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DEP: 2020 DIRECCION DE NUEVAS CREACIONES	EVE: 1 REGISTRO/DEPOSITO/CONCESION/DEPOSITO
TRA: 2 PATENTES DE INVENCION	FOLIOS: 21
ACT: 519 PRESENTACION	

DIRECCIÓN DE NUEVAS CREACIONES

2020
Bogotá, D.C.,

Abogado(a)/Señor(a)
ERNESTO CAVELIER FRANCO

Asunto: Radicación: 11-98760- -6
Trámite: 2
Evento: 1
Actuación: 519
Oficio: 1417
Folios: 21

Patente de Invención	X			Patente de Modelo de Utilidad	
Vía Nacional	X				
Vía PCT (Fase Nacional)		Capítulo I		Capítulo II	
No. Solicitud Internacional					
Fecha de Presentación Internacional					

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 No 11-098760-00000-0000	04/Agosto/2011
Reivindicaciones:	Radicado No 11-098760-00000-0000	04/Agosto/2011

2. Objeto de la invención

De acuerdo con lo establecido en el numeral 1.2.2.2 el Título 10 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. En este caso se estudiaran todas las reivindicaciones del radicado No 11-098760-00000-0000.

Título de la solicitud: “Composición microbiana empelada para disminuir la incidencia de diarreas y mejorar el desempeño fisiológico de terneros y su método de tratamiento”.

El problema planteado en esta solicitud consiste en la necesidad de proporcionar una composición para disminuir diarreas e incrementar el desarrollo corporal de *Bos taurus* y *Bos indicus*.

Para resolver este problema técnico la solicitud en estudio proporciona una composición que comprende uno o más cultivos de microorganismos seleccionados del grupo *Bacteroides succinogenes*, *Fibrobacter succinogenes*, *Ruminococcus albu*, *Ruminococcus flavefaciens*, *Streptococcus bovis*, *Succinomonas amylophilica*, *Prevotella bryantii*, *Anaerovibrio lypolytica*, *Butyrivibrio fibrosolvens* y *Methanobacterium Ruminantium*.

El objeto de la presente solicitud se relaciona con la Clasificación Internacional de Patentes (CIP): A23K 1/16, A23K 1/18, C12N 1/20, C12F 1/04 y C12P 7/40

3. No es una invención (Art 15 D 486 literales (b))

El objeto de las reivindicaciones 1-5, 13, 14 y 14 no se considera una invención, de acuerdo con el Art citado.

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

El objeto de la reivindicación 6, 7 y 9 no es patentable de acuerdo con el Art citado, dado que corresponde a un método de tratamiento. Se le aclara a la solicitante que a pesar de que las reivindicaciones 6 y 7 se encabezan en término de composiciones define como método de tratamiento.

5. De la solicitud de patente

Reivindicaciones (Art 30 D 486, Art 22 PCT)

La reivindicación 1 no es clara porque el término “uno o más” es impreciso, por lo cual no permite distinguir la invención del estado de la técnica. Se sugiere sustituirlo por un término preciso.

La reivindicación 10 presenta una falsa dependencia con los microorganismos presentes en la composición de la reivindicación 1, dado que esta reivindicación consiste en los ingredientes y sus proporciones para un medio de cultivo de crecimiento.

La reivindicación 12 presenta una falsa dependencia con la reivindicación 1, porque esta reclama un proceso de fermentación materia muy diferente a la composición microbiana.

La reivindicación 1 no se encuentra sustentada en la descripción porque el término “uno o más” da la posibilidad de un gran número de combinaciones de microorganismos que a su vez lleva a un gran número de composiciones, por cual se le sugiere a la solicitante restringir la solicitud a una razonable generalización del tipo de composiciones que ha sido probado, es decir que se encuentran debidamente sustentados.

Las reivindicaciones 1-5 no son claras porque se encabezan con el termino composición pero se define en término de microorganismos. Si la solicitante

pretende reclamar una composición debe definir cada uno de los ingredientes y las proporciones de los mismos, y que la referencia a uno o más microorganismos no permite definir los ingredientes específicos de la composición.

La reivindicación 12 no es clara porque se refiere a un proceso para la obtención de microorganismos, se le aclara a la solicitante que un proceso está descrito por una secuencia lógica de etapas y condiciones a controlar en el crecimiento microbiano.

El capítulo descriptivo contiene dos reivindicaciones con el número 14. Por favor realizar la corrección respectiva.

Descripción (Art 28 D 486)

La descripción no es clara porque la información que contiene no permite que el la persona del oficio normalmente versada en la materia pueda comprender el problema técnico y la solución aportada por la invención.

Se requiere al solicitante hacer las aclaraciones necesarias.

6. Unidad de invención (Art 25 D 486)

La solicitud no cumple con el requisito de unidad de invención porque se refiere a 2, grupos inventivos.

1° Una composición que comprende uno o más cultivos de microorganismos seleccionados del grupo *Bacteroides succinogenes*, *Fibrobacter succinogenes*, *Ruminococcus albu*, *Ruminococcus flavefaciens*, *Streptococcus bovis*, *Succinomonas amylofílita*, *Prevotella bryantii*, *Anaerovibrio lypolítica*, *Butyrivibrio fibrosolvens* y *Methanobacterium Ruminantium*.

2° Un medio de cultivo para el crecimiento de microorganismos compuesto por: extracto de levadura 2-5g/L, peptona 2-66g/L, glucosa 2-40g/L, rezazurin 0,5-2mL/L, bicarbonato de sodio 3-10g/L, cisteína-HCl 0,5 – 3 g/L, ácidos grasos volátiles (AGV) 0,2-0,6 mL/L, 100 -200mL/L de solución de hidrógeno fosfato de potasio al 3%, 100-200mL/L de solución de sales.

3° Un proceso para la obtención de microorganismos *Bacteroides succinogenes*, *Fibrobacter succinogenes*, *Ruminococcus albu*, *Ruminococcus flavefaciens*, *Streptococcus bovis*, *Succinomonas amylofílita*, *Prevotella bryantii*, *Anaerovibrio lypolítica*, *Butyrivibrio fibrosolvens* y *Methanobacterium Ruminantium*.

Se sugiere presentar las solicitudes divisionales que considere necesarias, o limitar la solicitud inicial a una sola invención, eliminando de ella las otras invenciones.

7. Determinación del Estado de la Técnica

El estado de la técnica se determinó con base en la fecha de presentación de la solicitud de patente nacional: agosto 8 de 2011 y se encontraron los siguientes documentos que anticipan el objeto de esta solicitud:

Ítem	Documento	Título	Fecha de Publicación*
D1	WO2002007741	"Probiotic recolonisation therapy"	25/Julio/2001

D2	Proceedings of the 30th Western Nutrition Conference Winnipeg, Manitoba	"Optimizing Efficiency of Animal Production"	Septiembre 2009
D3	Handbook of Microbiological Media, Third Edition, Volumen 1 Ronald M Atlas	"Handbook of Microbiological Media"	2004
D4	Animal Feed Science and Technology 77 (1999) 61–71	"Production of Ruminococcus flavefaciens growth inhibitor(s) by Ruminococcus albus"	14/Agosto/1998
D5	WO010124147	"Compositions and methods for methane production"	28/Octubre/2010

El documento D1¹ divulga una composición para el tratamiento de enfermedades crónicas asociadas con la distribución anormal de microorganismos en el tracto gastrointestinal de mamíferos, estas composiciones comprenden microorganismos no patógenos seleccionados del grupo de *Bacteroides*, *Eubacteria*, *Fusobacteria*, *Propionibacteria*, *Lactobacilli*, *Anaerobios cocci*, *Ruminococcus*, *E Coli*, *Gemmiger*, *Desulfomonas*, *Peptostreptococcus* y *Fungi* (Pág. 1).

El documento D2² divulga procesos para la optimización de la eficiencia en la producción animal, por el uso de probióticos (Pág. 2).

El documento D3³ enseña medios de crecimiento de microorganismos que comprenden medios de crecimiento de microorganismos anaerobios. (Pág. 67)

El documento D4 divulga un medio de cocultivo selectivo para el recuento de la cepa *R albus* 7 y *Ruminococcus flavefaciens* B1. (Pág. 61)

El documento D5⁴ divulga un proceso para para la producción de metano por medio de la fermentación ejercida por microorganismos del rumen como: *Ruminocaccus albus*, *R. flavefaciens*, *Streptococcus bovis*; *Megasphaera elsdenii*; *Butyrivibrio fibrisolvens* (Reivindicación 18).

8. Examen de Patentabilidad (Art 14 D486)

Novedad (Art 16 D486)

La reivindicación 12 consiste en "El proceso para la obtención de los microorganismos de la composición de la reivindicación 1 caracterizado porque tiene un tiempo de fermentación entre 0,5 y 10 horas, un pH entre 4,5 y 7,5 una temperatura de operación de 26° y 35 °C y una agitación constante entre 100 y 400 rpm "

1 Url: http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=WO&NR=0207741A1&KC=A1&FT=D&ND=3&date=20020131&DB=EPODOC&locale=en_EP
2 Url: <http://www.westernnutritionconference.ca/2009-Proceedings.pdf>
3 Url: <http://books.google.com.co/books?id=aWsF-e8tPzIC&pg=PA1&dq=handbook+de+agares&hl=es&sa=X&ei=rJ69UoGIDo3skQeTuYGgAQ&ved=0CC0Q6AEwAA#v=onepage&q=Fibrobacter%20succinogenes&f=false>
4 Url: <https://www.google.com.co/patents/WO2010124147A1?cl=en&dq=fermentation+process+with+Ruminococcus+albus,+Streptococcus+bovis&hl=es&sa=X&ei=vwbHUqioLtTokQejqoCABQ&ved=0CDcQ6AEwAA>

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

Características esenciales	D5
Proceso de fermentación	Fermentación
Tiempo de fermentación entre 0,5 a 10 horas	Muestras tomadas a diferentes tiempos (Párr.[00176])
pH 4,5 y 7,5	pH 6,5 (Párr.[00173])
Temperatura de operación 26°Cy 35°C	Temperatura 35°C (Párr.[00173])
Agitación entre 100 y 400 rpm	Agitación 300 rpm en reactor de agitación (Párr.[00173])

Las características esenciales de la composición de la reivindicación 11 habían sido divulgadas previamente en el documento D3, el cual revela un proceso para la obtención de metano por medio de la fermentación causada por microorganismos del rumen como: *Ruminococcus albus*, *R. flavefaciens*, *Streptococcus bovis* y *Butyrivibrio fibrisolvens* (Reivindicación 18).

En consecuencia, la reivindicación 11 no es nueva o carece de novedad, según lo divulgado en el documento D5.

Nivel inventivo (Art18 D486)

La reivindicación 8 consiste en “La composición de acuerdo a las reivindicaciones anteriores caracterizada porque viene en tabletas, líquido, gel u otra forma farmacéutica aceptable”

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

Características esenciales	D1	D2
La composición de acuerdo a las reivindicaciones anteriores caracterizada porque viene en tabletas, líquido, gel u otra forma farmacéutica aceptable	Tabletas, capsulas, formulaciones liquidas (Pág. 7 Línea 20-31)	No menciona
Una composición que comprende uno o más cultivos de microorganismos seleccionados del grupo de:	Una composición farmacéutica que comprende uno o más microorganismos no patogénicos o patogénicos atenuados como (Pág. 6 Línea 3-5):	Probióticos
<i>Bacteroides succinogenes</i> , <i>Fibrobacter sussinogenes</i> <i>Ruminococcus albu</i> , <i>Ruminococcus flavefaciens</i> , <i>Streptococcus bovis</i> , <i>Succinomonas amyolitica</i> , <i>Prevotella bryantii</i> , <i>Anaerivibrio lypolitica</i> , <i>Butytrivibrio fibrosolvens</i> y <i>Methanobacterium</i> <i>Ruminantium</i>	Grupo <i>Bacteroides</i> , <i>Eubacteria</i> , <i>Fusobacteria</i> , <i>Propionibacteria</i> , <i>Lactobacilli</i> , <i>Anaerobios cocci</i> , <i>Ruminococcus</i> , <i>E Coli</i> , <i>Gemmiger</i> , <i>Desulfomonas</i> , <i>Peptostreptococcus</i> y <i>Fungi</i> (Pág. 6 Línea 5-6) Microorganismos como: <i>Ruminococcus flavefaciens</i> (Pág. 16 Línea 12), <i>Ruminococcus albus</i> (Pág.11)	<i>Prevotella bryantii</i> (Pág. 5), <i>Butytrivibrio</i> <i>fibrosolvens</i> (Pág. 6) <i>Streptococcus bovis</i> (Pág. 67)

El documento D1 se considera el estado de la técnica más cercano a la invención porque divulga composiciones para el tratamiento de desórdenes asociados con la presencia anormal o distribución anormal de micro flora en el tracto gastrointestinal (Pág. 1 Línea 1-5), donde dichas composiciones comprenden microorganismos no patogénicos o patogénicos atenuados (Pág. 6 Línea 3-5) en forma de tabletas, capsulas, formulaciones liquidas (Pág. 7 Línea 20-31) que

contienen los grupos *Bacteroides*, *Eubacteria*, *Fusobacteria*, *Propionibacteria*, *Lactobacilli*, *Anaerobios cocci*, *Ruminococcus*, *E Coli*, *Gemmiger*, *Desulfomonas*, *Peptostreptococcus* y *Fungi* (Pág. 6 Línea 5-6). Estas composiciones a su vez comprenden microorganismos como: *Ruminococcus flavefaciens* (Pág. 16 Línea 12) y *Ruminococcus albus* (Pág.11), utilizadas para el tratamiento o prevención de la diarrea (Pág. 3).

El documento D2 divulga las funciones de microorganismos utilizados como probióticos en animales rumiantes.

La diferencia entre la invención, definida en la reivindicación 1 y la composición divulgada en el documento D1 consiste en la presencia de *Prevotella bryantii*, *Butyrivibrio fibrosolvens* y *Streptococcus bovis* en la composición.

El efecto que logra el incluir los microorganismos *Prevotella bryantii*, *Butyrivibrio fibrosolvens* y *Streptococcus bovis* es favorecer la flora bacteriana del tracto gastrointestinal.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo proporcionar composiciones para el tratamiento de desórdenes gastrointestinales en becerros”.

Sin embargo, la utilización de composiciones probióticas para la prevención de desórdenes gastrointestinales como la diarrea, ya está divulgada en el documento D1 que se refiere a composiciones que comprenden microorganismos no patogénicos o patogénicos atenuados (Pág. 6 Línea 3-5) como: *Ruminococcus flavefaciens* (Pág. 16 Línea 12) y *Ruminococcus albus* (Pág.11) para el tratamiento de desórdenes gastrointestinales. A su vez el documento D2 divulga las actividades ejercidas por los microorganismos *Prevotella bryantii* (Pág.5), *Butyrivibrio fibrosolvens* (Pág. 6) y *Streptococcus bovis* (Pág. 67) en el rumen de mamíferos, donde la *Prevotella bryantii* favorece la prevención de acidosis, en cuanto el *Butyrivibrio fibrosolvens* permite la producción de ácidos linoleicos conjugados. Por lo anterior una persona versada medianamente versada en la materia tendría la suficiente motivación de incluir los microorganismos *Prevotella bryantii*, *Butyrivibrio fibrosolvens* y *Streptococcus bovis* en las composiciones divulgadas en el documento D1 conociendo la funcionalidad de los mismos en el tracto gastrointestinal divulgadas en el documento D2.

En consecuencia, la persona del oficio normalmente versada en la materia, incluiría los microorganismos *Prevotella bryantii*, *Butyrivibrio fibrosolvens* y *Streptococcus bovis* del documento D2 en las composiciones divulgadas en el documento D1 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

Conclusión:

La reivindicación 8 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18).

Por su parte las reivindicaciones 13-15 no mencionan características adicionales por tanto carecen de altura inventiva.

La reivindicación 10 consiste en “Un medio de cultivo para el crecimiento de los microorganismos de la composición de la reivindicación 1 caracterizado porque está compuesta por: Extracto de levadura 2-5g/L, Péptona 2-6g/L, Glucosa 2-40g/L, Rezazurin 0,5-2mL/L, Bicarbonato de sodio 3-10g/L, Cisteína HCl 0,5-3 g/L, Ácidos grasos volátiles (Agv) 0,2 -0,6 mL/L, Solución de Hidrógeno fosfato de Potasio 3% 100-200mL/L, 100-200mL/L de solución sales”

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

Características esenciales	D3	D4
Un medio de cultivo	Un medio de cultivo para anaerobios (Pág. 667)	Medio de crecimiento de <i>Ruminococcus flavefaciens</i> (Pág. 63)
Extracto de levadura 2-5g/L	Extracto de levadura 0,5g	No menciona
Peptona 2-6g/L	Peptona	No menciona
Glucosa 2-40g/L	Glucosa	No menciona
Rezaurin 0,5-2mL/L	Rezaurin 0,4mL	Resazurin 0.3mL
Bicarbonato de sodio 3-10g/L	Bicarbonato de sodio 3-10g/L	Solución de carbonato de sodio
Cisteína HCl 0,5-3 g/L	Cisteína 0,05g	Cisteína 0,4%
Ácidos grasos volátiles (Agv) 0,2 -0,6 mL/L	No menciona	Ácidos grasos volátiles
Solución de Hidrógeno fosfato de Potasio 3% 100-200mL/L	Hidrogeno fosfato de potasio 50mL/100mL	No menciona
100-200mL/L de solución sales	Solución de sales 50mLpor cada 100mL	Soluciones minerales

El documento D3 se considera el estado de la técnica más cercano a la invención porque divulga medios de crecimiento de microorganismos anaerobios que comprende extracto de levadura, peptona, glucosa, rezaurin, bicarbonato de sodio, cisteína, hidrogeno fosfato de potasio y solución de sales (Pág. 667).

El documento D4 divulga un medio de cocultivo selectivo para el recuento de la cepa *Ruminococcus albus* 7 y *Ruminococcus flavefaciens* B1 que contiene ácidos grasos volátiles. (Pág. 61)

La diferencia entre la invención, definida en la reivindicación 10 y el medio de crecimiento divulgado en el documento D3 consiste en la presencia de ácidos grasos volátiles.

El efecto que logra el incluir los ácidos grasos volátiles es favorecer el crecimiento de los microorganismos *Bacteroides succinogenes*, *Fibrobacter succinogenes*, *Ruminococcocis albbus*, *Ruminococcocis flavefaciens*, *Streptococcus bovis*, *Succionomonas amyolitica*, *Prevotella bryantii*, *Anaerovibrio lypoliticam* *Butytrivibrio fibrosolvens* y *Methanobacterium ruminantium*.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: “cómo proporcionar un medio de crecimiento de los microorganismos *Bacteroides succinogenes*, *Fibrobacter succinogenes*, *Ruminococcocis albbus*, *Ruminococcocis flavefaciens*, *Streptococcus bovis*, *Succionomonas amyolitica*, *Prevotella bryantii*, *Anaerovibrio lypoliticam* *Butytrivibrio fibrosolvens* y *Methanobacterium ruminantium*”.

Sin embargo la utilización de ácidos grasos para el crecimiento de *Ruminococcus flavefaciens* ya se encontraba divulgada en el documento D4 a su vez documento D3 divulga un medio de crecimiento microorganismos anaerobios como: el *Fibrobacter succinogenes*, *Ruminococcocis albbus*, *Streptococcus bovis*, *Succionomonas amyolitica*, *Prevotella bryantii*, *Anaerovibrio lypolitica* *Butytrivibrio fibrosolvens* y *Methanobacterium ruminantium*”. Por lo tanto una persona versada en la materia incluiría los ácidos grasos divulgados en el documento D4 en el medio de cultivo divulgado en el documento D3 para favorecer el crecimiento de microorganismo *Ruminococcus flavefaciens*

En consecuencia, la persona del oficio normalmente versada en la materia,

incluiría los ácidos grasos del documento D4 en los medios de crecimiento divulgadas en el documento D3 para llegar así al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia.

Conclusión:

La reivindicación 10 cumple el requisito de novedad (Art. 16), pero no cumple con el requisito de nivel inventivo (Art. 18).

Por su parte la reivindicación 11 no menciona características adicionales por tanto carecen de altura inventiva.

9. Conclusión

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

Ítem	Documento	Reivindicaciones afectadas	Requisito que afecta
D1	WO2002007741	8 y 13-15	Nivel inventivo
D2	Proceedings of the 30th Western Nutrition Conference	8 y 13-15	Nivel inventivo
D3	Handbook of Microbiological Media, Third Edition, Volumen 1 Ronald M Atlas	10-11	Nivel inventivo
D4	Animal Feed Science and Technology 77 (1999) 61–71	10-11	Nivel inventivo
D5	WO 2010124147	12	Novedad

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 2014-02-11

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ELSEVIER

Animal Feed Science and Technology 77 (1999) 61–71

ANIMAL FEED
SCIENCE AND
TECHNOLOGY

Production of *Ruminococcus flavefaciens* growth inhibitor(s) by *Ruminococcus albus*

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Abstract

While trying to develop a most probable number (MPN) selective medium for the enumeration of *Ruminococcus albus* 7 and *Ruminococcus flavefaciens* B1a in coculture, it was observed that when cultures of the two organisms were mixed for testing the media, growth of *R. flavefaciens* B1a was inhibited. Subsequent studies indicated that *R. albus* 7 produced an inhibitory substance that was present in cell-free culture filtrates and the extent of inhibition increased with the quantity of *R. albus* filtrate. *R. albus*, MO2a and MO3g were also tested for inhibitory activity against *R. flavefaciens* B1a, B34b, R13e2 and C1a, *Fibrobacter succinogenes* S85 and *Butyrivibrio fibrisolvens* H17c. *R. flavefaciens* B1a and C1a were found to be inhibited by all strains of *R. albus* and strain B34b was inhibited by *R. albus* MO2a and MO3g. The inhibitory substance(s) was heat-labile and destroyed by protease. When *R. albus* strains were cocultured with a proteolytic organism isolated from the rumen, *B. fibrisolvens* H17c, inhibitory activity was decreased or completely destroyed. These results suggest that the inhibitory material is proteinaceous and may be a bacteriocin-like compound. © 1999 Elsevier Science B.V.

Keywords: Rumen; Bacteria; Growth Inhibition; Ruminococci

1. Introduction

When several types of bacteria are grown together in the same medium, antagonistic interactions sometimes occur. With closely related species, this antagonism can result from the production of specific bactericidal proteins called bacteriocins (Tagg et al., 1976; Barefoot and Klaenhammer, 1983; Barefoot and Nettles, 1993). Although growth inhibition by bacteriocins has been studied in detail in the food and dairy industries, little

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is known about their possible production by rumen bacteria. Iverson and Mills (1976) surveyed 47 strains of *Streptococcus bovis* for bacteriocin-like activity. Of the 23 *S. bovis* strains in their study which were isolated from the rumen, only one produced any inhibitory substances. Although not considered to be a major genus in the rumen, five strains of ruminal staphylococci have been observed to produce antimicrobial activity which, to varying extents, also inhibited three strains of *Ruminococcus* and a ruminal isolate of *E. coli* (Lauková and Mareková, 1993).

In a more complete study, Odenyo et al. (1994) reported that *Ruminococcus albus* 8 released an antagonistic substance which inhibited the growth of *R. flavefaciens* FD-1 when both organisms were cocultured in either cellobiose or cellulose medium. Adding pronase E or α -chymotrypsin into cell-free culture supernatants from *R. albus* 8 destroyed the antagonistic activity, while boiling at 100°C for 10 min did not. Growth of *Fibrobacter succinogenes* was not affected in coculture with *R. albus* 8.

Forty-nine strains of *Butyrivibrio fibrisolvens*, isolated from the rumen of various animal species at widely separated geographical locations, were tested for the production of bacteriocin-like compounds (Kalmokoff et al., 1996; Kalmokoff and Teather, 1997). Inhibitory activity against other *Butyrivibrio* strains was produced by 25 of the isolates; however, the extent of inhibition varied considerably between strains. The inhibitors from 18 of the isolates were sensitive to digestion by both pronase and chymotrypsin, but were resistant to gastric proteases like pepsin and trypsin. Differences in both the extent of inhibition and sensitivity to proteases, suggested that a number of different inhibitors may be produced. At least 12 strains (six different genera) of other ruminal bacteria were inhibited to varying degrees by the *Butyrivibrio* produced inhibitors. The bacteriocin-like inhibitor from one isolate was purified and identified as a single peptide with a molecular mass of approximately 4000 Da. This bacteriocin, butyrivibriocin AR10, is the first to be isolated from an anaerobic ruminal bacteria.

Fondevila and Dehority (1996) observed a somewhat different type of inhibition in forage cellulose digestion when *F. succinogenes* and *R. flavefaciens* were cocultured. The inhibitory activity was only produced when the organisms were cocultured and was stable to autoclaving at 121°C for 20 min.

During some preliminary experiments in our laboratory, it was observed that *R. albus* 7 inhibited the growth of *R. flavefaciens* B1a when they were cocultured. The present study was undertaken to investigate this inhibitory activity and included the following objectives: (1) to determine whether the inhibitory activity of *R. albus* 7 was cell associated or released into the medium; (2) to determine if inhibition was concentration dependent; (3) to survey other strains of *R. albus* for production of inhibitors; (4) to test other strains of *R. flavefaciens* and different species for susceptibility to this inhibitory activity; and (5) to study properties of the inhibitor(s).

2. Materials and methods

2.1. Organisms and media

Organisms used for these studies were strains 7, MO2a and MO3g of *Ruminococcus albus*, strains B1a, B34b, R13e2 and C1a of *Ruminococcus flavefaciens*, *Fibrobacter*

Table 1
Composition of selective MPN media for *Ruminococcus flavefaciens* B1a and *Ruminococcus albus* 7

Ingredient	Selective MPN medium for:	
	B1a	7
Mineral solution A (ml) ^a	60	60
Mineral solution B (ml) ^a	60	60
Resazurin solution (0.0001%) (ml) ^a	0.3	0.3
Vitamin mixture (ml) ^a	3	3
Trypticase (g)	0.5	0.5
Sucrose (g)	3	0
Cellobiose (g)	0	3
Clarified ruminal fluid (ml) ^b	30	0
2-Methylbutyric acid (2.56%) (ml)	0	1
Volatile fatty acid mixture (ml) ^c	1.35	0
Sodium carbonate solution (0.4%) (ml)	10	10
Cysteine–HCl solution (0.05%) (ml)	10	10
Distilled water (ml)	125.35	155.7

^aScott and Dehority (1965).

^bObtained from strained ruminal fluid centrifuged at $12\,500 \times g$ for 20 min.

^cCaldwell and Bryant, 1966.

succinogenes S85 and *Butyrivibrio fibrisolvens* H17c. All organisms were isolated from the rumen. Characteristics of these strains have been described previously (Bryant and Doetsch, 1954; Bryant et al., 1958; Dehority, 1963, 1966, 1986). Anaerobic techniques were similar to those described by Hungate (1950), with the modifications of Dehority (1969). Selective Most-Probable-Number (MPN) media, based on nutritional requirements and differences in substrates utilized, were formulated to determine concentrations of *R. albus* 7 and *R. flavefaciens* B1a in mixed culture (Table 1). Inoculum for each organism was grown overnight (18–24 h) in the same selective medium, except the carbohydrate concentration was reduced to 0.5%. The selective B1a roll tube agar medium was identical to selective B1a MPN medium, except it contained 2% agar. Strain 7, B1a and a mixture of 7 and B1a were diluted with anaerobic dilution solution (ADS) and inoculated into selective 7 and B1a media separately.

Bacterial growth in the MPN assay was determined by a decrease of at least 0.2 unit in medium pH after 7 days of incubation at 38°C and the numbers were determined from a most-probable number table. All experiments were repeated at least three times.

2.2. Culture filtrates and washed cells

R. albus culture filtrates were prepared by centrifuging the inoculum medium at $12\,500 \times g$ for 20 min and then filtering the supernatant through a 0.2 µm pore size membrane filter (Acrodisc; Gelman Sciences, Ann Arbor, MI). The sedimented *R. albus* cells were washed with 9 ml of sterile ADS, centrifuged at $12\,500 \times g$ for 20 min, and the supernatant was discarded. The washing procedure was repeated twice before adding

enough ADS to bring the volume back to that of the original inoculum culture. All procedures were conducted under anaerobic conditions.

2.3. Location of inhibitory activity in culture

One ml of an 18–24 h *R. flavefaciens* B1a culture was serially diluted in ADS and inoculated into selective B1a MPN medium as a control. One ml of *R. albus* 7 cell-free culture filtrate and 1 ml of strain B1a inoculum were added to a tube containing 8 ml of ADS. The mixture was serially diluted to 10^{-10} and inoculated into selective B1a MPN medium. One ml each of resuspended *R. albus* 7 washed cells and strain B1a inoculum were also serially diluted in ADS and inoculated into selective B1a roll tube agar medium. Four roll tubes were used at each dilution (10^{-5} – 10^{-8}). An equivalent concentration of resuspended *R. albus* 7 cells were also inoculated into selective 7 MPN medium to test the viability of *R. albus* 7 cells after centrifugation and washing.

2.4. Concentration studies

To test whether the extent of inhibition was based on concentration, 1, 2, 2.5, 3 and 3.5 ml of *R. albus* 7 culture filtrate were mixed with 1 ml of *R. flavefaciens* B1a inoculum and the volume was made up to 10 ml with ADS. These mixtures were serially diluted and inoculated into selective B1a MPN medium.

2.5. Survey of inhibitory activity in various cultures

Three ml of culture filtrates from *R. albus* 7, MO2a and MO3g (prepared as described earlier) were added to 1 ml aliquots of inocula from several different organisms (*R. albus* 7, MO2a and MO3g, *R. flavefaciens* B1a, B34b, C1a and R13e2, *F. succinogenes* S85, and *B. fibrisolvens* H17c) in order to test the possible inhibitory interactions. The mixtures were serially diluted with ADS. Inoculum and MPN media for all of the additional organisms was the selective cellobiose medium (*R. albus* 7) listed in Table 1, with the volatile fatty acid mixture replacing 2-methylbutyric acid. Sucrose medium, with the addition of clarified ruminal fluid, was utilized for growth of strain B1a.

2.6. Properties of inhibitor(s)

To investigate the general characteristics of the inhibitory substance(s), three assay systems were used, that is, *R. albus* 7 filtrate and *R. flavefaciens* B1a, *R. albus* MO2a filtrate and *R. flavefaciens* B34b, *R. albus* MO3g filtrate and *R. flavefaciens* C1a. Filtrates of *R. albus* 7, MO2a and MO3g were autoclaved at 121°C for 20 min or mixed with a non-specific protease from *Streptomyces griseus* (1000 µg/ml of filtrate) (Sigma, St. Louis, MO) and incubated at 37°C, pH 7.5 for 2 h. After treatment, filtrates were added with *R. flavefaciens* B1a, B34b and C1a inocula, respectively. Possible degradation of the inhibitory substance(s) by a proteolytic organism isolated from the rumen was also examined. Preliminary experiments had verified that none of the *R. albus* strains inhibited

growth of *B. fibrisolvans* H17c. Using 0.5 ml of 24 h cellobiose broth cultures for all organisms as inocula, each of the three strains of *R. albus* (7, MO2a and MO3g) were cultured by themselves (negative controls), or cocultured with *B. fibrisolvans* H17c for 18–24 h. Filtrates of the cocultures and negative controls were filter sterilized, added with the same test organisms as before and numbers were determined with a MPN assay. *R. flavefaciens* B1a, B34b and C1a inoculum cultures acted as the controls.

2.7. Statistical analysis

Data were converted into logarithm values before a paired-*t*-test was used to detect the level of significance (Steel and Torrie, 1980; Minitab Reference Manual, 1991).

3. Results

3.1. Enumeration of *R. albus* 7 and *R. flavefaciens* B1a in a mixture

The MPN media formulated for enumeration of *R. albus* 7 and *R. flavefaciens* B1a were selective enough to prevent growth of the other strain (Table 2). Concentrations of *R. albus* 7, determined alone or in a mixture with B1a, were not different ($p > 0.29$) when measured in selective 7 MPN medium. Growth of B1a was not observed in three of four replicates when the 7 and B1a mixture was assayed in selective B1a MPN medium. However, it should be noted that the lowest dilution counted was 10^{-6} , and the possibility cannot be overlooked that growth occurred in the first three replicates at a lower dilution. The mean of all four replicates for bacterial growth in the 7 plus B1a mixture was significantly lower than the value for B1a measured alone ($p < 0.02$). These data suggested that growth of B1a was somehow inhibited in the presence of 7.

If these two organisms could be grown in a solid medium without direct contact, it was possible that inhibition might not occur since diffusion of the inhibitory substance(s) could be slower through a solidified medium. Using the same selective B1a MPN medium with agar added, the concentration of B1a in a mixture of 7 plus B1a was determined in roll tubes. No colonies developed after 7 days incubation (data not shown), suggesting that inhibition occurred at the time the cultures were mixed.

Table 2

Enumeration of *Ruminococcus albus* 7 and *Ruminococcus flavefaciens* B1a, alone or in coculture, in selective MPN medium^a

Organism(s)	Bacterial concentration (no $\times 10^7$ /ml)	
	MPN medium for 7	MPN medium for B1a
<i>R. albus</i> 7	16.8 ± 9.1^b	0 ^d
<i>R. flavefaciens</i> B1a	0 ^c	53.0 ± 17.4^c
<i>R. albus</i> 7 + <i>R. flavefaciens</i> B1a	13.0 ± 5.7^b	0.02 ± 0.02^d

^aMean values $\pm$ SE of four replicates.

^{b,c}Means in the same column without a common superscript differ ($p < 0.001$).

^{d,e}Means in the same column without a common superscript differ ($p < 0.02$).

3.2. Location of inhibitor(s)

Numbers of strain B1a, measured in the presence of 1 ml of strain 7 culture filtrate ($1.5 \pm 0.9 \times 10^5$), were significantly lower ($p < 0.01$) than that of B1a measured alone ($63.1 \pm 32.3 \times 10^7$). In contrast, when strain B1a and resuspended 7 cells were mixed and inoculated into selective B1a roll tube agar medium, numbers of B1a did not differ from those of the control ($26.7 \pm 10.9 \times 10^7$). Washed *R. albus* 7 cells were completely viable when inoculated into selective 7 MPN medium (data not shown). Based on these results, it was concluded that the inhibition to B1a was probably caused by a soluble substance produced by strain 7.

3.3. Levels of filtrate

When 1, 2, 2.5, 3 and 3.5 ml of *R. albus* 7 culture filtrate were added with strain B1a, viable numbers of strain B1a decreased quadratically ($R^2 = 0.91$). Compared to the control without added filtrate, $33.8 \times 10^7/\text{ml}$, concentrations were 1.63×10^5 , 1.11×10^4 , 5.97×10^3 , 4.53×10^3 and 1.3×10^3 , respectively. Thus, inhibition was concentration-dependent. Three ml of *R. albus* 7 culture filtrate added to 1 ml of B1a inoculum reduced the numbers of B1a by 99.99%. This ratio, three parts of filtrate to one part of inoculum culture, was used in all subsequent inhibition studies.

3.4. Response of various cultures to inhibitor(s)

No inhibition was found between the three strains of *R. albus* (7, MO2a and MO3g) when culture filtrates from the different strains were added to the test inoculum (data not shown). Concentrations of *R. flavefaciens* strains B1a and C1a were lower ($p < 0.05$) in the presence of culture filtrates from *R. albus* 7, MO2a and MO3g, (Table 3). Strain B34b was inhibited ($p < 0.05$) by culture filtrates from *R. albus* MO2a and MO3g, but not by 7 ($p > 0.34$). In contrast, *R. flavefaciens* strain R13e2 was not inhibited by culture filtrates from any of the *R. albus* strains (7, $p > 0.59$; MO2a, $p > 0.66$; MO3g, $p > 0.42$). Compared to the controls, no differences in numbers were observed when 3 ml of *R. albus* 7, MO2a and MO3g culture filtrate was added to *F. succinogenes* S85 or *B. fibrisolvens* H17c.

3.5. Effect of heat, protease or coculture with a proteolytic organism

The effect of autoclaving culture filtrates from *R. albus* strains 7, MO2a and MO3g upon enumeration of *R. flavefaciens* B1a, B34b and C1a, respectively, is shown in Table 4. For strains B1a and B34b, concentrations determined in the presence of the autoclaved filtrates clearly did not differ from the controls ($p > 0.44$, $p > 0.67$). For C1a, addition of the autoclaved filtrate actually increased ($p < 0.01$) bacterial concentration.

When the three *R. albus* filtrates were treated with protease before being added to inocula of the susceptible organisms (strains B1a, B34b and C1a), no differences in numbers were observed ($p > 0.67$, $p > 0.21$ and $p > 0.22$) compared to their controls

Table 3
Effects of *Ruminococcus albus* culture filtrates (strains 7, MO2a and MO3g) on final concentrations of *R. flavefaciens* B1a, B34b, C1a and R13e2, *Fibrobacter succinogenes* S85 and *Butyrivibrio fibrisolvens* H17c, measured with an MPN assay^a

Organism	Bacterial concentrations (no $\times 10^7$ /ml)					
	<i>R. albus</i> 7		<i>R. albus</i> MO2a		<i>R. albus</i> MO3g	
	Control	Filtrate	Control	Filtrate	Control	Filtrate
<i>R. flavefaciens</i> B1a	63.1 $\pm$ 32.3 ^b	0.015 $\pm$ 0.009 ^c	66.5 $\pm$ 43.3 ^b	<0.001 ^c	136 $\pm$ 52 ^b	0.008 $\pm$ 0.008 ^c
<i>R. flavefaciens</i> B34b	181.0 $\pm$ 138	47.8 $\pm$ 25.7	234 $\pm$ 113 ^b	0.17 $\pm$ 0.13 ^c	319 $\pm$ 138 ^b	0.15 $\pm$ 0.14 ^c
<i>R. flavefaciens</i> C1a	31.5 $\pm$ 11.1 ^b	<0.001 ^c	51.9 $\pm$ 31.8 ^b	<0.001 ^c	82.5 $\pm$ 25.9 ^b	0.01 $\pm$ 0.01 ^c
<i>R. flavefaciens</i> R13e2	5.5 $\pm$ 2.0	4.8 $\pm$ 2.4	40.5 $\pm$ 34.7	12.5 $\pm$ 5.9	14.2 $\pm$ 4.9	11.9 $\pm$ 6.4
<i>F. succinogenes</i> S85	10.7 $\pm$ 6.9	17.6 $\pm$ 12.7	184 $\pm$ 139	183 $\pm$ 139	19.1 $\pm$ 4.9	18.4 $\pm$ 5.6
<i>B. fibrisolvens</i> H17c	102 $\pm$ 69.1	54.0 $\pm$ 21.4	549 $\pm$ 293	532 $\pm$ 307	174 $\pm$ 65.7	422 $\pm$ 337

^aMean values $\pm$ SE of three replicates, (four replicates were run for *R. flavefaciens* C1a with MO3g filtrate).

^{b,c}For each *R. albus* species, means in the same row without a common superscript differ ($p < 0.05$).

Table 4

Effect of autoclaving *R. albus* culture filtrates on their inhibitory activity against growth of *R. flavefaciens* B1a, B34b and C1a^a

<i>R. flavefaciens</i> strain	<i>R. albus</i> filtrate (strain)	Bacterial concentration (no $\times 10^7$ /ml)
B1a		63.1 $\pm$ 32.3 ^b
B1a	+	0.015 $\pm$ 0.009 ^c
B1a	+, Autoclaved (7)	41.6 $\pm$ 18.4 ^b
B34b		76.4 $\pm$ 16.9 ^e
B34b	+	0.016 $\pm$ 0.013 ^f
B34b	+, Autoclaved (MO2a)	59.5 $\pm$ 16.9 ^e
C1a		28.7 $\pm$ 13.9 ^b
C1a	+	<0.001 ^c
C1a	+, Autoclaved (MO3g)	63.0 $\pm$ 30.3 ^d

^aMean values $\pm$ SE, four replicates for B1a and three replicates each for B34b and C1a.

^{b,c,d}For each *R. flavefaciens* strain, means without a common superscript differ ($p < 0.01$).

^{e,f}Means without a common superscript differ ($p < 0.05$).

(Table 5). The protease enzyme added by itself (data not shown) had no effect on bacterial concentrations.

Previous experiments (Table 3) had demonstrated that none of the three *R. albus* strains inhibited growth of *B. fibrisolvens* H17c. Compared to the controls, no significant differences in the concentrations of *R. flavefaciens* B1a, B34b and C1a were observed ($p > 0.21$, $p > 0.95$ and $p > 0.22$) when culture filtrates from cocultures of individual *R. albus* strains (7, MO2a and MO3g) and *B. fibrisolvens* H17c were added (Table 6). This would suggest that the inhibitory substance(s) was degraded by this highly proteolytic organism. Another possible explanation for this loss or decrease of inhibitory activity might be a simple absorption or adsorption of the inhibitor by the second organism in the coculture. One might also question whether the *R. albus* strains grew and thus produced

Table 5

Effect of treating *R. albus* culture filtrates with a proteolytic enzyme^a on their inhibitory activity against growth of *R. flavefaciens* B1a, B34b, C1a^b

<i>R. flavefaciens</i> strain	<i>R. albus</i> filtrate (strain)	Protease treated	Bacterial concentration (no $\times 10^7$ /ml)
B1a			36.4 $\pm$ 6.2 ^c
B1a	+		<0.001 ^d
B1a	+	+	30.2 $\pm$ 6.2 ^c
B34b			59.5 $\pm$ 16.9 ^c
B34b	+		0.03 $\pm$ 0.01 ^d
B34b	+	+	95.8 $\pm$ 31.4 ^c
C1a			38.3 $\pm$ 35.7 ^c
C1a	+		<0.001 ^d
C1a	+	+	41.1 $\pm$ 34.3 ^c

^aNon-specific protease from *Streptomyces griseus*.

^bMean values $\pm$ SE of three replicates.

^{c,d}For each *R. flavefaciens* strain, means without a common superscript differ ($p < 0.05$).

Table 6

Effects of coculturing *R. albus* with a proteolytic organism (*B. fibrisolvens* H17c) upon the inhibitory activity of culture filtrates against *R. flavefaciens* B1a, B34b and C1a^a

Culture or coculture filtrate			
<i>R. flavefaciens</i> strain	<i>R. albus</i> strain	<i>B. fibrisolvens</i> H17c + <i>R. albus</i> (strain)	Bacterial concentration (no $\times 10^7$ /ml)
B1a	–	–	76.4 $\pm$ 16.9 ^b
B1a	+ (7)	–	<0.001 ^c
B1a	–	+ (7)	36.4 $\pm$ 6.2 ^b
B34b	–	–	116 $\pm$ 65.9 ^b
B34b	+ (MO2a)	–	<0.001 ^c
B34b	–	+ (MO2a)	76.4 $\pm$ 16.9 ^b
C1a	–	–	36.4 $\pm$ 6.2 ^b
C1a	+ (MO3g)	–	<0.001 ^c
C1a	–	+ (MO3g)	21.0 $\pm$ 10.8 ^b

^aMean values $\pm$ SE of three replicates.

^{b,c}For each *R. flavefaciens* strain, means without a common superscript differ ($p < 0.01$).

inhibitor(s) when cocultured with strain H17c. However, abundant growth of all *R. albus* strains was observed microscopically in the cocultures.

4. Discussion

In this study, three strains of *R. albus*, 7, MO2a and MO3g, were found to produce inhibitory substance(s) which showed a narrow but specific inhibitory spectrum against several strains of the closely related species *R. flavefaciens*. Loss or a decrease of inhibitory activity by treatment of culture filtrates with either heat or a proteolytic enzyme, would suggest the presence of a bacteriocin-like substance. Odenyo et al. (1994) reported that *R. albus* 8 produced a bacteriocin-like substance which inhibited the growth of *R. flavefaciens* FD-1, but not *F. succinogenes* S85. They suggested that bacteriocin production is a mechanism used by *R. albus* to compete for nutrients. The present findings extend our knowledge on inhibition between these species to three additional strains of *R. albus* and four different species of *R. flavefaciens*. It is of interest that the strain of *R. flavefaciens* (R13e2) isolated from reindeer in Alaska was not inhibited by any of the *R. albus* strains, not even those originally isolated from musk-oxen in Alaska (MO2a and MO3g). One possible difference in this study from that of Odenyo et al. (1994) is that the inhibitory substance(s) in the present study was destroyed by autoclaving for 20 min, while they found no loss in activity after boiling for 10 min. However, characteristics of bacteriocins produced by different bacterial strains within the same bacterial genus and species, can vary considerably both physically and chemically (Tagg et al., 1976; Geis et al., 1983; Kalmokoff and Teather, 1997).

For survival, bacteriocin-producing organisms must establish a specific immunity to their own and possibly homologous bacteriocins. The immunity obtained by the producer cells seems to be induced following the adsorption of the bacteriocin to the cell surface and is a result of the synthesis of a specific immune substance. Tagg et al. (1976)

suggested that this specific immune substance would form a firm complex with the bacteriocin resulting in inactivation of the bacteriocin. Since the inhibitor producing strains of *R. albus* in this study were not auto-inhibitory, nor cross inhibitory, it might be presumed that either they lacked appropriate cell receptors or produced an immune substance which complexed with the bacteriocin-like compounds and masked or inactivated them.

Since *R. flavefaciens* B34b was inhibited only by *R. albus* MO2a and MO3g, and not by strain 7, the bacteriocin-like substances produced by *R. albus* MO2a and MO3g might differ from that of *R. albus* 7. Another possible explanation is that *R. flavefaciens* B34b does not possess a functional cell receptor to the *R. albus* 7 inhibitory substance (Plate and Luria, 1972).

Although bacteriocin-like substances would be expected to be produced in vivo, this study suggests the possibility that they subsequently could be destroyed by the action of proteolytic organisms in the rumen. However, the fact that the proteolytic species tested in this study, *Butyrivibrio fibrisolvens*, also produces bacteriocin-like inhibitory activity would further complicate the overall picture (Kalmokoff et al., 1996). Although these bacteriocin-like substances might be expected to have a limited effect on the total rumen fermentation, it is possible that they could play a role within the environmental niche which occurs at the surface of plant particles.

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