GOS and FOS) and control group. At the end of the experiment weight gain the rats supplemented with prebiotics was similar (ranged ~ 110.53 to 120.64g) compared with controls. Moreover, there was no statistically significant difference (p>0.05) in weight gain between rats supplemented with prebiotics.

The statistical analysis revealed that the food intake non-significant differences between different groups (p>0.05) supplemented whit prebiotics and control. The food intake were similar between groups supplemented with prebiotics, the food intake ranged ~10–12 g/d No statistical significance differences were observed in Feed conversion rate (FCR) in groups supplemented with prebiotics (0.25) and control group (0.26). FCR was similar for rats supplemented

with prebiotic (0.25). Rats remained healthy and no mortality was was recorded over experimental period.

The means of the absolute weights of after 42 days supplementation with prebiotics are presented in Table 1. There was no significant difference (p>0.05) in the absolute weights of organs (thymus, spleen, and liver) between the control groups and supplemented with prebiotics. In addition, no significant difference was observed on the absolute weights of collected organs from the different groups of animals supplemented with prebiotics (Table 1).

Table 1- Growth performance and absolute weight of organs of Wistar albino's rats supplemented with prebiotics for 42 days.

Performance parameters	Control (Maltodextrin)	Prebiotic GOS	Prebiotic FOS	*p*-values
Initial body weight (g)	388.80±18.45	392.60±12.45	408.20±20.50	**0.053**
Body weight gain (g)	112.44±10.39	117.36±15.56	110.2±12.40	**0.065**
Total Food intake (g)	420.40±0.21	426.50±0.32	430.20±0.15	**0.054**
[1]Feed conversion ratio	0.26±0.04	0.25±0.05	0.25±0.12	**0.632**
[2] Weight organs (g)				
Thymus	33.80±2.60	33.20± 3.20	30.10±2.30	**0.523**
Spleen	74.60±3.10	73.10±1.40	70.50±2.70	**0.852**
Liver	148.20±1.20	140.30±1.80	146.40±1.50	**0.837**

Values are expressed as Mean ± SD (n = 12). Groups did not differ, p>0.05.
[1] Feed conversion ratio = weight gain (g)/food intake (g).
[2] Absolute weights of organs (g)

3.2 - Cecal Short-Chain Fatty Acid Concentrations, Molar Ratios and Cecal pH

The supplementation of prebiotics resulted in higher (p<0.05) cecal concentrations of acetate, propionate, butyrate compared with the control (Table 3). Total cecal SCFA pools were higher (p<0.05) from supplementation of GOS and FOS compared with control. The cecal SCFA (acetate, propionate and butyrate) were higher (p< 0.05) in rats supplemented with FOS compared with the control and GOS.

The supplementation with FOS resulted in higher (p<0.05) cecal butyrate concentrations compared with GOS treatment. The concentration of butyrate from 14.3 ± 3.7 µmol/g cecal content in the FOS group to 27.9 ± 12.6 µmol/g in the rats suplemented wiht GOS (p<0.01). Total cecal SCFA were dramatically higher (p<0.05) as a result of FOS consumption, which GOS consumption.

The cecal SCFA concentration in the rats supplemented with FOS and GOS are in the order from: acetate, propionate and butyrate respectively. The molar ratio (%) of acetate, propionate and butyrate were 60.33; 24.42 and 15.25, respectively in cecum of rats supplemented with FOS and 62.57; 20.80 and 16.63, respectively in rats supplemented with GOS. The supplementations of GOS induce such a high molar ratio of butyrate when compared with FOS.

Cecal pH was lower (p<0.05) in rats supplemented GOS and FOS com pared with control. Nevertheless, the cecal ph values were similar between FOS and GOS.

Table 2 - Short-chain fatty acids (SCFA) concentration in cecal rats supplemented with prebiotics[3-1.]

Item	Treatments			
Cecal SCFA (µmol/g cecal content)	**Control (Maltodextrin)**	**FOS**	**GOS**	***p*-value**
Acetato	15.22±2.69[a]	52.08±10.10[b]	36.97±7.93[c]	$p< 0.001$
Propionato	4.02±1.05[a]	19.67±5.78[b]	12.29±2.73[c]	$p< 0.005$
Butyrate	2.65±2.39[a]	14.58±3.45[b]	9.82±3.62[c]	$p< 0.005$
Total SCFA	21.89±11.10[a]	86.33±21.20[b]	59.08±16.50[c]	$p< 0.001$
Cecal pH	7.40[a]	6.10[b]	6.20[b]	$p< 0.001$

Values are mean ± SD (n=12)
FOS, fructo-oligosaccharides; GOS, Galacto-oligossacharides.
[abc] Means in the same row not sharing superscript letters differ ($p<0.05$).
SCFA = short chain fatty acids
*Total SCFA= acetate+propionate+butyrate.

3.3 - Intestinal Morphometric Parameters of Duodenum, Jejunum and Ileum.

Morphological measurements of the duodenum, jejunum and ileum of rats are apresented in Table 3. No significant differences ($p>0.05$) were observed in the villus height, crypt depth, and villus height:/crypt depth (VH:CD) ratio between the group's suplemented wiht prebiotics (FOS and GOS) compared with control group. Moreover, results were similar between the groups supplemented with prebiotics.

In the Jejunum the villus height was significantly higher ($p>0.05$) and crypt depths were significantly lower ($p>0.05$) for the rats supplemented whit prebiotics (FOS and GOS) compared with control (Table 3). No significant differences ($p>0.05$) were observed in the villus height, crypt depth between groups supplemented with prebiotics. The VH:CD depth ratio was significantly higher

(p<0.05) for groups of animals supplemented with prebiotics. However, the differences in VH:CD ratio were not significant between groups supplemented with prebiotics.

The Villus height and VH:CD ratio at the ileum were significantly higher (p<0.05) in the rats supplemented with prebiotic when compared with rats of control group. The crypt depths at the ileum were significantly lower for the rats suplemented with prebiotics compared with control group (p<0.05). The villus height, crypt depth and VH:CD ratio was similar (p>0.05) for both groups of animals supplemented with prebiotics (Table 3).

Table 3- Effects of FOS and GOS on the morphology of the intestinal mucosa in the duodenum, jejunum and ileum.

	Groups			
Duodenum	**Control (Maltodextrin)** (250mg/kg)	**FOS** (250mg/kg)	**GOS** (250mg/kg)	***p*- value**
Villus height (mm)	3.30 ± 0.49[a]	3.32 ± 0.57[a]	3.21±0.61[a]	0.822
Crypt depth (mm)	1.52 ± 0.24[a]	1.56 ± 0.26[a]	1.58 ± 0.41[a]	0.853
VH : CD ratio	2.25±0.18[a]	2.20±0.29[a]	2.22±0.25[a]	0.836
Jejunum				
Villus height (mm)	3.28 ± 0.50[b]	5.08 ± 0.69[a]	5.16 ±0,69[a]	0.032
Crypt depth (mm)	1.43 ± 0.26[a]	2.45 ± 0.34[b]	2.32 ± 0,22[b]	0.030
VH : CD ratio	1.38±0.18[b]	2.32±0.32[a]	2.26±0,34[a]	0.026
Ileum				
Villus height (mm)	3.28 ± 0.50[b]	5.08 ± 0.69[a]	5.16 ±0,69[a]	0.032
Crypt depth (mm)	1.43 ± 0.26[a]	2.45 ± 0.34[b]	2.32 ± 0,22[b]	0.030
VH : CD ratio	1.38±0.18[b]	2.32±0.32[a]	2.26±0,34[a]	0.026

Results are expressed as Mean ± SD (n= 12)
[a–b]Means within a row with different letters differ significantly ($p< 0.05$).
*Count in area equivalent to 10 villosities (in triplicate)
VH:CD = villus height/crypt depth

4 - Discussion

There has been a growing interest in the nutritional and physicochemical properties of the Prebiotics. One reason for that interest is the recent development of the industrial production in order to satisfy the demand of these functional food ingredients. The purpose of this work was to study the effects of supplementation of prebiotics on growth performance, organ weight and on the morphology of the small intestine in rats Wistar.

In the present study, results showed no significant differences among treatments, in feed intake, body weight, weight gain and feed conversion. Our results, in agreement with previous studies, showed that when GOS was added in diets to Broiler Chickens [20] and diets to pig growing [21]. Prebiotic supplementation did not significantly affect body weight, body weight gain and feed intake. However, other researchers have previously demonstrated significant increases in body weight gain in broilers receiving diets supplemented with prebiotics [22, 23].

The improvement in feed intake by dietary prebiotic supplementation often resulted in improved growth performance. However, in the present study, using the prebiotics specifics GOS and FOS, no differences were observed in feed intake and consequently in body weight and body weight gain between treatments.

Inconsistent results in response to prebiotic supplementation may be due to differences in methods of preparing the supplement, different experimental condition, doses of prebiotic applied, animal species and study population (e.g. in age, weight or breed) composition of diets, duration of supplementation or other environmental conditions [22].

Previous studies, reported no significant difference in organ weight (the spleen, liver and kidneys) in rats supplemented with 5% FOS during 23 days [24]. Results were similar to the ones we found in the present study. Nevertheless, was observed significant difference in lymphoid organs weight (thymus, spleen and bursa of Fabricius) in birds after supplementation with oligochitosans during 21 days [25].

In our study no pathological abnormalities were observed macroscopically in terms of external morphology, color and organ texture when they were collected to measure weight. These results are in agreement with previous studies, since they did not observed any microscopic and macroscopic anormalities in organs such as liver, pancreas, kidneys, spleen, and heart in rats supplemented with 5 to 10 % of FOS [26, 27]. The absence of significant difference among groups for the organ weight added to the absence of mortality suggested that prebiotic GOS was not toxic.

The present study demonstrated that supplementation with prebiotics FOS and GOS increased cecal SCFA concentrations in rats. Previous studies reported that intake of selected oligosaccharides, especially FOS and GOS, improved concentrations of total cecal SCFAs including butyrate [28].

Campbell et al [18], studying the effects of oligofructose, FOS and XOS at the 6% dietary level, on concentration of cecal SCFA, demonstrated the cecal SCFA were higher (p<0.05) in rats consuming oligosaccharides compared with the control and cellulose diet. The oligofructose and fructooligosaccharides containing diets resulted in higher cecal butyrate concentrations compared with the control, cellulose and xylooligosaccharide diets.

Similar results were reported by Younes et al. [29], demonstrated the rats fed FOS and XOS at the 7.5% dietary level to elevate the cecal total SCFA. Butyrate was those fed XOS, while these oligosaccharides differ in their chemical composition; they probably are fermented similarly by the microbiota of rats because of the presence of similar constituents (glucose and fructose) for fermentation [30].

Human colonic bacteria ferment oligosaccharides to short-chain fatty acids (SCFA), mainly acetate, propionate, and butyrate. SCFA contribute to normal large bowel function and prevent pathology through their actions in the lumen and on the colonic musculature and vasculature and through their metabolism by colonocytes. Butyrate, in particular, is thought to play a role in maintaining a normal colonocyte population [31].

In vitro studies as well as animal studies indicate that in particular propionate and butyrate have the potential to support the maintenance of a healthy gut and to reduce risk factors that are involved in the development of gut inflammation as well as colorectal cancer [32].

In the present study, were observed that butyrate was produced in a higher concentration in rats supplemented with FOS and GOS compared with control. Butyrate is a preferred substrate for colonocytes and appears to promote a normal phenotype in these cells. Fermentation of some oligossacharides types favors butyrate production.

The cecal pH values were lower ($p<0.05$) in cecum of the rats supplemented with oligosaccharides FOS and GOS compared with the control group. An inverse relationship between the pH and SCFA concentration in the cecal contents of rats

were observed in this study. The pH values are lowered significantly and SCFC concentrations are increased in rats supplemented with oligosaccharides. Lower pH values (and raised SCFA) are believed to prevent the overgrowth of pH-sensitive pathogenic bacteria [31].

The histological study showed that the supplementation of prebiotics FOS and GOS increased the villus height and VH: CD villus ratio and decreased crypt depth in the jejum and ileum compared with controls. However, no effects of suplementation of prebiotics were observed for villus height and crypt depth at the duodenum. No statistically significant differences were observed in the villus height, crypt depth and VH: CD ratio between the prebiotics FOS and GOS.

The villus play a crucial role in the digestion and absorption processes of the small intestine, as villus increase surface area and are the first to make contact with nutrients in the lumen [33]. Higher values of crypt depth indicate more proliferative cell function to guarantee a suitable rate of epithelial renewal and high demand for new tissue [34].

The crypts of the villus contain several specialized cells such as absorptive cells, goblet cells, and regenerative cells that are responsible for the production of mucus and the replacement of old cells [35].

Previous studies, reported that the inulin-containing diet resulted in greater villus height jejunal ($p<0.05$) and deeper crypts ($p<0.01$) than in control birds without affecting villus:crypt depth ratio [36]. Similarly, studies using supplementation of the prebiotic FOS, reported morphological differences (mucosal thickness, villus height, crypt depth and villus/crypt ratio) in broilers [37].

In weaned piglets, studies using feed without additives as well as feed containing an antibiotic, a probiotic, a prebiotic and a symbiotic, observed that the prebiotics provided an increase in the villus height and crypt depth in pigs fed with a diet with the prebiotic than in those receiving a diet with the probiotic [38].

Similar results were observed in study the effect of the prebiotic mananoligosacharide (MOS) on the small intestine in broiler chicken, they concluded that the addition of MOS to the diets increased the longitudinal muscular and mucosa thickness resulting in a higher nutrient absorption surface area in the small intestine of broiler chicken at 21 and 42 days of age [39].

The increases in villus height/crypt depth ratio were observed of the three segments in the small intestine of rats supplemented with prebiotics. The villus height/crypt depth ratio is a useful criterion for estimating the likely digestive capacity of the small intestine [40].

A decrease in the villus height/crypt depth ratio is considered deleterious for digestion and absorption, and vice versa. Prebiotics that increases the villus height/crypt depth ratio might also increase the hydrolytic capacity of the epithelium [41].

The influence of the prebiotics on the intestinal mucosa can be related to the fact that they reach the colon, suffer bacterial action of the micro-flora and are fermented. This fermentation activity results in volatile short-chain fatty acids (SCFA), especially acetate, propionate, and butyrate [42, 28].

That supplementation of oligossacharides FOS and GOS, improved concentrations of total cecal SCFAs including butyrate, the preferred energy source for colonocytes, resulting in a trophic effect in small intestine.

These results were reported by Koruda et al. [30] in studies with rats observed the trophic effects of SCFA on epithelial cell proliferation. One of the most important properties of SCFA is their trophic effect on the intestinal epithelium. These trophic properties have important implications, particularly for patients receiving enteral or parenteral nutrition, and in maintaining the mucosal defence barrier against invading organisms [43].

These SCFA are the prime substrates for the energy metabolism in the colonocyte and they act as growth factors to the healthy epithelium. In normal cells butyrate has been shown to induce proliferation at the crypt base, enhancing a healthy tissue turnover and maintenance. In inflamed mucosa butyrate stimulates the regeneration of the diseased lining of the gut. In neoplastic cells butyrate inhibits proliferation at the crypt surface, the site of potential tumour development [43, 44].

The data indicate that FOS and GOS supplementation (250mg/kg/d) produced higher SCFA concentration in cecum and decrease pH. Furthermore, the supplementations resulted in an increase in the villus height and decreased crypt depth of intestinal mucosa of rats. These results represent a valuable morphometric reference for future studies of small bowel in human.

5 - Conclusion

In conclusion, the GOS produced by β-galactosidase from *Scopulariopsis* sp is likely to exert protective effects on the small intestine mucosa in rats. Therefore, this product might be promising as a food ingredient for functional foods. However, more studies should be performed *in vivo* to confirm their protective effects on the intestinal mucosa.

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Non-digestible Oligosaccharides: influence on Concentration of Short-Chain Fatty Acids, Microbial Population and Intestinal Morphlogy of Male Rats

Non-digestible Oligosaccharides: Influence on Concentration of Short-Chain Fatty Acids, Microbial Population and Intestinal Morphlogy of Male Rats

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Abstract

This study evaluated the concentrations of cecal SCFAs, cecal and fecal pH, concentrations of microbial population, small intestinal mucosal morphology of several oligosaccharides, using a rat model. Forty male Wistar rats were randomly assigned to one of the following five treatments for 42 days: Rats were respectively supplemented by gavage of Inulim, fructo-oligosaccharides (FOS) galacto-oligosaccharides and GOS/FOS in ratio of 9:1 [250 mg/(Kg body weight/day). Control group were give gavage of physiological saline solution. The growth performance was not affected by suplemmentation of oligosaccharides. The concentration SCFAs in rats cecum were significantly increased (p<0,05) by intake oligosaccharides, especially GOS/FOS mixture ratio 9:1. The pH cecal and fecal o was significantly lower in rats receiving the oligosaccharides. The Cecal *bifidobacterium*, *lactobacillus* and total anaerobes were higher whereas total aerobes were lower in rats supplemented with oligosaccharide compared with rats the control group. The oligosaccharides significantly increased (p<0.05) villus height, crypt/villus ratio and total mucosal thickness and and decreased crypt depth at the jejunum and ileum. In the present study, the indigestible oligosaccharides are fermentable in the gut, by providing SCFA, trophic effects on small intestine, lowering pH, and increasing bifidobacteria may be beneficial in improving gastrointestinal health. In conclusion, supplementation of indigestible oligosaccharides may be beneficial to gastrointestinal health.

Keywords: Oligosaccharides, short-chain acids, *Bifidobacterium, Lactobacillus*, intestinal morphology, rats.

1 – Introduction

Oligosaccharides are carbohydrates with a low degree of polymerization (DP) and consequently low molecular weight. They have been variously defined as including anything from 2 to 20 monosaccharide units. The concept of no-digestible (or nondigestible or unavailable) oligosaccharides (NDOs) originates from the anomeric C atom (C1 or C2) of the monosaccharide units of some dietary oligosaccharides has a configuration that makes their osidic bounds nondigestible to the hydrolytic activity of the human digestive enzyme (Roberfroid & Slavin, 2000).

The NDOs possess important physicochemical and physiological properties, and are claimed to behave as dietary fibers and prebiotics. (Mussato & Mancilha, 2006). Commercially, oligosaccharides are produced by enzymic processes either by hydrolysis of polysaccharides or synthesis from smaller sugars. Because of their prebiotic properties, oligosaccharides have received much recent attention as functional food ingredients (Roberfroid, 1998; Cummings et al, 2001).

NDO pass through the small intestine tract. In the large intestine, are selectively fermented by bifidobacteria and lactobacilli, stimulating their growth, both of which are considered beneficial intestinal bacteria. (Cherbut et al., 1994; Roberfroid et al., 2010).

The increased levels of beneficial bacteria in the gut resulting from the consumption of indigestible oligosaccharides are associated with a number of positive effects in the digestive and immune systems. These include the development of the mucosal barrier, production of short-chain fatty acids which reduce pH in the gut, activation of the immune system, metabolism of bile acids, and synthesis of several vitamins (Bruzzese et al., 2006).

Prebiotics oligosaccharides currently commercialized as food ingredients include: fructo-oligossacharides, galacto-oligosaccharides, lactosucrose, isomalto-oligosaccharides, gentio-oligossacharides and xilo-oligossacharides (Rastall, 2002), However, only Inulin, Galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) have an established prebiotic status, and their health effects have been extensively studied (Gibson et al., 2004; Roberfroid, 2007).

"Fructans" is a general term used for naturally occurring plant oligo – and polysaccharides. Belonging to fructans inulin (IN) and fructo-oligosaccharides (FOS) are plant-derived carbohydrates with the benefits of soluble dietary fiber and different chain lengths (degree of polymerization). (Lopez-Molina et al., 2005). GOS were recently defined as "a mixture of oligosaccharides occurring naturally in human milk, the commercial products are synthesized by the action of B-galactosidase on lactose (Rabiu et al., 2001).

Galactooligosaccharides and fructooligosaccharides have been used to stimulate Bifidobacteria, and several human studies have demonstrated the prebiotic effect of these compounds (Gibson et al., 1995; Bouhnik et al, 1997).

The genus *Bifidobacterium*, a major bacterial group in the gastrointestinal tract (GIT), accounts for up to 25% of the total culturable bacteria in adults (Sgliir et al, 1998) and are generally considered to be health promoting and beneficial (Rao, 1999). Much recent research has focused on bifidobacteria to establish the importance of these bacteria in influencing certain normal functions of the intestinal tract and in exploring their role in human health and diseases (Mitsuoka et al., 1990).

However, several important factors influence the total oligosaccharide effects in the bowel, particularly the nature of the oligosaccharides, the dose, the duration of the treatment, the place where their fermentation mainly occurs (proximal or distal colon) and the initial composition of intestinal microbiota (Delzene, 2003).

There is a growing commercial interest in new non-digestible oligosaccharides for functional food ingredients. Also, it is essential intense scientific research for evaluating their functional traits. In this sense, the current study aimed evaluating prebiotic properties of the GOS produced by β-galactosidase from *Scopulariopsis* sp and compare prebiotic properties of GOS with commercially available prebiotic FOS (Raftilose®, Beneo, Belgium) and Inulin (Oraft-Raftiline ® Beneo, Belgium).

Was examined, the influence of different indigestible oligosaccharides: Inulin, FOS and GOS, on the concentrations of cecal SCFAs, cecal and fecal pH, concentrations of cecal microbial population and intestinal morphology in Wistar rats.

2 - Materials and Methods

2.1 - Oligosaccharides

Two commercial oligosaccharides, GOS produced from lactose and GOS/FOS mixture were investigated for their effects on the intestine physiology of rats. The FOS (Raftilose P95) contain 95% oligofructose with Degree of Polymerisation (DP) between 2 and 8 and 5% of glucose, fructose and sucrose were obtained from ORAFTI (Belgium). The inulin (Oraft-Raftiline HPX, BENEO 100%) is a mixture of oligo- and polysaccharides with DP between 2 and 60. The commercial oligosaccharides Inulin and FOS were kindly donated by Orafti-Active Food Ingredients (Belgium). The galacto-oligossacharides (GOS) mixture used in this study was produced by transgalactosylation using β-galactosidase from *Scopulariopsis* sp on lactose. The main component of GOS is 4' galactosyllactose and contains of the several oligosaccharides species (disaccharides, trisaccharides and tetrasaccharides) and lactose, glucose and galactose (Santos et al. 2009).

2.2 - Animal and Treatments

Forty male Wistar rats (Center of Bioterism – CEMIB. Unicamp (Campinas-SP, Brazil) weighing 385±12g was assigned randomly to one of five groups

(8rats/group). Treatment included: Control group were give gavage of physiological saline solution [2 mL/ (kg BW.d)]. Iunlin, FOS, GOS and GOS/FOS mixture in a ratio of 9:1 groups were rendered gavage of 250mg/mL corresponding oligosaccharides solution [2mL/ (kg BW.d)], respectively. The animals were allowed free access to water and commercial diet (PURINA®)

During the period of adaptation (5 d), the rats were housed two per cage in a temperature-controlled room (22℃) with a 12-h cycl e of light and darkness. Following the adaptation phase, rats were housed individually in plastic boxes. The experimental protocol was reviewed and approved by the Institutional Committee for Ethics in Animal Experimentation (CEEA/IB/UNICAMP). The experimental lasted 42 days, and individual food consumption and weight gains of the rats were recorded daily.

2.3 - Sample Collection

At the termination of the experiment, the rats were anaesthetised with subcutaneous injection of ketamine-xylazine-acepromazine cocktail (1.4 ml/kg body wt) and killed by exsanguination through a cardiac puncture. After laparotomy, the cecum and colon with contents was removed. The Cecal contents were collected, pH measured, and a 0.5g aliquot immediately processed for SCFA analysis. The remaining cecal contents were immediately placed into a sterile assay tube for bacterial enumeration. After removal of the appropriate samples, the small intestines were cleaned with saline solution, and immersed in the fixing solution for subsequent histological analysis. The pH was measured in the fresh

stool sample using pHmeter (Pack ph 21) by homogenizing 2.0g of fecal sample in 3 w/v sterile H2O (pH 7.0) in a 10 ml centrifuge tube.

2.4 Determination of the Concentrations of SCFA

Cecal SCFA concentrations were determined in duplicate using the method described by of Smirick-Tjardes et al. (2003) with modification. Cecal contents were acidified with 250 g/L metaphosphoric acid using a mixture of 0.4g sample:0.8 mL acid:2.8 mL distilled H2O and centrifuged at 25,900 1g for 20 min. 2), precipitated at room temperature for 30 min, and then centrifuged at 25,900 × g for 20 min. The supernatant was decanted and frozen at −20℃ in microfuge tubes. After freezing, the supernatant was thawed and centrifuged in microfuge tubes at 13,000 × g for 10 min. The supernatant was used in GC/MS analysis.

The cecal digesta was analysed for SCFA concentration with the gas chromatography GC/MS (Agilent 5975C, inert MSD) equipped with a Nukol capillary columm (Supelco, 30m x 0.25mm x 0.25µm film thickness). The injection was performed in the splitless mode for 1 min at 220℃. Oven temperature was programmed at 100℃ for 5 min, then increase to 200 ℃ at 8℃.min $^{-1}$ and held for 1 min at the final temperature. Injector and detector temperature was 200℃; the carrier gas was nitrogen at a flow rate of 25 mL/min. Volatile free acid standard mix (Supelco4697-U) was used as internal standard. Caecal SCFA pool size was calculated as the product of SCFA concentration in cecal digesta mass.

2.5 - Bacterial Enumeration

Bacterial enumerations were determined using the method described by Pan et al. (2009). Samples for enumeration of selected genera of cecal bacteria were serially diluted 10-fold with peptone-water immediately after collection; 100 µl of the appropriate dilutions were inoculated onto duplicate plates using selective media for the enumeration of different bacteria. Bacteria were counted on plate count agar – PCA (Merck; total aerobes) Wilkins –chalgren agar (Difico; total anaerobes), Mac-Conkey agar (Merck; *Enterobacteriaceae*), de Man, Rogosa and Sharp – MRS agar (Oxoid; *Lactobacillus*), and MRS agar (Oxoid; *Bifidobacterium*)

Enumeration of *Bifidobacterium* was determined using the method described by Moriya et al (2006). The Commercial MRS medium (Oxoid) was sterilised at 121ºC for 15min and was added the inhibitors and the aminoacid L-cysteine added. The inhibitors concentration in the final medium were: lithium chloride 3 mg/ mL, nalidixic acid 15 µ/mL, neomycin sulphate 100 µg/mL and paramomycin sulphate 200 µg/mL. The L-cysteine concentration was 0.5 g/mL in the final medium.

Plates were incubated at 37ºC aerobically or in anaerobic conditions (Anaerogen® - anaerobiosis generator from Oxoid), for 24 or 72 h as appropriate. Plates containing 20 to 200 colonies were enumerated. The microbial count data were expressed as colony forming units/g wet sample. The selectivity of the growth conditions was confirmed by microscopic examination.

2.6 - Preparation of Tissues

The small intestine was carefully dissected distally to the stomach and proximal to the cecum. Transversal sections (2cm) of the duodenum, jejunum, and ileum were washed in saline solution, and immersed in a fixing solution (formol 5%) for 24h for subsequent histological examination. The histological cuts were dehydrated in a series of crescent concentrations of alcohol, diafinized in xylol, and immersed into paraffin. Sections were cut (5 µm) on a microtome (Leica RM 2145) cut, and then stained with hematoxylin and eosin (HE staining).

2.7 - Morphological Analysis

For the morphometric study of intestinal mucosa, ZoomBrowser EX Imaging System software was used in conjunction with a microscope (Carl Zeiss, Germany) fitted with a video camera (Sony). The video camera transferred the image from the microscope to the computer screen. After capturing the images, the morphometric analysis was performed using the Image Tool Software.

The height of the villus and the depth of the crypts and Total mucosal thickness were measured in well-guided longitudinal sections. The villosities length was measured the extension between the junction crypt-villi and the villosities ends. The crypt depth was measured between the junction crypt-villi and the crypt base. Total mucosal thickness were measured the extension from the top of the villus to the border over the muscularis mucosae (Figure 1). A total of 10 intact crypt-villus units were selected in triplicate for each intestinal cross-section (30 measurements for each sample)

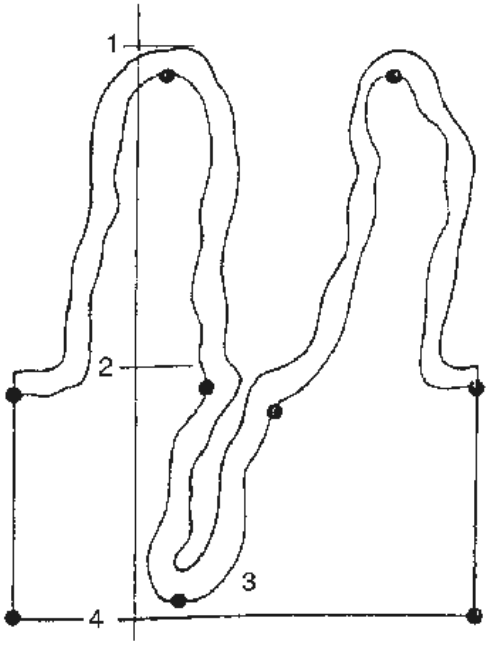

Figure 1- Schematic diagram of an intestinal villus showing how the intestinal mucosal levels were measured in well-guided longitudinal sections (Pires et al., 2003). (**1-2**) Villus height, (**2-3**) Crypt depth

2.8 - Statistical Analyses

One-way analysis of variance was performed using the GRAPHPAD PRISM 5.0 version (graphPad software, Inc., La Jolla, CA, USA). Differences among means were tested using the Tukey test. Values of less than 0.05 ($p<0.05$) were considered significant.

3 - Results and Discussion

3.1 - Effects of Supplementation on Body Weight and Feed Intake

No significant differences in feed intake or weight gain were noted throughout the study. The body weight and feed intake of the rats did not differ among the control, and groups Supplemented with oligosaccharide (data not shown). Feed intake was unaffected by oligosaccharides. After 42 days of gavage, feed intake ranged ~20–22 g/d with an average of 21.4 g/d and fluctuated daily, the groups supplemented with oligosaccharides (Inulin, FOS and GOS), the total ration consumption was 0.858; 0.862 and 861kg respectively per rats and in the control group, and it was 0.862 kg. No statistical differences in feed intake between groups Supplemented with oligosaccharide. In addition, there was no significant difference (p>0.05) in food conversion ratio between the groups supplemented with oligosaccharides and control group.

Campbell et al. (1997) and Sakaguchi et al. (1998) also observed no difference in feed consumption in rats with the oligosaccharides added to the feed. There is considerable variation in the results reported by other researchers, since Vesna et al. (2007) reported on lower feed consumption in broilers supplemented with mannan-oligosaccharides (MOS) Similar results were obtained by Rosen (2007) reported on average lower feed consumption for birds fed MOS vs. controls,

Initial body weights ranged from 384 to 386g in the groups Supplemented with oligosaccharides with final body gain from 445 to 452g. The body weights

increased over time for all groups supplemented and control. The body gain was similar between control groups (450.5g) and supplemented. Morever, no significant differences in body weight were noted in the groups supplemented with different oligosaccharides.

These results seem to confirm observations made by Campbell et al. (1997), showed that No significant differences in food intake and body weight in rats suplemented with different oligosaccharides (Oligofructose, FOS and XOS) and control group. Similar results were obtained by Younes et al. (1995), no observed significant differences in food intake or weight gain in rats feed diets containing 7.5% two oligosaccharides (fructo-oligosaccharide or xylo-oligosaccharide).

These results indicate that the effects of oligosaccharides on body weight and food intake might be affected by differences in the animal model, animal gender, experimental period, supplemental method, and dose and type of oligosaccharide used

3.2 - Cecal SCFAs Concentrations and pH Analysis

All oligosaccharides significantly increased (p<0.05) the cecal concentration of total SCFA, acetate, propionate, and butyrate compared to the control (Table 1). The cecal concentration of total SCFAs was greatest (p<0.05) for the FOS compared with inulin and GOS. Moreover, the mixture of GOS/FOS had results in similar concentration of total SCFA compared with inulin, FOS and GOS.

Table 1- Concentration of short-chain fatty acids (SCFAs) in cecal contents and pH values in rats supplemented with different oligosaccharides for 42 days.

Item	**Treatment**					
SCFAs (µmol/g)	**Control**	**Inulin**	**FOS**	**GOS**	**GOS/FOS (9:1)**	***p* values**
Acetate	6,88±1,99[a]	28,18±11,82[b]	52,05±13,01b[c]	34,24±15,46[b]	33,75±6,96[b]	*p*<0.0001
Propionate	3,78± 0,65[a]	9,49± 5,43[b]	26,36±3.57b[c]	9,34± 4,63[b]	10,45± 5,72[b]	*p*<0.0020
Butyrate	2,64±1,93[a]	7,33± 3,8[b]	12,75± 9,26b[c]	7,33± 3,80[b]	7,28± 2,69[b]	*p*<0.0042
*Total SCFA	13,30±2.19[a]	45,00±11.47[b]	91,16±19.96b[c]	50,83±15.02[b]	51,49±17.16[b]	*p*<0.0032
Fecal pH	7.7±0.28[a]	6.3±0,26[b]	6.2±0,18[b]	6.4±0,34[b]	6.2±0.39[b]	*p*<0.0001
Cecal pH	7.3±0.32[a]	5.7±0.52[b]	5.5±0.44[b]	6.0±0.37[b]	5.8±0.52[b]	*p*<0.0001

FOS: fructo-oligosaccharides; GOS: Galacto-oligosaccharides.
Results are show as mean± SD (means of 8 rats).
[abc] Means in the same row not sharing superscript letters differ (*p*<0.05).
*Total SCFA =Acetate+Propionate+Butyrate

All the three major short chain fatty acids (acetate, propionate, and butyrate) were significantly increased (*p*<0.05) the cecal concentration by oligosaccharides. FOS resulted in greatest (*p*<0.05) concentrations of acetate among oligosaccharides. The mixture GOS/FOS resulted in a similar concentration of acetate (*p*<0.05) compared with inulin and GOS.

Propionate production was higher (*p*<0.05) in rats supplemented with oligosaccharides. However, FOS resulted in greatest (*p*<0.05) concentrations of propionate among oligosaccharides. Propionate production was similar between oligosaccharides inulim, GOS and GOS/FOS mixture.

Oligosaccharides resulted in greatest (p<0.05) concentrations of butyrate compared to the control. The butyrate concentration was higher (p<0.05) in rats supplemented with FOS. However the Butyrate production was similar for inulim, GOS and GOS/FOS mixture. Butyrate is the preferred energy substrate of colonocytes. In the present study the cecal SCFAs concentration are in the order predicted from that equation, i.e., acetate > propionate > butyrate.

Differences in the relative proportions of acetate, propionate and butyrate were observed between 5 groups experimental. The cecal SCFAs are produced in molar ratio (52:28:20) respectively in control group; inulin (62:21:17) respectively, FOS (57:23:20) respectively, GOS (67:18:15) respectively, GOS/FOS (65:20:15). Acetate was the predominant SCFA in the cecum in all rats. Molar ratios of SCFA are probably a more reliable indicator of dietary changes than SCFA concentrations (Scheppach, 1994). The characterization of changes in the concentration of microbial products such as SCFA may reflect dietary effects and provides an indication of microbial Activity (Kleessen et al., 1997).

Fecal pH was lower (p<0.05) in rats supplemented with oligosaccharides compared with control. Similarly, Cecal pH was lower (p<0.05) in rats supplemented with oligosaccharides compared with control. Moreover, cecal pH was dramatically lower (p<0.05) that fecal pH in rats supplemented with oligosaccharides.

In the present study, three types of oligosaccharides, inulin FOS and GOS were used to study the potential effects of on SCFA formation, using a rat model. The

oligosaccharides, inulin, FOS and GOS increased cecal concentration of cecal SCFA, especially FOS. The production rate and level of SCFA in the colon depends on the composition of the microbiota, the substrate availability, and the gut transit time. (Wong & Jenkins, 2007).

The values of cecal SCFA concentration from this study are comparable with previous studies. Pan et al. (2009) investigating the effects of oligosaccharides FOS, GOS, MOS and COS on concentration of cecal SCFA demonstrated that SCFA in mice cecum were significantly increased by intake oligosaccharides.

In studies employing different oligosaccharides, using a rat model, similar observations have been made by Campbell et al (1997), Nilsson et.al (2005) and Juskiewicz et al. (2006). However, in humans production of total colonic SCFA is difficult to determine. Human experimentation has been confined largely to fecal measurements, which are also limited, because more than 95% of the SCFA are rapidly absorbed and metabolised by the host. Probably for these reasons various studies were not able to show effects of different fermentable substrates on fecal SCFA concentrations (Topping & Clifton, 2001).

The major fermentation products of indigestible oligosaccharides metabolism in large bowel are short chain fatty acids (SCFAs), mainly acetate, propionate and butyrate, are usually produced in a molar ratio of 60:20:20, respectively (Cummings, 1981). However, the concentrations and proportions of SCFA are dependent not only on the counts and activities of the bacterial species but also on

the environmental conditions such as luminal pH and the absorption rate of the SCFA in the gut (Cummings and Macfarlane, 1991).

SCFA are rapidly absorbed from the colonic lumen by passive diffusion and anion exchange, and only 5-10% is excreted in the feces (Ruppin et al., 1980). Butyrate is the most interesting of the SCFA since; it regulates cell growth and differentiation of colonocyte. In addition to this trophic effect, butyrate stimulates the immunogenicity (sensitivity to the immune response) of the cells. The colonic microbiota has a considerable influence on the immune system of the host (Bornet et al., 2002). Propionate is largely taken up by the liver. Acetate enters the peripheral circulation to be metabolized by peripheral tissues (Wong et al., 2006; Hijova & Chmelarova, 2007).

The SCFA acidify the luminal pH which suppresses the growth of pathogens and stimulation of sodium and water absorption, they also influence intestinal motility (Scheppach, 1994). The SCFAs have been associated with reduced risk of developing gastrointestinal disorders, including reduction of cancer risk, increase in mineral absorption, improvement in bowel habit, control of serum lipid and cholesterol level (Hijova &Chmelarova, 2007).

The pH of cecum was significantly lower in rats receiving the oligosaccharides and GOS/FOS mixture in compared with control group. The fecal pH decreased after oral supplementation with oligosaccharides. The influence of the mixture GOS/FOS on the change in fecal pH was significant ($p<0.05$). The reduction in pH value was

a result of microflora action utilizing oligosaccharides and producing fermentation end-products like lactic and short-chain fatty acids

In rats, fermentation is essentially localized in the cecum. The cecum has the highest level of SCFA available for absorption/utilization. As a result of fermentation, pH decreases. There are several results available indicating that SCFA and pH influence the physiological role of intestinal cells.

3.3 - Effects of Oligosaccharides on Microbial Population

The effects of supplementation of the oligosaccharides on cecal microflora are presented in Table 2. In the present study, the cecal concentration of *Bifidobacterium* and *Lactobacillus* were greatest (p<0.05) as a result of suplementation of oligossacharides. However, there was no significant difference in and *lactobacillus* counts among the oligosaccharides groups.

Total aerobes were lower (p<0.05) due to ingestion of oligosaccharides compared with control group, whereas total anaerobes were higher (p<0.05) for rats suplemented with oligossacharides compared with control group. Cecal concentrations of the *E.coli* were lower (p<0.05) for rats supplemented with oligossacharides compared with control group.

Table 2- Effects of oligosaccharides in cecal microbial population.

log10 CFU/g wet contents	Treatment					
	Control	**Inulim**	**FOS**	**GOS**	**GOS/FOS (9:1)**	***p*- values**
Bifidobacterium	7.8±0.22[a]	9.2±0.36[b]	9.8±0.25[b]	9.4±0.32[b]	9.8±0.20[b]	0.0001
Lactobacillus	6.2±0.20[a]	8.4±0.23[b]	8.6±0.52[b]	8.5±0.55[b]	8.2±0.36[b]	0.0122
E.coli	7.8±0.55[a]	6.2±0.36[b]	6.3±0.58[b]	6.5±0.28[b]	6.3±0.61[b]	0.0138
Total aerobes	8.4±0.62[a]	7.2±0.26[b]	6.8±0.52[b]	6.6±0.56[b]	6.9±0.38[b]	0.0001
Total anaerobes	7.8±0.2[a]	10.2±0.6[b]	10.5±0.7[b]	10.1±0.3[b]	10.2±0.8[b]	0.0001

FOS: fructo-oligosaccharides; GOS: Galacto-oligosaccharides, CFU, colony-forming units.
Results are show as mean±SD, n=8.
Means in a row without a common letter differ, $p<0.05$

In the present study, no significant differences were observed in in cecal population of the *Bifidobacterium* and *Lactobacillus* in group supplemented with mixture of GOS/FOS (9:1). Results were similar when compared with others oligosaccharides (inulin, FOS or GOS).

Infant formula supplementation with a prebiotic mixture (90% short-chain galacto-oligosaccharides (scGOS) and 10% long-chain FOS (lcFOS) leads to increased numbers of the fecal *Bifidobacterium* and *Lactobacillus*. Several studies demonstrated that this mixture modulated the entire microbiota closer to the intestinal flora composition found in breast-fed infants (Moro et al., 2002).

Marked differences in the rat cecal and colonic microflora in response to different indigestible oligossacharides also were reported by other authors (Campel et al.1993; Pan et al 2009).

Pan et al. (2009) reported significant differences in the cecal microbiota of rats supplemented whit oligosaccharides. After 14 d of gavage, the concentrations of *Bifidobacterium* and *Lactobacillus* in the cecum were higher (P<0.05) in each oligosaccharide group than in the control group. These results confirm that indigestible oligossacharides has the potential for changing the composition of the intestinal microflora.

It was demonstrated that the intakes of indigestible oligosaccharides FOS, oligofructose and xylooligosaccharide led to significant increase in *Bifidobacterium* and *Lactobacillus* (Campbell et al., 1997).

Several in vivo studies demonstrate that diets that supply oligosaccharides (e.g., inulin, FOS, and GOS) selectively increase the intestinal tract population of bifidobacteria in animals and humans (Delzenne, & Roberfroid, 1994, Hsu et al., 2004; Hsu et al., 2004). The indigestible oligossacharides may benefit gastrointestinal tract health via fermentation and proliferation of desirable bacterial species (Campbell et al., 1997).

A number of physicochemical and microbial factors can influence the pattern and the extent of fermentation of appropriate bacterial substrates. These include competition for nutrients, the physicochemical environment of the large gut (e.g., oxidation-reduction potential, pH, SCFA, and lactate concentrations), various host conditions (e.g., intestinal motility, antibacterial compounds), metabolic interactions

among bacteria, and changes in the fermentation strategy of bacterial species (Macfarlane & Cummings, 1991; Kleessen et al., 1997).

Also, a study by Tuohy et al. (2001) demonstrated that that the dose and the duration of oligosaccharide intake, the place where fermentation mainly occurs (proximal or distal colon), as well as the initial composition of fecal flora, are important factors influencing the extent of the prebiotic effect, i.e. the increase in bifidobacteria.

The colonic microflora should change in response to gross nutritional shifts (e.g., weaning), progressive change (such as aging), or variations in food intake. In aged persons, Escherichia coli, streptococci, and clostridia increase and bifidobacteria decrease further (Mitsuoka et al., 1996; Topping & Clifton, 2001).

The effect of oligosaccharides on histological measurements of the duodenum, jejunum and ileum mucosa of rats is summarized in Table 3. In the present study, no significant differences in villus height in the duodenum were observed in rats supplemented whit oligosaccharides. However, the villus height was significantly increased ($p<0.05$) after 42 days oral supplementation with oligosaccharides versus control.

No significant differences were observed in villus height among groups supplemented with oligosaccharides or mixture of GOS/FOS. The crypts of the villus contain several specialized cells such as absorptive cells, goblet cells, and

regenerative cells that are responsible for the production of mucus and the replacement of old cells (Ayabe et al., 2000; Solis de los Santos et al., 2007).

In the present study, we observed significant increases in crypt depth in the jejunum and ileum in rats supplemented whit oligosaccharides compared with control group. However, there was no difference in crypt depth in the duodenum among groups supplemented and control. Crypt depth was similar (p>0.05) and jejunum and ileum between groups supplemented with oligosaccharides and mixture GOS/FOS.

According to Silva et al. (2010), higher values of crypt depth indicate more proliferative cell function to guarantee a suitable rate of epithelial renewal and high demand for new tissue.

Table 3. Effects of oligosaccharides on the morphology of the intestinal mucosa at different sites in the small intestine.

	Treatment				
Site	**Control**	**Inulin**	**FOS**	**GOS**	**GOS/FOS**
Villus height (mm)	3.48±2.26	3.42±2.87	3.17±2.75	3.46±2.14	3.28±2.75
Duodenum	3.79±3.20	5.79±2.17	5.58±4.15	5.89±3.75	5.87±4.10
Jejunum	3.53±1.20	5.38±3.35	5.74±2.75	5.62±3.73	5.69±3.33
Ileum					
Crypt depth (mm)					
Duodenum	1.36±0.78	1.34±0.56	1.28±0.55	1.39±0.74	1.16±0.52
Jejunum	1.82±2.15	2.06±5.30	2.34±6.03	2.19±3.65	2.06±2.12
Ileum	1.76±8.30	2.28±6.45	2,10±8.01	2.19±6.23	2.30±8.08
Villus height/ crypt depth					
Duodenum	1.58±0.06	1.62±0.07	1.42±0.03	1.75±0.8	1.55±0.06
Jejunum	1.78±2.21	2.86±2.36	2.38±2.57	2.65±4.75	2.84±2.45
Ileum	1.48±3.75	2.53±8.20	2.44±7.32	2.65±4.72	2.37±4.40

Results are show as mean±SD
Means within a row with different letters differ significantly (p<0.05).
Means represent 8 rats per treatment; there was one sample for each of the three intestinal segments per rat, three cross-sections per sample, 24 cross-sections for each of the three intestinal segments per treatment, and 10 measurements per cross-section for a total of 240 measurements for each of the three intestinal segments per treatment.

The crypt/villus ratio in the jejunum and ileum were significantly higher for the rats supplemented with oligosaccharides versus control. The ratio villus height to crypt length in the normal small intestine varies from about 3:1 to 5:1.

This result concurs with results found in earlier experiments. Xu et al. (2003) no observed significant differences for villus height, crypt depth, or microvillus height at the duodenum. By contrast, addition of 4.0g/ kg FOS significantly increased ileal villus height, jejunal and ileal microvillus height, and villus-height-to-cryptdepth ratios at the jejunum and ileum and decreased crypt depth at the jejunum and ileum

Solis de los Santos et al. (2005) observed significantly increased in villus height and and crypt depth the duodenum and Ileum in broilers supplemented with prebiotics. Similarly, Budiño et al. (2005). It was found that the density and length of duodenal villus was higher in pigs fed a diet with the prebiotic than in those receiving a diet with the probiotic.

Silva et al. (2009) evaluated the effect of yeast extract or prebiotic on performance and intestinal morphometry of broiler chickens and observed the prebiotic increased in the villus height and crypt depth in the three intestinal regions. Nevertheless, data in the present study disagree from those reported by Leforestier et al. (2009) studying the effect of the GOS ingestion on small intestinal mucosal morphology in mice, no detectable effect on the intestine villus height.

Dietary factors increase the height of intestinal villi as well as the number and depth of crypts (Mroz 2001) Nutritional factors, such as short-chain organic acids, may directly affect intestinal morphology, stimulating the proliferation of intestinal epithelial cells (Sakata et al. 1995; Ichikawa et al. 2002).

Oligosaccharides indigestible increased the production rate of SCFA (acetate, propionate and butyrate) in the colon. The SCFA are metabolites of bacterial fermentation, which stimulate epithelial cell proliferation in the gut (Ichikawa et al., 1999). The most important of these short-chain fatty acids is butyrate.

Butyrate plays a key role in colonic epithelium homeostasis, is rapidly absorbed in the epithelium and is thought to stimulate proliferation, is a principal energy source for epithelial cell, also believed to protect against colon cancer as it inhibits DNA synthesis and induces cell differentiation (Gonçalves et al., 2011). By producing a greater concentration of butyrate, the preferred energy source for colonocytes, a trophic effect may be resulted within the gastrointestinal tract (Pan et al., 2009).

It is likely that the trophic effects of the oligossacharides are due to the ability of the oligossacharides create a more favorable intestinal microbial environment and are not a direct action of oligossacharides on the intestinal tissue (Xu et al., 2003).

The intestinal tract can be anatomically divided into two well-defined segments: the small intestine and the large intestine or colon. The small intestine is subdivided

into three proximal-distal segments: the duodenum, jejunum, and ileum. The absorptive surface area of the small intestine is dramatically increased by numerous finger-like protrusions that point toward the lumen, the so-called villi, and invaginations into the submucosa known as the crypts of Lieberkühn.

The mucosa of the large intestine lacks villi; crypts invaginate deep into the submucosa, Crypts are responsible for the proliferation of epithelial cells (Barker et al., 2008). The small intestinal epithelium consists of four principal cell types deriving from one multipotent stem cell: enterocytes, goblet, enteroendocrine, and Paneth cells. (Sancho et al. 2003)

Accordingly, the trophic effect of indigestible oligosaccharides on the small intestine is assigned the production of SCFAs, principally butyrate. SCFA are metabolized rapidly by colonocytes and are major respiratory fuels and trophic to the small bowel and colon (Topping & Clifton, 2001).

In vivo studies with rats have documented the trophic effects of SCFA on epithelial cell proliferation (Frankel et al. 1994, Koruda et al. 1988). These trophic properties have important physiological implications in addition to maintaining the mucosal defense barrier against invading organism (Salminen et al., 1998).

These findings suggest that oligosaccharide supplementation could modify the population and metabolic characteristics of the gastrointestinal bacteria, which

might in turn modulate enteric functions and provide resistance to colorectal cancers (Buddington et al., 2002).

The beneficial action of indigestible oligosaccharides on the villus height and crypt depth in jejunum and ileum was probably influenced by production of SCFA (acetate, propionate and butyrate) in the colon.

These results preliminary suggest that GOS produced by β-galactosidase from *Scopulariopsis* sp have prebiotic potential, could have potential in the development of new functional product. However, further studies of the effects of these oligosaccharides on intestinal microbiota, morphological of the small intestinal mucosa and concentration of SCFA are necessary to sustain this beneficial health effects.

4 - Conclusions

In conclusion, from this study we can conclude that GOS produced by β-galactosidase from *Scopulariopsis* sp can exert beneficial effects in the gastrointestinal tract, by providing SCFA, lowering pH, increasing bifidobacteria and beneficial changes in intestinal architecture.

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CONCLUSÕES GERAIS E PERSPECTIVAS FUTURAS

O presente trabalho foi desenvolvido com o objetivo de avaliar as propriedades funcionais e efeitos prebióticos dos galacto-oligosacarídeos (GOS) produzido pela ação da enzima β-galactosidase produzida pelo fungo *Scopulariopsis* sp. Os experimentos foram realizados *in vitro e in vivo* utilizando ratos wistar como modelo animal e permitiram as seguintes conclusões.

● Os GOS produzido pela ação da enzima β-galactosidase extraída do fungo *Scopulariopsis* sp demonstrou características prebióticas. Não foi hidrolisado pelas enzimas digestivas, foi seletivamente fermentado no colon pelas bactérias fecais e convertido em ácidos graxos de cadeia curta: acetato, butirato e propionato (analisados por cromatografia gasosa).

● De acordo com os resultados obtidos os GOS não produziram efeitos adversos (tóxicos) mesmo quando ingerido em doses elevadas no modelo experimental utilizado.

● A suplementação de GOS promoveu um aumento significativo na população de bifidobacterias e lactobacilos e inibiu o crescimento de bacteria patogênica (*E.coli*), e aumentou a concentração de ácidos graxos de cadeia curta no ceco e conseqüentemente promoveu uma redução no pH cecal e fecal

● O estudo histológico demonstrou que a suplementação de GOS promoveu um aumento na altura das vilosidades e na relação altura de vilo/profundidade de cripta e diminui a profundidade das criptas no jejuno e íleo, promovendo mudanças benéficas na arquitetura intestinal, sendo uma das características para análise de absorção de nutrientes associadas a produção de AGCC.

● Desta forma, os GOS produzidos pela β-galactosidase por *Scopulariopsis* sp promoveu varias alterações fisiológicas benéficas no trato-gastrointestinal.

● De acordo com os resultados obtidos, pode se concluir que os GOS produzidos pela β-galactosidase por *Scopulariopsis* sp pode ser uma nova fonte de ingrediente alimentar funcional, pois apresenta características cientificamente comprovada de propriedades funcionais e efeitos prebióticos.

● No entanto, novos estudos são necessários para verificar quais os oligossacarídeos presentes são responsáveis pelos efeitos benéficos à saúde, já que se trata de uma mistura diferente aos demais.

ANEXO I - Parecer do Comitê de Ética

UNICAMP

CEEA/Unicamp

ANEXO I

Comissão de Ética na Experimentação Animal
CEEA/Unicamp

CERTIFICADO

Certificamos que o Protocolo nº **1707-1**, sobre **"Estudo sobre os efeitos da suplementação de prebióticos (frutooligossacarídeos e galactooligossacarídeos) sobre as funções fisiológicas de ratos Wistar"**, sob a responsabilidade de **Profa. Dra. Gláucia Maria Pastore / Adriane Cristina Garcia Lemos**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal – CEEA/Unicamp em **15 de dezembro de 2008**.

CERTIFICATE

We certify that the protocol nº **1707-1**, entitled **"Study on the effects of the prebiotics suplementation (frutooligosacchides and galactooligosaccharides) about the physiologic functions of rats Wistar"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **December 15, 2008**.

Campinas, 15 de dezembro de 2008.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

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