

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado
EMILIO FERRERO

ASUNTO	Radicación	08 002394
	Trámite	11
	Evento	378
	Actuación	519
	Oficio	
	Folios	9

05566

Patente de Invención	☒	Patente de Modelo de Utilidad	☐
Vía Nacional	☐		
Vía PCT (Fase Nacional)	☒	Capítulo I	☒
		Capítulo II	☐
No. Solicitud Internacional	PCT/FR2006/001687		
Fecha de Presentación Internacional	11 Julio/2005		

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:	Folios 134 a 155	11/Enero/2008
Reivindicaciones:	Folios 156 a 163	11/Enero/2008
Dibujos:	Folios 165 a 167	11/Enero/2008
Prioridad:	FR 0507528	13/Julio/2005

2. Análisis de la Prioridad

Se acepta la reivindicación de Prioridad porque:
Esta 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

Título de la solicitud: "Productos alimenticios fermentados que contienen cepas probióticas y método de preparación de los mismos"

Expediente 08 02394 1er Art 45

El problema planteado en esta solicitud es la necesidad de proporcionar un proceso para obtener un producto fermentado que comprende bacterias lácticas y bifidobacterias el cual va a mantener la población de bifidobacterias durante la conservación hasta su uso y así éste llegue a satisfacer los criterios médicos acerca de la dosis diaria recomendada para una persona.

Para resolver este problema técnico la solicitud en estudio proporciona un proceso para obtener un producto fermentado y durante el proceso se busca elevar la población de bifidobacterias, que se mantengan en buen estado fisiológico y con una velocidad de supervivencia significativa durante la conservación hasta su uso; se apoya del fenómeno simbiótico presente en entre las bacterias para promover el crecimiento de estas.

El objeto de la presente Solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A 23 C 009/123

4. De la Solicitud de Patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

Las reivindicaciones siguientes no son claras porque:

- Las reivindicaciones 1 a 4, 6, 7, 9, 11, 14 a 19 y 23 utilizan los términos: "*más de aproximadamente*", "*menor de aproximadamente*", "*aproximadamente*", "*en particular*", "*por lo menos*"; son imprecisos, por lo cual no permite distinguir la invención del estado de la técnica.
- La reivindicación 1 reclama como característica técnica esencial un resultado a obtener "*más de aproximadamente $5 \cdot 10^7$, en particular más de aproximadamente 10^8 bifidobacterias por gramo de producto alimenticio fermentado por un periodo de conservación de por lo menos 30 días, en particular por lo menos 35 días*", lo cual no es permitido porque la reivindicación incluiría no sólo la solución propuesta por el solicitante, sino todas las alternativas presentes o futuras que lleguen a ese resultado. Se sugiere remplazarlo por las características esenciales estructurales del producto alimenticio fermentado, que contribuyen a la obtención de ese resultado. Lo anterior, también aplica para la reivindicación 4, 20 y 24.
- La reivindicación 2 define una relación que debe cumplir el producto como es "*la proporción del número de bifidobacterias contenidas en el producto alimenticio fermentado al final del período de conservación, al número de bifidobacterias contenidas en el producto alimenticio fermentado al inicio del período de conservación de por lo menos 30 días, en particular por lo menos 35 días es de aproximadamente 0.2 a aproximadamente 0.8, en particular aproximadamente 0.3 a aproximadamente 0.7, en particular aproximadamente 0.4 a aproximadamente 0.5*", sin embargo esta relación no es una característica técnica de un producto sino de un proceso "de conservación".

Al respecto, no debe olvidarse que las reivindicaciones de producto y de procedimiento son diferentes y, por ello, deben redactarse de manera independiente en el pliego reivindicatorio.

Sobre las mismas, el Tribunal Andino de Justicia ha dicho lo siguiente:

"El objeto de la invención de producto esta constituido por un cuerpo cierto destinado a llenar una necesidad industrial que el estado de la técnica aun no ha satisfecho. En cambio, el objeto de la invención de procedimiento se encuentra conformado por "una serie o sucesión de operaciones que inciden sobre una materia (sólida, líquida o gaseosa) que se encamina a la obtención de una cosa o un resultado". (Otero Lastres, José Manuel. Ob. Cit)¹.

- La reivindicación 3 presenta un preámbulo diferente del de la reivindicación 1 que también es de producto, lo cual no es permitido. Las reivindicaciones del mismo objeto (producto) deben tener el mismo preámbulo, ya que este normalmente coincide con el título de la invención y sirve de introducción informativa sobre el objeto que va a ser protegido presentando la información técnica conocida en el estado de la técnica del objeto que se pretende proteger. Es así que de acuerdo a lo anterior existe una incoherencia con la materia que se considera novedosa en la reivindicación 1 que en la reivindicación 3 se encuentra en el preámbulo "*conservado por un período de conservación de por lo menos 30 días, en particular por lo menos 35 días*".
- La reivindicación 3, en el preámbulo, no es clara porque hace referencia a una temperatura, siendo que el objeto de la reivindicación es de producto, y la temperatura es una característica de un proceso. Lo anterior, también aplica para la reivindicación 6 que afirma que el producto es "*preparado*", no se permite caracterizar un producto por su método de obtención. Igualmente para la reivindicación 25 "*es obtenido a partir del proceso*".
- Las reivindicaciones 1, 3 y 4 mencionan la cantidad de bacterias por gramo de producto, lo cual no es lo usualmente utilizado en el campo técnico de esta invención dado que el conteo de células viables se realiza por ml de muestra.
- La reivindicación 8 hace referencia a un efecto que se produce por la selección de las bacterias, lo cual no es permitido. Las reivindicaciones deben caracterizar un producto por la forma o constitución del mismo y las partes que lo componen. No se aceptan reivindicaciones que caractericen resultados, usos, ventajas o efectos. Por lo anterior, la reivindicación 8 debe eliminarse del capítulo en cuestión.
- La reivindicación 10 tiene como característica técnica esencial un uso que se le da al producto alimenticio, lo cual no es permitido.
- Las frases "*sustancia de partida*", "*temperatura de inicio*", "*temperatura final*", en la reivindicación 11, son frases de carácter general que sugieren de forma

imprecisa que la protección se extiende a otras posibles variaciones o modificaciones.

- En las reivindicaciones 11, 20, 21 y 22 que caracterizan un proceso no se muestran los parámetros de cada una de las etapas; en las etapas de enfriamiento y almacenamiento no se especifica una transformación del producto que demuestre que estas dos etapas son diferentes. La etapa de pasteurización no define parámetros que la caractericen.

5. Unidad de invención (Art 25 D 486)

La Solicitud no cumple con el Requisito de Unidad de Invención porque se refiere a dos Grupos Inventivos.

Los Grupos Inventivos están definidos por las siguientes características esenciales:

- Grupo I: Reivindicaciones 1-10. Producto alimenticio fermentado no firme que contiene bacterias lácticas. Como características particulares contiene además bacterias probióticas, específicamente bifidobacterias.
- Grupo II: Reivindicaciones 11-25. Un proceso para preparar un producto alimenticio fermentado. Como características técnicas particulares se tiene una serie de etapas: pasteurización, siembra, fermentación, enfriamiento intermedio, almacenamiento y enfriamiento final.

Los grupos I y II inventivos se diferencian porque la característica particular del grupo I se refiere a las bifidobacterias contenidas en el producto y el grupo II se refiere a un proceso para preparar un producto alimenticio fermentado; en ningún momento se muestra que este proceso sea específico para preparar el producto de la invención que se reclama en el grupo I.

Se le sugiere al solicitante restringir la materia a patentar en un solo concepto común inventivo especificando el avance tecnológico sobre el estado de la técnica. Esto se logra entre otras formas definiendo las características esenciales de la invención y mostrando las otras características particulares como dependientes, pero sin perjuicio de la concisión de las nuevas reivindicaciones tanto independientes como dependientes.

El solicitante también puede realizar una o más de las divisionales de acuerdo a los grupos de conceptos técnicos inventivos presentados en esta solicitud, esto claro, sin ampliar el objeto inicial de la invención.

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: 13 de julio de 2005 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Expediente 08 02394 1er Art 45

Item	Documento	Título	Fecha de Publicación*
D1	FR2842707	Yaourt et son procede de production	30/Enero/2004
D2	Libro 1	Survivability and beta-galactosidase activity of bifidobacteria stored at low temperatures.	1998,

D1<http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=FR&NR=2842707A1&KC=A1&FT=D&ND=6&date=20040130&DB=EPODOC&locale=en> EP divulga a un yogurt y el proceso para su producción; el yogurt contiene bacterias lácticas en compañía de bifidobacterias. (Pág.18, líneas 1 – 18)

D2Libro 1 divulga la capacidad de las bifidobacterias para sobrevivir durante el periodo de almacenamiento en frio. (Abstract), (Folio 209 - 226).

7. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 1 consiste en *“Un producto alimenticio fermentado no firme que contiene fermentos que contiene bacterias lácticas, caracterizado porque comprende más de aproximadamente $5 \cdot 10^7$ en particular más de aproximadamente 10^8 bifidobacterias por gramo de producto alimenticio fermentado por un periodo de conservación de por lo menos 30 días, en particular por lo menos 35 días”*.

Comparación de las características técnicas esenciales, con las del Estado de la Técnica:

Características esenciales	D1
Producto fermentado	Producto fermentado
comprende más de aproximadamente $5 \cdot 10^7$ en particular más de aproximadamente 10^8 bifidobacterias por gramo de producto.	población de Bifidobacterias animalis de $1,4 \cdot 10^8$ bifidobacterias/ml (Pág. 16, ejemplo 3)

La característica esencial que se muestra en la reivindicación 1 había sido divulgada previamente en D1, el cual produce un yogurt (Pág. 1, Párr. 5) y revela que comprende una población del género bifidobacterias animalis en una cantidad de $1,4 \cdot 10^8$ (Pág. 16, ejemplo 3).

En relación a las reivindicaciones dependientes 5, 7 y 8, el documento D1 determina:

- Bifidobacterias animalis (Pág. 16, Tabla 1).
- Bacterias lácticas, lactococcus lactis (Pág. 16, Tabla 1).

- La proporción de bacterias en la mezcla es de 1:1 a 1:2; lo que podría ser 2% de bacterias A (bifidobacterias) y 2% de bacterias B (lácticas) (Págs. 8, Párr. 4 y Pág. 9, Párr. 1).

Conclusión:

Las reivindicaciones dependientes 5, 7 y 8 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)

La reivindicación 11, menciona: *"Un proceso para la preparación de un producto alimenticio fermentado a partir de una sustancia de partida, caracterizado porque comprende las siguientes etapas sucesivas:*

-una etapa de siembra de una sustancia de partida, opcionalmente pasteurizada, mediante inoculación con fermentos de siembra que contienen aproximadamente $4 \cdot 10^6$ a aproximadamente $1 \cdot 10^7$ bifidobacterias por ml de sustancia de partida, con el fin de obtener una sustancia sembrada,

-una etapa de fermentación de la sustancia sembrada obtenida en la etapa precedente, de tal manera que la temperatura al inicio de la fermentación es de aproximadamente 36 a aproximadamente 38°C, la temperatura al final de la fermentación es de aproximadamente 37 a aproximadamente 39°C y el tiempo de fermentación es de aproximadamente 8 a aproximadamente 11 horas, con el fin de obtener una sustancia fermentada,

-una etapa de enfriamiento intermedio de la sustancia fermentada obtenida en la etapa precedente, de tal manera que el tiempo de enfriamiento intermedio es de aproximadamente 1 hora 30 minutos a aproximadamente 2 horas y la temperatura de enfriamiento intermedia es de aproximadamente 18°C, con el fin de obtener una sustancia pre-enfriada,

-una etapa de almacenamiento de la sustancia pre-enfriada obtenida en la etapa precedente, de tal manera que el tiempo de almacenamiento es menor a aproximadamente 15 horas, con el fin de obtener una sustancia almacenada,

-una etapa de enfriamiento final de la sustancia almacenada obtenida en la etapa precedente, de tal manera que la temperatura al inicio del enfriamiento final es menor de aproximadamente 21°C y la temperatura al final del enfriamiento final es de aproximadamente 2 a aproximadamente 6°C, para obtener un producto alimenticio fermentado."

Comparación de las características técnicas esenciales, con las del Estado de la Técnica:

Características esenciales	D1	D2
Etapa de siembra (aproximadamente $4 \cdot 10^6$ a aproximadamente $1 \cdot 10^7$)CFU/ ml	Población de Bifidobacterias animalis de $1,4 \cdot 10^8$ bifidobacterias/ml (Pág. 16, Tabla 1, ejemplo 3)	Bifidobacterias(bifidum, breve, animalis), inoculadas $>1 \cdot 10^7$ /ml (Pág.87, Párr. 2)
Etapa de fermentación desde Aproximadamente 36 a aproximadamente 38°C La temperatura al final de la fermentación es de aproximadamente 37 a aproximadamente 39°C	Temperatura de fermentación 30 a 40°C (Pág. 9, Párr. 2).	37°C (Pág. 76, Párr. 2)
Aproximadamente durante 8 a 11 horas	durante 5 a 10 h (Pág. 9, Párr. 2).	No se menciona
Etapa de enfriamiento intermedia Aproximadamente a 18°C Aproximadamente durante (1h y 30min) a 2h	No se menciona	Almacenamiento hasta por 14 días (tabla 3 y 4), (Pág. 76, Línea 25); y posterior almacenamiento a 4°C (Pág. 85, discusión).
Etapa de almacenamiento es menor a aproximadamente 15 horas		
Etapa de enfriamiento final Menor de aproximadamente 21°C Aproximadamente (2 a 6)°C		

Los documentos D1 y D2 se consideran el estado de la técnica cercano a la invención definida en la reivindicación 1 porque divulgan el proceso de producción de un yogur utilizando bacterias lácticas y bifidobacterias (D1, abstract) y la habilidad de las cepas de bifidobacterias para sobrevivir hasta por 14 días (D2, abstract).

La diferencia entre la invención, definida en la reivindicación 11 y el procedimiento de D1 se encuentra en la etapa de enfriamiento y almacenamiento; en tal sentido, en la reivindicación 11 se trabaja un enfriamiento intermedio a 18°C, un almacenamiento de 15 h y un enfriamiento final menor de 21°C; es decir, rangos de temperatura entre (21 a 2)°C.

La diferencia entre la invención, definida en la reivindicación 11 y el procedimiento de D2 se encuentra en la etapa de enfriamiento y almacenamiento en donde se trabaja con rangos de temperatura entre (21 a 2)°C según la reivindicación 11.

El efecto que logra el incluir la etapa de enfriamiento y almacenamiento es el mantenimiento de la población de bifidobacterias en todo el periodo de conservación del producto alimenticio fermentado.

Por lo tanto, el Problema Técnico Objetivo que pretende resolver esta invención se

puede formular así cómo proporcionar un proceso para obtener un producto fermentado que comprende bacterias lácticas y bifidobacterias, el cual va a mantener la población de bifidobacterias durante la conservación hasta su uso.

Sin embargo, en el estado de la técnica ya se había solucionado el problema técnico objetivo según lo descrito en D2, el cual muestra el efecto benéfico sobre la población de bifidobacterias en etapas de enfriamiento y almacenamiento a 4°C hasta por 14 días de almacenamiento. (Pág. 87, Párr. 2); y por lo cual se indica que algunas cepas de bifidobacterias pueden sobrevivir en un alta población cuando se exponen a bajas temperaturas durante el almacenamiento del producto alimenticio fermentado (Pág. 88, Párr. 1), (Pág. 87, Párr. 2).

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría la exposición a bajas temperaturas que se indica en D2 en el procedimiento de D1 para llegar así al objeto de la reivindicación 11 de la solicitud. Por lo cual se considera obvia.

En relación a las reivindicaciones dependientes 13, 17, 21, 22, 24 el documento D1 determina:

- Una etapa de esterilización antes de la etapa de enfriamiento entre (70 a 90)°C durante 5 a 30 min. (Pág. 5, Párr. 4).
- La etapa de homogenización y adición de aditivos, las divulga D1 en el (Pág. 5, Párr. 4 y 3).
- Bifidobacterias animalis (Pág. 16, Tabla 1).
- Bacterias lácticas, lactococcus lactis (Pág. 16, Tabla 1).
- Etapa de adición de los aditivos (Pág. 16, Fig. 1).

Las reivindicaciones dependientes 12 a 25 no mencionan alguna característica que hagan inventiva al proceso de la reivindicación 11, por lo cual se considera que no son inventivas.

8. **Conclusión:**

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

Item	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	FR2842707	1, 5, 7, 8, 13, 17, 21, 22, 24, 25	Novedad
D2	Libro 1	11, 16, 23	Nivel inventivo

Este Despacho encuentra que a nivel de trámite nacional se encuentra la solicitud de número de expediente 08-2395 con el título: “*Productos alimenticios fermentados que contienen cepas probióticas y su proceso de preparación*”, y dicha solicitud se encuentra en trámite nacional con la misma fecha de presentación nacional (13 de

julio de 2005). Se le sugiere al solicitante revisar que la materia que se reclama en la actual solicitud se encuentre excluida dentro del alcance de la otra solicitud.

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 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 para 60 días y presentarla por escrito

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

11 ABR. 2012

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Food Biotechnology

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/lfbt20>

Survivability and β -galactosidase activity of bifidobacteria stored at low temperatures

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Available online: 09 Dec 2009

To cite this article: Todd H. Dechter & Dallas G. Hoover (1998): Survivability and β -galactosidase activity of bifidobacteria stored at low temperatures, Food Biotechnology, 12:1-2, 73-89

To link to this article: <http://dx.doi.org/10.1080/08905439809549944>

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SURVIVABILITY AND β -GALACTOSIDASE ACTIVITY OF BIFIDOBACTERIA STORED AT LOW TEMPERATURES

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ABSTRACT

This work evaluated the ability of strains representing six species of *Bifidobacterium* with probiotic potential to survive and maintain β -galactosidase activity through a two-step, low-temperature storage period. Cultures were also evaluated for their ability to ferment skim milk and retain viability during storage at 4°C. *Bifidobacterium longum* ATCC 15707, *B. breve* 15700, and *B. bifidum* 29521 maintained the greatest viabilities at $> 1 \times 10^7$ CFU/mL, and *B. infantis* 15702 maintained the highest β -galactosidase activity at > 1 U/ml (with $< 1 \times 10^5$ CFU/mL) after -60 to 4°C storage. In fermented skim milk, *B. breve* 15700, *B. bifidum* 29521, and *B. animalis* 25527 tolerated a final product pH of 4.75 with $> 1 \times 10^6$ CFU/mL remaining after 14 days of storage at 4°C. Overall, it was found that highest levels of β -galactosidase activities did not necessarily correlate to the highest plate-count populations.

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INTRODUCTION

The use of *Bifidobacterium* in cultured or culture-containing milks received greater attention at the end of the 1970s due to increased knowledge of the intestinal ecology of bifidobacteria and the improvements in anaerobic technology (Reuter, 1990). More recent, world-wide interest in nutraceutical foods, especially in prebiotic compounds (bifidus growth factors) and probiotic cultures has led to a rapid incorporation of bifidobacteria for commercial use as dietary adjuncts. In the United States, an increasing number of yogurt manufacturers are adding bifidobacteria into their product formulations. Other examples of bifidobacteria-amended foods include cottage cheese, sour cream, lite ice creams, and orange juice (Gorski, 1994).

A major factor in the development of any culture-amended food is the ability of the product to deliver its beneficial effects. Maintenance of normal intestinal microflora, antimicrobial activity, synthesis of vitamins, and increased calcium absorption are some of the benefits attributed to bifidobacteria (Hughes and Hoover, 1991); however, these health benefits may not be available if the number of viable organisms after processing, distribution, and storage of the product is less than one million per milliliter of product (Kurmann and Rasic, 1991).

Proper strain selection is additionally important in the manufacture of bifidobacteria-amended dairy products. Desirable characteristics of selected strains of bifidobacteria include excellent viability through long-term low-temperature storage at low or high pH, and rapid growth rate and acidification of milk for fermented varieties. Low-temperature storage viability in dairy products has been found to work well for most strains of bifidobacteria, although the low pH of some products can cause problems (Klaver *et al.*, 1993; Medina and Jordano, 1994; Samona and Robinson, 1994; Lankaputhra *et al.*, 1996). The use of strains of bifidobacteria of human origin are most popular and thought to be necessary; however, many European products contain animal-origin bifidobacteria, especially *B. animalis* (Ishibashi and Shimamura, 1993).

It has been suggested that products such as yogurt, cultured milks, and nonfermented culture-containing dairy products are able to provide an additional benefit to lactose maldigestors, people with reduced lactase activity in the gastrointestinal tract (Lin *et al.*, 1991;

Savaiano *et al.*, 1984). The starter cultures used in these products may contain sufficient levels of β -galactosidase (β -gal) to digest intestinal lactose, thus eliminating the possible abdominal discomfort associated with lactose maldigestion. Bifidobacteria are known to have relatively high levels of β -gal activity (Chevalier *et al.*, 1990; Desjardins *et al.*, 1990), yet only a limited number of studies have been conducted with relevance to the β -gal activity of bifidobacteria used in dairy products (Hekmat and McMahon, 1992; Hughes and Hoover, 1995; Desjardins *et al.*, 1991). It has been shown that ingestion of large numbers of lactic acid bacteria do not necessarily improve lactose malabsorption in malabsorbers (Hove *et al.*, 1994).

In the studies presented here, the ability of bifidobacteria to remain viable and maintain β -gal activity after a two-step low-temperature storage in a nonfermented milk product was evaluated. Additionally, bifidobacteria were evaluated for their ability to ferment skim milk and remain viable during storage at refrigerated temperatures.

MATERIALS AND METHODS

Culture Conditions

Freeze-dried preparations of *Bifidobacterium* were purchased from the American Type Culture Collection (Rockville, MD). Strains representing six species of *Bifidobacterium* were used in this study: *B. breve* ATCC 15700; *B. infantis* ATCC 15702; *B. adolescentis* ATCC 15703; *B. longum* ATCC 15707; *B. bifidum* ATCC 29521; and *B. animalis* ATCC 25527. Working stocks were prepared by adding 1 mL of fresh culture to 1 mL of sterile glycerol with subsequent storage at -60°C in 15 x 45 mm glass screw-cap vials (Wheaton Scientific; Millville, NJ). Cultures were revived in sterile lactobacilli MRS broth (Difco; Detroit, MI) and incubated for 24 h at 37°C in an anaerobic chamber (Coy Laboratory Products; Grass Lake, MI) containing an atmosphere of 85% nitrogen, 10% carbon dioxide, and 5% hydrogen. Before each experiment, each culture was revived from its stock by two transfers into sterile MRS broth with 18-h incubations. Both 10% (wt/vol) skim milk (SM; Difco) and MRS broth were sterilized by use of an autoclave (15 min, 121°C).

Two-step, Low-temperature Storage Study

For the preparation of concentrated cultures, the strains of bifidobacteria were grown anaerobically for 10 h at 37°C in 50 mL of sterile MRS broth. Cultures were aseptically transferred to two sterile 30-mL polycarbonate centrifuge tubes (Sorvall), balanced, and centrifuged at 91,500 g for 30 min at 4°C in a Model RC70 ultracentrifuge (Sorvall). Supernatants were removed by aspiration and both cell pellets were resuspended by mild vortex in a total volume of 15-mL cold, sterile 10% SM. Into each of four 15 x 45 mm glass screw-cap vials was dispensed 4.0 mL of concentrated cell suspension. Samples from the first vial of each strain were immediately assayed for cell viability and β -galactosidase activity (day 0). The other three vials were frozen at -60°C. On days 1, 14, and 28, vials were removed from storage and samples were assayed for cell viability and β -galactosidase (β -gal) activity.

In the preparation of nonfermented milk containing bifidobacteria, 0.5 mL of thawed cell suspensions (after 28 days of storage at -60°C), were aseptically transferred to bottles containing 50 mL of cold, sterile 10% SM. Cell viability and β -gal activity of each nonfermented milk product were assayed. Bottles were then stored at 4°C and samples taken on days 1, 7, and 14 to determine cell viability and β -gal activity.

Fermented Milk Preparation and Subsequent Storage

For each strain of *Bifidobacterium*, two bottles containing 50 mL of sterile 10% SM were inoculated with 0.5 mL of the respective 18-h culture. All bottles were placed in the anaerobic chamber at 37°C. After 24 h, one bottle was removed for each strain and samples of the one-day fermented milks were assayed for cell viability and pH. The one-day fermented milks were then placed at 4°C for storage. The remaining bottles were removed from anaerobic incubation after 48 h, and the two-day fermented milks were assayed as above before they were placed at 4°C for storage. Samples of each product were also assayed on days 1, 7, and 14 of storage.

Enumeration of Viable Cells and pH Measurements

Samples (1.0 mL) of nonfermented and fermented milks were diluted in 0.1% peptone water (bacto-peptone; Difco), spread-plated (0.1 mL) in duplicate onto MRS agar (MRS broth

plus 1.5% bacto-agar; Difco) and incubated for 48 h at 37°C anaerobically. Colonies were counted with the aid of a bacteria colony counter (Model 500A; Exotech Inc.; Gaithersburg, MD). Aliquots (1.0 mL) were removed, diluted with 9.0 mL of distilled water, and the pH of each sample read with a digital ionalyzer (Orion Research Inc.; Boston, MA).

Assay of β -Galactosidase Activity

β -Galactosidase activity was measured by use of *o*-nitrophenyl- β -galactopyranoside (β -oNPG; Sigma Chemical Co., St. Louis, MO) as a substrate. Hydrolysis of β -oNPG results in the release of *o*-nitrophenol (oNP), a highly chromogenic compound which can be detected spectrophotometrically at 420 nm.

To 4.0 mL of 0.02 M sodium phosphate buffer (NaPB, pH 7.0) was added 1.0 mL of *Bifidobacterium* culture in milk. Samples were centrifuged (Model Centra-4B; International Equipment Co.) in PC Oak Ridge Tubes (Nalgene) for 10 min at 9,000 *g*. The supernatant was decanted and to each pellet 1.0 mL of Triton X-100 (1% vol/vol in distilled water; Sigma) and 2.0 mL of NaPB was added. Samples were vortexed vigorously to resuspend the pelleted material. The suspension was incubated in a 37°C water bath to allow for cell permeabilization. After 15 min, 1.0 mL of 15 mM β -oNPG was added to each sample, and quickly inverted to mix. After 10 min at 37°C, the reaction was terminated by adding 1.0 mL of 0.5 M sodium carbonate (Sigma). Samples were centrifuged for 5 min to remove cell debris, and release of oNP was determined at 420 nm. Units of activity were expressed as micromoles of oNP released per minute (1 U = 1 μ mol/min).

Statistical Evaluation of Data

Data for viability and β -galactosidase activity for each of the six strains were evaluated by analysis of variance for a split-plot design. The least-significant-difference test was used to compare means (SAS Institute, 1985).

RESULTS

Frozen Storage of Concentrated Cultures

Freezing and storage of concentrated cultures at -60°C had no significant effect ($P < 0.05$) on the viability of *Bifidobacterium longum* 15707, *B. bifidum* 29521, and *B. breve* 15700 (Table

1). Each of these strains had $> 1 \times 10^9$ CFU/mL after 28 days of storage at -60°C . *B. animalis* 25527 had its only significant decrease in viability from days 14 to 28, and 8.9×10^8 CFU/mL remained after storage at -60°C . *B. infantis* 15702 showed significantly lower plate counts on day 14 than on day 1, and on day 28 as compared to day 14. These decreases represented a 0.3 $\log_{10}$ -cycle reduction to 2.2×10^9 CFU/mL. The only strain that exhibited significant losses in viability through the entire 28-day period was *B. adolescentis* 15703. These decreases represented a 0.7 $\log_{10}$ -cycle decrease to 1.4×10^8 CFU/mL.

The stability of β -galactosidase activity through 28 days of frozen storage varied among the six species of *Bifidobacterium* (Table 2). No significant differences were observed for *B. infantis* 15702 that maintained activity of greater than 100 U/mL. Both *B. breve* 15700 and *B. bifidum* 29521 showed significant reductions in β -gal activity between 14 and 28 days of storage, and their activities were just under 1 U/mL after 28 days at -60°C . Three strains exhibited erratic enzyme activity through storage at -60°C . Both *B. animalis* 25527 (final activity 64 U/mL) and *B. adolescentis* 15703 (final activity 44 U/mL) showed significant increases between days 0 and 1, and then significant reductions after 14 days of storage. *B. longum* had significantly lower activity on day 1 than on day 0, but showed a significant increase after 14 days to 84 U/mL.

Refrigerated Storage of Nonfermented Bifidus Milks

Viability of *Bifidobacterium* at 4°C in nonfermented milk prepared with cells after storage at -60°C is shown in Table 3. *B. breve* 15700 and *B. bifidum* 29521 showed no significant decrease in viability through 14 days, although significant increases were observed between 0 and 7 days and 7 and 14 days. Greater than 2×10^7 CFU/mL remained for both cultures. For *B. longum* 15707, viability on day 7 was significantly lower than on day 0, and on day 14 as compared to day 7. Yet, $> 3 \times 10^7$ CFU/mL remained after 14 days at 4°C . *B. animalis* 25527 had significantly lower viability between days 0 and 1 and day 7. After 14 days of storage at 4°C , 6.5×10^6 CFU/mL of strain 25527 remained. *B. infantis* 15702 showed significantly less viability with each successive storage period, and *B. adolescentis* 15703 showed

TABLE 1. Viability of Concentrated Cultures of *Bifidobacterium* during Storage at -60°C.

days at -60°C	Log ₁₀ CFU/ml ^a of strain:					
	<i>B. breve</i> 15700	<i>B. infantis</i> 15702	<i>B. adolescentis</i> 15703	<i>B. longum</i> 15707	<i>B. bifidum</i> 29521	<i>B. animalis</i> 25527
0	9.22 ^b	9.60 ^b	8.79 ^b	9.81 ^b	9.38 ^b	9.05 ^b
1	9.22 ^b	9.50 ^{bc}	8.63 ^c	9.81 ^b	9.38 ^b	9.02 ^b
14	9.22 ^b	9.47 ^c	8.30 ^d	9.81 ^b	9.33 ^b	9.01 ^b
28	9.17 ^b	9.33 ^d	8.14 ^e	9.81 ^b	9.28 ^b	8.95 ^c

^a Each value is the average log₁₀ CFU per milliliter from four trials determined on MRS agar.
^{b,c,d,e} Values in the same column followed by different superscript letters differ significantly (P < .05).

TABLE 2. β -galactosidase Activity of Concentrated Cultures of *Bifidobacterium* during Storage at -60°C .

days at -60°C	Activity (U/ml) ^a of strain:					
	<i>B. breve</i> 15700	<i>B. infantis</i> 15702	<i>B. adolescentis</i> 15703	<i>B. longum</i> 15707	<i>B. bifidum</i> 29521	<i>B. animalis</i> 25527
0	1.68 ^b	107.27 ^b	37.08 ^b	98.47 ^b	1.94 ^b	48.49 ^b
1	1.61 ^b	105.50 ^b	48.95 ^d	60.69 ^d	2.43 ^b	72.65 ^c
14	1.60 ^b	108.44 ^b	52.93 ^d	78.79 ^c	1.89 ^b	75.64 ^c
28	.95 ^c	106.55 ^b	43.72 ^{bc}	83.48 ^{bc}	.93 ^c	64.04 ^c

^a 1 U is $1\mu\text{mol}$ of o-nitrophenol released per min per ml of sample. Each value is the average from four trials.

^{b,c,d} Values in the same column followed by different superscript letters differ significantly ($P < .05$).

TABLE 3. Viability of *Bifidobacterium* at 4°C in Nonfermented Milk Prepared from Cells after 28 d of Storage at -60°C.

days at 4°C	Log ₁₀ CFU/ml ^a of strain:					
	<i>B. breve</i> 15700	<i>B. infantis</i> 15702	<i>B. adolescentis</i> 15703	<i>B. longum</i> 15707	<i>B. bifidum</i> 29521	<i>B. animalis</i> 25527
0	7.19 ^b	7.49 ^b	6.39 ^b	7.61 ^b	7.17 ^b	6.96 ^b
1	7.16 ^{bc}	7.24 ^c	5.85 ^c	7.59 ^{bc}	7.15 ^{bc}	6.96 ^b
7	7.26 ^c	6.14 ^d	5.78 ^c	7.56 ^c	7.22 ^c	6.87 ^c
14	7.32 ^d	4.57 ^e	4.83 ^d	7.50 ^d	7.27 ^d	6.81 ^c

^a Each value is the average log₁₀ CFU per milliliter from four trials determined on MRS agar.
^{b,c,d,e} Values in the same column followed by different superscript letters differ significantly (P < .05).

significantly less viability on day 1 than on day 0 with another significant drop evident between days 7 and 14. Both cultures had $< 1 \times 10^1$ CFU/mL after 14 days at 4°C.

β -Gal activity after refrigerated storage again varied among strains (Table 4). *B. breve* 15700 and *B. adolescentis* 15703 showed no significant difference in β -gal activities through 14 days of storage. *B. infantis* 15702 exhibited significantly less activity on day 1 than on day 0 with no further reductions observed thereafter. *B. longum* 15707 and *B. animalis* 25527 showed significantly lower activities from day 0 to day 7 with no further reductions observed. *B. bifidum* 29521 showed a significant decrease in activity between days 0 and 1, but higher β -gal activities were observed after further storage at 4°C. Overall, β -gal activities exhibited by bifidobacteria in these nonfermented bifidus milks were lower than activities observed for the same strains in concentrated form.

Milks Fermented with Bifidobacteria

The ability of each species of *Bifidobacterium* to acidify 10% SM and remain viable through 14 days of storage at 4°C was examined. Previous studies have used milks, frozen dairy desserts, and ice creams prepared at standard pHs for those products (Biavati *et al.*, 1992; Laroia and Martin, 1991; Hekmat and McMahon, 1992; Klaver *et al.*, 1993). Milks fermented for 24 h resulted in good growth and acidification by *B. breve* 15700, *B. longum* 15707, *B. animalis* 25527, and *B. bifidum* 29521. The pH of the above bifidus milks dropped from an initial reading of 6.60 to 5.11, 5.20, 5.27, and 5.22, respectively, and remained steady through 14 days of storage at 4°C. *B. longum* 15707 showed sensitivity to the level of acidity in the fermented milk, indicated by a 1.7 $\log_{10}$ -cycle decrease to 9.5×10^6 CFU/mL after 14 days of storage (Fig. 1). *B. animalis* 25527 only decreased 0.3- $\log_{10}$ cycle to a final count (14 days) of 1.0×10^8 CFU/mL. *B. breve* 15700 and *B. bifidum* 29521 were observed to be the most resistant to the conditions of acidity and refrigerated storage as shown by average reductions of < 0.10 - $\log_{10}$ cycles after 14 days of storage. Both milks that were fermented for 24 h by *B. adolescentis* 15703 and *B. infantis* 15702 showed substantially less growth, acid production, and stability during storage. Previous studies (not published) showed that these two strains had very slow growth rates in 10% SM, and were also very sensitive to high levels of acidity.

TABLE 4. β -galactosidase Activity of *Bifidobacterium* at 4°C in Nonfermented Milk Prepared from Cells after 28 d of Storage at -60°C.

days at 4°C	Activity (U/ml) ^a of strain:					
	<i>B. breve</i> 15700	<i>B. infantis</i> 15702	<i>B. adolescentis</i> 15703	<i>B. longum</i> 15707	<i>B. bifidum</i> 29521	<i>B. animalis</i> 25527
0	.58 ^b	5.31 ^b	.71 ^b	5.41 ^b	.21 ^{bc}	1.48 ^b
1	.52 ^b	1.79 ^c	.58 ^b	3.29 ^c	.13 ^b	.93 ^c
7	.46 ^b	1.56 ^c	.63 ^b	1.25 ^d	.32 ^{cd}	.45 ^d
14	.42 ^b	1.50 ^c	.52 ^b	1.10 ^d	.35 ^d	.36 ^d

^a 1 U is 1 μ mol of o-nitrophenol released per min per ml of sample. Each value is the average from four trials.

^{b,c,d} Values in the same column followed by different superscript letters differ significantly (P < .05).

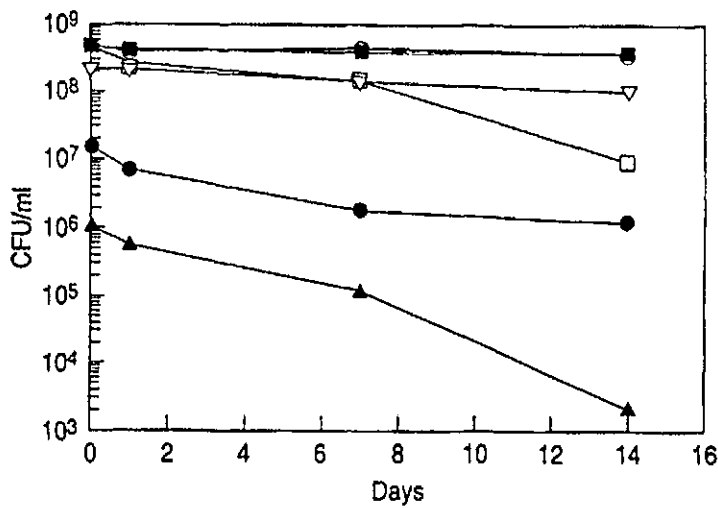


Figure 1. Counts on MRS agar of 24-h fermented bifidus milks stored at 4°C: *Bifidobacterium breve* 15700 (○); *B. infantis* 15702 (▲); *B. adolescentis* 15703 (●); *B. longum* 15707 (□); *B. bifidum* 29521 (■); and *B. animalis* 25527 (▽).

Populations of *B. adolescentis* 15703 declined by 1.1- $\log_{10}$ cycles at pH 5.9 after 14 days to 1.3×10^6 CFU/mL. *B. infantis* 15702, which showed no actual growth during fermentation for 24 h, had reductions of 2.7- $\log_{10}$ cycles at pH 5.8 after 14 days of storage to 2.2×10^3 CFU/mL.

Milks that were fermented with bifidobacteria for 48 h to enable greater acid production before storage at 4°C had similar patterns of viability throughout storage (Fig. 2). *B. breve* (fermented to pH 4.7) and *B. bifidum* (pH 4.8) had < 0.10- $\log_{10}$ -cycle reductions through 14 days at 4°C with $> 4.0 \times 10^8$ CFU/mL remaining. These two strains were able to tolerate the higher levels of acid seen after 48 h of fermentation and subsequent storage at 4°C as compared to the levels of acid observed after only 24 h of fermentation. *B. animalis* (pH 4.9) showed a greater sensitivity to acid than the above strains with a total reduction of 0.3- $\log_{10}$ cycles to 5.4×10^7 CFU/mL after 14 days of storage. This decrease was no greater than that observed for milk fermented for 24 h with this strain. *B. longum* (pH 4.7) showed much less tolerance to acid when observed during storage of the 48-h fermented milk. A 3.5- $\log_{10}$ -cycle reduction to 9.2×10^4 CFU/mL was observed after 14 days of storage at 4°C, nearly 2- $\log_{10}$ cycles greater than the milk fermented for 24 h with *B. longum*. Both *B. adolescentis* and *B. infantis* showed

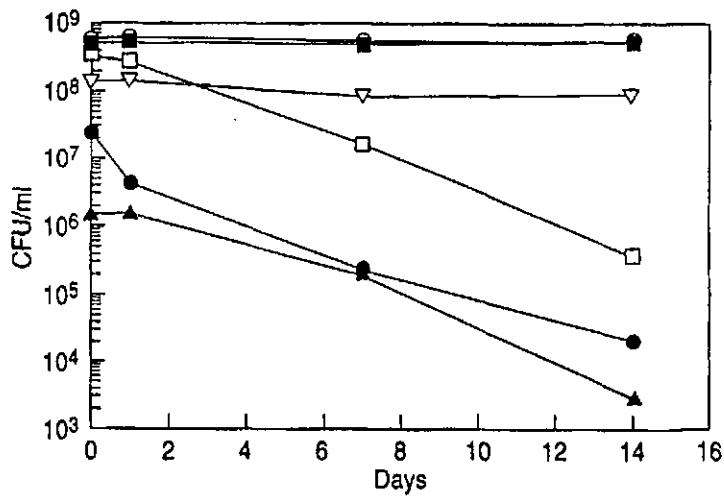


Figure 2. Counts on MRS agar of 48-h fermented bifidus milks stored at 4°C: *Bifidobacterium breve* 15700 (○); *B. infantis* 15702 (▲); *B. adolescentis* 15703 (●); *B. longum* 15707 (□); *B. bifidum* 29521 (■); and *B. animalis* 25527 (▽).

greater reductions in viability and less acid production than the milks fermented for 48 h by other strains. At a pH of 5.4, *B. adolescentis* was reduced 3.6-log₁₀ cycles to 3.3 x 10³ CFU/mL, significantly greater than the drop observed at pH 5.9 for the milk fermented for 24 h. At pH 5.5, *B. infantis*, which had < 1 x 10⁶ CFU/mL at day 0, had 3.2 x 10² CFU/mL remaining after 14 days at 4°C. This represented a slightly greater reduction of viable cells than the losses observed at the higher pH (5.8) 24-h fermented milk.

DISCUSSION

It is a desirable characteristic for strains used in nonfermented and fermented dairy products to retain their viability through processing and long-term storage. Storage of *Lactobacillus acidophilus* and *Bifidobacterium* spp. in frozen yogurts, ice creams, and fermented dairy desserts has been previously evaluated for delivering high numbers of viable organisms to consumers (Holcomb *et al.*, 1991; Laroia and Martin, 1991; Hekmat and McMahon, 1992). The small losses in viability observed for the six strains of *Bifidobacterium* through storage at -60°C and subsequent storage at 4°C suggest that these bacteria are stable under these handling and

storage conditions such that high numbers of viable cells in a nonfermented product can be commercially realized. Although anaerobic, the strains of bifidobacteria in our study were able to survive with no efforts to maintain anaerobiosis during storage.

If nonfermented milks containing *Bifidobacterium* species are to ameliorate the problems of lactose digestion, it would be advantageous for the organisms to maintain high β -galactosidase activity with refrigerated storage. Studies have shown that some strains of bifidobacteria have relatively high levels of this enzyme (Chevalier *et al.*, 1990; Desjardins *et al.*, 1990; Hughes and Hoover, 1995). Our study showed similar results; however, we found significant reductions in β -gal activities occurring after exposure to the two-step, low-temperature storage sequence for all six strains of *Bifidobacterium* that were examined. These reductions may raise concerns regarding the stability of the activity of β -gal in cultures added to products held at low temperatures. During storage of concentrated cultures at -60°C , we found four strains with relatively high β -gal activity; however, three of these strains displayed erratic enzyme activity upon storage. Significant elevations or reductions in activity observed within the first day of storage may be the result of cells adapting at a different rate to low temperature. No such phenomenon was seen with cultures of *L. acidophilus* taken from room temperature to a sub-optimal freezing temperature (Gilliland and Lara, 1988). Additionally, once the cultures of bifidobacteria were inoculated into nonfermented 10% SM, β -gal activities were observed at much lower levels than were seen in the concentrated cultures. This may be the result of assaying for β -gal activity with greatly diminished cell counts in a fluid milk system, where interference may occur from milk proteins. Lin *et al.* (1989) used EDTA to solubilize extracellular milk proteins, but Somkuti and Steinberg (1979) showed that EDTA is a strong inhibitor of β -gal activity. Therefore, EDTA was not included in our assay procedure, and without removal of the interfering proteins, the levels of β -gal observed at day 0 in the fluid milk system were lower than expected.

It has been noted that $> 1 \times 10^6$ cells of bifidobacteria per milliliter of product should be consumed each day to deliver the often described potential benefits (Kurmann and Rasic, 1991); however, such a high concentration of viable bifidobacteria may not be needed for the specific

benefit of associated with β -galactosidase activity. For example, after the two-step, low-temperature storage of *B. infantis* 15702, there were $< 1 \times 10^5$ CFU/mL from a starting concentration of $> 1 \times 10^9$ CFU/mL. Yet, this strain had higher β -galactosidase activity than the other strains, including *B. longum* 15707, which had the highest count after storage ($> 1 \times 10^7$ CFU/mL). Apparently some cells of *B. infantis* were unable to form colonies but still demonstrate β -gal activity. This phenomenon has also been seen with strains of *L. acidophilus* (Gilliland and Lara, 1988). Thus it would appear that $< 1 \times 10^6$ viable cells/mL of product are necessary to realize improved lactose tolerance based on β -gal activity.

If bifidobacteria are to contribute to the fermentative process of cultured products, the strains must be capable of surviving conditions of low pH and refrigerated storage. Our survey of milks fermented with bifidobacteria showed varied sensitivities to pH by cultures of bifidobacteria. The method of directly inoculating bifidobacteria to ferment milk could be used with *B. bifidum* 29521, *B. breve* 15700, and *B. animalis* 25527 because these strains had $> 1 \times 10^7$ CFU/mL in milks fermented to pHs as low as 4.75 and stored for 14 days at 4°C. The other strains showed significant lower survivability in high levels of acid and storage at 4°C.

We observed that variability exists in growth and survival of bifidobacteria in milk. Desjardins *et al.* (1990) showed that fast-growing bifidobacteria in milk were mainly of infant origin, whereas slow-growing strains such as *B. adolescentis* were of adult origin. These results suggested a relationship between the environmental and nutritional conditions of the intestinal origin of bifidobacteria and their ability to grow in milk; however, in our study, fast-growing strains were mainly of adult origin, and slow-growing strains included *B. infantis* 15702 of infant origin. *B. animalis* 25527, isolated from rat feces, also grew well in milk and survived refrigerated storage in high numbers. Klaver *et al.* (1993) showed that the viability of most strains of *Bifidobacterium* was lost rapidly during storage in a fermented milk product. At pH 5.5, $< 1 \times 10^3$ CFU/mL remained viable after 14 days at 4°C for *B. infantis* 15702. Our results were similar, with a slightly higher concentration of viable cells remaining after storage. Other strains of *B. infantis* have been shown to survive pH levels of 4.0 (Biavati *et al.*, 1992; Klaver *et al.*, 1993).

The work presented here has indicated that some strains of *Bifidobacterium* can survive in high numbers when exposed to low temperatures, aerobiosis, and low pH; conditions the cultures may be exposed to during processing and storage of culture-amended dairy products. It would be difficult at this point to select one particular culture for use in a culture-amended dairy product because of the wide variability observed under the different conditions of storage. Each application or product requires strain characterization to select the isolate or species of *Bifidobacterium* that would be most effective in its intended commercial use.

ACKNOWLEDGMENTS

This work was supported by Dairy Management Inc. We thank Cindy Stewart for her review of the manuscript.

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