

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO	
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DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 378 FASE NACIONAL INCLUIDO CAPITULO I
TRA: 11 PCT-PATENTE DE INVENCION CAPITULOS I Y II	FOLIOS: 5
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
GERMAN CASTILLO GRAU

Asunto: Radicación: 12-188273- -7
 Trámite: 11
 Evento: 378
 Actuación: 519
 Oficio: 18983
 Folios: 5

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional					
Vía PCT (Fase Nacional)	X	Capítulo I	X	Capítulo II	
No. Solicitud Internacional	PCT/US2011/038346				
Fecha de Presentación Internacional	27/05/2011				

Como resultado del examen de fondo realizado, con fundamento en el Artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se comunica:

1. Documentos estudiados

Descripción:	Radicado N°12-188273-00000-0000	23/Octubre/2012
Reivindicaciones:	Radicado N°12-188273-00000-0000	23/Octubre/2012
Dibujos(Figuras 1 a 3)	Radicado N°12-188273-00000-0000	23/Octubre/2012
Prioridad:	US 127790666 US 127790666	28/Mayo/2010 28/Mayo/2010

2. Análisis de la Prioridad

Se acepta la reivindicación de prioridad porque ésta solicitud fue presentada dentro del plazo establecido (Art. 9 D 486) y su contenido técnico corresponde con el de la prioridad reivindicada.

3. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 del Capítulo Primero del Título X de la Circular Única para efectos del examen de patentabilidad, se tendrá en cuenta la tasa pagada por el solicitante al momento de comenzar el examen para las reivindicaciones adicionales; esto es que cuando la solicitud contenga más de diez (10) reivindicaciones y no se haya pagado la tasa complementaria, sólo se estudiarán las cubiertas por la tasa pagada.

Título de la solicitud: “Composiciones Nutricionales”

El problema planteado en esta solicitud consiste en la necesidad de una composición para bebés y niños que aumente el sistema inmunológico.

Para resolver este problema técnico la solicitud en estudio proporciona una composición de acuerdo al radicado N°12-188273-00000-0000.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A61K 31/ 716, A61P 37/00, A23L 1/29, A23C 9/13.

4. Excepción a la Patentabilidad (Art 20 D 486 literal (d))

El objeto de las reivindicaciones 19 y 20 no es patentable de acuerdo con el Art citado, porque se refiere a administrar un sucedáneo de la leche materna en un método de tratamiento.

5. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones 1 y sus dependientes no son concisas ya que no especifican los constituyentes de la composición por sus nombres genéricos o estructuras química que permita diferenciar a la composición de las conocidas en el arte previo. Es importante recordar que las fuentes de determinado grupo de alimentos (grasas, carbohidratos y proteínas) son infinitas lo mismo que los compuestos que pertenecen a dichos grupos.

No se entiende cual es la diferencia entre la reivindicación 1 y la 10, observándose que se repite la materia reclamada, innecesariamente. Además se observa que la reivindicación 10 es más general que la 1.

La expresión “cerca de” de la reivindicación 2, no es concisa.

Las reivindicaciones 3 y 14 contemplan la expresión “nutricionalmente completa” la cual es vaga ya que no precisa ninguna característica técnica esencial, adicional, que diferencie a la composición reivindicada de las conocidas en el arte previo.

Las reivindicaciones 4, 5, 15 y 16 mencionan las expresiones “por lo menos un prebiótico” y “por lo menos un probiótico” los cuales no son concisos ya que dichas expresiones comprenden un infinito número de alimentos, que tengan hoy y a futuro esa funcionalidad, tomando en consideración el significado de dichas expresiones:

Alimentos probióticos son aquellos adicionados con microorganismos vivos que permanecen activos en el intestino y ejercen importantes efectos fisiológicos que, ingeridos en cantidades suficientes, pueden tener efectos beneficiosos como contribuir al equilibrio de la microbiota (microflora) intestinal del huésped y potenciar el sistema inmunitario.

Los prebióticos son una especie de alimentos funcionales, definidos como: ingredientes no digeribles que afectan beneficiosamente al organismo mediante la estimulación del

crecimiento y actividad de una o varias cepas de bacterias en el colon, mejorando la salud. La definición de prebiótico es literalmente como "promotores de vida" (contrario a antibiótico), este tipo de alimentos se muestra en forma de productos lácteos.

Las reivindicaciones 6, 7,17 y 18 aunque se encabecen por la palabra composición se refieren a la administración de una composición, lo cual no es una característica técnica de una composición.

La reivindicación 8, no es concisa por el hecho de incluir la expresión "un producto nutricional para niños", ya que esta no especifica de que producto se trata.

Se requiere al solicitante hacer las aclaraciones y/o correcciones necesarias acorde a lo soportado en la descripción.

6. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de prioridad: mayo 28 de 2010, y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D1	WO02/47612	Dietary Supplement compositions	Junio 20 de 2002
D2	US6168821	Glucan containing nutritional product and method of making the same	Enero 2n de 2001

D1¹ divulga una composición que comprende calostro (proteínas, grasa y carbohidratos), lactoferrina, glucano 1,3 y pectina de cítricos, además enseña que dicha composición se puede combinar con un complejo de sacáridos y que esta combinación proporciona un conjunto sinérgico de proteínas, péptidos, gliconutrientes que puedan ayudar a lograr una salud óptima a través de un sistema inmune inmunomodulado.

D2² divulga una composición que comprende una composición en forma de jugo de frutas y vegetales enriquecidos que además comprende B-glucanos en un porcentaje entre el 0.001% y 0.5%.

7. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 1 consiste en una composición nutricional para un niño que comprende: una fuente de grasas; una fuente de carbohidratos; una fuente de proteínas y una fuente de B-1,3-glucano.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1
Una composición nutricional para un niño , que comprende	Una composición nutricional que comprende
Una fuente de grasas	Calostro
Una fuente de carbohidratos	Pectina
Una fuente proteínas	lactoferrina
Una fuente B-1,3-glucano	B-1,3-glucano

Las características esenciales de la composición de la reivindicación 1 habían sido divulgadas previamente en el documento D1, el cual enseña una composición que

1http://worldwide.espacenet.com/searchResults?compact=false&PN=WO0247612&ST=advanced&locale=en_EP&DB=EPODOC
2http://worldwide.espacenet.com/searchResults?compact=false&PN=US6168821&ST=advanced&locale=en_EP&DB=EPODOC

comprende calostro (proteínas, grasa y carbohidratos), lactoferrina, glucan 1,3 y pectina de cítricos (pág.19, ln. 18 a 20)

En consecuencia, la reivindicación 1 no es nueva o carece de novedad, según lo divulgado en el documento D1.

Las reivindicaciones dependientes 2 - 5 y 7 -8, 10 a 13 15 a 18 no mencionan alguna característica que haga nueva a la composición de la reivindicación 1, por lo cual se considera que no son nuevas.

Nivel inventivo (Art18 D486)

Para las modalidades que puedan ser nuevas se tiene que:

La reivindicación 2 consiste en una composición nutricional para un niño que comprende: una fuente de grasas; una fuente de carbohidratos; una fuente de proteínas y una fuente de B-1,3-glucano que esta entre cerca de 0,010g y cerca de 0,050g por 100g de composición nutricional.

Comparación de las características técnicas esenciales, con las del estado de la técnica:

Características esenciales	D1	D2
Una composición nutricional para un niño , que comprende	Una composición nutricional para un niño , que comprende	Una composición nutricional para un niño , que comprende
Una fuente de grasas	Una fuente de grasas	Una fuente de grasas
Una fuente de carbohidratos	Una fuente de carbohidratos	Una fuente de carbohidratos
Una fuente proteínas	Una fuente proteínas	Una fuente proteínas
Una fuente B-1,3-glucano cerca de 0,010% y cerca de 0,050%	Una fuente B-1,3-glucano	Una fuente B-1,3-glucano 0.001% a 0.5%

El D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 2 porque divulga una composición que comprende calostro (proteínas, grasa y carbohidratos), lactoferrina, glucano 1,3 y pectina de cítricos (pág.19, ln. 18 a 20), además enseña que dicha composición se puede combinar con un complejo de sacáridos (como un polisacárido de glucosa, glucanos, divulgada en WO98/06418) y que esta combinación proporciona un conjunto sinérgico de proteínas, péptidos, gliconutrientes que puedan ayudar a lograr una salud óptima a través de un sistema inmune inmunomodulado.

La diferencia entre la invención, definida en la reivindicación 1 y la composición de D1 es que contiene B-1,3-glucano cerca de 0,010% y cerca de 0,050%.

A pesar de la diferencia no es posible evidenciar un efecto técnico sorprendente.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo modificar la composición de D1 para lograr una composición alternativa a la ya conocida en este arte previo”.

Sin embargo, D2 revela una composición en forma de jugo de frutas y vegetales enriquecida que además comprende B-glucanos en un porcentaje entre el 0.001% y 0.5% (col.5 a 10).

Además, se observa que los resultados mostrados por las figuras 1 a 3 y comentados en los párrafos 60 a 62, no son inesperados dado lo divulgado en el arte previo D1 sobre que la composición que divulga combinada con un complejo de sacáridos (como un polisacárido de glucosa, glucanos) produce un conjunto sinérgico de proteínas, péptidos, gliconutrientes con el efecto de ayudar a lograr una salud óptima a través de un sistema inmune inmunomodulado, como ocurre con los neutrófilos, entre otros

efectos, que conllevan a optimizar el sistema inmune (ver tabla I, pág. 20 a 23).

Por otro lado, ya se conoce que el beta-1,3-glucan es un modulador del sistema inmune³ y que los prebióticos son ampliamente utilizados en alimentos para infantes porque tienen un relevante efecto biológico en el desarrollo postnatal del sistema inmune⁴.

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría el B-glucano en un porcentaje entre el 0.001% y 0.5% (col.5 a 10) del documento D2 en la composición divulgada en el documento D1 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

Puesto que las reivindicaciones dependientes 3- 5 y 7-8, 10-13, 15-18 no mencionan características inventivas adicionales tampoco se pueden considerar inventivas.

8. Conclusión

Los requisitos de Patentabilidad de la presente solicitud están afectados así:

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO02/47612	2 - 5 y 7 -8, 10 a 13 15 a 18	Novedad Nivel inventivo
D2	US6168821	3- 5 y 7-8, 10-13, 15-18	Nivel inventivo

³ Yeast beta-1, 3-glucan and its use against anthrax infection and in the treatment of cancer (2002).Anexo al concepto

⁴Structural and functional aspects of prebiotics used in infant nutrition (2008). Anexo al concepto.

En cumplimiento del artículo 45 de la Decisión 486 de la Comisión de la Comunidad Andina, se le indica que debe dar respuesta dentro del término de sesenta (60) días contados a partir del día siguiente de la fecha de notificación de la presente comunicación. En caso de no dar respuesta al presente requerimiento, o si a pesar de la respuesta subsistieran los impedimentos para la concesión, se denegará la patente.

Se invita al solicitante se sirva indicar, si así lo considera, su renuncia al término faltante de sesenta (60) días para dar respuesta a este requerimiento y presentarla por escrito.

Finalmente, se le recuerda al solicitante que si como respuesta a este requerimiento llegara a modificar el capítulo reivindicatorio y éste contenga más de diez (10) reivindicaciones, no pagará la tasa de modificación voluntaria pero sí la correspondiente al pago por reivindicación adicional.

Notifíquese el presente oficio conforme a lo dispuesto en el numeral 5.2, literal e), del capítulo quinto del título primero de la Circular Única No. 10 de 2001.



JOSÉ LUIS SALAZAR LÓPEZ
Director de Nuevas Creaciones (c)

El anterior requerimiento fue notificado por fijación en lista, de fecha 2013-10-08

Elaboró: Lyna Del Carmen Mordecay Dunoyer
Revisó: Gloria Jacqueline Alfonso Roza

Yeast β -1,3-glucan and Its Use Against Anthrax Infection and in the Treatment of Cancer

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Clinical Assistant Professor,
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In this edition of *JANA*, the paper by Vetvicka *et al.* makes an important contribution to our scientific understanding of the nutraceutical stimulation of the immune system in the treatment of both infectious disease and cancer. While abundant evidence demonstrates the ability of β 1,3-glucans to activate macrophages and neutrophils when given intravenously or intraperitoneally, there has been little information concerning its efficiency when given orally.

In their study, Vetvicka *et al.* used oral β 1,3-glucan (Imucell™ WGP Beta Glucan) from a yeast source in mice infected with *Bacillus anthracis*. With the high incidence of complications associated with anthrax vaccines, an alternative approach is badly needed in this era of bioterrorism threat. Dr. Ken Alibek, a top-ranking scientist at the Russian bioweapons labs, stated that because of the number of possible bioweapon agents available, something other than mass inoculations would be needed. He suggested non-specific immune stimulation. The most effective form of non-specific immune stimulation is macrophage activation.

The anthrax bacillus secretes two toxins, edema toxin and lethal toxin. Edema toxin stimulates an outpouring of fluid, especially into the lungs. Lethal toxin, inhibits neutrophil phagocytosis and triggers destructive intracellular reactions that destroy macrophage cells. Of primary interest is the fact that anthrax lethal toxin inhibits the macrophages from releasing their immune messengers, primarily IL-1, IL-2, IFN-gamma, and TNF-alpha.

Of particular importance in combating infection is the cytokine TNF-alpha. Vetvicka *et al.* demonstrated that yeast-derived β 1,3-glucan given orally stimulates TNF-alpha

release from the macrophage, apparently overcoming inhibition by anthrax lethal toxin. This would account for the high survival figures in the β 1,3-glucan-treated animals. Some previous studies found no increase in TNF-alpha but a significant increase in IL-1 β .¹ Other researchers have demonstrated increased TNF-alpha in response to β -glucan stimulation.²

My own review of the literature confirms their statement that the most effective source of β 1,3-glucan is from *Saccharomyces cerevisiae*, the one chosen by most researchers. Purity of the product is vital, since protein contaminants, as seen in the earlier-used source Zymosan, can cause untoward immune reactions.

β 1,3-glucan also stimulates phagocytosis of neutrophils. In one study, the killing efficiency of neutrophils was increased 20- to 50-fold.³ This is important since the capsular antigen poly-D-glutamic acid from the anthrax organism inhibits neutrophil phagocytosis. It is the two lethal toxins and the capsular antigen that makes the anthrax organism especially deadly. In addition, β 1,3-glucan has been shown to increase clearance of bacteria by the reticuloendothelial system. Thus far, no other solutions have solved this problem.

As for β 1,3-glucan's effects on tumor growth, several studies have shown a significant effect on tumor growth in animal models.^{4,5} Early studies using immune stimulation found occasional tumor growth enhancement. This was later found to be secondary to stimulation of blocking antibody production. A safer and more effective method of immune stimulation is directed at cellular immunity, in particular the stimulation of T-helper cells and NK cells.

β 1,3-glucan has been shown to increase lymphocyte production, NK cell activation, and activation of macrophages. Several studies have also demonstrated the role played by cytokines in inhibiting tumor growth; again, particular interest is in TNF- α release.⁶ Of interest also is the role played by IL-1 β , which is increased by β 1,3-glucan as well. Interleukin 1 β has been shown to enhance mobilization of PMLs in the bone marrow and enhance their chemotactic ability. In addition, IL-1 β increases the lymphocyte count and increases their activity.⁷

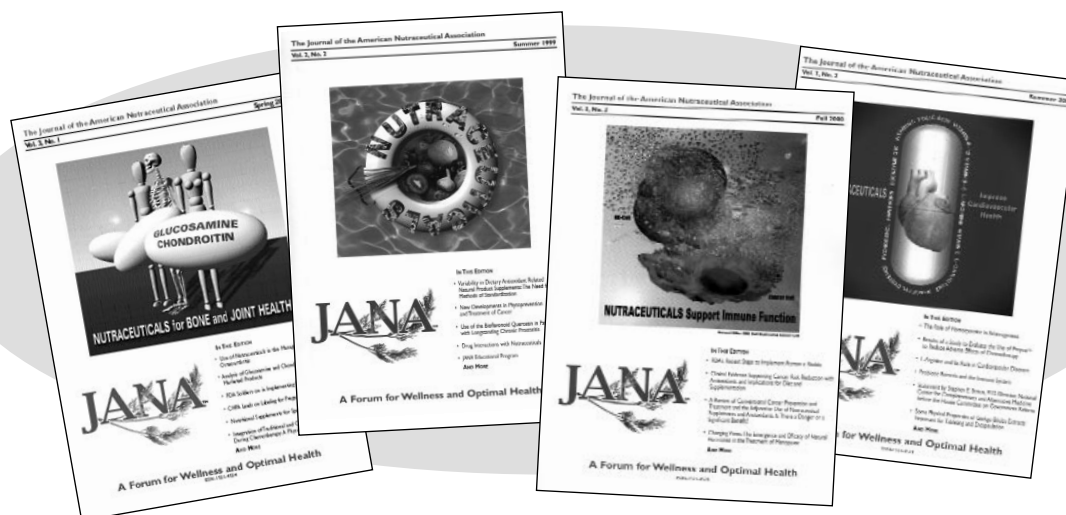
The use of β 1,3-glucan is of special interest in the cancer patient undergoing chemotherapy and/or radiation treatment, since β -glucans have shown a remarkable ability to accelerate hematopoietic recovery in both sublethally and lethally irradiated mice, even when given after the radiation dose. It can also stimulate recovery of the bone marrow following chemotherapy, something vital to restricting tumor growth and preventing infectious complications during treatment.

While data provided in the research by Vetvicka and co-workers is preliminary and needs to be confirmed by a larger controlled trial, this is an important pilot study, in that it demonstrates the effectiveness of oral β 1,3-glucan in treating both infectious agents and tumors.

REFERENCES

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Pilot Study: Orally-Administered Yeast β 1,3-glucan Prophylactically Protects Against Anthrax Infection and Cancer in Mice

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ABSTRACT

β 1,3-glucans from various bacterial, mushroom, yeast, and cereal sources have been established as immunomodulators. In the present paper we demonstrate that orally-administered yeast β 1,3-glucan (WGP Beta Glucan) had significant effects as a prophylactic treatment to reduce the mortality of anthrax infection in mice. In addition, the same type of treatment also inhibited the growth of cancer cells *in vivo*. The mechanism of action involves the stimulation of three important cytokines: IL-2, IFN- γ , and TNF- α . These results provide preclinical evidence for the beneficial effects of orally-administered yeast β 1,3-glucan.

INTRODUCTION

β 1,3-glucan's role as a biologically active immunomodulator has been well documented for over 40 years. First interest in the immunomodulatory properties of polysaccharides was raised after experiments showing that a crude yeast cell preparation stimulated macrophages via activation of the com-

plement system.¹ Further work identified the immunomodulatory active component as β 1,3-glucan.² Numerous studies have subsequently shown that β 1,3-glucans, either particulate or soluble, exhibit immunostimulating properties, including antibacterial and anti-tumor activities.^{3,4}

β 1,3-glucans can be isolated from almost every species of yeast. β 1,3-glucan derived from *Saccharomyces cerevisiae* (Baker's yeast) has been the most extensively studied. β 1,3-glucan forms a significant part of the yeast cell wall, together with mannan, proteins, lipids, and small amounts of chitin. In addition to yeast, β 1,3-glucans can be isolated from bacteria, mushrooms, algae, or cereal grains. The structure of the β 1,3-glucan depends on both source and type of isolation. Different physicochemical parameters, such as solubility, primary structure, molecular weight, and branching play a role in the biological activities of β 1,3-glucans.⁵

Original studies on the effects of β 1,3-glucan on the immune system focused on mice. Subsequent studies demonstrated that β 1,3-glucan has strong immunostimulating activity in a wide variety of other species, including earthworms, shrimps, fish, chicken, rats, rabbits, guinea pigs, sheep, pigs, cattle, and humans (for review see reference 6). Based on these results it has been concluded that β 1,3-glucan represents a type of immunostimulant that is active across the evolutionary spectrum, likely representing an evolutionarily-conserved innate immune response directed against fungal pathogens.⁷⁻⁹

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More than 800 publications have reported that β 1,3-glucans, either soluble or particulate, exhibit immunomodulatory properties. Despite the extensive investigations, no consensus on the source, size, and other properties of β 1,3-glucan has been achieved. In addition, numerous concentrations and routes of administration have been tested, including intraperitoneal, subcutaneous, and intravenous applications. For decades, oral treatment with β 1,3-glucan has been on the periphery of interest, despite the fact that it represents the most convenient route. In the last decade, however, a renewed interest in human application brought about important studies of orally-administered β 1,3-glucan.

In this paper we report on the activity of orally-delivered yeast β 1,3-glucan (WGP Beta Glucan) in infectious disease and tumor animal model systems. Although the anti-infective properties of β 1,3-glucans have been already established, the majority of this work has been done with parenterally-administered β 1,3-glucans, and the number of bacteria tested were very limited. Due to the recent threats of bioterrorism, we tested the effects of orally-delivered yeast β 1,3-glucan on infection with *Bacillus anthracis*. Similarly, the anti-tumor effects of β 1,3-glucans are well established, and most of the work has been done with parenterally-administered fungal β 1,3-glucans, or oral administration of crude mushroom glucan preparations. In this study we tested the effects of oral administration of highly purified yeast β 1,3-glucans on tumor growth.

MATERIAL AND METHODS

Yeast β 1,3-glucan

A highly purified particulate yeast-derived β 1,6-branched β 1,3-glucan (Imucell™ WGP Beta Glucan, Biopolymer Engineering, Inc., Eagan, Minn.) was used in all experiments. The glucan was suspended in water at indicated concentrations.

Bacteria

Bacillus anthracis strain Vollum 1B (USAMRIID, Ft. Detrick, Md) was used for preparation of spores by harvesting from blood agar plates followed by heat shock (80°C for 11 minutes). Aliquots diluted in phosphate buffered saline (PBS) were stored at -80°C. A well-established model of anthrax infection in mice was used.¹⁰

Mice

Female, 6-wk-old BALB/c mice were purchased from Charles River Laboratories, St. Constant, Quebec, or male 6-wk-old BALB/c mice were purchased from Clea Japan, Inc. For anthrax experiments, mice were maintained inside the biosafety level 3 laboratory at DRES. Care and handling of mice followed guidelines set out by the Canadian Council on Animal Care.

Tumor cell line

Mouse intestinal tumor cell line Colon26 was passaged

in vivo in BALB/c mice.¹¹ Twenty-one days after inoculation, the tumors were excised, gently teased over stainless steel screens, washed in RPMI 1640 medium and resuspended in PBS (1x10⁶ viable cells/ml).

Anthrax-protective prophylactic effects

Experimental groups (10 mice/group) were gavaged daily (days -7 to 0) with 0.1 ml of water containing either 0, 2 mg/kg or 20 mg/kg of WGP Beta Glucan. On day 0, 60 minutes after the final oral treatment, mice were infected subcutaneously with an LD₅₀ dose of 85 ± 11 anthrax spores/animal. Confirmation of the doses was determined by seeding 0.1 ml of the same suspension on blood agar plates. All experimental animals were monitored twice daily post-infection for 10 days. At day 10, mortality had plateaued and the experiment was ended.

Tumor-protective effects

Tumor cells (1x10⁵ viable cells in 0.1 ml) were injected into the abdominal wall of all animals on day 0. Groups of mice (8 mice/group) were gavaged daily with water or WGP Beta Glucan (28.4 mg/kg) for 21 consecutive days (days 1-21). Twenty-one days after tumor administration animals were sacrificed, tumors excised, and tumor weight measured.

Chemicals

Fetal calf serum and RPMI 1640 medium were purchased from Gibco BRL (Rockville, MD), HEPES, Concanavalin A, and lipopolysaccharide were purchased from Sigma (St. Louis, MO).

Cytokines

For evaluation of cytokine production, we incubated purified spleen cells from each animal (2x10⁶ cells/ml in RPMI 1640 medium with 5% FCS) in wells of a 24-well tissue culture plate. After addition of 1 µg of Concanavalin A or 10 µg of LPS per well, cells were incubated for 72 hr in a humidified incubator (37°C, 5% CO₂). At the endpoint of incubation, supernatants were collected, filtered through 0.45 µm filter and tested for the presence of IL-2, IFN-γ and TNF-α. Levels of cytokines were measured by commercial ELISA in accordance with the protocol for Cytoscreen™ of BioSource International, Inc. (Camarillo, CA).

RESULTS

Orally-administered WGP Beta Glucan treatments showed significant anthrax-protective anti-infective effects. Under our experimental conditions, 5 out of 10 control mice survived the anthrax infection. Compared to this 50% control survival rate, prophylactic daily oral doses of WGP Beta Glucan (2 or 20 mg/kg) increased survival to 100% (Figure 1).

Orally-administered WGP Beta Glucan treatments also showed tumor-protective effects on tumor size and vascularization (Figure 2). Table 1 shows the effects of oral administration of WGP Beta Glucan on tumor weight. These

results show the beneficial effects of WGP Beta Glucan, as evidenced by a statistically significant decrease (-21%) in tumor weight at day 21 from 0.66 +/- 0.06 g in control mice to 0.52 +/- 0.06 g in β 1,3-glucan-treated mice ($P < 0.05$). In addition, the control animals were observed to be inactive and crouching, and had reduced body temperature in comparison to the WGP Beta Glucan-treated animals.

Evidence of orally-administered WGP Beta Glucan immunomodulatory activity was also demonstrated through effects on the production of three different cytokines, IL-2, IFN- γ , and TNF- α (Table 2). The production of these three cytokines was measured after a 72 hr *in vitro* incubation of spleen cells isolated from control and WGP Beta Glucan-administered animals. For all three tested cytokines, oral administration of WGP Beta Glucan resulted in significantly-increased cytokine levels (IL-2 (2.3-fold), IFN- γ (4.4-fold), and TNF- α (2.2-fold), $P < 0.05$) over control animals.

DISCUSSION

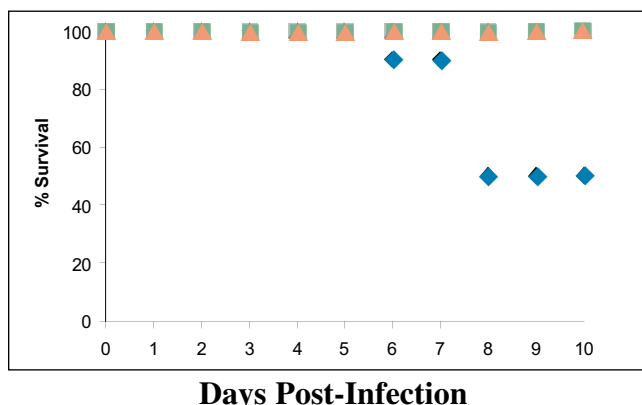
β 1,3-glucan is widely used as a dietary supplement, with well-established stimulating effects on the immune defense system.^{6,12} A large body of published data supports this use. Browder *et al.* described strongly decreased septic morbidity with β 1,3-glucan administration.¹³ A series of well-documented multicenter blind studies demonstrated that β 1,3-glucan-treated patients had significantly lower infection rates.^{14,15} Positive effects were also found in patients after cardiopulmonary bypass,¹⁶ and inhibition of

antiviral activity has been found in HIV-infected patients.¹⁷ Some β 1,3-glucans are routinely used in patients for tumor immunotherapy.^{18,19}

The majority of the previous work has dealt with injected β 1,3-glucans. Only a limited number of investigations have focused on oral administration. Suzuki *et al.* demonstrated significant activation of peritoneal macrophages by orally-delivered β 1,3-glucan.^{20,21} In a subsequent communication they reported an enhancement of alveolar macrophage function by oral delivery of β 1,3-glucan,²² probably mediated via the Peyer's patches in the intestinal wall. Oral administration of lentinan has been found to increase the number of T helper cells in blood of lentinan-fed rats.²³ To further study the mechanism of action of orally-administered β 1,3-glucans, Ikuzawa *et al.* studied the fate and tissue distribution of Krestin.²⁴ Based on these promising reports, latter attention focused on effects of β -glucans delivered *per os*. The summation of this work suggests that β 1,3-glucans function by stimulating host immune defense mechanisms, primarily macrophages, neutrophils, and NK cells.

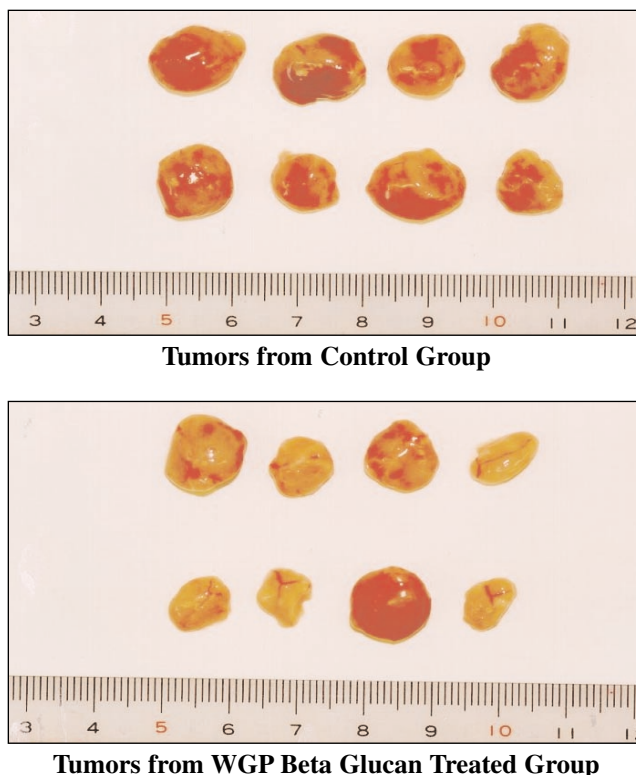
With the threat of bioterrorism in the United States

Figure 1. Anthrax-protective effect of daily oral prophylactic administration of WGP Beta Glucan.



Groups of 10 Balb/c mice were gavaged daily (days -7 to 0) with 0.1 ml of water as a control (◆) or 0.1 ml water containing 40 (■) or 400 µg (▲) of WGP Beta Glucan per mouse (2 or 20 mg/kg). On Day 0, one hour after the last prophylactic oral dosing, animals were infected subcutaneously with an LD₆₀ dose of *B. anthracis* spores. Animals were observed daily until the end of the study and survival time recorded. The percentage survival was calculated from the ratio of surviving animals each day to the total number of challenged animals in each group (n = 10). *P values were determined using a Fisher exact test (daily prophylactic 2 and 20 mg/kg; $P = 0.016$).

Figure 2. Tumor-protective effect of daily oral administration of WGP Beta Glucan.



Tumor cells were implanted into all animals by abdominal wall injection on Day 0. Groups of mice (8 mice/group) were gavaged daily with water or WGP Beta Glucan (28.4 mg/kg) for 21 consecutive days (days 1 to 21). Twenty-one days after tumor administration animals were sacrificed, tumors excised and photographed.

Table 1. Effect of oral administration of WGP Beta Glucan on tumor growth

	Control	WGP Beta Glucan
Tumor Weight (g)	0.66 +/- 0.06	0.52 +/- 0.06*

At day 21 after tumor cell administration animals were sacrificed, tumors excised, and weighed. *P values were determined using a student's T-test ($p < 0.05$).

becoming a reality, we tested the hypothesis that oral yeast β 1,3-glucan could be used as a protective agent against anthrax infection. The preclinical results described in this report demonstrate that orally-administered WGP Beta Glucan has strong anthrax-protective effects. Oral WGP Beta Glucan treatment significantly increased the number of surviving animals as well as prolonged survival time of lethally-infected animals. Dose ranging studies to date have demonstrated that daily prophylactic doses of 2-20 mg/kg WGP Beta Glucan provides a maximal anthrax-protective effect in mice. Ongoing dose ranging studies are being conducted to determine the minimal WGP Beta Glucan dose required to protect mice against a lethal anthrax infection. Based on the presented preclinical data, WGP Beta Glucan shows promise as a prophylactic treatment to support the immune system and reduce the risk of anthrax infection.

Oral WGP Beta Glucan treatment also reduces the threat of cancer, slowing down the progression of tumor growth in a preclinical colon cancer model. This observation extends the large body of preclinical and clinical work done in Japan demonstrating the oral anti-tumor activity of mushroom β 1,3-glucans²⁵⁻²⁷ to yeast β 1,3-glucan. These published studies have demonstrated that β 1,3-glucan immunotherapy leads to the activation of the innate immune cells (macrophages, neutrophils (PMN) and natural killer (NK) cells), the stimulation of tumoricidal activities, production of cytokines, and the generation of enhanced cell-mediated responses. Suzuki and colleagues have reported the stimulation of activated macrophages by the administration of SSG²² and NK-type lymphokine-activated killer cells by the combined administration of lentinan and IL-2.²⁸ The stimulation of tumoricidal activities in PMN by a linear bacterial β 1,3-glucan has also been reported.^{29,30} A number of clinical studies have demonstrated synergy between oral β 1,3-glucan immunotherapy, and traditional radiation and chemotherapeutic cancer treatment options.^{31, 32}

At present we do not fully understand the mechanisms mediating the anthrax and tumor-protective effects of WGP Beta Glucan. We believe that through specific interactions between the β 1,3-glucan active component of WGP Beta Glucan and β 1,3-glucan receptors on M-cells within Peyer's patches in the intestinal mucosa that a systemic signal provided by cytokines is elicited by the gut-associated lymphatic system that stimulates the innate immune system

Table 2. Effects of oral administration WGP Beta Glucan on cytokines

Cytokine	Control	WGP Beta Glucan
IL-2	9.7 +/- 0.5 pg/ml	23.4 +/- 2.1 pg/ml*
IFN-γ	107.8 +/- 8.4 pg/ml	475.8 +/- 42.3 pg/ml*
TNF-α	487.8 +/- 58.2 pg/ml	1083.5 +/- 44.6 pg/ml*

Twenty-one days after tumor cell administration, animals were sacrificed, spleens excised, and spleen cells purified from each animal. Spleen cells from each animal were cultured with 1 μ g of Concanavalin A or 10 μ g of LPS for 72hr. Culture supernatants were collected, filtered through 0.45 μ m filter, and tested for presence of IL-2, IFN- γ , and TNF- α . *P values were determined using a student's T-test ($P < 0.05$ for IL-2, IFN- γ , and TNF- α).

components (macrophages, neutrophils, and NK cells) to a higher functional level, increasing the first line of host defense mechanisms. For these experiments we focused on three important cytokines, IL-2, IFN- γ , and TNF- α . All of these cytokines play an important role not only in physiological processes, but also in bioregulation of host defense reactions. IL-2 is a cytokine produced by activated CD4 and some CD8 T lymphocytes. In addition to being the major T cell growth factor, IL-2 also stimulates: growth and differentiation of cytotoxic T cell precursors, NK cells, differentiation of activated human B-lymphocytes, and activation of monocytes. TNF- α is a pleiotropic cytokine secreted primarily by monocytes/macrophages and T lymphocytes, respectively. TNF- α is the principal mediator of natural immunity against gram-negative bacteria and a key mediator of inflammatory responses and septic shock.³³ IFN- γ , sometimes called immune interferon, is produced mainly by T lymphocytes as a result of antigenic or mitogenic stimulation. The activities of IFN- γ are many, including induction of MHC expression, macrophage activation, and effects on the differentiation of lymphocytes.

In this paper we report important biological activities of yeast-derived β 1,3-glucan (Imucell™ WGP Beta Glucan). Oral administration of WGP Beta Glucan increased the production of three important cytokines (IL-2, IFN- γ , and TNF- α), inhibited growth of cancer cells *in vivo*, and provided a prophylactic defense against anthrax infection in mouse models. Despite our current lack of knowledge about the precise mechanisms through which oral β 1,3-glucan mediates its protective effects, these anti-tumor and anti-infective properties of yeast-derived WGP Beta Glucan presented in this report suggest that further study is warranted to understand these benefits of β 1,3-glucans.

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Structural and Functional Aspects of Prebiotics Used in Infant Nutrition^{1,2}

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Abstract

Breast-feeding is associated with several benefits. Among them, the balanced postnatal development of the immune system is 1 of the key functions of breast-feeding. Although this effect is of multifactorial origin, it is widely accepted that the entire intestinal microbiota of breast-fed infants represents an important stimulating factor of the postnatal development of the immune system. The effect of breast-feeding on the intestinal microbiota can not be attributed to a single compound, but there is accumulating evidence that human milk oligosaccharides play a crucial role. Because there is a broad consensus that the intestinal microbiota plays an important physiological role for the host, many attempts have been made to influence the intestinal flora by dietary interventions. This article summarizes results of intervention studies in which nonmilk oligosaccharides have been used to mimic the prebiotic effect of breast-feeding. A second focus has been related to the question of whether the prebiotic activity has beneficial effects on the postnatal development of the immune system. The data clearly demonstrate that prebiotics of nonmilk origin can mimic the prebiotic effect of breast-feeding, and this has positive consequences for the postnatal development of the immune system. J. Nutr. 138: 1818S–1828S, 2008.

Introduction

Human milk is considered to be the ideal nutrition for term infants because it provides all necessary nutrients for normal growth and development. The quantity and quality of nutrients are adapted to the functional maturation of the gastrointestinal tract as well as the metabolic state of the infant, so that relatively low concentrations of nutrients fulfill the requirements of the infant. In addition, human milk contains components that survive—partially or completely—intestinal digestion and provide functional capacity (1,2). There is a broad consensus that breast-fed infants grow and develop differently than infants with artificial feeding (3). Breast-fed infants have a reduced incidence of allergic or atopic diseases (4–6) as well as of infections (7–9) in comparison to bottle-fed infants, indicating a major impact of breast-feeding on the development of the immune system. Further potential benefits of breast-feeding, such as reduced incidence of diabetes mellitus type I (10), better cognitive

functions (11), and lower blood pressure (12), have been discussed.

The prevalence of atopic diseases has steadily increased during recent decades in the developed countries. Thus, they represent a major public health problem, particularly during infancy and childhood in most areas (13). There is increasing evidence that the composition of the intestinal microbiota plays a key role in the postnatal development of the immune system (14,15). Before birth, the infant's gut is sterile. During vaginal delivery, the natural colonization of the infant starts with bacteria mainly from the vaginal and intestinal microbiota of the mother. For the further development of the intestinal microbiota of the infant, the diet plays an important role (16). During breast-feeding, the composition of the gut microbiota develops within a short period and becomes dominated by bifidobacteria, whereas formula-fed infants without prebiotics develop a flora of a more adult type (17).

Because of the importance of the intestinal microbiota for the development of the gut physiology and the immune system, many attempts have been made to mimic the intestinal microbiota of breast-fed infants in bottle-fed infants.

The composition of the intestinal microbiota can be influenced either by administration of living health-promoting bacteria that survive the gastrointestinal tract, exert their biological activity by interaction with the surface of the small intestine, and colonize the colon (18) or by application of dietary ingredients that are nondigestible during the passage through the small intestine, reach the colon, and stimulate selectively health-promoting colonic bacteria (19,20) or by combining both principles in a “synbiotic” approach (21).

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This article summarizes the current knowledge on the influence of human milk oligosaccharides (HMOs)⁶ on the development of intestinal microbiota, the possibilities of mimicking this function with oligosaccharides of nonmilk origin, and the potential beneficial effects of prebiotic oligosaccharides on the postnatal development of the immune system.

Structure of HMOs

The oligosaccharides in human milk are characterized by an enormous structural diversity. There are great variations in concentration and composition among individuals and during the course of lactation (22). They appear as free structures or are conjugated to macromolecules such as glycoproteins, glycolipids, and others (23–25). This article focuses on the structure and function of free oligosaccharides in human milk.

The monomers of HMOs are D-glucose, D-galactose (Gal), N-acetylglucosamine, L-fucose (Fuc), and sialic acid (N-acetylneuraminic acid). There is evidence that >1000 distinct molecules exist in the HMO fraction (23,24).

Oligosaccharides appear in human milk at concentrations up to 10 g/L. The molecules are synthesized in the breast starting with lactose at the reducing terminus. The core molecule is characterized by repetitive attachment of Gal and N-acetylglucosamine, in β -glycosidic linkage to lactose. The α -glycosidic linkages of Fuc to the core molecule characterize the neutral fraction, and the additional linkage to sialic acid characterizes the acidic fraction of HMOs (25). Specifically, the attachment of Fuc is based on the secretor/Lewis blood group status of the individual mother (26).

Physiological functions of HMOs

There are many different functions attributed to HMOs (23–25,27), which may explain the great variety of their structures. In this article, the prebiotic function (mainly related to the neutral fraction of HMOs), the antiadhesive properties that protect the epithelial surface from attachment of pathogens (mainly related to the acidic fraction of HMOs), and possible direct interactions of HMOs with the immune system are discussed in the next sections.

Prebiotic effect of HMOs. The prebiotic effect of human milk was intensively investigated over the last century. In particular, several milk proteins, such as lactoferrin and lactalbumin, or urea as part of the nonprotein nitrogen fraction have been described as “bifidogenic factors” (28). Although the effect of human milk on the postnatal development of the intestinal microbiota cannot be attributed to a single ingredient, there is evidence that HMOs play a key role (23,27–29).

Because the human intestine expresses no luminal enzymes to cleave the α -glycosidic linkages of Fuc and sialic acid as well as the β -glycosidic linkages in the core molecule, they are resistant to enzymatic cleavage in the intestine (30–32). As a result of their low digestibility, HMOs are still detectable in feces of breast-fed infants (33). On the other hand, many intestinal bacteria express glycosidases to metabolize HMOs (34).

Recent studies on the genome of bifidobacteria (35) and other intestinal bacteria (*Bacteroides*, *Actinobacteria*, and others) (36) have revealed the particular adaptation of the bacterial metabolism to the environment provided by the host. Bifidobacteria are highly endowed with glycohydrolases able to metabolize

monosaccharides, which constitute molecules of HMOs (37). Thus, the high portion of the genome dedicated to sugar metabolism in bifidobacteria might explain their large presence in the colon, reflecting a specific adaptation to this highly competitive ecological niche, especially in breast-fed infants.

Data so far available on HMOs have focused mainly on their role as growth and proliferation enhancement factors for bifidobacteria. Ward et al. (38) demonstrated in vitro studies using HMOs as a sole carbon source that *Bifidobacterium longum* and *Bifidobacterium infantis* showed significantly higher fermentation of the tested HMOs compared with the fermentation observed with *Escherichia coli*. These data clearly confirm that bifidobacteria can indeed utilize complex carbohydrates such as HMOs, supporting the hypothesis that these substances selectively amplify the bacterial population in the infant's intestine.

All these data provide strong evidence that many HMOs are preferentially synthesized to be metabolized by intestinal microbiota.

In summary, although it is not possible to calculate exactly the quantities of oligosaccharides digested by the intestinal microbiota, the available data indicate that the majority of HMOs are used as prebiotic substrates rather than as nutritional substrates.

Direct interactions of HMOs with immune cells and bacteria. Apart from their prebiotic effect, there is also evidence that HMOs act as receptor analogs to inhibit the adhesion of pathogens on the epithelial surface (39). Specific binding of HMOs to bacterial structures that mediate the adhesion on the epithelial surface is seen as a passive defense of the host. There are many different target structures (24), which might partially explain the great variety of structures of HMOs.

There is also the possibility that HMOs interact directly with immune cells (40–42). Direct effects can be the result of interactions of the HMOs with selectins (43), dendritic cell specific C-type lectin (44), integrins (45), and other target receptors such as Toll-like receptors (46). In an in vitro study in which human white blood cells separated from cord blood were incubated with fractions of neutral and acidic HMOs [separated from pooled human milk (47,48)], particularly acidic HMOs resulted in a decrease of activated or regulatory T cells (40).

Because HMOs are resistant to digestion, they can pass the intestinal wall in small amounts (~1% of intake) and can be detected in the urine of breast-fed infants (33). The appearance in the plasma and the distribution across the whole body might be 1 factor for a systemic effect on the immune system of HMOs. However, this hypothesis needs further investigation.

Clinical evidence of beneficial effects of HMOs. The infant at birth is extremely vulnerable to infections and therefore needs particular protection and support.

Howie et al. (7) investigated 618 pairs of mothers and their infants to evaluate the influence of early diet on incidence of infectious symptoms. They could demonstrate that infants who were breast-fed for >13 wk had a lower incidence of gastrointestinal (2.9% vs. 15.7%) and of respiratory infections (25.6% vs. 37.0%) compared with bottle-fed infants. The reduction of infections was maintained even beyond the period of breast-feeding, indicating an immune modulation effect of breast-feeding. In summary, breast-feeding has been shown to enhance the development of the immune system of the newborn, resulting in protection against enteric and respiratory infections (7,9,49,50).

⁶ Abbreviations used: DP, degree of polymerization; FOS, fructo-oligosaccharides; Fuc, L-fucose; Gal, D-galactose; GOS, galacto-oligosaccharides; HMOs, human milk oligosaccharides; lc, long chain; sc, short chain.

Breast-feeding also appears to protect infants from the development of atopic diseases (51,52) and is associated with a reduced incidence of immune-mediated diseases demonstrating evidence that breast-feeding stimulates the postnatal development of the immune system (53–55).

Although human milk protects the infant from infections in a very complex way (54), there is evidence that HMOS is 1 important factor of breast milk to strengthen the infant's immune system.

Non-human-milk oligosaccharides

There is a wide range of molecule size distribution within the HMOS fraction. Since 1980, oligosaccharides have been defined as carbohydrates with a degree of polymerization (DP) up to 10. However, oligosaccharides have recently been variously defined as a DP ranging from 2 to 20 or more. Recently, the IUB-IUPAC Joint Commission on Biochemical Nomenclature stated that the borderline between oligo- and polysaccharides cannot be drawn too strictly. However, the term oligosaccharide is commonly used to refer to defined structures as opposed to a polymer of unspecified length. The same approach is used for oligosaccharides of non-human-milk origin as long as they have defined structures (56,57).

There are still many open questions regarding the relation between the structure of oligosaccharides and their biological function (23). Because HMOS have been identified as functional compounds in human milk, many efforts have been taken to mimic these functions by alternative compounds. Oligosaccharides from milk of domestic animals as well as several oligosaccharides of nonmilk origin have been investigated.

Oligosaccharides from animal milks. Free oligosaccharides are natural constituents of all mammal milks. In comparison to human milk, the concentrations of oligosaccharides in these milks are much lower, and their structure is less complex (24,58).

In the neutral fraction of animal milk oligosaccharides, in contrast to HMOS, linkages to Fuc are very rare with a few exceptions, whereas linkages of Gal or GalNAc are dominant. In addition, Gal and GalNAc can be detected in α -glycosidic linkage at the nonreducing terminus. Sialic acid is the most important structural element in the acidic fraction of the animal milk oligosaccharides. The oligosaccharides from domestic animals have been extensively reviewed by Uraschima et al. (59). Based on the structures of these oligosaccharides, it can be assumed that they might also be effective as prebiotics in humans. They might also provide antiadhesion properties to prevent the adhesion of pathogens on the epithelial surface, and direct interactions with immune cells can not be excluded. Despite all these theoretical advantages, the preparation of these compounds is difficult, and therefore, large-scale production is not yet commercially available. Consequently, no clinical trial has been published so far using fractions of animal milk oligosaccharides as prebiotics.

Nonmilk oligosaccharides. Another alternative is the use of nonmilk oligosaccharides. These can be found in bacteria, fungi, and plants and derive from hydrolysis of dietary polymers during digestion. Technologically, they can be extracted from natural sources or be synthesized from monomers and/or small oligosaccharides or derived from hydrolysis of natural polymers.

Prebiotic effects during infancy have been investigated for galacto-oligosaccharides (GOS) (60,61), short-chain fructo-oligosaccharides (scFOS) (62–71), inulin (72,73), lactulose

(74,75), and combinations such as a mixture of scGOS and lactulose (76), a mixture of scFOS and long-chain (lc)FOS (77), galacturonic acid oligosaccharides in combination with scGOS and lcFOS (78), and a mixture of scGOS and lcFOS (79–104) (see also **Tables 1** and **2**).

There are several other carbohydrates under investigation in regard to their possible prebiotic function, such as pectins, resistant starch, xylo-oligosaccharides, soybean oligosaccharides, or isomaltulose (24,105–107). However, there are no data available with respect to their prebiotic properties in infancy.

The counts of fecal bifidobacteria, their percentage among the total bacteria, and the production of SCFA are generally accepted measurements to detect a prebiotic effect. On the basis of these markers, sufficient data are available only for GOS and FOS to classify them as prebiotics (20,108). Therefore, the next section focuses on these 2 prebiotics.

Structure of GOS and FOS. The GOS are synthesized from lactose via an enzymatic transgalactosylation using a β -galactosidase mainly of bacterial origin (109). These GOS consist of a chain of galactose monomers, usually with a glucose monomer on the reducing terminus, with a DP much less than 10 monomers.

Fructans are linear or branched fructose polymers that are either β 2–1-linked inulins or β 2–6-linked levans. The inulin-type fructans can easily be extracted from plant sources and have widely been used as ingredients for dietary products. In the current literature, the term FOS refers to the inulin-type fructans. In the natural sources of FOS, the molecule size is widespread [DP ranging from 2 to >60 (110)]. Because the biological activity of prebiotics depends on the molecular size (111), it is particularly important to consider the molecular size distribution for reviewing clinical data on fructans. lcFOS are prepared from inulin from which the scFOS (DP 2–6) have been largely removed and consequently contain predominantly large molecules with a DP between 7 and 60.

Digestibility of GOS and FOS. Because nondigestibility in the small intestine and selective fermentation by the intestinal microbiota are prerequisites of any prebiotic effect of dietary ingredients (20), human studies have been performed to address this issue.

In fructose-sensitive patients fed lcFOS, no side effect could be detected demonstrating the low or absent digestibility of lcFOS (112). In adult patients with ileostomata, scGOS are still detectable after passage of the small intestine (113). In term infants fed a formula supplemented with scGOS/lcFOS, both prebiotics could be detected in the feces (81). Although it is difficult to quantify the percentage of substrate that reaches the colon, it can be speculated that the majority reaches the colon. This assumption is supported by analyses of the bacterial fermentation products of intestinal microbiota. The mixture of scGOS/lcFOS results in similar SCFA profiles than HMOS in vitro (114) as well as in term infants (83).

This is in line with in vitro experiments in which the fermentation of GOS and FOS by bifidobacteria and lactobacilli has been studied (114–117).

Prebiotic function of GOS and FOS. GOS as supplement to a formula stimulated the counts of bifidobacteria and lactobacilli in 2 studies (60,61). In contrast, the data from studies in which scFOS were investigated are inconsistent (62–70). Only 2 small studies have been published using inulin as supplement (72,73). Both studies demonstrate a bifidogenic effect of inulin.

TABLE 1 Clinical trials with prebiotic oligosaccharides in term infants (nutritional intervention during the first year of life)

Prebiotic compound ¹	Basic nutrition ¹	Target group and age	Study groups (n)	Main outcome	Reference
scGOS, 20 g/L	IMF Intact CMP	Healthy term infants	Prebiotics (43) Control (17) BM reference (20)	Increased counts of bifidobacteria and lactobacilli	Yahiro et al. (60)
scGOS, 24 g/L	IMF Intact CMP	Healthy term infants 0–6 mo	Prebiotics (69) Control (52) BM reference (26)	Increased counts of bifidobacteria and lactobacilli, decreased fecal pH	Ben et al. (61)
scFOS, 45 g/L	FOF Intact CMP	Infants with antibiotic treatment 6–24 mo	Prebiotics (57) Control (56)	Increased counts of bifidobacteria after antibiotic treatment	Brunser et al. (62)
scFOS, 0.55 g/15 g	Cereals	Healthy infants 4–24 mo	Prebiotics (63) Control (60)	Decreased severity of diarrhea diseases (no microbiology)	Sarveeda et al. (63) Tschernia et al. (64)
scFOS, 15 and 30 g/L	IMF Intact CMP	Healthy term infants 2–12 wk	Prebiotics, 1.5 g (28) Prebiotics, 3.0 g (30) BM reference (14)	No clear effect on counts of bifidobacteria, softer stools (dose dependent)	Euler et al. (65)
scFOS, 0.75 g/25 g	Cereals	Healthy infants 4–12 mo	Cereals + FOS (27) Placebo (29)	Softer stools, no effect on fecal pH (no microbiology)	Moore et al. (66)
scFOS, 0.55 g/15 g	Cereals	Healthy infants 6–12 mo	Prebiotics (239) Control (276)	No influence on clinical course and incidence of diarrhea, no effect on vaccination response (no microbiology)	Duggan et al. (67)
scFOS	IMF Intact CMP	Healthy term infants 6–24 mo	Prebiotics (10) Control (12)	Trend for higher counts of bifidobacteria and decrease in potential pathogens, no persists after intervention	Waligora-Dupiet et al. (68)
scFOS, 1.0, 2.0, 3.0 g/day	IMF Intact CMP	Healthy term infants 0–6 wk	Prebiotics, 1.0 g (13) Prebiotics, 2.0 g (11) Prebiotics, 3.0 g (12) Controls (17)	Increased number of stools, no bifidogenic effect, no influence on faecal pH	Guesry et al. (69)
scFOS, 15 and 30 g/L	IMF Intact CMP	Healthy term infants 0–14 wk	Prebiotics, 1.5 g (72) Prebiotics, 3.0 g (74) Control (66)	Safe and less complication (no microbiology)	Bettler et al. (70)
Inulin, 1.0 g/d	IMF Intact CMP	Healthy term infants 5–24 wk	Inulin and placebo as crossover (14)	Increased counts of bifidobacteria and lactobacilli, softer stools	Kim et al. (72)
Inulin, 0.75, 1.0, and 1.25 g/d	IMF Intact CMP	Healthy term infants 5–12 mo	Prebiotics, 0.75 g/d (10) Prebiotics, 1.0 g/d (9) Prebiotics, 1.25 g/d (9) Controls (8)	Tendency of increased short chain fatty acid production, significant influence on mineral absorption (no microbiology)	Yap et al. (73)
Lactulose, 5 and 10 g/L	IMF Intact CMP	Healthy term infants 0–6 mo	Lactulose and control as crossover (6)	Increased counts of bifidobacteria, reduced fecal pH	Nagendra et al. (74)
Lactulose, 6 g/d	IMF Hydrolyzed CMP	Infants with allergic symptoms 1–36 mo	Prebiotics (12)	Increased counts of bifidobacteria, improvement of symptoms	Rinne et al. (75)
scGOS/lactulose, 4 and 8 g/L	IMF Intact CMP	Healthy term infants 0–6 mo	Prebiotics, 4 g/L (74) Prebiotics, 8 g/L (76) Controls (76)	Softer stools and increased stool frequency (no microbiology)	Ziegler et al. (76)
scFOS/inulin, 1.0 g/25 g	Cereals	Healthy term infants 0–12 mo	Prebiotics (24) Control (25)	Increased postvaccination IgG measles antibody plasma levels	Firmansyah et al. (77)
AOS, 2 g/L and 2 g/L + 6 g/L scGOS/lcFOS	IMF Intact CMP	Healthy term infants 0–6 mo	Prebiotic AOS (16) Prebiotic AOS + scGOS/lcFOS (15) Control (15)	Increased counts of bifidobacteria with GOS/FOS/AOS, decreased fecal pH	Fanaro et al. (78)
scGOS/lcFOS, 8 g/L	IMF, partially hydrolyzed protein	Infants with constipation 3–20 wk	Prebiotic (20) Control (18)	Reduced hardness of stool (no microbiology)	Bongers et al. (79)
scGOS/lcFOS, 4 and 8 g/L	IMF Intact CMP	Healthy term infants 0–4 mo	Prebiotics, 4 g (28) Prebiotics, 8 g (28) Control (29)	Increased counts of bifidobacteria and lactobacilli, decreased fecal pH, effect dose dependent	Moro et al. (80) Moro et al. (81)

(Continued)

TABLE 1 Continued

Prebiotic compound ¹	Basic nutrition ¹	Target group and age	Study groups (n)	Main outcome	Reference
scGOS/lcFOS, 8 g/L	IMF, partially hydrolyzed protein	Healthy term infants 0–6 mo	BM reference (15) prebiotic (28) placebo (29)	Increased counts of bifidobacteria, softer stools	Schmelze et al. (82)
scGOS/lcFOS, 8 g/L	IMF Intact CMP	Healthy term infants 0–6 mo	BM reference (15) Prebiotic (21) Placebo (20)	Increased counts of bifidobacteria and lactobacilli, dominance of <i>B. infantis</i> , short chain fatty acid pattern as in breast-fed infants	Knol et al. (83) Haarman and Knol (84) Haarman and Knol (85)
scGOS/lcFOS, 4 g/L	IMF Intact CMP	Healthy term infants 1–12 wk	Prebiotics (34) Control (32)	Trend for higher counts of bifidobacteria, reduced counts of clostridia	Costalos et al. (86)
scGOS/lcFOS, 8 g/L	IMF, partially hydrolyzed protein	Infants with minor gastrointestinal problems 9–12 mo	Prebiotics (604)	Reduction of gastrointestinal problems (no microbiology)	Salvino et al. (87)
scGOS/lcFOS, 8 g/L	IMF, partially hydrolyzed protein	Infants with minor gastrointestinal problems 9–12 mo	Prebiotics (55) Control (40)	Reduction of gastrointestinal problems (no microbiology)	Salvino et al. (88)
scGOS/lcFOS, 8 g/L	IMF Intact CMP	Term infants at weaning 4–12 mo	Prebiotic (10) Control (10)	Increased counts of bifidobacteria	Scholtens et al. (89)
scGOS/lcFOS, 6 g/L	IMF Intact CMP	Healthy term infants 0–4 mo	Prebiotic (19) Control (19)	Reduced fecal pH, increased fecal short chain fatty acids, increased fecal sIgA; no significant higher counts of bifidobacteria compared with controls	Bakker-Zierikzee et al. (90) Bakker-Zierikzee et al. (91)
scGOS/lcFOS, 8 g/L	IMF, extensively hydrolyzed protein	Healthy term infants at risk for allergy 0–6 mo	Prebiotic (102) Control (104)	Increased counts of bifidobacteria, reduced incidence of atopic dermatitis, reduced incidence of infections, anti allergic serum antibodies	Moro et al. (92) Arslanoglu et al. (93) Garrssen et al. (94) Arslanoglu et al. (95)
scGOS/lcFOS, 6 g/L	IMF Intact CMP	Healthy term infants 0–26 wk	Prebiotic (86) Control (90)	Increased counts of bifidobacteria, increased fecal sIgA	Alliet et al. (96)
scGOS/lcFOS, 4 g/L	IMF Intact CMP	Healthy term infants 0–12 wk	Prebiotic (14) Control (19)	Increased counts of bifidobacteria	Decsi et al. (97)
scGOS/lcFOS, 8 g/L	IMF Intact CMP	Healthy term infants 0–6 mo	Prebiotic (8) Control (8) BM reference (8)	Increased counts of bifidobacteria <i>Bifidobacterium</i> microbiota close to breast-fed infants	Rinne et al. (98)
scGOS/lcFOS, 8 g/L	IMF Intact CMP	Healthy term infants 0–4 wk	Prebiotic (20) Observation study	Increased counts of bifidobacteria and lactobacilli	Penders et al. (99)
scGOS/lcFOS, 4 g/L	IMF Intact CMP	Healthy term infants, 0–12 mo	Prebiotic (162) Control (164)	Decreased rate of infection (recurrent upper respiratory tract infection, diarrhea)	Bruzzese et al. (100)

¹ IMF, infant milk formula; FOF, follow-on formula; AOS, acidic oligosaccharides deriving from pectin; CMP, cow milk protein.

In recent years different mixtures have been studied. In particular, a combination of scGOS/lcFOS (ratio 9:1) (Immunofortis, Numico, Wageningen, The Netherlands) has been extensively studied, as has a mixture of scFOS with lcFOS (see Tables 1 and 2).

There are several reasons to investigate a mixture of oligosaccharides instead of individual components (118). One is the fact that the composition of the entire intestinal microbiota is very complex (17), which might require different substrates for the development of the entire microbiota. A second reason is the great variability of oligosaccharide structures in human milk (24), which also indicates that several structures are necessary to stimulate microbiota typical for breast-fed infants.

Because the interaction among dietary components and the intestinal ecosystem is very complex, the matrix of the food might influence the effectiveness of oligosaccharides. The type and concentration of proteins have been discussed as factors modulating the intestinal microbiota [intensively reviewed by

Coppa et al. (28)]. Most studies in infants have been performed with cow-milk-based formulas with different qualities and quantities of protein. Prebiotics have also been successfully added to solid weaning food or cereals (Tables 1 and 2). Prebiotic effects have also been seen in adults consuming a typical Western diet (119). Thus, there is evidence that the prebiotic effect is independent of the type of food used as basis for the nutrition.

Effect of colonic fermentation of GOS and FOS. The bifidogenic effect is often associated with a reduction of the stool pH and changes in the SCFA pattern. Lower fecal pH has been described in a study using GOS (61) and in studies using a mixture of scGOS/lcFOS (78,80,83,90), but scFOS were not able to influence fecal pH (66,69).

As mentioned above, supplementing an infant formula with the mixture of scGOS/lcFOS resulted in a pattern of SCFA in feces corresponding to that found in the feces of breast-fed

TABLE 2 Review of clinical trials with prebiotics in preterm infants (nutritional intervention during the first year of life)

Prebiotic compound	Basic nutrition	Target group and age	Study groups (n)	Main outcome	Reference
scFOS, 4 g/L	Preterm formula, Intact CMP ¹	Healthy preterm infants 0–21 d	Prebiotics (36) Controls (20)	Increased counts of bifidobacteria within 1 wk of intervention	Kapiki et al. (71)
GOS/lcFOS, 10 g/L	Preterm formula, Intact CMP	Healthy preterm infants 0–21 d	Prebiotics (15) Controls (15) Reference (13)	Increasing counts of bifidobacteria, reduction of hardness of stools, reduction of counts of fecal pathogens	Boehm et al. (101) Knol et al. (102)
GOS/lcFOS, 8 g/L	Preterm formula, Intact CMP	Healthy preterm infants 0–21 d	Prebiotic (10) Controls (10)	Reduction of gastrointestinal transit time; reduction of stool viscosity (no microbiology)	Mihatsch et al. (103)
GOS/lcFOS, 8 g/L	Preterm formula, Intact CMP	Healthy preterm infants 0–21 d	Prebiotic (10) Controls (10)	Statistically significant but small effect on reduction of gastric emptying time (no microbiology)	Indrio et al. (104)

¹ CMP, cow milk protein.

infants (83). Yap et al. (73) found a tendency to increased SCFA production in infants fed a formula supplemented with inulin alone. SCFA represent fermentation products of bacteria in the colon, and they are therefore an important characteristic feature of the entire intestinal microbiota (120). Thus, it can be assumed that SCFA profiles similar to the profiles found in breast-fed infants reflect similarities of the entire microbiota between breast-fed infants and infants fed a prebiotic formula.

There are several results available indicating that SCFA and pH influence the physiological role of intestinal cells.

In an in vitro model in which epithelial cells (T 84 cell line) were combined with myofibroblast cells (CDD-18 Co cell line) in a coculture, SCFA as they appear in the feces of breast-fed infants were able to stimulate mucin-2 production and improve the gut barrier integrity (121). The effect of SCFA on growth of pathogens as well as of commensals has been studied in vitro at pH 7.5 (typical fecal pH in formula-fed infants) and at pH 5.5 (typical fecal pH in breast-fed infants). SCFA inhibit the growth of pathogens in a dose-dependent manner but did not affect the growth of commensals. This effect was seen only at pH 5.5 but not at pH 7.5, indicating that achieving the same pH and SCFA pattern found in stools from breast-fed infants by prebiotics results in reduced growth of pathogens (68,80,99,122).

Additionally, SCFA might play a role in the regulation of intestinal motility (123,124).

Selectivity of the prebiotic effect of GOS and FOS. There is evidence that early colonization with specific microbiota might be associated with the development of allergic symptoms later in life. Bjorksten et al. (125) found that allergic infants were less often colonized by lactobacilli and bifidobacteria than nonallergic infants. Additionally, it was found that allergic infants had more adult-like species in their fecal flora, including *Bifidobacteria adolescentis*, compared with healthy infants in whom *B. bifidum*, *B. infantis*, and *B. breve* predominated (126). Also, in Japanese infants suffering from atopic dermatitis, similar findings have been reported (127). This suggests that different bacterial species may have different functional effects on the immunological reaction of the host. Specific modulation of the composition of the intestinal microbiota by the use of prebiotics is therefore expected to have a functional impact on the immune system.

Consequently, studies focusing on the effect of prebiotics on the development of the different species of bifidobacteria (80,95) have been performed. In these studies, it could be demonstrated that the prebiotic mixture of scGOS/lcFOS promoted *B. infantis* and depressed *B. adolescentis*. In a study in term infants (80), *B. adolescentis* dominated on d 5 of life (70%), but the percentage was reduced to ~20% during a 6-wk breast-feeding period. During this period, *B. infantis* increased. The same changes occurred in the group fed the prebiotic mixture but not in the group fed formula without prebiotics.

In summary, the experimental data as well as the results of clinical trials prove that prebiotic substances with a structure different from the structure of HMOS are able to influence the intestinal microbiota toward a composition as found in breast-fed infants.

Effect of GOS and FOS on immune system: results of animal studies. There is accumulating evidence that the interaction between the intestinal microbiota and the gut plays an important role for the postnatal development of the immune system. However, the interactions among intestinal epithelial, immune cells, and the different species of the intestinal microbiota are very complex and not fully understood (128,129).

Following the Process for the Assessment of Scientific Support for Claims on Foods recommendation (130,131), studies in mice are recommended to substantiate conclusions related to immunological effects of dietary compounds. The experimental data concerning the immune modulatory effect of prebiotics have been intensively reviewed by Vos et al. (46).

In mice, it could be shown that scGOS/lcFOS were bifidogenic in a dose-dependent manner, result in a reduction of the fecal pH and in a fecal SCFA pattern as found in human infants, and support the relevance of the animal data for the human situation (132).

In the mouse vaccination model, the prebiotic mixture of scGOS/lcFOS significantly stimulated the vaccination response in a dose-dependent manner and modulated the immune system toward a Th1-dominated immune response. This effect occurred only if the nutritional intervention with prebiotic started before the first vaccination. This was not seen when the prebiotics were fed after the first vaccination, indicating that the use of

prebiotics for prevention is more relevant than for a treatment approach.

In the same experiments, different classical fiber mixtures in a dose similar to the scGOS/lcFOS mixture have been tested. There was no effect of these fibers on the measured biomarkers of the immune system, indicating that different nondigestible carbohydrates react differently with respect to intestinal flora and immune function (132).

There are also data available concerning the effect of this specific prebiotic mixture on the allergic reaction in a mouse model using ovalbumin as antigen. Feeding the prebiotic mixture of scGOS/lcFOS significantly reduced the allergic reaction against ovalbumin (133).

Based on the link between immune status and cancer development, Pierre et al. (134) observed in Min mice depleted in CD4⁺ and CD8⁺ lymphocytes that dietary scFOS provided a mechanism of tumor surveillance effective against the development of colon tumors.

In summary, the animal data allow the conclusion that certain prebiotics are able to modulate the immune system of the mice and provide preventive effects with regard to the development of infectious as well as allergic diseases. This effect seems mainly mediated by modulation of the intestinal microbiota.

Effect of GOS and FOS on the immune system: results of human studies. There is increasing evidence that the interaction between the intestinal microbiota and the intestinal epithelial and immune cells plays a key role in the postnatal development of the immune system (135–137). The results of the animal experiments support the hypothesis that the establishment of intestinal microbiota in formula-fed infants similar to that found in breast-fed infants will result in a development of the immune system comparable to the development in breast-fed infants.

Saavedra et al. (63) reported that the supplementation of weaning food with scFOS (0.55 g/15 g cereals; intake 1.2 g/d) was associated with a reduced rate of infectious episodes. No effects of scFOS on clinical course and incidence of diarrhea have been found by Duggan et al. (67).

Firmansyah et al. (77) reported increased ($P < 0.05$) postvaccination IgG antibodies in plasma in infants fed with cereals supplemented with a mixture of scFOS and lcFOS.

Moro et al. (80) reported a reduced cumulative incidence of atopic dermatitis according to the international recommended diagnostic criteria (131) in a group of high-risk infants fed a formula supplemented with scGOS/lcFOS compared with a non-supplemented formula (9.8% vs. 23.1%; $P = 0.014$) and a reduced rate of episodes of infections (47 vs. 21; $P = 0.01$). The prebiotic supplementation resulted in a significant reduction of plasma levels of total IgE, IgG1, IgG2, and IgG3, but no effect on IgG4 was observed, indicating that the prebiotic mixture induced an antiallergic immune globulin profile in this cohort of infants at risk (91). More recently, the 2-y follow-up data have been reported that further support the hypothesis that the prebiotic formula resulted in a reduced incidence of allergic symptoms (90).

In a study performed in a healthy population of 326 term infants (97), the supplementation of a formula with prebiotic mixture scGOS/lcFOS results in a reduced incidence of infectious symptoms during the first years of life (acute diarrhea, 0.13 ± 0.39 vs. 0.26 ± 0.53 episodes/child annually, $P = 0.02$; >3 episodes of upper respiratory tract infections, 22/169 vs. 36/173, $P = 0.06$; number of children who received more than 2 antibiotics courses/y, 32/84 vs. 59/87, $P < 0.01$).

In summary, the available data from human trials are completely in accord with the data derived from animal experiments demonstrating the immune modulatory effect of prebiotics. There are indications that the effects are specific for each prebiotic ingredient. Consequently, the European Food Safety Agency requested for approval of a new prebiotic ingredient (or combination of ingredients) specific studies to demonstrate its safety and efficiency (138).

There is evidence that prebiotics have a significant and biologically relevant effect on the postnatal development of the immune system. The most conclusive data exist for a mixture of scGOS/lcFOS. The mechanism behind the immune modulatory effects of the studied prebiotic oligosaccharides is not yet fully understood. However, findings in the human trials confirm the results obtained from animal models. The data indicate that prebiotics can serve as an effective and safe tool to strengthen the immune system during infancy, which might offer a new method to prevent infections and allergy. However, long term studies are needed to test the hypothesis that the influence of dietary factors on the immune system early in life might have beneficial consequences later in life.

Other articles in this supplement include references (139–148).

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