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Identificación de Interacciones tipo Adhesina-Carbohidrato en la Adherencia de *Lactobacillus* Probióticos a la Mucosa Intestinal de Lechones

PRESENTADA POR

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Es el ojo de la ignorancia el que asigna un color
fijo e incambiable a cada objeto
Paul Gauguin

No estudio por saber más,
sino por ignorar menos
Sor Juana Inés de la Cruz

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CONTENIDO

COMUNICACIONES CIENTÍFICAS

RESUMEN

En la flora intestinal del cerdo, el género *Lactobacillus* es de los más abundantes. Debido a sus beneficios en la salud animal es que se utilizan como probióticos. Sin embargo, los mecanismos por los que se adhieren a la mucosa intestinal no están totalmente elucidados. Los objetivos de este trabajo se enfocaron a identificar las especies de *Lactobacillus* en el intestino delgado de lechones y realizar una caracterización probiótica parcial de las mismas. Posteriormente, identificar los carbohidratos y proteínas que intervienen en la adhesión de dichas cepas a la mucosa intestinal. Por último, cuantificar la fuerza de adhesión de tres cepas a la mucosa y a proteínas de membrana de los glóbulos de grasa de la leche (MFGM por sus siglas en inglés), que también contienen mucinas, utilizando pinzas ópticas.

Se obtuvo el intestino delgado de seis lechones recién destetados. De ahí se aislaron e identificaron cepas de *Lactobacillus*, además se colectó la mucosa mediante raspado del mismo. La evaluación probiótica parcial consistió en cuantificar la resistencia de las cepas al tránsito gastrointestinal, evaluar sus propiedades de superficie y su antagonismo contra *E. coli* K88.

Para identificar los carbohidratos participantes en la adhesión de las cepas probióticas a la mucosa, se realizaron ensayos de manchas (dot blot en inglés) de inhibición de la adhesión con diferentes azúcares y glicoproteínas. Para identificar la masa molecular de las proteínas de mucosa, involucradas en la adhesión, se realizaron transferencias de las mismas (western blot en inglés) a membranas de nitrocelulosa y se pusieron en contacto con bacterias marcadas con biotina para visualizar la interacción. Por último, para cuantificar la fuerza de adhesión de tres cepas de *Lactobacillus* a mucinas intestinales y MFGM, se utilizaron pinzas ópticas, con las que se puede atrapar una sola célula bacteriana y ponerla en contacto con diferentes superficies.

Se identificaron especies como *Lb. salivarius*, *Lb. reuteri* y *Lb. mucosae*, a las que se les evaluó sus propiedades probióticas. Se encontraron 8 cepas que cumplieron con los criterios para ser consideradas

como benéficas. De ellas se seleccionaron 3 para realizar ensayos de manchas con mucosa intestinal de lechones y transferencias de mucinas a membranas de nitrocelulosa.

Se observó que las masas moleculares de las proteínas de la mucosa intestinal de lechones con las que interaccionaron las cepas probióticas se encuentran en un rango de 10 a 50 kDa. La mucina gástrica porcina inhibió la adhesión de la cepa 6 a la mucosa intestinal de lechones mientras que galactosa y manosa fueron los inhibidores para las cepas 9 y 10. Las tres bacterias mostraron fuerte interacción con los epítopes de los receptores intestinales para *E.coli* K88, Gal(α1-3)Gal y Gal(β1-4)GlcNAc acopladas a albúmina de suero bovino.

En la cuantificación de la fuerza de adhesión, se observó que la cepa 10 fue de tamaño pequeño como para ser despegada con el láser una vez que estuvo unida a las microesferas recubiertas con mucina intestinal o MFGM. Solamente en una ocasión se logró remover a la bacteria de las mucinas y la fuerza de separación calculada fue de 10 piconewton (pN). Dos cepas de origen no porcino, no se adhirieron a las proteínas intestinales. Sin embargo, su fuerza de adhesión a MFGM fue de 25 y 6 pN. Ambas fueron de mayor longitud que la cepa 10, por lo que su remoción con el láser fue más eficiente.

En conclusión, se lograron aislar cepas de *Lactobacillus* con valor probiótico potencial, a partir del intestino de lechones recién destetados. En las cepas 6, 9 y 10 se demostró el establecimiento de interacciones tipo adhesina-carbohidrato durante su proceso de adhesión a la mucosa intestinal. Los monosacáridos y oligosacáridos reconocidos por las cepas aisladas de lechón coinciden con los reconocidos por *E. coli* K88. Lo anterior es un indicio de que las cepas pueden competir con el patógeno por sitios de adhesión en el intestino del cerdo. Sin embargo, se requieren otros estudios para probarlo. Por otro lado, a pesar de que se cuantificó la fuerza de adhesión, utilizando pinzas ópticas, entre algunos *Lactobacillus* y esferas recubiertas con MFGM, deben superarse inconvenientes técnicos para que este instrumento pueda utilizarse con células de cualquier tamaño.

INTRODUCCIÓN

Los esfuerzos constantes por producir alimentos de origen animal de mejor calidad y al menor costo posible, han llevado a la búsqueda de mejores combinaciones de nutrientes, así como al desarrollo de aditivos que incrementen el nivel de producción de los animales. Dichos esfuerzos han conducido al uso de sustancias promotoras del crecimiento, como hormonas, antibióticos y agentes antimicrobianos, que permiten tener bajo control la ganancia de peso de los animales. Sin embargo, estas prácticas producen residuos tóxicos en las carcasas y resistencia a los antibióticos por parte de patógenos que pudieran afectar la salud del cerdo (Doyle, 2001)

En los últimos años la utilización de bacterias probióticas se ha visto favorecida como una opción para disminuir el uso de sustancias promotoras del crecimiento. Estos microorganismos benéficos se multiplican en el aparato digestivo para conformar una flora intestinal favorable. Además, pueden ayudar a la promoción del crecimiento del huésped, disminuyendo la aparición de algunas enfermedades, principalmente aquellas de origen diarreico (Maxwell y Stewart, 1995).

Los géneros bacterianos utilizados como probióticos tanto en humanos como en animales son: *Lactobacillus*, *Bifidobacterium*, *Bacillus*, *Streptococcus*, *Pediococcus* y *Enterococcus*. Los géneros que han tomado mayor relevancia debido a sus posibles propiedades nutricionales y terapéuticas son *Lactobacillus* y *Bifidobacterium*, ya que forman parte de la microflora normal del tracto gastrointestinal de los mamíferos (Dunne *et al.*, 1999). Algunas especies de *Lactobacillus* se asocian en simbiosis con el huésped ayudándolo en múltiples aspectos como prevención de la implantación de bacterias patógenas, producción de sustancias como bacteriocinas y ácidos orgánicos, estimulación la respuesta del sistema inmune, entre otros (Naidu *et al.*, 1999).

El efecto benéfico de los probióticos está relacionado con su habilidad para interactuar y adherirse a la mucosa y/o epitelio intestinal. La adhesión es uno de los principales criterios de selección puesto que es un pre-

requisito para la colonización y se debe a la interacción de fuerzas atractivas y repulsivas entre las superficies participantes. La composición de la pared celular bacteriana, así como la naturaleza de la superficie a la cual se adhiere, afectan este fenómeno, pero el origen molecular de cada una de las interacciones no está bien definido (Greene y Klaenhammer, 1994; Bibiloni *et al.*, 1999).

La adhesión bacteriana se ha descrito principalmente para géneros patógenos como *E. coli* y *Vibrio cholerae*. Estos microorganismos se mantienen en el tracto intestinal en contra del flujo del peristaltismo debido a que *E. coli* posee fimbrias (adhesinas), las cuales son estructuras proteicas que reconocen carbohidratos a nivel de mucosa y epitelio; mientras que *V. cholerae* produce una toxina de origen proteico, la cual se une a glicoesfingolípidos en el intestino delgado. Mediante ese reconocimiento ocurre la adhesión con una posterior colonización causante de infecciones gastrointestinales (Varky *et al.*, 1999). Se cree que lo mismo podría ocurrir con las bacterias probióticas, pero en lugar de causar infecciones gastrointestinales, pudiera existir una competencia por los sitios de adhesión, dando lugar a lo que se conoce como exclusión competitiva (Ouwehand y Conway; 1996; Jin *et al.*, 2000; Laux *et al.*, 2005).

Por otro lado, algunas bacterias gram positivas, entre las que se encuentran las de valor probiótico, poseen proteínas de superficie (conocidas como S-layer en inglés), las cuales se cree tienen una fuerte relación con las propiedades de adhesión. Aunque esto es una hipótesis aún, estudios realizados con *Lb. acidophilus* y *Lb. crispatus* que tienen la capa proteica rodeando a la pared celular son adherentes (Sara y Uwe, 2000).

El objetivo de este trabajo es verificar la existencia de interacciones tipo proteína-carbohidrato entre cepas potencialmente probióticas de *Lactobacillus* y mucinas, ambas aisladas de intestino delgado de lechón.

ANTECEDENTES

La porcicultura está considerada como una actividad pecuaria importante generadora de empleos e ingresos económicos, en muchos países. Este tipo de producción beneficia además a otras industrias, como a los productores de alimentos balanceados y de granos forrajeros (Berreira, 1996). En México, Sonora ocupa el segundo lugar en la producción de carne de cerdo de alta calidad en el país (SAGARPA, 2003). Debido a la trascendencia económica de la actividad y a que casi la totalidad de la carne producida en el estado se exporta a Oriente y Norteamérica, los productores buscan constantemente formas de mejorar su eficiencia productiva encaminada a aumentar los índices de salud y crecimiento de los animales (Mantecón y Ahumada, 2000, Bermúdez *et al.*, 2001).

La rentabilidad de la producción porcina está determinada principalmente, por el éxito o fracaso de la transición de la leche de la madre a dietas secas, sin que ocurra reducción del crecimiento o aparición de enfermedades. El potencial de crecimiento de los lechones es alto inmediatamente después del destete, pero el limitado consumo de alimento, aunado a un sistema digestivo inmaduro, impide que se alcance este potencial (Doyle, 2001).

El destete temprano de los lechones (entre los 10 a 21 días) es cada vez más frecuente, ya que maximiza el rendimiento reproductivo. Sin embargo, esto trae consigo un aumento en los problemas nutricionales e inmunológicos, lo que se refleja en un mayor número de animales enfermos (Alle y Touchette, 2000). Los problemas más sobresalientes que afectan la rentabilidad de las explotaciones porcinas son los desórdenes gastrointestinales. El padecimiento de afecciones diarreicas en animales jóvenes y adultos supone, además de una mayor tasa de mortalidad, una menor ganancia de peso. La etiología de estos procesos es compleja ya que pueden intervenir agentes de naturaleza muy diversa (virus, bacterias y parásitos). Aunque la mayoría de estos microorganismos pueden afectar a los cerdos de todas las edades, las infecciones de mayor repercusión en las

dos primeras semanas de vida suelen estar causadas por *Escherichia coli* K88, *Clostridium perfringens*, el rotavirus y el coronavirus de la gastroenteritis transmisible (Ouwehand y Conway, 1996; Nousiainen y Setälä, 1998).

Con la privación de los anticuerpos del calostro y el cambio de alimentación asociado al destete, adquieren una mayor relevancia las enfermedades ocasionadas por *Salmonella* y *E. coli* K88 y K99 (Fedorka *et al.*, 1999; Mantecón y Ahumada, 2000). En los últimos años, la tecnificación de la producción porcina ha impactado considerablemente la sanidad de los cerdos, particularmente en las fases de neonatos y destetados. La mayoría de los problemas de infecciones entéricas ocurren desde el nacimiento a los 60 Kg., de allí en adelante la inmunidad frente a las infecciones endémicas se establece en la granja y los focos de diarrea se ven solo cuando nuevas infecciones se introducen en la granja o cuando cerdos susceptibles se introducen en locales infectados (Arenas *et al.*, 1999).

El uso tanto médico, como veterinario de los antibióticos, ha propiciado la aparición de cepas bacterianas resistentes a antimicrobianos. Las bacterias resistentes que son patógenas para el humano, pueden causar enfermedades difíciles de tratar; incluso las no patógenas representan un alto riesgo en la salud, ya que pueden transferir los genes de resistencia a antibióticos a otras bacterias patógenas. El problema aumenta debido al descubrimiento de que el uso de algunos antibióticos en cantidades subterapeúticas, mejora la ganancia en peso del animal (Doyle, 2001).

Durante años se han evaluado las posibles alternativas al uso subterapeútico de antibióticos y que produzcan los mismos resultados que los antimicrobianos. Se ha señalado que los cambios en los patrones alimenticios de las granjas y la introducción de otros componentes en el alimento pueden ser una solución que compense el efecto de los antimicrobianos y maximice la producción (Fuller, 1999). Entre los promotores de salud sugeridos como sustitutos de los antimicrobianos, están los que producen una exclusión competitiva de las bacterias patógenas, como los probióticos, las enzimas, los inmunomoduladores, los ácidos

orgánicos y otros suplementos como minerales, vitaminas, ácidos grasos, etc. Estos compuestos pueden ayudar a la promoción del crecimiento, a mejorar la eficiencia y a disminuir algunas enfermedades, principalmente aquellas de origen diarreico (Thomke, 1998; Hughes y Heritage, 2004).

Probióticos

Un probiótico es un adyuvante dietario de origen microbiano que beneficia la fisiología del huésped, modulando la inmunidad de la mucosa y la inmunidad sistémica. También mejoran el balance nutricional y microbiano del tracto intestinal (Naidu *et al.*, 1999). Entre los organismos probióticos se encuentran las bacterias lácticas, algunas de las cuales han sido utilizadas desde la antigüedad para la elaboración de diversos productos fermentados. Metchnikoff (Wood, 1999) fue de los primeros investigadores en proponer los efectos benéficos saludables relacionados con el consumo regular de leches fermentadas con bacterias lácticas. Posteriormente se ha observado que estas bacterias se encuentran implicadas en la modulación del sistema inmune de su huésped y en la protección contra el ataque de microorganismos patógenos (Naidu, 1999; Tannock, 1999).

Las bacterias lácticas con actividad probiótica son generalmente flora entérica que juega un papel benéfico en el ecosistema del tracto gastrointestinal (Naidu *et al.*, 1999). Los principales géneros bacterianos probados como probióticos y aditivos de alimentos en cerdos son *Lactobacillus* (*Lb. acidophilus, Lb. fermentum, Lb. bulgaricus*) y *Enterococcus* (*E. faecium, E. thermophilus*), *Bacillus* sp, algunas cepas de *Bifidobacterium*, levaduras (*Saccharomyces cereviseae*) y *Aspergillus* sp. Los probióticos se mezclan con la dieta sólida o bien se utilizan como suplementos en la leche (Nousiainen y Setälä, 1998). Se ha observado que para cerdos, los mejores resultados se obtienen utilizando de probióticos bacterianos (Nousiainen y Setälä, 1998; Olivares, 2000; Doyle, 2001).

Criterios de Selección

La seguridad de las bacterias ácido lácticas utilizadas en alimentos funcionales es de gran importancia. En general, están consideradas como GRAS (Generalmente Reconocidas Como Seguras). Según los estudios realizados son especies no patógenas, sin embargo, es importante confirmar todas las regulaciones necesarias para considerar a una bacteria como probiótico (Salminen *et al.*, 1998).

Algunos de los criterios más importantes que debe cumplir un microorganismo para que se le pueda considerar como probiótico son:

1.- Criterio de Origen: Se debe aplicar a la especie de donde se aisló

2.- Debe tener una identificación bioquímica

3.- Debe tener un efecto antagónico contra bacterias patógenas

4.- No debe ser oportunista, aún cuando el hospedero se encuentre inmunosuprimido

5.- Debe estimular el sistema inmunológico, mejorando la resistencia a patógenos

6.- Debe adherirse a la mucosa intestinal para ejercer su efecto probiótico (Gibson y Fuller, 2000).

Mecanismos de Acción Propuestos

Aunque algunos no se han comprobado del todo, estos son los mecanismos que explican mejor el efecto probiótico de diferentes microorganismos:

- Competencia por nutrientes: los nutrientes que llegan al intestino grueso sin digerir son alimento para las bacterias intestinales, algunos de ellos son nutrientes selectivos de bacterias benéficas (prebióticos), por lo tanto, estos microorganismos compiten con los patógenos no sólo por espacio físico, sino también por los nutrientes disponibles.
- Actividad antimicrobiana: los probióticos producen una variedad importante de sustancias que inhiben microorganismos patógenos. Estas sustancias incluyen la producción de ácidos grasos de cadena corta, la producción de bacteriocinas, peróxido de hidrógeno y

piroglutamato con actividad antimicrobiana, que eliminan microorganismos indeseables de la microflora como *E. coli*, *Streptococcus* y *Salmonella*.

- Efectos inmunomoduladores: incluyen el aumento en número y actividad de macrófagos, monocitos, neutrófilos (con consecuente aumento de la actividad fagocitaria) expresión de citoquinas ante la presencia de antígenos (IL-1, IL-2, e interferón gamma), secreción de inmunoglobulina A por células de la mucosa intestinal.
- Adhesión: los microorganismos presentan la propiedad de adherirse a receptores que se encuentran en la mucina del epitelio intestinal por medio de adhesinas y fimbrias. Este mecanismo permite que microorganismos benéficos compitan por el espacio físico con los patógenos.

No todos los microorganismos presentan las mismas acciones sobre el huésped, y la industria suele utilizar combinaciones de dos o más microorganismos para favorecer la respuesta benéfica en el huésped (Piccagli, 2002).

Adhesión de Bacterias Probióticas al Tracto Gastrointestinal de Cerdo

Las bacterias se adhieren a sustratos mediante interacciones de tipo iónico, hidrofóbico, puentes de hidrógeno, etc. Con frecuencia, la adhesión es un evento estereoespecífico, en el que las bacterias se unen a su sustrato sólo si posee un cierto tipo de receptor. Un ejemplo de estereoespecificidad es la que se da entre una lectina y un carbohidrato. En microbiología, a las proteínas de origen bacteriano que reconocen carbohidratos se conocen como adhesinas. La adhesión es un conjunto de factores que incluye la presentación, orientación y accesibilidad de los receptores y adhesinas. Además, requiere de la participación de diferentes moléculas, que interactúan de manera secuencial para superar las fuerzas repulsivas (Ofek y Doyle, 1994).

En el tracto gastrointestinal es común que las bacterias reconozcan los oligosacáridos complejos que se encuentran en las mucinas y/o células del epitelio. Sin embargo, la mayoría de los estudios de adhesión están enfocados a tratar de entender el mecanismo de patogenicidad de las bacterias Gram negativas, principalmente de *E. coli* y se conoce poco acerca de este tipo de interacciones en bacterias Gram positivas (Ouwehand *et al.*, 2001).

La mayoría de las bacterias Gram negativas expresan múltiples adhesinas con diferentes especificidades por oligosacáridos complejos de las células eucariotas. Diferentes investigaciones indican que patógenos del lechón como *E. coli* K88 y *Salmonella choleraesuis*, se unen a receptores específicos tanto en el epitelio intestinal como en la mucosa. *E. coli* K88 expresa una adhesina con masa molecular de 210 a 230 kDa, que reconoce ácido siálico en mucosa. Así mismo, también se sabe que expresa otra adhesina que se une a oligosacáridos complejos con galactosas terminales (Francis *et al.*, 1998; Grange *et al.*, 1998). *Salmonella choleraesuis* posee fimbrias (adhesinas) específicas para manosa. (Firon *et al.*, 1983). De manera similar, ya que los probióticos también se adhieren a mucosa y epitelio intestinal, se esperaría que reconocieran glicoproteínas u oligosacáridos complejos del tracto intestinal y compitieran por un sitio de unión con otras bacterias; lo cual es un mecanismo de adhesión propuesto, (Maxwell y Stewart, 1995).

En bacterias Gram negativas, las adhesinas son subunidades proteicas de pilis, conocidas como fimbrias, que se encuentran alrededor de la célula. Presentan un diámetro de 5 a 7 nm y pueden extenderse de 100 a 200 nm de longitud. El dominio de reconocimiento de carbohidratos, generalmente se encuentra en la punta del pili (Varky *et al.*, 1999).

En bacterias Gram positivas, las proteínas de superficie (S-layer) son el grupo más grande de adhesinas, las cuales son monoméricas. Sin embargo, otras moléculas como polisacáridos y lípidos también pueden tener funciones adhesivas (Figura 1) (Talay, 2005). Las proteínas de superficie existen como arreglos bidimensionales que cubren completamente

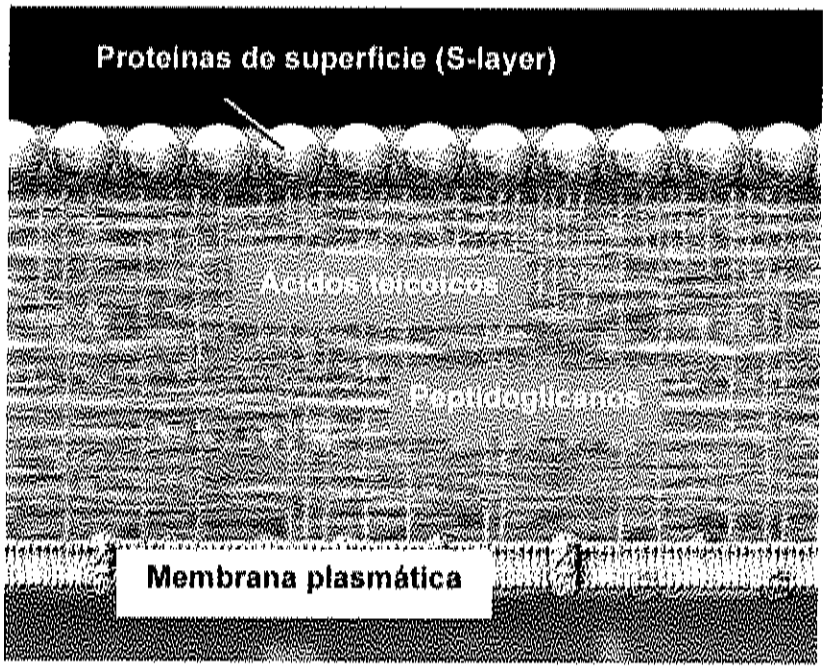

Figura 1. Pared celular de bacteria Gram positiva mostrando la presencia de proteínas de superficie. Disponible en: http://www.rkm.com.au/CELL/Bacteria/bacterialimages/Gram-positive-wall-500.jpg).

la superficie bacteriana. Están compuestas de una sola subunidad de (glico) proteína, cuya masa varía de 40 a 200 kDa y se encuentran ensambladas en arreglos con simetría oblicua, cuadrada o hexagonal (Figura 2). La mayoría de las proteínas de superficie tienen un pI de 4 a 6 y contienen cerca del 40% de aminoácidos hidrofóbicos pero muy pocos con grupos sulfuros (Smit *et al.*, 2001).

En bacterias patógenas como *Streptococcus*, *Staphylococcus* y *Listeria* la función de las proteínas de superficie está bien definida como moléculas de adhesión durante el proceso infectivo. En bacterias comensales del tracto intestinal de mamíferos también pueden jugar un papel muy importante durante la colonización. Sin embargo se debe tener en consideración que son de peso molecular menor y altamente básicas (pI≥ 9), por lo que se necesita realizar más estudios al respecto (Smit *et al.*, 2001; Talay, 2005).

Estudios de competencia entre bacterias patógenas y algunos probióticos muestran que las interacciones que median su adhesión con el tracto gastrointestinal pueden ser de origen proteína-carbohidrato (adhesina-receptor). Lo anterior se sugiere debido a que fue posible inhibir, *in vitro*, la adhesión de las bacterias a mucosa intestinal de diferentes mamíferos, empleando carbohidratos, glicoproteínas o mediante tratamiento con proteasas (Fontaine *et al.*, 1994; Tuomola *et al.*, 2000; Gao y Meng, 2004).

En lo que se refiere a bacterias probióticas, uno de los criterios de selección se enfoca en su adherencia a la mucosa intestinal, para que puedan ejercer un efecto benéfico. La presencia de adhesinas ya se ha demostrado en *Bifidobacterium pseudolongum*. Esta bacteria presentó actividad hemaglutinante que fue inhibida en presencia de monosacáridos y de glicoproteínas con galactosa expuesta, incluyendo mucina gástrica bovina y porcina, así como de algunos azúcares libres. Sin embargo, no se ha(n) aislado ni caracterizado la(s) adhesina(s) (Meng *et al.*, 1998). Por otro lado, *Lactobacillus fermentum* se adhiere a la mucina intestinal de cerdo de 35 días, sin embargo, no se menciona hacia qué carbohidratos tiene afinidad aunque la proteína ya fue aislada y caracterizada (Rojas *et al.*, 2002).

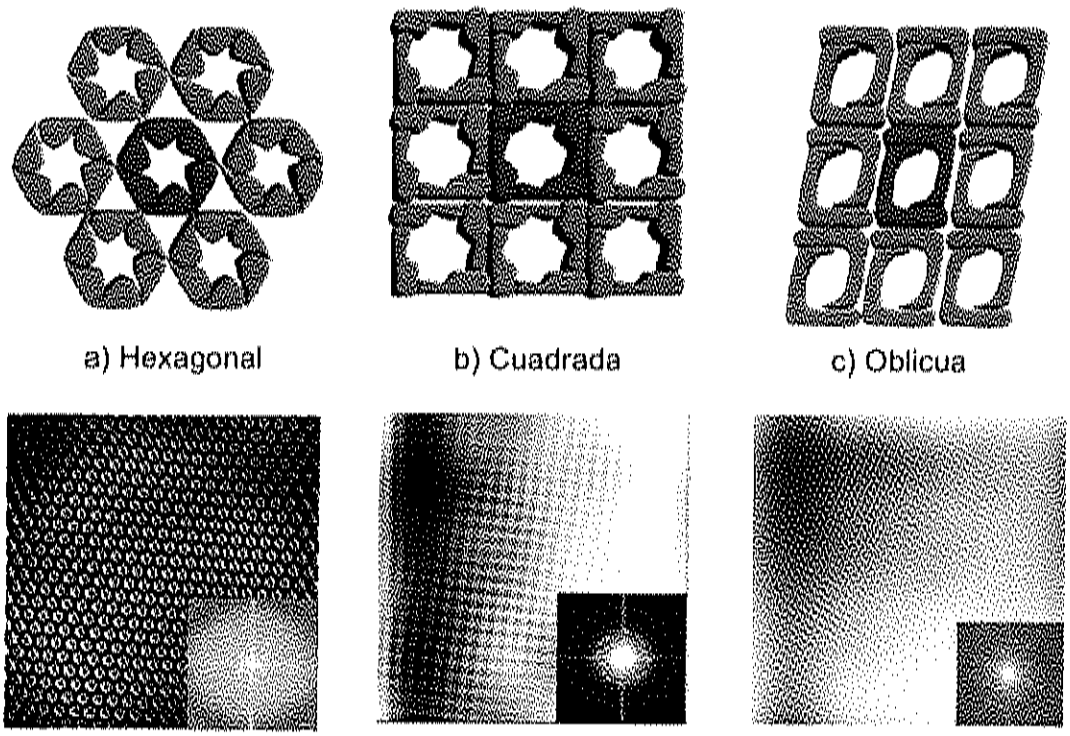

Figura 2. Micrografías electrónicas de los arreglos de las proteínas de superficie

Fuente: Agave BioSystems, 2005

Mucinas

La mucosa es la cubierta más externa del tracto gastrointestinal. Sus funciones fundamentales son la lubricación y protección de las células epiteliales contra agresiones químicas, mecánicas y degradación bacteriana. Funciona como una barrera física contra los microorganismos patógenos evitando que éstos se adhieran, colonicen e invadan el epitelio. Esta formada principalmente de mucinas, que son glicoproteínas de alto peso molecular capaces de formar geles viscoelásticos. Además contiene proteínas, lípidos y sales inorgánicas en menor proporción. La composición exacta, así como el espesor de la capa mucosa varían considerablemente dependiendo de la ubicación anatómica y del estado fisiopatológico del animal (Mechref y Novotny, 2002).

Las mucinas del intestino delgado están formadas por multímeros, unidos mediante enlaces disulfuros. Los monómeros de proteínas varían considerablemente en tamaño, desde 300 aminoácidos hasta más de 13,000. En ellas prevalecen residuos de serina, treonina y prolina, en una proporción importante (Pérez y Hill, 1999). A través de uniones O-glicosídicas, en los aminoácidos serina y treonina, se establecen enlaces con cadenas oligosacáridas de distinta composición y tamaño (entre 2 y 20 azúcares con un promedio de 8). En menor cantidad se encuentran cadenas oligosacáridas unidas mediante enlaces *N*-glicosídicos. En su composición el tipo de carbohidratos se limita a galactosa, manosa, fucosa, N-acetilglucosamina, N-acetilgalactosamina y ácido neuramínico (ácido siálico) que se agrupan en diversas combinaciones. Estos carbohidratos constituyen del 70 al 90% del peso de la glicoproteína (Rodríguez *et al.*, 2000; Laux *et al.*, 2005).

Se ha sugerido que la exposición de ciertos oligosacáridos del tracto intestinal del cerdo en las distintas etapas de desarrollo del animal está involucrada en el aumento de la enterovirulencia y de la unión de patógenos a los animales recién nacidos. Lo anterior debido a que la expresión de oligosacáridos complejos se ve influida por la edad del animal (Mikelsaar *et al.*, 1998; Dai *et al.*, 2000). Esto provoca que las bacterias patógenas que se

adhieren a los receptores oligosacáridos de la mucina y las células epiteliales de los lechones, pudieran no tener afinidad por los receptores de los animales adultos y viceversa. Por otro lado, cabría esperar que, si las bacterias probióticas presentan adhesinas, podrían competir por los receptores específicos del intestino, previniendo la adhesión de los patógenos (Maxwell y Stewart, 1995).

Proteínas de Membrana de Grasa de la Leche (MFGM)

Las proteínas que se encuentran en la membrana de los glóbulos de la grasa de la leche (MFGM por sus siglas en inglés) de bovino se han identificado y clasificado de acuerdo a su movilidad en geles de poliacrilamida. Las mas abundantes son la mucina MUC1, la Xantina deshidrogenada/oxidasa (XDH/XO), la mucina MUC15, la CD 36, la Butirofilina (BTN), las proteínas positivas al Ácido Periódico/Schiff 6/7 (PAS 6/7) y la Adipofilina (ADPH) (Mather, 2000).

Las proteínas más importantes desde el punto de vista de adhesión bacteriana son MUC15 y MUC1 debido a que están altamente glicosiladas y se expresan en las células apicales de casi todos los tejidos epiteliales de los mamíferos. Son proteínas integrales de la membrana, no formadoras de gel y su función fisiológica no está totalmente explicada. Se ha propuesto que pueden actuar como una barrera en las células expuestas, protegiéndolas de daños físicos y de microorganismos invasivos, igual que las mucinas intestinales (Pallesen *et al.*, 2001; Pallesen *et al.*, 2002).

MUC15 es una glicoproteína de masa molecular entre 95 y 100 kDa. Los carbohidratos presentes en la molécula constituyen más del 67% del peso húmedo. Aunque las estructuras oligosacáridas de esta glicoproteína no han sido determinadas, la presencia de *O*-glicanos ha sido confirmada por tratamiento de la proteína con *O*-glicosidasas, que liberan Galβ1-3GalNAc de los residuos de serina y treonina. Se han identificado aproximadamente 22 sitios de O-glicosilación y 11 de *N*-glicosilación (Pallesen *et al.*, 2002).

La proteína MUC1 es otra proteína altamente glicosilada, cuya masa molecular oscila entre los 156 a193 kDa. Aproximadamente el 50% del peso húmedo de la mucina está determinado por la presencia de carbohidratos. Fucosa, galactosa, manosa, *N*.Acetilglucosamina, *N*.Acetilgalactosamina y ácido siálico son los que están presentes en mayor proporción. Principalmente se han identificado *O*-glicanos unidos a esta proteína, en la que se han identificado secuencias lineales y ramificadas de residuos de *N*-Acetillactosamina (Mather, 2000).

Al ser tan similares en el contenido de carbohidratos, las glicoproteínas de la leche de bovino y las intestinales pueden ser utilizadas como modelos para estudiar fenómenos de adhesión, tanto de bacterias patógenas como probióticas. Cabe destacar que las glicoproteínas lácteas se pueden obtener más fácilmente que las intestinales (Mather, 2000; Tuomola *et al.*, 2000).

Ensayos de Adhesión *In vitro*

Muchos de los estudios sobre adhesión bacteriana a mucosa intestinal, se han realizado en líneas celulares como Caco-2 y HT-29-MTX. Lo anterior presenta la limitante de que dichas células son transformadas (cancerosas) por lo que presentan cambios muy importantes en los patrones de glicosilación de los oligosacáridos de sus membranas plasmáticas. En las investigaciones que han utilizado este tipo de células, los resultados pudieran no reflejar lo que sucede *In vivo* (Vesterlun *et al.*, 2005). Blum *et al.* (1999) realizaron una comparación de diferentes investigaciones que utilizaron estas células transformadas en estudios de adhesión de probióticos. Encontraron que los resultados obtenidos por diferentes laboratorios, utilizando la misma cepa probiótica, diferían dramáticamente al variar las condiciones de adhesión.

Otros estudios han empleado tejidos de animales maduros, por lo que es difícil extrapolar los resultados a lo que sucede en animales jóvenes o recién nacidos (Sarem *et al.*, 1996; Tuomola *et al.*, 1999). Debido a que la adhesión bacteriana está involucrada en muchos sectores de la vida y la

salud, el desarrollo de métodos para medirla es un área importante dado que puede afectar los resultados (Vesterlun *et al.*, 2005).

Las bacterias probióticas son utilizadas para reforzar la capacidad de defensa natural de la microflora comensal del intestino. Uno de los beneficios que ofrecen es inhibir la adhesión de patógenos. Sin embargo, el mecanismo no está bien establecido. Como ya se mencionó anteriormente, las bacterias patógenas poseen adhesinas que reconocen carbohidratos en la mucosa y/o epitelio. Dentro de ese reconocimiento se encuentran involucradas interacciones de tipo específico y no específico con las que se adhieren y colonizan el tracto intestinal. En los microorganismos probióticos la presencia de adhesinas, su papel en la colonización y desplazamiento de patógenos en el intestino es un área poco explorada. Es importante continuar con los estudios y comprobar si en la mayoría de las bacterias probióticas, están presentes las mismas interacciones al llegar al tracto intestinal o si esto es una característica intrínseca de cada cepa (Dunne *et al.*, 1999; Naidu *et al.*, 1999; Tannock, 1999; Vesterlun *et al.*, 2005).

PLANTEAMIENTO DEL PROBLEMA

La composición y el metabolismo de la microflora intestinal afectan el rendimiento de los animales de granja, especialmente en los más jóvenes que están sujetos a un ambiente estresante. La colonización de los compartimentos del intestino por bacterias comensales, a través de la asociación con la mucosa, sirve como la primera barrera de defensa contra organismos patógenos (Hardy, 2002).

La mucosa intestinal selecciona y elimina el material potencialmente peligroso para el organismo, mientras que permite el paso de componentes nutricionales inocuos. Su función principal es evitar la adhesión y penetración de patógenos en el intestino. El grosor y la composición del moco que cubre las microvellosidades contribuyen a la defensa de la superficie contra la adhesión y penetración de antígenos (Mechref y Novotny, 2002). Se ha encontrado que la composición de la mucosa intestinal en animales recién nacidos tiene una menor relación molar de proteínas y carbohidratos donde se observa un escaso contenido de fucosa y N-acetilgalactosamina. Lo anterior posiblemente limita la defensa superficial contra agentes nocivos. Mediante la inoculación de bacterias benéficas en el intestino para contrarrestar la acción de las patógenas, se puede dar el fenómeno conocido como exclusión competitiva (Maxwell y Stewart, 1995).

La colonización de la microflora intestinal en el huésped se basa en su capacidad para adherirse a la mucosa y/o epitelio y contribuir a la exclusión competitiva. El mecanismo de adhesión no está elucidado del todo pero es un requisito para asegurar su permanencia y de esa manera ejercer una acción benéfica más directa (Maxwell y Stewart, 1995; Hardy, 2002). Esta tesis se enfocó en determinar las propiedades probióticas de cepas de *Lactobacillus* aisladas de lechón y verificar la existencia de interacciones adhesina-carbohidrato con la mucosa intestinal. Además se propuso evaluar la interacción de las cepas con receptores intestinales para *E.coli* K88 y cuantificar su fuerza de adhesión a la mucosa y MFGM en escala de piconewton (pN), ya que en ambas hay presencia de mucinas.

OBJETIVOS

General

Caracterizar parcialmente la adhesión, mediada por interacciones adhesina-carbohidrato, de cepas probióticas de *Lactobacillus* a la mucosa intestinal de lechones.

Particulares

a) Amplificar y secuenciar un segmento del gen 16S rARN de cepas de *Lactobacillus* aisladas de intestino delgado de lechones recién destetados, para identificar las especies predominantes.

b) Evaluar el potencial probiótico de cepas de *Lactobacillus* aisladas del intestino delgado lechones, para su posible uso en la promoción de la salud de estos animales.

c) Identificar los carbohidratos y masas moleculares de las proteínas que favorecen la adhesión de *Lactobacillus* a las mucinas del intestino delgado de lechones, mediante ensayos de manchas y transferencia de proteínas de la mucosa a membranas de nitrocelulosa.

d) Cuantificar en unidades de piconewton (pN) la fuerza de adhesión de *Lactobacillus* a mucinas intestinales de lechones y proteínas de membrana de los glóbulos de grasa de la leche, utilizando las pinzas ópticas como herramienta principal.

METODOLOGÍA

Para realizar esta investigación se sacrificaron 6 lechones sanos de 10 días de nacidos para obtener el intestino delgado. De ahí se aislaron cepas de *Lactobacillus* además de obtener la mucosa mediante raspado del mismo. Para identificar las especies presentes se realizó un patrón de fermentación de 18 carbohidratos y la secuenciación de un segmento de 492 pb del gen 16S rARN. La caracterización probiótica parcial se realizó con recuentos en placa de la sobrevivencia de las cepas, aisladas e identificadas, a pH bajo y en presencia de sales biliares (simulación del tránsito gastrointestinal). También se evaluaron las propiedades de superficie como autoagregación e hidrofobicidad y se realizaron ensayos de antagonismo contra *E. coli* K88 (**Artículo 1** pp 4-8).

Una vez caracterizadas como probióticas potenciales las cepas de *Lactobacillus* (**Artículo 1** pp 4-8), se seleccionaron 3 de ellas de la especie *Lb. salivarius*, se marcaron con biotina y se realizaron ensayos de adhesión a la mucina intestinal de lechones. Para identificar los carbohidratos participantes en dicha adhesión se realizaron ensayos de manchas (dot blot) y de inhibición de la adhesión a mucosa con diferentes azúcares y glicoproteínas. Para identificar las masas moleculares de las proteínas de las mucinas intestinales, tanto de lechón como de cerdo adulto, que interaccionan con las bacterias se realizaron transferencias de las mismas (western blot) a membranas de nitrocelulosa (**Artículo 2** pp 29-32).

Por último, para cuantificar la adhesión de las cepas a las mucinas intestinales y MFGM se utilizaron pinzas ópticas, las cuales consisten de un microscopio invertido que posee un rayo láser capaz de atrapar una sola célula bacteriana, sin causarle daño alguno, y ponerla en contacto con diferentes tipos de superficies. Con este equipo se evaluó la fuerza de adhesión de las bacterias en escala de piconewton (pN) con ayuda de la ecuación de fuerza de fricción $F=3\pi\eta\delta v\kappa$ (**Artículo 3** pp 50-53).

RESULTADOS Y DISCUSIÓN

En este trabajo se aislaron 62 cepas de *Lactobacillus* del intestino delgado de 6 lechones sanos. Se encontró que la especie predominante fue *Lb. salivarius*, seguida por *Lb. reuteri* y finalmente *Lb. mucosae* (**Artículo 1** pp 8-9) Robredo y Torres (2000) y Roos y Jonsson, (2000) reportaron las mismas especies en el tracto intestinal de cerdos adultos; sin embargo no mencionan las proporción de cada una. Lo anterior indica que las especies aisladas se mantienen en el intestino a lo largo de la vida del animal, lo cual es deseable si ejercen un efecto benéfico en la salud (Saarela *et al.*, 2000).

Los microorganismos probióticos deben resistir factores adversos en el tracto gastrointestinal como la acidez del estómago y las sales biliares excretadas en el duodeno para posteriormente adherirse a la mucosa y/o epitelio. 20 cepas resistieron pH bajo y presencia de sales biliares con más del 45% de sobrevivencia, siendo *Lb. salivarius* la especie más resistente (**Artículo 1** pág. 9). En general, se han reportado resultados variables en cuanto a la resistencia de este género bacteriano a esas dos condiciones adversas. Algunas investigaciones previas muestran que esto es una característica intrínseca de cada cepa y no de la especie (Clark y Martin, 1994; Chung *et al.*, 1999).

Por otro lado, otra característica probiótica que se evaluó fueron las propiedades de superficie autoagregación e hidrofobicidad, las cuales son una medida de la habilidad que muestra una cepa para adherirse a líneas celulares (Del Re *et al.*, 2000). Se encontró que de 20 cepas evaluadas, 8 de ellas mostraron características autoagregativas e hidrofóbicas en $\geq$ 40%. De esas cepas 6 correspondieron a *Lb. salivarius*, 1 a *Lb. reuteri* y 1 a *Lb. mucosae* (**Artículo 1** pp 9-10). De acuerdo con Pérez *et al.* (1998), 40% de agregación e hidrofobicidad es lo mínimo necesario para considerar a una cepa con habilidades de adhesión para ejercer probiosis. La habilidad para autoagregarse aunada a una superficie celular hidrofóbica puede ser utilizada como una evaluación preliminar para identificar cepas potencialmente adherentes.

A las 8 cepas con características de autoagregación e hidrofobicidad se les evaluó su efecto antagónico de manera cualitativa y cuantitativa contra el patógeno de lechones *E. coli* K88. Se observó que los cultivos completos (células + sobrenadante) de las 8 cepas presentaron un efecto adverso con halos de inhibición del patógeno mayores a 20 mm. El análisis cuantitativo mostró que las cepas de *Lactobacillus* inhibieron a K88 en una proporción mayor al 97% (**Artículo 1** pág. 10). *E. coli* K88 está identificada como uno de los principales agentes etiológicos de las diarreas en lechones. Se ha sugerido que los probióticos pueden coagregar bacterias patógenas y producir sustancias antimicrobianas como ácidos orgánicos y bacteriocinas. Otro mecanismo de acción propuesto es la competencia por sitios de adhesión en el tracto intestinal, pero esto no está comprobado (Ouwehand *et al.*, 1999; Doyle, 2001).

Una vez que se evaluaron las propiedades probióticas de las cepas de *Lactobacillus* aisladas de lechón, se seleccionaron 3 de la especie *Lb. salivarius* con la finalidad de buscar interacciones de tipo adhesina-carbohidrato mediante ensayos de manchas (dot blot). Los ensayos se realizaron determinando la interacción entre las bacterias biotiniladas y mucinas tanto de lechón como de cerdo adulto. Se encontró que la cepas 9 y 10 tuvieron gran afinidad por las mucinas de lechón mientras que la cepa 6 no muestra diferencias entre mucinas de lechón o de cerdo adulto (**Artículo 2** pag. 32).

Por otro lado, los ensayos de transferencia de proteínas (western blot) mostraron una mayor interacción de las cepas 9 y 10 con mucinas de lechón que con las de cerdo adulto. Resultados similares se observaron con la cepa 6, pero la diferencia entre adulto y lechón fue menor que con las otras dos bacterias (**Artículo 2** pp. 32-33). Ross and Jonsson (2002) encontraron que una proteína de superficie de *Lb. reuteri* 1063 puede adherirse a proteínas de mucosa de cerdo adulto con pesos moleculares entre 15 y 45 kDa, pero también a componentes de alto peso molecular que no entran al gel. Nuestras cepas reconocen proteínas de 10 a 40 kDa en mucinas de cerdo adulto mientras que en la mucosa de lechón reconoce proteínas entre 10 y

50 kDa. Dai *et al.*, (2000) propuso que oligosacáridos complejos presentes en las superficies intestinales pueden ser receptores para la adhesión bacteriana. Ouwehand *et al.*, (1999) no encontraron relación entre edad y adhesión de una cepa probiótica de *Lactobacillus* a mucosa intestinal. Sugieren que no existe cambio en los receptores intestinales de humanos con la edad. Esto muestra que cada cepa posee sus características probióticas que no son extrapolables a otras de la misma especie.

Explorando los posibles carbohidratos responsables de la adhesión de estas tres cepas a la mucosa intestinal de lechones, encontramos que todas mostraron gran afinidad por los oligosacáridos receptores intestinales para *E.coli* K88, Gal(α1-3)Gal y Gal(β1-4)GlcNAc conjugados a albúmina de suero bovino (**Artículo 2** pág. 33). Esto es un fuerte indicio de que estas bacterias pueden competir *In vivo* con el patógeno por un sitio de unión en el intestino delgado y colonizarlo (Grange *et al.*, 2002).

Al realizar los ensayos de manchas encontramos que los carbohidratos que disminuyen la adhesión de las cepas 9 y 10 a las mucinas de lechón fueron D-manosa y D-galactosa en concentración de 2 mg/mL. La adhesión de la cepa 6 no se vio disminuida por monosacáridos pero sí por mucina gástrica porcina en concentración de 2 mg/mL (**Artículo 2** pág. 33). Gao y Meng (2004) encontraron que galactosa, arabinosa, manosa y otros monosacáridos inhibieron reacciones de hemaglutinación de una cepa de *Lactobacillus*, mientras que ninguna glicoproteína lo hizo. Caso contrario a lo que encontraron Ross y Jonsson (2002). Con una proteína de superficie proveniente de *Lb. reuteri* 1063 que se adhiere a mucosa intestinal de cerdo, se encontró que mucina, fetuina y asialofetuina inhibieron su adhesión a la mucosa, mientras que ningún monosacárido tuvo ese efecto.

La adhesión de bacterias probióticas a la mucosa intestinal parece ser un proceso mucho más complejo de lo que se creía. Consiste de interacciones de múltiples adhesinas cuyas estructuras están en espera de ser determinadas. También puede requerir de la participación de otros tipos de interacciones diferentes a las de adhesina-carbohidrato para superar las fuerzas repulsivas (Ofek y Doyle, 1994).

Para investigar las moléculas involucradas en la adhesión a la mucosa de lechones, las tres cepas de *Lactobacillus* se expusieron a diferentes tratamientos. La cepa 6 disminuyó su adhesión con periodato y proteasas, mientras que las cepas 9 y 10 se vieron afectadas sólo por las enzimas (**Artículo 2** pag. 33). Los tratamientos enzimáticos afectaron la adhesión de las tres cepas sugiriendo que las moléculas responsables fueron proteínas. Los carbohidratos de la pared celular de la cepa 6 pueden estar parcialmente involucrados dado que la cepa tratada con periodato se adhirió menos que el control. Tuomola *et al.*, (2000) observó que la adhesión de *Lb. gasseri* a mucosa intestinal de humanos involucró moléculas sensibles al periodato. *Lb. acidophilus* BG2FO4 involucró moléculas sensibles a proteasas y a periodato y *Lb. acidophilus* NCFM/2 moléculas sensibles a proteasas. Se encontraron tres mecanismos de adhesión diferentes, lo que indica que varias moléculas están involucradas en el proceso de adhesión.

Finalmente, se realizó un experimento en el que se trató de cuantificar la fuerza de adhesión de tres cepas de *Lactobacillus*, a la mucosa intestinal de lechones y MFGM. Las cepas utilizadas en este experimento fueron la cepa 10 (*Lb. salivarius*), NCFM (*Lb. acidophilus*) y 33199 (*Lb. gallinarum*). De un gran número de intentos por adherir a las cepas NCFM y 33199 a las microesferas recubiertas con mucinas, no se observó que éstas se unieran. La cepa 10 se adhirió pero no pudo ser removida por las pinzas ópticas, excepto en una ocasión en la que la fuerza de adhesión calculada fue de 10 pN (**Artículo 3** pág. 53). Al parecer, cuando la cepa se adhiere a la esfera recubierta con las mucinas, es imposible que ésta no interfiera con el láser debido al tamaño pequeño de la bacteria (Simpson *et al.*, 2002-2004).

En los ensayos realizados con microesferas recubiertas con MFGM se observó lo mismo para la cepa 10. Se adhirió pero no pudo ser removida por el láser. Lo contrario sucedió para las cepas NCFM y 33199, que mostraron una fuerza de adhesión a estas proteínas de 25 y 6 pN respectivamente (**Artículo 3** pág. 54). Ambas cepas fueron de mayor longitud que la 10, por lo que su remoción con el láser fue más eficiente.

En conclusión, con este trabajo se logró aislar cepas de *Lactobacillus* con valor probiótico potencial, a las cuales se les confirmó que interacciones específicas de tipo adhesina-carbohidrato tienen lugar durante su proceso de adhesión a la mucosa intestinal de lechones. También se puede concluir que existen indicios de que *In vivo* puede existir una competencia por receptores intestinales con *E. coli* K88, ya que ambos géneros reconocen dos estructuras que involucran al carbohidrato galactosa.

Por otro lado, a pesar de que las pinzas ópticas son una gran herramienta para cuantificar fuerzas de adhesión, todavía se deben superar algunos inconvenientes técnicos para que puedan utilizarse con bacterias o células de cualquier tamaño. Lo que se debe destacar de este experimento es que se confirmó la especificidad de las especies hacia sus sustratos. Es decir, ninguna cepa de *Lactobacillus* que no fuera de origen porcino se adhirió a las mucinas intestinales de lechón.

REFERENCIAS BIBLIOGRÁFICAS

1.- Alle G.L.y Touchette K.J. (2000) Efectos de la Nutrición Sobre la Salud Intestinal y el Crecimiento de Lechones. XV Curso de Especialización. Avances en Nutrición y Alimentación Animal. FEDNA. Barcelona, España. pp 55-70.

2.- Arenas A., Huerta B., Maldonado A., Tarradas C., Astorga R., Luque I., Borge C. y Perea A. (1999). Síndromes Entéricos del Cerdo. Ciencias Veterinarias. Marzo.

3.- Bermúdez C., Miranda-Vázquez L., Espinosa-Plascencia A., Valenzuela-Quintanar A. y Vázquez-Moreno L. 2001. Residuos de sulfonamidas en músculos de porcinos sacrificados en la región noroeste de México. Revista Científica de la Facultad de Ciencias Veterinarias. Venezuela. p. 127-132.

4.- Berreira, M. 1996. Panorama internacional de la producción de carne de cerdo. Claridades Agropecuarias. Junio. 34, 21-24.

5.- Bibiloni, R., Pérez, P.F. y De Antoni, G.L. 1999. Factors Envolved in Adhesión of Bifidobacterials Strains. Anaerobe. 5, 483-485.

6.- Blum S., Reinero R., Schiffrin E.J., Crittenden R., Mattila-S. T., Ouwehand A., Salminen S., von Wright, Saarela M., Saxelin M., Collins K y Morelli L. 1999. Adhesion studies for probiotics: need for validation and refinement. TRENDS IN Food Sci. Technol. 10, 405-410.

7.- Chung, H., Kim, Y.B., Chun, S.L. y Ji, G.E. 1999. Screening and Selection of acid and bile resistant bifidobacteria. Int. J. Food Microbiol. 47, 25-32.

8.- Clark, P.A. y J Martin, H. 1994. Selection of Bifidobacteria for use as dietary adjunts in cultured dairy foods: III – Tolerance to simulated bile concentrations of human small intestine. Cult. Dairy Prod. J. 29, 18-21.

9.- Dai D., Nanthkumar N., Newburg D. y Walter W. 2000. Role of Oligosaccharides and Glycoconjugates in Intestinal Host Defense. J. Pediatric Gastroenterol. Nutr. 30 (Suppl) 2, 23S-33S.

10.- Del Re, B., Sgorbat,i B., Miglioli, M. y Palenzona, D. 2000. Adhesion, Autoaggregation and Hydrophobicity of 13 Strains of *Bifidobacterium longum*. Lett. Appl. Microbiol. 31, 438-442.

11.- Doyle, M.E. 2001. Alternatives to antibiotic use for growth promotion in animal husbrandy. Food Research Institute. Abril 2001.

12.- Dunne, C., Murphy L., Flynn S. y O'Mahony L. 1999. Probiotics: from myth to reality. Demostration of funcionality in animal models of disease in human trials. Antonnie van Leeuwenhock. 76, 279-292.

13.- Fedorka-Cray P., Bailey J., Stern N., Cox N., Ladely S. y Musgrove M. 1999. Mucosal competitive exclusion to reduce *Salmonella* in swine. J. Food Protec. 62, 1376-1380.

14.- Firon, F., Ofek I. y Sharon Sharon N. 1983. Carbohydrate specificity of the surface lectin of *E. coli, Klebsiella pneumoniae* and *Salmonella typhimurium*. Carbohydr. Res. 120, 235-249.

15.- Fontaine I., Alssi E. y Bouquelet S. 1994. In Vitro Binding of *Bifidobacterium bifidum* DSM 20082 to Mucosal Glycoproteins and Hemagglutinating Activity. Current Microbiol. 28, 325-330.

16.- Francis D., Grange P., Zeman D., Baker D., Sun R. y Erikson A. 1998. Expression of Mucin-Type Glycoprotein K88 Receptors Strongly Correlates with Piglet Susceptibility to K88' Enterotoxigenic *E. coli*, but Adhesion to Brush Borders Does Not. Infect.Immu. 66, 4050-4055.

17.- Fuller, R. 1999. Probiotics in farm animals. In Probiotics-A critical review. Ed. G.W. Tannock. Horizon Scientific, Wymondham. UK. pp 15-22.

18.- Gao W. y Meng Q. 2004.Lectin Activity and Chemical Characteristics of *E.coli*, *Lactobacillus spp* and *Bifidobacterium spp* from Gastrointestinal Mucosa of Growing Pigs. Asian-Aust. J. Anim. Sci. 17, 863-868.

19.- Gibson G.R. y Fuller R. 2000. Aspect of *In vitro*. Research Approachs Directed Toward Identifyng Probiotics and Prebiotics for Human Use. Am. Soci. for Nutr. Sci. P. S391-S395.

20.- Grange P., Erickson A., Anderson T. y Francis D. 1998. Characterization of the Carbohydrate Moiety of Intestinal Mucin-Type Sialoglycoprotein Receptors for the K88ac Fimbrial Adhesin of *Eschericchia coli*. Infec. Immun. 66, 1613-1621.

21.- Grange P., Mouricout M., Levery S., Francis D. y Eriksson A. 2002. Evaluation of receptor binding specificity of *Escherichia coli* K88 fimbrial adhesin variants using porcine serum transferrin and glycosphingolipids as model receptors. Infec Immun. 70, 2336-2343.

22.- Greene J. y Klaenhammer T. 1994. Factors Involved in Adherence of Lactobacilli to Caco-2 Cells. Appl. Environm. Microbiol. 60, 44874494.

23.- Hardy, B. 2002. Nutraceutical concepts for gut health pigs. Nutrivision Inc. Fairmont, Minnesota. www.nutrivisioninc.com Acceso Mayo 2003.

24.- Hughes, P. y J. Heritage. Antibiotic growth-promoters in food animals. http://www.fao.org/DOCREP/ARTICLE/AGRIPPA/555_EN.HTM. acceso 09/12/04.

25.- Jin L., Marquardt R. y Zhao X. 2000. A strain of Enterococcus faecium (18C23) Inhibits Adhesion of Enterotoxigenic K88 to Porcine Small Intestine Mucus. Appl. Environm. Microbiol. 66, 4200-4204.

26.- Laux D., Cohen P. y Conway T. 2005. Role of the Mucis Layer in Bacterial Colonization of the Intestine. En Colonization of Mucosal Surfaces. Ed. ASM Press. Washington DC, USA. pp. 199-206.

27.- Mantecón, T. y A. Ahumada. 2000. Diarrea mecánica de porcino en lactación y postdestete. Mundo Ganadero. 119.

28.- Mather I. 2000. A Review and Proposed Nomenclature for Major Proteins of the Milk Fat Globule Membrane. J. Dairy Sci. 83, 203-247.

29.- Maxwell F.y Stewart C. 1995. The Microbiology of the Gut and the Role of Probiotics. En The Neonatal Pig *Development and Survival*. Ed. Cab International. University of Leeds, UK. pp. 155-177.

30.- Mechref Y. y Novotny M.V. (2002). Structural Investigations of Glycoconjugates at High Sensitivity. Chemical Reviews: 102, 321-369.

31.- Meng, Q., Kerley, M.S., Russel, T.J. y Allee, G.L. 1998. Lectin-like activity of *Escherichia coli* K88, *Salmonella choleraesuis* and *Bifidobacterium pseudolongum* of Porcine Gastrointestinal Origin. J. Anim. Sci. 76, 551-556.

32.- Mikelsaar M., Turi M., Lencner H., Kolts K., Kirch R. y Lencner A. 1998. Interrelations between Mucosal and Luminal Microflora of Gastrointestine. Nahrung. 31, 449-456.

33.- Naidu, A.S., W.R. Bidlack y R.A. Clemens. 1999. Probiotics spectra of lactic acid bacteria (LAB). Critical Rev. Food Sci. Nutr. 38, 13-126.

34.- Nousiainen, J. y J. Sëtala. 1998. Lactic Acid Bacteria as Animal Probiotic In Lactic Acid Bacteria. Microbiology and Functional Aspects. Salminen S. & A. Von Wright ed. Marcel Dekker. NY. pp 45-57.

35.- Ofek, I. y Doyle, R.J. 1994. Bacterial Adhesion to Cells and Tissues. Chapman & Hall, New York. pp. 80-84.

36.- Olivares, R. 2000. El uso de los microorganismos en la nutrición humana. Los porcicultores y su entorno. Julio-Agosto, 59-63.

37.- Ouwehand A. y Conway P. 1996. Purification and Characterization of a Component Produced by *Lb. fermentum* that Inhibits the Adhesion of K88 Expressing to Porcine Ileal Mucus. J. Appl. Bacteriol. 80, 311-318.

38.- Ouwehand, A.C., Kirjavainen P., Shortt C. y Salminen S. 1999. Probiotics: mechanism and established effects. Int. Dairy J. 9, 43-52.

39.- Ouwehand A., Tuomola E., Tölkkö S. y Salminen S. 2001. Assessment of Adhesion Properties of Novel Probiotic Strains to Human Intestinal Mucus. Int. J Food Microbiol. 64, 119-126.

40.- Pallesen L., Andersen M., Nielsen R., Berglund L., Petersen T., Rasmussen L. y Rasmussen J. 2001. Purification of MUC1 from Bovine Milk Fat Globules and Characterization of a Corresponding Full-Length cDNA Clone. J. Dairy Sci. 84, 2591-2598.

41.- Pallesen L., Berflund L., Rasmussen L., Petersen T. y Rasmussen J. 2002. Isolation and Characterization of MUC15, a novel Cell Membrane-Associated Mucin. Eur.J. Biochem. 69, 2755-2763.

42.- Pérez, P. F., Minnaard, Y., Disalvo, A. y De Antoni, G. L. 1998. Surface Properties of Bifidobacterial Strains of Human Origin. Appl. Environ. Microbiol. 64, 21-26.

43.- Pérez J. y Hill Robert. 1999. The Structure and Assembly of Secreted Mucins. J. Biol. Chem. 274, 31751-31754.

44.- Piccagli M. 2002. Alimentos Probióticos. Serie en Línea. http://www.fuedin.org/c_ateneos_nutricion/junio2002/alimentos probioticos.html Acceso en Octubre 2003.

45.- Robredo B. y Torres Carmen. 2000. Bacteriocin production by *Lb. salivarius* of Animal Origin. J Clin Microbiol. 38, 3908-3909.

46.- Rodríguez I., Cerezo A. y Salem I. 2000. Sistemas de Liberación Bioadhesivos. Ars Pharmaceutica. 41, 115-128.

47.- Rojas M., Ascencio F. y Conway P., 2002. Purification and Characterization of a Surface Protein from *Lactobacillus fermentum* 104R that Binds to Porcine Small Intestinal Mucus and Gastric Mucin. Appl. Environ. Microbiol. 68, 2330-2336.

48.- Roos S. y Jonsson H. 2002. A high-molecular-mass cell surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. Microbiology. 148, 433-442.

49.- Saarela M., Mogensen G., Fondén R., Matto J. y Sandholm T.M. 2000. Probiotic bacteria : safety, functional and technological properties. J. Biotech. 84, 197-215.

50.- SAGARPA. 2003. www.sagarpa.gob.mx/. 12 de marzo del 2003.

51.- Salminen S., Ouwehand A. e Isolauri E. 1998. Clinical Applications of Probiotic Bacteria. Int. Dairy J. 8, 563-572

52.- Sara M. y Uwe B. 2000. S-Layer Proteins. J. Bacteriol. 182, 859-868.

53.- Sarem, F., L.O. Sarem-Damerdji y J.P. Nicolas. 1996. Comparison of the adherence of *Lactobacillus* strains to Caco-2 and Int-407 human intestine cell lines. 22, 439-442.

54.- Simpson KH, Bowden MG,, Höök M y Anvari B. 2002. Measurement of Forces Between *S. epidermis* and Fibronectin-Coated surfaces Using Optical Tweezers. Lasers in Surgery and Medicine.52, 31-45.

55.- Simpson KH, Bowden MG, Peacock J, Arya M, Höök M y Anvari B. 2004. Adherence of *Staphylococcus aureus* fibronectin binding protein A mutants: an investigation using optical tweezers. Biomolecular Engineering. 21, 105-111.

56.- Smit E., Oling F., Demel R., Martinez B. y Powels P. 2001. The S-layer of *Lactobacillus acidophilus* ATCC 4356: Identification and Characterization of Domains Responsible for S-protein Assembly and Cell Wall Binding. J. Mol. Biol. 305, 245-257.

57.- Talay S. 2005. Gram-positive Adhesins. Contrib. Microbiol. 12, 90-113.

58.- Tannock G. 1999. Probiotics *A critical Review*. Ed. Horizon Scientific Press. University of Otago, Dunedin New Zealand. pp 45-50.

59.- Thomke, S. y K. Elwinger. 1998. Growth promotants in feeding pigs and poultry. III. Alternatives to antibiotic growth promotants. Ann. Zootech. 4, 245-271.

60.- Tuomola E., Ouwehand A. y Salminen S. 1999. Human ileostomy glycoproteins as a model for small intestine mucus to investigate adhesion of probiotics. Lett Appl. Microbiol. 28, 159-163.

61.- Tuomola E., Ouwehand A. y Salminen S. 2000. Chemical, physical and enzymatic treatments of probiotic lactobacilli and their adhesion to human mucus glycoproteins. Int. J. Food Microbiol. 60, 75-81.

62.- Varki A., Cummings R., Esko J., Freeze H., Hart G. y Marth J. 1999. Essentials of Glycobiology. Ed. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, N. York, EUA. pp 85-101.

63.- Vesterlund S., Paltta J., Karp M. y Ouwehand A. 2005. Measurement of Bacterial Adhesion–*In vitro* Evaluation of Different Methods. J. Microbiol. Meth. 60, 225-233.

64.- Wood B. 1999. The Lactic Acid Bacteria in Health and Disease. Ed. Scientific Press. University of Otago, Dunedin New Zealand. pp 69-77.

ARTÍCULO 1

Evaluation of Probiotic Properties in *Lactobacillus* isolated from Small Intestine of Piglets

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Abstract

Lactobacillus genus has been related with beneficial effects in human and animal health. Oral administration of probiotic bacteria must resist gastrointestinal transit, in order to colonize the intestinal mucus and offer an antagonistic effect against pathogenic microorganisms. The aim of this work was to select *Lactobacillus* strains isolated from small intestine of piglets based on the characteristics of resistance to low pH and bile salts, surface properties and antagonistic effect against the pathogen of piglets, *E. coli* K88. To identify *Lactobacillus* species, a fragment of 16S rRNA gene of the strains was amplified and sequenced. Sequences were compared with information in the data base available in nucleotide-nucleotide BLAST in NCBI web page. Low pH, bile salts resistance and antagonistic activity were quantified by viable count in plates. Surface properties were measured using a spectrophotometer at 600 nm.

Sixty-two *Lactobacillus* strains were isolated from small intestine of piglets and 3 species were identified: *Lb. salivarius* (42 strains), *Lb. reuteri* (19) and *Lb. mucosae* (1). We found that 20 *Lactobacillus* strains resisted low pH and bile salt, 8 of them were adherents and they inhibited *in vitro* the growth of *E. coli* K88. In conclusion, our results showed that 8 strains have potential probiotic value, according resistance to gastrointestinal tract, surface properties and antagonistic characteristics. *Lb. salivarius* was the specie that fulfill the criterion to be identified as possible probiotic microorganisms.

Key words: health of piglets, *Lactobacillus*, probiotic characteristics

Introduction

The pig breeding is a profitable activity in many countries. Sometimes, the economy of this activity is affected by infectious diarrhea in neonatal pigs (Gusils *et al.*, 2002). *E. coli* K88 has been identified as one of the main causal agents of this illness. Those bacteria invade mucosal cells and produce enterotoxins that cause diarrhoea and death of infected pigs (Meng *et al.*, 1998). Commonly antibiotics are used to prevent and/or eliminate these infections, unfortunately low control or antibiotic misuses are frequent practices. The worst disadvantage of these practices is bacterial resistance developed to these substances, for this reason it is important to look for alternatives against antibiotics usage (Gibson and Wang, 1994). Probiotics can be administered to prevent infectious diseases, to strengthen the barrier function of the gut microflora and for a non-specific enhancement of the immune system (Gusils *et al.*, 2002).

Probiotic microorganisms are those capable of colonizing their hosts with beneficial effects. Oral administration of this bacteria helps to maintain microbiota balance, preventing or remedying the appearance of gastrointestinal infections (Gómez-Gil *et al.*, 1998). *In vitro* studies have shown that *Lactobacillus* can offer protection against some human pathogens, like *E. coli* O157:H7, *Salmonella* and *Clostridium* (Yusof *et al.*, 2000, Gopal *et al.*, 2001). Also it has been found that lactic acid producer species like *B. infantis* and *B. choerinum*, isolated from the colon of pigs, have an antagonistic *in vitro* effect against *E. coli* K88 and *Salmonella choleraesuis* (Corona, 2003). Previous facts suggest that beneficial bacteria can be a feasible solution to diminish incidence of intestinal diseases in piglets.

Lactobacillus and *Bifidobacterium* belong to the normal human and animal microbiota. In this way their species are widely studied as probiotic. In animals, it has been observed that the rate of growth has increased with a better food conversion, and probiotics are helpful for this conversion (Jonson and Conway, 1992). Probiotics neither generate antimicrobial resistance nor

produce toxic compounds in carcass (Fuller, 1999). Strains need to survive acidic conditions in the stomach, and bile salts in duodenum, in order to exert their beneficial effects in the gut. Therefore, bile tolerance is considered one of the most important properties of probiotic microorganisms, because it allows them to survive and to colonize the gastrointestinal tract by mucus and/or enterocytes adhesion (Gómez-Zavaglia. *et al.*, 2002).

Adherence to the intestinal mucus layer is another important selection criterion for probiotic microorganisms, because it is a requirement for the bowel colonization. Adherence constitutes the first defence mechanism against pathogens invasion. Passing through the small intestine takes about 2.5 hours, but it is faster in the duodenum than the colon, therefore bacterial adherence to mucus and/or enterocytes for colonization is necessary (Yusof *et al.*, 2000; Rinkinen *et al.*, 2003).

The aim of this work was to select *Lactobacillus* strains isolated from small intestines of piglets, based on their characteristics of resistance to low pH and bile salts, surface properties and antagonism against *E. coli* K88 as a first requirement for their possible use as probiotics.

Materials and Methods

Isolation of Lactobacillus strains

Six healthy weaned piglets were slaughtered. Small intestine was divided into three portions in aseptic conditions: duodenum, jejunum and ileum. Each portion was separately placed in tubes with MRS broth (Difco-Becton Dickinson & Company, Sparks, MD, USA) pH 6.0 with cysteine hydrochloride 0.5 gr/L (J.T. Baker, Phillipsburg, NJ), 2,3,5-triphenyltetrazolium chloride (TTC) 25 ppm (Merck, Darmstadt, Ge), sodium propionate 0.3% w/v (Sigma, St. Louis MO, USA), lithium chloride 0.2% w/v (Sigma, St Louis, MO, USA) and antibiotics, as nalidixic acid 20 ppm (Sanofi-synthelabo Edo. México, Mex.), kanamycin 50 ppm (Sigma, St. Louis MO, USA) and polymyxin B sulphate 8.5 ppm. (Sigma, St. Louis MO, USA). Cultures were incubated at 37° C in

anaerobic jars by 48 h. After that cultures were seeded in plates with MRS agar (Difco-Becton Dickinson & Company, Sparks, MD, USA) pH 6.0 with cysteine, without antibiotics and incubated at 37° C in anaerobic conditions by 48 h (Corona, 2001). From those plates, white and creamy colonies were selected. Gram stain, motility and catalase assay were done as a first screening.

Phenotypic and Genotypic identification by 16S rRNA gene sequence analysis

To identify the species of *Lactobacillus*, the strains were proved for the fermentation of carbohydrates. Sugars tested were L-arabinose, lactose, celobiose, melezitose, raffinose, sorbitol, starch, xilose, manose, fructose, galactose, sucrose, maltose, trehalose, melibiose, mannitol, inulin y salicin (Sigma, St. Louis MO, USA). Fermentation test was made in tryptone peptone yeast broth (TPY) (Difco-Becton Dickinson & Company, Sparks, MD, USA) supplemented with 1% carbohydrate and bromocresol purple as pH indicator (Scardovi, 1986).

In the genotypic identification, DNA from strains was isolated (De los Reyes *et al.*, 1992). PCR was performed in a Thermal cycler (Perkin Elmer, Wellesley MA, USA). PCR primers used for this experiment were 27F and 519R reported previously. They amplified a 492 bp fragment from 16S rRNA gene (Lane, 1996). A typical reaction used the following programme involving a initial denaturation of 3 min at 94° C, 30 cycles of 94° C for 30 s, 55° C for 60 s and 72° C for 30 s. The final cycle was 72° C for 10 min. The PCR products were analyzed on 1.2% agarose gels (Sigma, St. Louis MO, USA). They were stained with ethidium bromide and observed in a UV transiluminator (Vilbert Loumart, Marne La Vallee, Francia). PCR products were purified with a GFX PCR DNA and Gel Band Purification Kit (Amersham, Piscataway NJ, USA). The purified products were sent to Arizona Research Laboratories (Tucson AZ, USA) for sequencing. Sequenced DNA was compared with information in the data base available in nucleotide-nucleotide BLAST in NCBI web page. Partial sequences were manually aligned using

DNAMAN (4.03 Lynnon BioSoft, Quebec Canada). A distance matrix and phylogenetic tree was generated using the Observing divergency method.

Resistance to pH 3.0 and bile salts

To test the resistance to pH 3.0 and conjugated porcine bile salts (CPBS) (0.5% w/v) (Sigma, St. Louis MO, USA), a modification of the proposed technique by Rodríguez *et al.* (2003) was performed. Strains were inoculated in 4 mL of MRS broth pH 6.0 and incubated in anaerobic conditions at 37° C by 48 h, then 2 mL were used for viable count on MRS agar pH 6.0 (Control). Remaining 2 mL were harvested 2600 x g at 4° C (GS-6R Beckman, USA). Supernatant was discarted and the pellet was resuspended in 2 mL MRS broth pH 3.0 with cystein. Cultures were incubated during 1 h at 37° C in aerobic conditions. After incubation, viable count was done using MRS agar pH 6.0 in previously described conditions At the same time, the cultures were inoculated for a viable count in plates of MRS with CPBS (0.5% w/v). In both cases, the plates were incubated in anaerobic jars at 37° C for 48 h. All plates were inoculated by duplicate.

Percentage of resistance to pH 3.0 and CPBS was determined using the equation of Kociubinsky *et al.* (1999) % Resistance = $100(CFU_{(pH\ 3.0\ or\ CPBS)} / CFU_{control})$.

Autoaggregation assay

Strains were grown in anaerobic conditions at 37° C for 48h in 3 mL of MRS broth pH 6.0 with cystein and they were harvested at 2400 x g. Supernatant was retained in a different tube. The pellet was washed twice with phosphate buffered saline (PBS) 0.02 M pH 7.4 and resuspended in same buffer until an optical density (O. D.) of 0.5 units at 600 nm (Spectronic 21D Milton Roy, USA) was reached. From this suspension 3 mL were harvested at 2400 x g. Supernatant was eliminated and cells were resuspended in their original broth, and incubated in aerobic conditions by 2 h at 37° C. 1 mL was taken from the superior part of the culture and the O.D. was measured. Finally, culture was shaken and total O. D. was measured. The autoaggregation (%

A) is expressed in the following equation 1 – (O. D. superior culture / O. D. total) x 100 (Del Re *et al.*, 2000). This experiment was done by triplicate.

Hydrophobicity (microbial adhesion to hydrocarbons)

Strains were grown in anaerobic conditions at 37 °C for 48 h in 3 mL of MRS broth pH 6.0 with cystein. Cultures were washed with PBS buffer and resuspended as described previously. 2 mL of bacterial suspension were transferred into another tube and 0.4 mL of xylene was added (Fluka, GmbH, Switzerland). Tubes were shaken for 2 min and reposed during 15 min. After that O.D. of aqueous phase at 600 nm was measured. O.D. decrease in aqueous phase was considered as a measurement of cells surface hydrophobicity (%H). %H was calculated according to the following equation $[(A_0–A)/ A_0] \times 100$. Where A_0 and A, were the absorbance before and after xylene extraction respectively (Del Re *et al.*, 1998; Gusils *et al.*, 2002; Mishra and Prasad, 2005).

Antagonism against Escherichia coli K88

Lactobacillus strains and *Escherichia coli* K88 were inoculated separately in MRS broth, pH 7.0 with cystein. Cultures were incubated by 24 h at 37° C in anaerobic jar. *Lactobacillus* strains were adjusted with MacFarland's nephelometer to approximately $24x10^8$ CFU/mL and *E. coli* K88 to $15x10^8$ CFU/mL. This last strain was massively inoculated on MRS plates pH 7.0 with cystein according to Hernández (2003). Four holes of 6 mm of diameter at similar distances were punched and filled with 70 µl of *Lactobacillus* culture. Plates were incubated at 37° C for 24 h in aerobic jars and growth inhibition was measured in milimeters (De Martinis *et al.*, 2002).

On the other hand, mixed cultures were prepared according to González *et al.*, (1993). 24 h cultures of *Lactobacillus* and *E. coli* K88 in MRS broth pH 7.0 with cystein were adjusted with MacFarland's nephelometer to $15x10^8$ CFU/mL. Pathogenic bacterium was diluted three times by serial dilutions. Equal volumes of both cultures in proportion 1000:1 (*Lactobacillus* : *E. coli*

K88) were mixed in 3 mL MRS broth pH 7.0 with cystein and incubated at 37° C by 6 h in anaerobic jars. Control culture contained only *E. coli* K88. After 6 h, a viable count in ENDO agar (Difco, Mexico) was done. Determination of antagonism percentage was calculated according the equation: % I = 100 [($T_{6\ control}$ – $T_{6\ mixed\ culture}$) / $T_{6\ control}$], where % I was the percentage of bacterial inhibition of each strain, $T_{6\ control}$ was the viable count obtained from the control and $T_{6\ mixed\ culture}$ was the viable count obtained from the mixed culture (González *et al.* 1993; Gusils *et al.*, 2002; García-Galaz *et al.*, 2004).

Statistical Analysis

ANOVA and Tukey-Kramer test were used for mean comparisons ($p<0.05$) in all experiments, with statistical package NCSS 6.0 (Hintze, 1997). Comparisons were carried out for species and strains.

Results

Isolation of Lactobacillus strains

Sixty-two *Lactobacillus* strains were isolated from small intestine of six healthy piglets. All of them grew in aerobic and 5% CO_2 conditions, were Gram positives rods, non motile and catalase negative as preliminary characteristics. From jejunum 33 strains were isolated, being different ($p<0.05$) respect to duodenum (10) and ileum (19) in bacteria gut distribution in piglet.

Phenotypic and Genotypic identification by 16S rRNA gene sequence analysis

At least a 492 bp fragment of the 5' region of the 16S rRNA gene was sequenced for all the strains. We found that by comparison of sequences in the NCBI data base, 35 strains showed 99% of identity with *Lb. salivarius* subsp. *salivarius*, 2 strains had 99% of identity with *Lb.salivarius* subsp.

salicinus, 5 strains had 99% of identity with *Lb. salivarius*, 19 strains showed 99% of identity with *Lb. reuteri* and 1 strain showed 98% of identity with *Lb. mucosae*. With the alignment, we found three species of *Lactobacillus* and with the phylogenetic tree we could observe the same three species grouped by separated. Group I correspond to *Lb. salivarius* (subsp *salivarius* and *salicinus* together), Group II to *Lb. mucosae* and Group III to *Lb. reuteri*. It is important to notice that the phylogenetic analysis was not enough in order to differentiate subsp. *salivarius* from subsp *salicinus* (Figure 1). There were no statistical differences ($p>0.05$) in species distribution, according to three analyzed portions in small intestine.

The isolates identified by partial sequence of 16S rRNA gene, were characterized by carbohydrate fermentation and it confirmed the genotypic identification.

Resistance to pH 3.0 and bile salts

There were no statistical differences ($p>0.05$), between the two main *Lactobacillus* species isolated (*Lb. salivarius* and *Lb. reuteri*) with respect to low pH and CPBS resistances (Table 1). Data of strains which did not survive these conditions are not shown. From all isolated strains, only 20 survived more than 45% this *in vitro* model. Survival at pH 3 is significant as ingestion with food or dairy products raises the pH in stomach to 3.0 or higher. Resistant strains belonged to three identified species, being *Lb. salivarius* the most common with 15 strains (Figure 1). Others strains showed good survival to low pH (more than 50 %), but they were discarded, because the resistance to CPBS was less than 0.1 %.

Autoaggregation and Hydrophobicity (microbial adhesion to hydrocarbons)

20 strains that survived to pH 3.0 and CPBS conditions more than 45 % were included to further characterization. They showed significant differences ($p<0.05$) in their autoaggregation and hydrophobicity properties. Strains 5, 6, 8, 9, 10, 11, 13, 18 and 20 showed an autoaggregation percentage superior to 40%, but strain 13 had less than 30% for hydrophobicity, reason for which

it was discarded like a potential probiotic. For hydrophobicity, strains 7, 15 and 19 showed less than 10 % (Figure 2). Altogether, 8 strains showed autoaggregation and hydrophobicity percentages superior to 40 %, from these, 6 correspond to *Lb. salivarius*, 1 to *Lb. reuteri* and 1 to *Lb. mucosae*. This infers that these strains posses autoaggregative and hydrophobic characteristics which is related with adhesion to epithelia.

Antagonism against Escherichia coli K88

8 strains that displayed autoaggregation and hydrophobicity superior to 40 % were included in this experiment for antagonism. Whole cultures were used and halos of inhibition more than 20 mm against *E. coli* K88 were observed. There were no significant differences ($p>0.05$) between strains.

Quantitative antagonistic analysis, including all of 8 strains showed pathogen inhibition more than 97 %. There were differences between strains ($p < 0.05$). Strains 6, 9, 10, and 20 showed values of inhibition superior to 98% (Figure 3). Antagonistic effect of *Lb. reuteri* strains, which were not resistant to low pH neither CPSB, and did not show surface properties in the required percentage, was quantified. They showed inhibition against *E. coli* K88 more than 96 % (data no shown).

Discussion

The gastrointestinal tract of pigs is a very complex microbial ecosystem. Lactobacilli are established early in piglet intestine, and although succession occurs throughout lifetime of the pigs, they may remain as one of the predominant elements of the bacterial community (De Angelis *et al.*, 2006).

In this work, sixty-two *Lactobacillus* strains were isolated from small intestine of weaned piglets, with the aim to characterize them as potential probiotics. One facultatively heterofermentative and two obligately heterofermentative species were found by partial sequencing of 16S rRNA gene: *Lb. salivarius*, *Lb. reuteri* and *Lb. mucosa*, respectively. Similar results were reported by Robredo and Torres (2000) and Roos *et al.*, (2000) in intestinal tract of

healthy adult's pigs. Those results indicate that isolated species are maintained in the intestine throughout the whole life of pigs. This is a desirable fact, if they exert probiotic effects (Saarela *et al.*, 2000; De Angelis *et al.*, 2006).

Probiotic microorganisms need to resist the adverse factors in the gastrointestinal tract when they pass through it, like the stomach acidity and bile salts, excreted in duodenum. García-Galaz, *et al.* (2004), mentions that 20 % of resistance to these two factors is enough to secure beneficial effects potential for probiotic *Enterococcus*. Nevertheless, for this investigation it was decided to select strains with a resistance more than 45 %, to assure that bacteria arrive in suitable concentration (6 to 8 logarithms/ g of consumed food) to the intestine, and exert their probiotic effect (Shah *et al.*, 1999).

In this work, all of three species were resistant to pH 3.0 and CPBS. Maxwell and Stewart (1995) found that *Lb. acidophilus*, *Lb. fermentum* and *Lb. lactis* were resistant to these adverse conditions in adult pigs. From those species, 20 strains survived the gastrointestinal transit more than 45 %. Gómez-Zavaglia *et al.* (1998) and Kociubinski *et al.* (1999) obtained resistant strains to gastrointestinal transit over 23%. Those species were *B. pseudolongum*, *B. infantis B. animalis* and *B. breve*. Aside, Ibrahim and Bezkorovainy (1993) worked with strains of *B. bifidum*, *B. breve*, *B. infantis* and *B. longum*, which were resistant to the adverse conditions of digestive tract. In general, variable results have been documented in respect the resistance of low pH and bile salts of the *Lactobacillus* and *Bifidobacterium* strains (Clark and Martin, 1994; Chung *et al.*, 1999; Mishra and Prasad, 2005). Previous results have shown a wide diversity in resistance between strains and this resistance is not characteristic of single specie.

It is important to notice that from sixty-two isolated strains, only 5 showed low resistance to pH 3.0 (less than 10 %). The *Lactobacillus* genus has optimal growth in pH 6.0. It is characterized by its capacity to produce lactic acid mainly, which creates atmospheres with pH up to 4.0, where they are able to remain viable by variable periods, depending on the activity of their H^+-ATPase (Matsumoto *et al.*, 2004).

The greater adverse effect was observed by the CPBS. Thirty-four strains showed to be less resistant (less than 0.1 %). It has been found that biliary salts hydrolases, produced by some *Lactobacillus* strains, are involved in this resistance. Some authors have reported that the conjugated salts, mainly the glycodeoxicolic acid, are lethal for this bacterial genus and the mortality rate increases as pH diminishes. Hydrolases enzymes, makes the deconjugated bile salts less soluble, and they are eliminated more easily in feces. Nevertheless, the previous hypothesis is not totally established (Grill *et al.*, 2000; Tanaka *et al.*, 2000; Kim *et al.*, 2004).

Another desirable property of probiotic bacteria is the colonization in intestinal wall. This colonization is necessary in order to exert its beneficial effects (Tuomola *et al.*, 2001). In probiosis, it is important to evaluate surface properties, like autoaggregation and hydrophobicity, because they are used as a measurement directly related to adhesion ability to enterocytic cellular lines (Pérez *et al.*, 1998; Del Re *et al.*, 2000).

Autoaggregation besides determines the capacity of the bacterial strain to interact with itself, in a nonspecific way. Aside, when that hydrophobicity is high (more than 40%), it indicates the presence of hydrophobic molecules in the bacterial surface, like surface array proteins; wall intercalated proteins, cytoplasmic membrane protein and lipids. When it is low (less than 40%), it indicates the presence of hydrophilic molecules, like teichoic acids and/or peptidoglycans (Ofek and Doyle, 1994; Pérez *et al.*, 1998; Bibiloni *et al.*, 1999; Bibiloni *et al.*, 2001).

In this work, 20 strains were resistant to gastrointestinal transit, 8 had values of autoaggregation and hydrophobicity superior to 40%. According to Del Re *et al.* (1998) and Pérez *et al.* (1998), this percentage is the minimum necessary for considering a strain with adhesion abilities to exert probiosis.

Lb. salivarius was the specie with better surface properties. Pérez *et al.* (1998) studied same surface properties with *B. breve* strains isolated from humans and no one of the 4 strains tested, were autoaggregatives or hydrophobics. Neither were adherent to cellular lines Caco-2. Time later,

Bibiloni *et al.*, (2001) related these non adherent strains with poor presence of protease-sensitive non polar like proteins molecules in their surface.

Del Re *et al.* (1998) studied the surface properties of 6 *B. suis* strains and found a great variation in its ability to adhere, and they concluded that the adhesion property is characteristic of each strain and cannot be generalized to species. Abitity to autoaggregate together with cell surface hydrophobicity could be used for preliminary screening to identify potentially adherent bacteria.

We isolated only one strain of *Lb. mucosae*. This strain shows good characteristics of resistance to low pH and CPBS as well as surface properties. The opposite happened with *Lb. reuteri*, which was the second predominant specie, but only one strain had good characteristics of hydrophobicity capacities.

To improve the probiotic characterization, 8 strains that showed surface properties over 40%, were tested for their antagonism *in vitro* against *E. coli* K88. All strains showed inhibition zones more than 20 mm (diameter) and antagonism percentages superior to 97 %. Gusils *et al.* (2002) found that *Lactobacillus* and *Enteroccocus* strains isolated from pig feces, do not inhibit the growth of *Yersinia enterocilitica*, *Salmonella choleraesuis*, *Salmonella typhimurium* and *Salmonella enteritidis* after 24 hours of plates incubation. The quantification of antagonism of strains in this work showed an *E. coli* K88 inhibition superior to 97% in 6 hours. According to antagonistic values, resistance to gastrointestinal transit and adherence factors of isolated strains, it is possible to infer that these bacteria could be used as an alternative for the treatment of diarrhea in piglets. Nevertheless, *in vivo* studies are necessary to confirm it.

E. coli K88 has been identified as one of the main bacterial producer diarrheas in new born pigs. It has been suggested that probiotics can coaggregate pathogenic bacteria and release antagonistic substances, like organic acids (lactic mainly) and bacteriocins. Also there are studies that indicate that they can compete for adhesion sites with several

microorganisms, but this is still not verified (Mulder *et al.*, 1997; Meng *et al.*, 1998; Ouwehand *et al.*, 1999; Doyle, 2001).

In conclusion, our results showed that 8 selected strains (*Lb. salivarius* mainly) have potential probiotic value, on the bases of the results obtained with resistance to gastrointestinal tract, surface properties and antagonistic characteristics. We found that the predominant specie, *Lb. salivarius*, shows the best characteristics to fulfill the criteria of a probiotic strain. In addition, it is recommendable to continue analyzing these strains according to the selection criteria, because a bacterium is considered probiotic when it demonstrates the stimulation of the immunological system and tests of adhesion to the pig mucosa and/or epithelium intestinal.

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References

1.- Bibiloni, R., Pérez, P.F. and De Antoni, G.L. 1999. Factors Envolved in Adhesión of Bifidobacterials Strains to Epithelial Cells in Culture. Anaerobe. 5, 483-485.

2.- Bibiloni, R., Pérez, P.F., Garrote, L. G., Disalvo, E.A. and De Antoni, G.L. 2001. Surface Characterization and Adhesive Properties of *Bifidobacteria*. Methods Enzymol. 336, 411-427.

3.- Chung, H., Kim, Y.B., Chun, S.L. and Ji, G.E. 1999. Screening and Selection of acid and bile resistant bifidobacteria. Int. J. Food Microbiol. 47, 25-32.

4.- Clark, P.A. and J Martin, H. 1994. Selection of Bifidobacteria for use as dietary adjunts in cultured dairy foods: III – Tolerance to simulated bile concentrations of human small intestine. Cult. Dairy Prod. J. 29, 18-21.

5.- Corona, V. 2001. Aislamiento e identificación de *Bifidobacterium* sp. a partir del contenido gástrico del cerdo. Thesis. University of Sonora. Sonora, Mexico

6.- Corona, V. 2003. Evaluación probiótica de especies de *Bifidobacterium* en cerdos lactantes. Thesis. Research Center in Food and Development. Sonora, Mexico

7.- De Angelis M., Siragusa S., Berloco M., Caputo L., Settanni L., Alfonsi G., Amerio M., Grandi A., Ragni A. and Gobetti M. 2006. Selection of Potential Probiotic lactobacilli from pig feces to be used as additives in pelleted feeding. Research Microbiol. 157, 792-801.

8.- De los Reyes C., Limsowtin G., Tailliez P., Séchaud L. and Accolas J. 1992. A *Lactobacillus helveticus*-specific DNA probe detects restriction fragment length polymorphisms. Appl. Environm. Microbiol. 58, 3429-3432.

9.- De Martinis, E., Alves, V. and Franco B. 2002. Fundamentals and perspectives for the use of bacteriocins produced by lactic acid bacteria in meat products. Food Reviews Int. 18, 191-208.

10.- Del Re, B., Busetto, A., Vignola, G., Sgorbati, B. and Palenzona, D.L. 1998. Autoaggregation and Adhesion Ability in a *Bifidobacterium suis* Strain. Lett. Appl. Microbiol. 27, 307-310.

11.- Del Re, B., Sgorbat,i B., Miglioli, M. and Palenzona, D. 2000. Adhesion, Autoaggregation and Hydrophobicity of 13 Strains of *Bifidobacterium longum*. Lett. Appl. Microbiol. 31, 438-442.

12.- Doyle, M.E. 2001. Alternatives to antibiotic use for growth promotion in animal husbrandy. Food Research Institute. April 2001.

13.- Fuller, R. 1999. Probiotics for farm animals. In: Probiotics. A critical Review. Horizon Scientific Press. Wymondham, U.K. pp. 15-43.

14.- García-Galaz, A., Pérez-Morales, R., Díaz-Cinco, M.E. and Acedo-Félix, E.2004. Resistance of *Enterococcus* strains isolated from pigs to gastrointestinal tract and antagonistic effect against *E. coli* K88. Rev. Latinoam. Microbiol. 46, 5-11.

15.- Gibson, G.R. and Wang, X. 1994. Regulatory effects of bifidobacteria on the growth of other colonic bacteria. J. Appl. Bacteriol. 77, 412-420.

16.- González, S.N., Apella, M.C., Romero, N.C., Nader de Macías, M.E. and Oliver, G. 1993. Inhibition of enteropathogens by Lactobacilli strains used in fermented milk. J. Food Prot. 56, 773-776.

17.- Gómez-Gil, B., Roque, A., Turnbull, J. and Inglis, V. 1998. A review on the use of microorganisms as probiotics. Rev. Latinoam. Microbiol. 40, 166-172.

18.- Gómez-Zavaglia, A., Kociubinski, G., Pérez, P. and De Antoni, G. 1998. Isolation and characterization of *Bifidobacterium* strains for probiotic formulation. J. Food Prot. 61, 865-873.

19.- Gómez-Zavaglia, A., Kociubinski, G., Pérez, P., Di Salvo, E. and De Antoni, G. 2002. Effect of bile on the lipid composition and surface properties of bifidobacteria. J. Appl. Microbiol. 93, 794-799.

20.- Gopal K.P., Prasad P., Smart John and Gill Harsharanjit. 2001. In vitro adherence of *Lactobacillus rhamnosus* DR20 and *Bifidobacterium lactis* DR10 strains and their antagonistic activity against an enterotoxigenic *Escherichia coli*. Int J Food Microbiol. 67, 207-216.

21.- Grill, J.P., Perrin, S. and Schneider, F. 2000. Bile salt toxicity to some bifidobacteria strains: role of conjugated bile salt hydrolase and pH. Can. J. Microbiol. 46, 878-884.

22.- Gusils, C., Bujazha, M. and González, S. 2002. Preliminary studies to design a probiotic for use in swine feed. Interciencia. 27, 409-413.

23.- Hernández, F. 2003. Algunos aspectos de investigación básica y aplicada sobre probióticos. Memoria electrónica del 3er Simposio Mexicano de Probióticos. México D.F.

24.- Hintze, J.L. 1997. NCSS 97 *user's guide-II [Computer software manual.]* Kaysville, UT: Number Cruncher Statistical Systems.

25.- Ibrahim, S.and Bezkorovainy, A. 1993. Survival of bifidobacteria in the presence of bile salt. J. Animal Sci. 62, 351-354.

26.- Jonson, E. and Conway, P. 1992. Probiotics for pigs. The Scientific Basis. R. Fuller ed. Chapman and Hall editorial. London. pp 84-93

27.- Kim, G.B., Yi, S.H. and Lee, B.H. 2004. Purification and characterization of three different types of bile salt hydrolases from *Bifidobacterium* strains. J. Dairy Sci. 87, 258-266.

28.- Kociubinski, G., Perez, P.F., Añon, M.C., and De Antoni, G.L. 1999. A method for the screening of lactic acid bacteria with high inhibitory power. J. Food Prot. 59, 739.

29.- Lane D.J. 1996. 16S/23S rRNA sequencing. In: Nucleic Acid Techiques In Bacterial Systematics. Ed. John Wiley and Sons. Chichester, England. pp 115-147.

30.- Matsumoto, M., Oishi, H. and Benno, Y. 2004. H^+ ATPase activity in *Bifidobacterium* with special reference to acid tolerance. Int. J. Food Microbiol. 93, 109-113.

31.- Maxwell F.J. and Stewart C.S. 1995. The Microbiology of the Gut and the Role of Probiotics In: The neonaltal Pig *Development and Survival* .Ed. Cab International. Leeds, UK. pp 155-177.

32.- Meng, Q., Kerley, M.S., Russel, T.J. and Allee, G.L. 1998. Lectin-like activity of *Escherichia coli* K88, *Salmonella* choleraesuis and *Bifidobacterium pseudolongum* of Porcine Gastrointestinal Origin. J. Anim. Sci. 76, 551-556.

33.- Mishra V. and Prasad D. 2005. Application of In vitro Methods for selection of *Lactobacillus casei* strains as Potential Probiotics. Int. J.Food Microbiol. 103, 109-115.

34.- Mulder, R.W.A.W., Havenaar, R. and Huis in 't Veld, H. 1997. Intervention strategies: the use of probiotics and competitive exclusion microfloras against contamination with pathogens in pigs and poultry. In: Probiotics 2: Application and Practical Aspects. R. Fuller editor. Chapman & Hall. pp. 187-205.

35.- Ofek, I. and Doyle, R.J. 1994. Bacterial Adhesion to Cells and Tissues. Chapman & Hall, New York. pp. 80-84.

36.- Ouwehand, A.C., Kirjavainen, P. V., Colette, S. and Salminen, S., 1999. Probiotics: Mechanisms and Established Effects. Int. Dairy J. 9, 43-52.

37.- Pérez, P. F., Minnaard, Y., Disalvo, A. and De Antoni, G. L. 1998. Surface Properties of Bifidobacterial Strains of Human Origin. Appl. Environ. Microbiol. 64, 21-26.

38.- Rinkinen, M., Westermarck, E., Salminen, S. and Ouwehand, A. 2003. Absence of host specificity for in vitro adhesion of probiotic lactic acid bacteria to intestinal mucus. Vet. Microbiol. 97, 55-61.

39.- Robredo B. and Torres Carmen. 2000. Bacteriocin production by *Lactobacillus salivarius* of Animal Origin. J Clin Microbiol. 38, 3908-3909.

40.- Rodríguez E., Arqués J., Rodríguez M., and Medina M. 2003. Reuterin Production by lactobacilli isolated from pig faeces and evaluation of probiotic traits. Letters Appl. Microbiol. 37, 259-263.

41.- Roos S., Karner F., Axelsson L. and Jonsson H. 2000. *Lactobacillus mucosae* sp. nov., a new species with in vitro mucus-binding activity isolated from pig intestine. Int J System Evol Microbiol. 50, 251-258.

42.- Saarela M., Mogensen G., Fondén R., Matto J. and Sandholm T.M. 2000. Probiotic bacteria : safety, funtional and technological properties. J. Biotech. 84, 197-215.

43.- Scardovi, V. 1986. Bergey's Manual of Systematic Bacteriology. Vol. 2. P. H. A. Sneath, N. S. Mair, M.E. Sharpe & J.G. Holt (editors) Williams and Wilkins (editorial). Baltimore, London, los Angeles.pp. 1418-1434.

44.- Shah, H., Lee, J., Pestka, J. and Unstund, Z. 1999. Viability of bifidobacteria in commercial dairy products during refrigerated storage. J. Food Prot. 63, 327-331.

45.- Tanaka, H., Hashiba, H., and Kok, J. 2000. Bile salt hydrolase of *B.longum*- biochemical and genetic characterization. Appl. Environ. Microbiol. 66, 2502-2512.

46.- Tuomola, E., Crittenden, R., Playne, M., Solauri, E. I. and Salminen, S. 2001. Quality assurance criteria for probiotic bacteria. Am. J. Clin. Nutr. 73 (Suppl), 393S-398S.

47.- Yusof, R.M., Haque, F., Ismail, M. and Hassan, Z. 2000. Isolation of *B. infantis* and its antagonistic activity against ETEC O157 and *Salmonella typhimurium* S-285 in weaning foods. Asia Pacific J. Clin. Nutr. 9, 130-135.

Figure Legends

Figure 1. Unrooted phylogenetic tree based on the Observed Divergency Method of partial 16S rRNA sequences of *Lactobacillus* isolates from small intestine of piglets. Horizontal bar represents 1% sequence divergence. Numbers indicate bootstrap values for branch point.
Accesion numbers of sequences obtained from NCBI correspond to the following species: AB289296.1 *Lb. salivarius* subsp. *salivarius*, AB289295.1 *Lb.salivarius* subsp. *salicinus*, DQ444477.1 *Lb. salivarius*, AB289270.1 *Lb. reuteri* and AF126738.1 *Lb. mucosae*.

Figure 2. Resistance to pH 3.0 (■) and conjugated porcine bile salts (■) (CPBS) of *Lactobacillus* strains isolated from small intestine of piglets.
Lb. salivarius from 1 to 15, *Lb. reuteri* from 16 to 19 and *Lb. mucosae* 20.

Figure 3. Percentages of autoaggregation (■) and hydrophobicity (■) of *Lactobacillus* strains that survived pH 3.0 and CPBS.
Lb. salivarius from 1 to 15, *Lb. reuteri* from 16 to 19 and *Lb. mucosae* 20.

Figure 4. Antagonism against *E. coli* K88 of *Lactobacillus* strains isolated from small intestine of piglets.
Lb. salivarius from 5 to 11, *Lb. reuteri* 18 and *Lb. mucosae* 20.

Figure 1

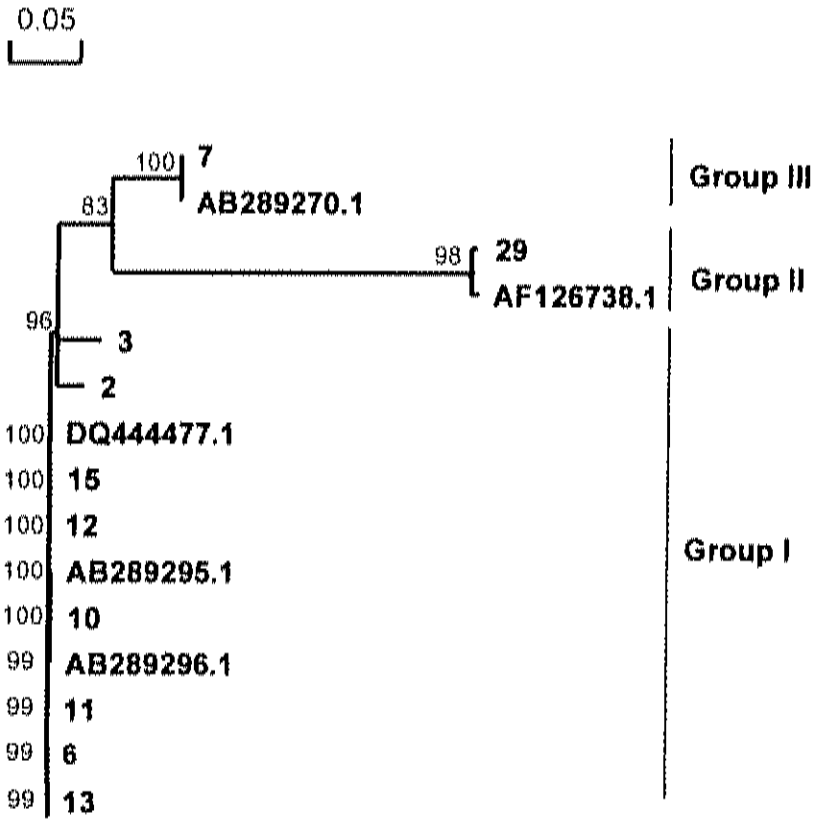

0.05
100
7
AB289270.1
83
98
29
AF126738.1
96
3
2
100
DQ444477.1
100
15
100
12
100
AB289295.1
100
10
99
AB289296.1
99
11
99
6
99
13
Group III
Group II
Group I

Table 1. Percentage of resistance to pH 3.0 and conjugated porcine bile salts (CPBS) of predominant species of *Lactobacillus* strains isolated from small intestine of piglets

Species	Resistance to pH 3.0	Resistance to CPBS
Lb. salivarius	66.5 ± 30	29 ± 35.5
Lb. reuteri	48.7 ± 39.2	16 ± 24.8

± indicates standard deviation

Figure 2

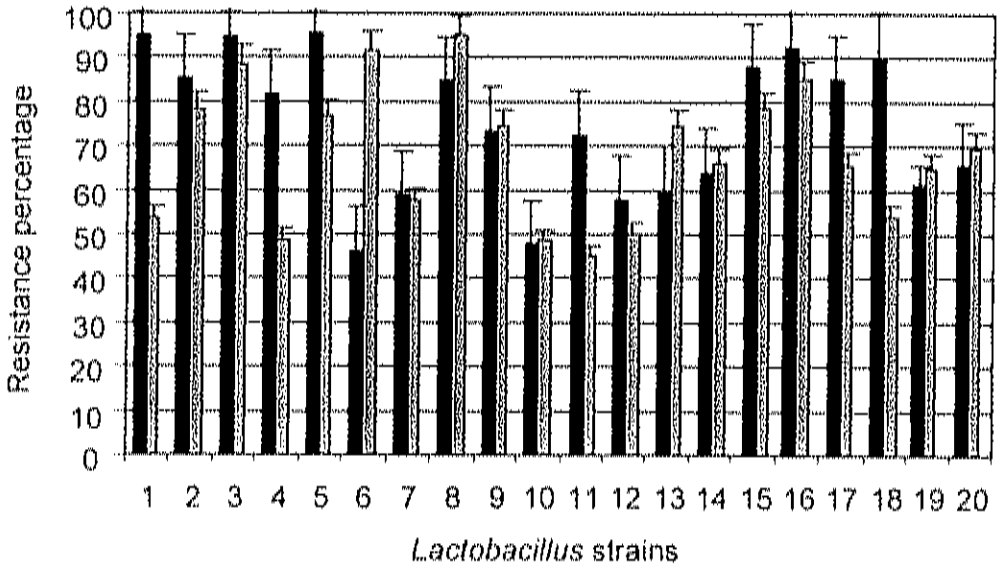

Resistance percentage
100
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Lactobacillus strains

Figure 3

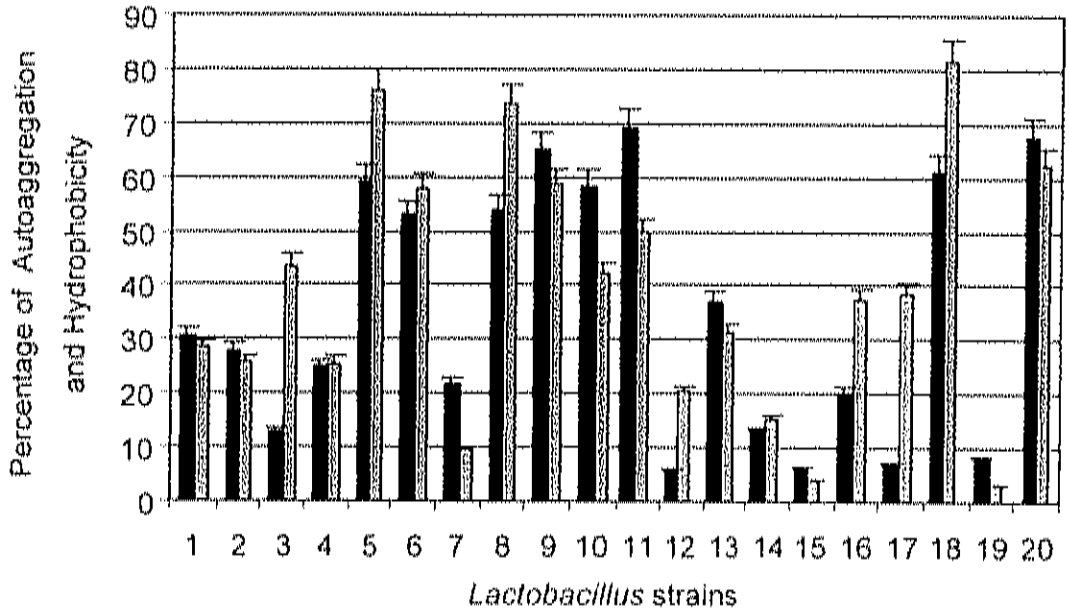
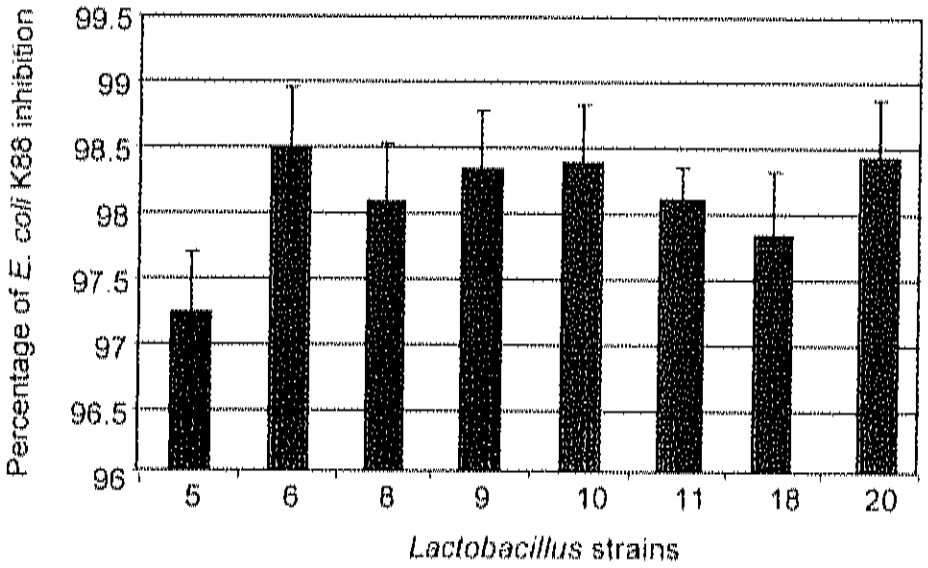

Percentage of Autoaggregation and Hydrophobicity
90
80
70
60
50
40
30
20
10
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Lactobacillus strains

Figure 4


Percentage of E. coli K88 inhibition
99.5
99
98.5
98
97.5
97
96.5
96
5
6
8
9
10
11
18
20
Lactobacillus strains

ARTÍCULO 2

Adhesion of *Lactobacillus salivarius* to Mucosa of Small Intestine of Piglets

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Abstract

For many of the proposed health effects of probiotic bacteria it is desirable that the microorganisms at least transiently can exert adherence to the gastro-intestinal tract. The aims of this study were to evaluate the adhesion of three *Lactobacillus salivarius* strains (6, 9 and 10) to intestinal mucosa of weaned piglets and to determine whether lectin-like activity exists. Dot and western blot assays were performed to investigate for bacterial adhesion. Some carbohydrates and glycoproteins were probed to interfere with the adhesion of strains to intestinal mucins and to determine if lectin-like activity exists. Strains 9 and 10 had greater interaction with mucins of piglets than with those of adult animals ($p<0.05$), whereas with the adhesion of strain 6 there were no differences ($p>0.05$). Western blot assays showed that, in piglets, the adhesion was observed in the 3 portions of the small intestine, whereas in adults it was variable according the different animal mucus proved. It was found that galactose and mannose were the carbohydrates that diminished ($p<0.05$) the interaction of strains 9 and 10 to intestinal mucosa, whereas pig gastric mucin affected the adhesion of strain 6. Treatments of strains 9 and 10 with proteases diminished ($p<0.05$) their adhesion to the mucins, whereas strain 6 was in addition affected by periodate. It was concluded that the three strains adhere to mucosa of the small intestine of piglets and they have lectin-like activity proteins that is involved in this adhesion.

Key words: *Lactobacillus salivarius*, adhesion, piglet mucins, carbohydrates

Introduction

Infectious diarrhea of neonatal pigs is one of the most common and economically devastating condition encountered in the animal industry (Gusils *et al.*, 2002). *Escherichia coli* K88 has been recognized as an important etiologic diarrheic agent since can cause death of infected pigs. Control of the number of pathogens in pig industry can be achieved by antibiotics but they have led to an imbalance of the beneficial intestinal florae and the appearance of resistant bacteria (Pascual *et al.*, 1999). According to this, in the last years exist an increasing scientific and commercial interest in using probiotics microorganisms to enhance animal health and feed conversion (Styriak *et al.*, 2003).

The use of probiotics has proved effectiveness in suppression of the attachment and multiplication of pathogens in the intestines of pigs (Meng *et al.*, 1998 Mukai *et al.*, 2002; Lee *et al.*, 2003).The colonization of different portions of the intestinal tract by probiotic microorganisms constitutes the first defensive barrier against the invasion of pathogenic microorganisms and is considered as an important characteristic for their health benefits evaluation (Lee, *et al.* 2003). Intestinal epithelial cells of the intestine are covered by a relatively thick (400 μm) mucus layer consisting of mucin, a 2-megadalton (MDa) gel-forming glycoprotein, and a large number of smaller glycoproteins, proteins, glycolipids and lipids (Piel *et al*, 2004).

Mucus layer has been implicated in interacting with bacteria in a number of ways. These include involvement of the mucus layer as an initial site for bacterial adhesion, as a protective barrier and as a source of nutrients and matrix for bacterial replication and colonization (Laux, 2005). Bacterial enteropathogens must traverse the mucus layer in order to approach and adhere to intestinal epithelial cells. For this reason, mucus interaction in intestinal colonization has been of interest to many investigators (Cohen and Laux, 1995). Cell adhesion is a multistep process involving contact of the cell membrane and interacting surfaces. Proteinaceous surface appendages, fimbriae, flagella or (S)-layers with affinity for mammalian extracellular matrix

or mucosa, have been extensively characterized for many pathogenic bacteria (Talay, 2005).

In probiotic bacteria the adhesion has been reported to be mediated by proteins and oligosaccharide moieties on the cell surface to interact with components of the mucus (Roos and Jonsson, 2002) but information about the mechanisms of bacterial colonization is limited. It is thought that attachment of probiotic bacteria to living cells can be mediated by lectins (carbohydrate-binding proteinaceous component of non-immune origin), located on bacterial surfaces and the oligosaccharide part of mucus layer (glycoproteins, glycolipids) can be a receptor for those lectins (Meng *et al.*, 1998; Gao and Meng, 2004).The aims of this study was to evaluate the adhesion of three *Lactobacillus salivarius* strains isolated in our laboratory (named 6, 9 and 10) to intestinal mucosa of weaned piglets and to determine whether lectin-like activity exist.

Materials and Methods

Bacterial strains and culture conditions

Three *Lactobacillus salivarius* strains (6, 9 and 10) were isolated of small intestine from 10 days-old piglets. Bacteria were stored at -20° C in 30% glycerol. They were previously characterized as potential probiotic .

For the subsequent experiments, individual strains were used to inoculate 10 mL MRS broth with cysteine hydrochloride 0.5 gr/L (Difco) and incubated for 18-24 h in 5% CO_2.

Mucin preparation

Pig intestinal mucin glycoproteins were isolated from each portion of small intestine (duodenum, jejunum and ileum) from healthy piglets (n=6) and adult pigs (n=6) by scrapping. Extraction and dual ethanol precipitation as described by Tuomola *et al.* (2000) was conducted.

Dot blot Assays

Bacteria were harvested from overnight cultures, washed twice with PBS (phosphate saline buffer) pH 7.2 (0.02M) and adjusted to turbidity 1.0 (approximately $1x10^9$ CFU/mL) using a wavelength of 450 nm. Then, the bacterial cells were Sulfo-*N*-hydrosuccinimide (NHS)-biotin labeled (Pierce Rockford IL, USA) as previously described Mukai *et al.* (2002).

Serial two fold dilution of mucins (2 mg/mL to 5 ng/mL of concentration in PBS buffer pH 7.2) was applied to nitrocellulose (BioRad, Hercules CA, USA). Membranes were blocked with 2% bovine serum albumin (Sigma, St Louis MO, USA) in PBS buffer pH 7.2 with 0.05% Tween 20 ((Sigma, St Louis MO, USA) for 2 h at room temperature, prior to the addition of biotinylated bacteria. Following overnight incubation at 4° C, membranes were washed five times with PBS buffer with 0.05% Tween 20, to remove unbound bacteria and developed with Avidin-Peroxidase and 3,3'-diaminobenzidine (Sigma, St Louis MO, USA) to detect adhesion (Ruhl *et al.*, 1996). Intensity of the dots was measured with a GS-700 Imaging Densitometer & Molecular Analyst Software Ver 2 (BioRad Hercules CA, USA). Experiment was performed by triplicate.

Dot blot assays as was described above were used to probe the adhesion of bacteria to glycoproteins asialofetuin, ovoalbumin and pig gastric mucin (Sigma, St Louis MO, USA) as well as the neoglycoproteins Galα(1-3)Gal-BSA and Gal(β1-4)GlcNAc-BSA (Calbiochem San Diego CA, USA) which are receptors for *E. coli* K88 in small intestine of piglets. Glycoproteins and neoglycoproteins were diluted by serial dilution at concentration 5 μg/mL (Meng *et al.*, 1998; Tuomola *et al.*, 2000). Experiment was performed by triplicate.

Western blot assays

SDS-PAGE Nonreducing in 10% acrylamide gels was performed with mucins of each portion of small intestine from piglets and adult animals. 50 μg of protein per lane were used in a Mini-Protean II electrophoresis chamber

(BioRad Hercules CA, USA). Half of the gel was used to stain with Comassie blue and other was used to transfer the separated proteins to a nitrocellulose membrane in a semidry electroblotter (Trans-Blot SD Semi-Dry Transfer Cell, BioRad Hercules CA, USA) at 0.06 V for 1 h. Non-specific protein binding sites on the membrane were blocked with bovine serum albumin (Sigma St. Louis MO, USA) 2% in PBS for 2 h at room temperature, prior to the addition of biotinylated bacteria. Following overnight incubation at 4° C, membranes were washed five times with PBS buffer to remove unbound bacteria and developed with Avidin-Peroxidase and 3,3'-diaminobenzidine (Sigma, St Louis MO, USA) to detect reactive bands (Rojas *et al.*, 2002).

Mucin adhesion inhibition assays

D(+)-Mannose, D(+)-Galactose, D-Lactose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine and pig gastric mucin (Sigma St. Louis MO, USA) were used, by serial dilution, in concentration 2 mg/mL as potential inhibitors of adhesion of the three bacterial strains to mucins of piglets.

Turbidity of bacterial cells was adjusted to an absorbance 1.0 (approximately $1x10^9$ CFU/mL) using a wavelength of 450 nm, and biotin-labeled. They were incubated with each potential inhibitor for 1 h at 37° C. Dot blot assays were conducted with immobilized piglet mucins (Meng *et al.*, 1998). Experiment was carried out by triplicate.

Enzymatic and chemical treatment of bacteria

Bacterial concentration was adjusted to a turbidimetric absorbance 1.0. In the enzymatic treatments, trypsin (2 mg/mL) and protease (0.4%) were probed. Sodium periodate (20 mM) (Sigma St. Louis MO, USA) was used as chemical treatment as described Tuomola *et al* (2000). The bacterial suspensions were incubated for 1 h at 37° C with each one of the treatments. After, the cells were centrifuged 2600 x *g* at 4° C and bacterial pellets were washed five times with PBS buffer pH 7.2. The final bacterial cell density was adjusted with the same buffer solution as before and then biotin-labeled as described Mukai *et al* (2002). Dot blot assays were conducted with

immobilized piglet mucins at concentration 5 μg/mL (Meng *et al.*, 1998). Experiment was performed by triplicate.

Statistical Analysis

ANOVA and Tukey-Kramer test were used for the mean comparisons ($p<0.05$) in all dot blot assays, with statistical package NCSS 6.0 (Hintze, 1997). Comparisons were carried out for strains treatments.

Results

In the current study piglet and adult pig intestinal mucus glycoproteins isolated from small intestine were used as a substratum for adhesion of potential probiotic *Lactobacillus* strains.

We observed bacterial adhesion in all the intestinal mucin concentrations probed (from 2 mg/mL to 5 ng/mL obtained by serial two fold dilution) but it was in 5 μg/mL where we could to observe differences in the color intensity of the dots for strains. For further analysis, that mucin concentration was used.

Strains 9 and 10 showed a significant difference in adhesion to piglet or adult mucus ($p<0.05$) (Figure 1) while strain 6 showed any preference. No differences were found in adhesion between duodenum, jejunum and ileum in piglets or adult pigs ($p>0.05$) (Data no shown).

On the other hand, western blot assays showed that strains 9 and 10 had interaction with more protein bands of piglet mucins than with those of adult pigs. With the strain 6 we observed a similar number of protein bands, in both intestinal mucins that interacted with the bacterium. We found that specific strains recognize specific (glyco)proteins in the intestine (Figure 2). Bacterial adhesion in adult pigs was dependent of the animal, because while with piglet mucins, we always observed adhesion to proteins of the three small intestine sections (duodenum, jejunum and ileum), with the adult mucins the interaction was observed just in one or two intestinal sections. There are

several hypothesis about that bacterial affinity to intestine can change with the animal age, but this is still studied.

In the adhesion experiment by dot blot assays to a 5 μg/ml concentration of glycoproteins and neoglycoproteins (K88 intestinal receptors); all of the three strains (6, 9 and 10) displayed high affinity to the last ones. There were differences in the bacterial interaction between glycoprotein and neoglycoprotein molecules ($p<0.05$) but there were not found differences in adhesion between strains ($p>0.05$) (Figure 3). Previous could to implicate that carbohydrates (mono or disaccharides) are the specific receptors of bacterial adhesins. Another explication is perhaps that the glycoproteins concentration used, was not enough to observe a strong interaction with *Lactobacillus* strains.

It was observed that strains 9 and 10 diminished their adhesion to intestinal piglet mucin with galactose and mannose (2 mg/mL), whereas pig gastric mucin (2 mg/mL) diminished the adhesion of strain 6 (Figure 4). Disaccharides as D-lactose (2mg/mL) or sucrose (2 mg/mL) were probed to inhibit the strains adhesion to intestinal mucins but they were no inhibitors (Data no shown).

To investigate bacterial actors involved in the adherence of strains to intestinal mucins, bacteria were exposed to chemical and enzymatic treatments prior to adhesion. Strain 6 diminished its adhesion with sodium periodate and proteolitic enzymes. Strains 9 and 10 were affected only by protease and trypsin (Figure 5). The involvement of proteins in adhesion was strongly supported by the results obtained after treatment with the enzymes. For strain 6, the nature of this lectin-like substance was suggested to be glycoprotein, because the treatment with metaperiodate resulted in a decreased adhesion or carbohydrate molecules in the bacterial surface are participating on strain binding to intestinal mucosa.

Discussion

Blot assays showed that strain 6 had not preference to adhere to adult or piglet intestinal mucus, whereas strains 9 and 10 had affinity to piglet mucins. With the results of the last two strains, we can think that the intestinal receptors for these *Lactobacillus* bacteria vary with the age of the animal, being greater in piglets. Nevertheless, the result for strain 6 indicates the opposite.

Glycans in intestinal mucus can be a niche for bacterial adhesion (Dai *et al.*, 2000). It is verified that for piglet pathogens as *E.coli* K88 and 987P, intestinal mucus can work as receptor for bacterial adhesion. The presence of these pathogens has been found to be age-dependent and usually their intestinal adhesion decrease with the age (Conway *et al.*, 1990; Grange *et al.*, 2002). With probiotic bacteria this is not still verified. Some strains diminish their presence with the host age, whereas others are in the lifetime of it. Kirjavainen *et al.*, (1998) found that *Lb. salivarius* adhered significantly better to newborn than 2 month old infant mucus. The previous observation contrast with the results of Ouwehand *et al.*, (1999) where they found absence of a correlation between age and adhesion of probiotic *Lactobacillus* strains to human mucus. They suggest that there is no change in the availability of receptors in humans with the age. This shows that each strain by its own merits and the extrapolation from related strains is not acceptable.

Furthermore, we found that specific strains recognize specific receptors in the mucus intestine of piglets. All of the three strains can bind to different glycoproteins of the three sections (duodenum, jejunum and ileum) in difference with K88 which can adhere better to duodenum mucins than ileum (Pedersen and Tannock, 1989; Grange *et al.*, 1998; Ramos-Clamont M., 2003). The last showed that receptor(s) for our *Lactobacillus* strains are present in all small intestines, while for K88 they are mostly in duodenum.

Gan and Marquardt (2000) isolated from piglets small intestine mucus two glycoproteins (receptors) for K88 of 26 and 41 kDa. Probably, our strains 9 and 10 are interacting with those ones because they showed interaction with

bands close those molecular masses, but strain 6 does not. The authors do not know if the proteins are in just one section of small intestine or in the three and they do not mention what kind of carbohydrates can be present, although a previous work mention that the receptor for K88 may contain D-galactosamine or D-galactosamine like residues (Metcalfe *et al.*, 1991). Other works demonstrated that K88 possess multiple receptors in mucus and brush borders and they can vary in monosaccharide distribution, chain length and sialic acid content leading to variations in migration during electrophoresis and, the appearance of bands in nitrocellulose membrane blots (Billey *et al.*, 1998; Jeyasingham *et al.*, 1999; Van de Broeck *et al*, 2000).

With the adult pig mucus the adhesion of *Lactobacillus* strains was observed poorly in duodenum and jejunum but it was not in ileum. It is possible with the age the receptors can change or disappear and for that the binding diminish (Conway *et al.*, 1990; Dean, 1990; Grange *et al.*, 2002). Ross and Jonsson (2002) found that a surface protein from *Lb. reuteri* 1063 can adhere to adult pig mucus with molecular masses between 15 and 45 kDa, but also to components with very high molecular mass that could no enter the gel. Our strains recognize proteins with molecular masses between 10 and 40 kDa but not those high molecular mass in adult pig mucus, whereas in piglet mucus they can bind to proteins between 10 and 50 kDa.

For to know the nature of the interactions between our strains and the piglet intestinal mucus, adherence properties were studied with different glycoproteins (Ovoalbumin, Asialofetuin and Pig gastric mucin). Bacteria showed poor adhesion at concentration of 5 μg/mL, but pig gastric mucin reduced the adhesion of strain 6 in intestinal mucin at concentration of 2mg/mL. Fontaine *et al.*, (1994) observed that *Bifidobacterium bifidum* DSM 20082 binds better to the O-glycosilproteins (like mucins) than N-glycosilproteins and they inhibited the adhesion to rat intestinal mucus in a dose-dependent manner. On the other hand, the strains can adhere strongly to the same intestinal receptors for K88, Galα(1-3)Gal-BSA and Galβ(1-4)GlcNAc-BSA, (Grange *et al.*, 2002). Maybe they can compete for a

site in the small intestine of piglets and colonize it, moving the pathogenic bacterium.

Strains 9 and 10 were similar to the one of Gao and Meng (2004). They found that galactose, arabinose, mannose and other carbohydrates were able to inhibit hemagglutination reactions with *Lactobacillus* spp. Sun *et al.*, (2006) found that *Lactobacillus plantarum* Lp6 strain has a mannose-specific adhesin loosely binding to cell surface components by noncovalent interactions. The protein adheres to rat small intestinal mucus. Our strains were able to diminish their adhesion to piglet mucins by mannose (2 mg/mL) or galactose (2 mg/mL) but not by glycoproteins. Strain 6 reduced its adhesion to piglet mucus using pig gastric mucin (2 mg/mL).

Ross and Jonsson (2002) found that some glycoproteins (mucin, fetuin and asialofetuin) can inhibit the adhesion to mucus material for *Lactobacillus reuteri* 1063, whereas any monosaccharide inhibited the adherence. Fontaine *et al.*, (1994) observed that mono-, di- and tetrasaccharides did not inhibit *Bifidobacterium bifidum* adherence to rat intestinal mucus whereas glycophorin A did it. By the previous studies, we can conclude that some strains recognize sugars and not glycoproteins as specific receptors of bacterial lectins (Sun *et al.*, 2006). In other cases the oligosaccharides play a very important role in the adhesion process (Ross and Jonsson, 2000). Sometimes the interaction between probiotic bacteria and intestinal mucus depends highly on peptidic structures rather than oligosaccharidic structures or simple carbohydrates that can inhibit adhesion, are forming part of a more complex carbohydrate receptor structure (Fontaine *et al.*, 1994).

Adhesion is a set of factors that includes the presentation, direction and accessibility of the receptors and adhesins. Adhesion of probiotic bacteria to mucins appears to be a much more complex process than previously envisioned, consisting of the interactions of multiple adhesins, and their structure remains to be determined. It may require the participation of a number of distinct surface constituents that interact in a sequential manner to overcome repulsive forces (Ofek and Doyle, 1994).

Enzymatic treatments decreased the adhesion of three strains 6, 9 and 10 suggesting that the determinants responsible for the adhesion were proteinaceus components located on the bacterial surface. Some studies have revealed the presence of protein arrays named S-layer (Smith *et al.*, 2001; Talay, 2005). It has been proposed that these proteins are involved in surface recognition, and they could be potential mediators in the initial steps involved in adhesion (Buck *et al.*, 2005). Also, carbohydrates in the bacterial surface as glycoproteins, exopolysaccharide or teichoic acids may be partially involved in the adhesion of strain 6 because periodate-treated cells adhere more poorly than control. Tuomola *et al.*, (2000) observed that *Lb. gasseri* ADH adherence to human intestinal mucus involved only periodate-sensitive factors. *Lb. acidophilus* BG2FO4 involved both protease-sensitive and periodate-sensitive factors and *Lb. acidophilus* NCFM/2 only protease sensitive factors.

Three different adhesion mechanisms were found, which indicated that several different bacterial structures might be involved in the adhesion. Kos *et al.*, (2003) found that glycoproteins in the S-layer of *Lb. acidophilus* M92 bind to lectins on the intestinal epithelial cells.

All of the three strains adhered to specific targets in the mucosa of the small intestine of piglets and they had lectin like activity proteins that are involved in this adhesion. In addition it is thought that the intestinal receptors for *Lactobacillus* vary with the age of the animal, being greater in piglets. But, apparently the previous depends on the strain tested.

Acknowledgments

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References

1.- Billey L., Erickson A. and Francis D. 1998. Multiple receptors on porcine intestinal epithelial cells for the three variants of *E.coli* K88 fimbrial adhesin. Vet Microbiol. 59, 203-212.

2.- Buck B., Altermann E. Svingerud T. and Klaenhammer T. 2005. Functional Analysis of putative adhesion factors in *Lb. acidophilus* NCFM. Appl. Environ. Microbiol. 71, 8344-8351.

3.- Cohen P. and Laux D. 1996. Bacterial Adhesion to and Penetration of Intestinal Mucus *in Vitro*. Methods Enzymol. 253, 309-314.

4.- Conway P., Welin A. and Cohen P. 1990. Presence of K88-Specific Receptors in Porcine Ileal Mucus Is Age Dependent. Infect. Immun. 58, 3178-3182.

5.- Dai D., Nanthkumar N., Newburg D. and Walter W. 2000. Role of Oligosaccharides and Glycoconjugates in Intestinal Host Defense. J. Pediatric Gastroenterol. Nutr. 30 (Suppl) 2, 23S-33S.

6.- Dean E. 1990. Comparison of receptors for 987P pili of enterotoxigenic *E. coli* in the small intestines of neonatal and older pigs. Infect Immun. 58, 4030-4035.

7.- Fontaine I., Aissi E. and Bouquelet S. 1994. In Vitro Binding of *Bifidobacterium bifidum* DSM 20082 to Mucosal Glycoproteins and Hemagglutinating Activity. Current Microbiol. 28, 325-330.

8.- Gan L.F. and Marquardt R. 2000. Isolatio, affinity purification and identification of piglet small intestine mucosa receptor for enterotoxigenic *E. coli* K88ac fimbriae. Infect Immun. 68, 564-569.

9.- Gao W. and Meng Q. 2004.Lectin Activity and Chemical Characteristics of *E.coli*, *Lactobacillus spp* and *Bifidobacterium spp* from Gastrointestinal Mucosa of Growing Pigs. Asian-Aust. J. Anim. Sci. 17, 863-868.

10.- Grange P.A., Erikson A.K., Anderson T.J., Francis D.H. 1998. Characterization of the carbohydrate moiety of intestinal mucin-type sialoglycoprotein receptors for the K88ac fimbrial adhesin of Escherichia coli. Infect Imunn. 66, 1613-1621.

11.- Grange P., Mouricout M., Levery S., Francis D. and Eriksson A. 2002. Evaluation of receptor binding specificity of *Escherichia coli* K88 fimbrial adhesin variants using porcine serum transferrin and glycosphingolipids as model receptors. Infect Immun. 70, 2336-2343.

12.- Gusils, C., Bujazha, M. and González, S. 2002. Preliminary studies to design a probiotic for use in swine feed. Interciencia. 27, 409-413.

13.- Hintze, J.L. 1997. NCSS 97 *user's guide-II [Computer software manual.]* Kaysville, UT: Number Cruncher Statistical Systems.

14.- Jeyasingham M., Butty P., King T., Begbie R. and Kelly D. 1999. E. coli K88 receptor expression in intestine of disease-susceptible weaned pigs. Vet Microbiol. 68, 219-234.

15.- Kirjavainen P., Ouwehand A., Isolauri E. and Salminen S. 1998. The ability of probiotic bacteria to bind to human intestinal mucus. Microbiol. Lett. 167, 185-189.

16.- Kos B., Suskovic J., Vukovic S., Simpraga M., Frece J. and Matosic S. 2003. Adhesion and Aggregation ability of probiotic strain *Lb. acidophilus* M92. J. Appl. Microbiol. 94, 981-987.

17.- Laux D., Cohen P. y Conway T. 2005. Role of the Mucis Layer in Bacterial Colonization of the Intestine. In Colonization of Mucosal Surfaces. Ed. ASM Press. Washington DC, USA. pp. 199-206.

18.- Lee Y., Puong K., Ouwehand A. and Salminen S. 2003. Displacement of bacterial pathogens from mucus and Caco-2 cell surface by lactobacilli. J. Medical Microbiol. 52, 925-930.

19.- Meng, Q., Kerley, M.S., Russel, T.J. and Allee, G.L. 1998. Lectin-like activity of *Escherichia coli* K88, *Salmonella choleraesuis* and *Bifidobacterium pseudolongum* of Porcine Gastrointestinal Origin. J. Anim. Sci. 76, 551-556.

20.- Metcalfe J., Krogfelt K., Krivan H., Cohen P. and Laux D. 1991. Characterization and Identification of a porcine small intestine mucus receptor for the K88ab fimbrial adhesin. Infect Immun. 59, 91-96.

21.- Mukai T., Asasaka T., Sato E., Mori K., Matsumoto M. and Ohori H. 2002. Inhibition of binding of *Helicobacter pylori* to the glycolipid receptors by probiotic *Lactobacillus reuteri*. Immun. Medical Microbiol. 32, 105-110.

22.- Ofek, I. and Doyle, R.J. 1994. Bacterial Adhesion to Cells and Tissues. Chapman & Hall, New York. pp. 80-84.

23.- Ouwehand A., Kirjavainen P., Gronlund M., Isolauri E., and Salminen S. 1999. Adhesion of probiotic micro-organisms to intestinal mucus. Int. Dairy J. 9, 623-630.

24.- Pascual M., Hugas M., Badiola J., Montfort J. and Garriga M. 1999. *Lactobacillus salivarius* CTC2197 prevents *Salmonella enteritidis* Colonization in Chickens. Appl. Environ. Microbiol. 65, 4981-4986

25.- Pedersen K. and Tannock G. 1989. Colonization of porcine intestinal tract by lactobacilli. Appl. Environ. Micorbiol. 55, 279-283.

26.- Piel C., Montagne L., Salgado P. and Lalles J. 2004. Estimation of ileal output of gastro-intestinal glycoprotein in weaned piglets using three different methods. Reprod. Nutr. Dev. 44, 419-435.

27.- Ramos Clamont Montfort. 2003. Inmunoglobulinas séricas porcinas: Purificación, generación de concentrados estables y caracterización parcial de la adhesión de patógenos porcinos a sus estructuras oligosacáridas. Doctoral thesis. Centro de Investigación en Alimentación y Desarrollo. Hermosillo, Sonora, México. pp 92-94

28.- Rojas M., Ascencio F. and Conway P. 2002. Purification and Characterization of a Surface Protein from *Lactobacillus fermentum* 104R that Binds o Porcine Small Intestinal Mucus and Gastric Mucin. Appl. Environ. Microbiol. 68, 2330-2336.

29.- Roos S.and Jonsson H. 2002. A high-molecular-mass cell surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. Microbiology. 148, 433-442.

30.- Ruhl S., Sandberg A., Cole M. and Cisar J. 1996. Recognition of Immunoglobulin A1 by Oral Actinomyces and Streptococcal Lectins. Infect. Immun. 64, 5421-5424.

31.- Smit E., Oling F., Demel R., Martinez B. y Powels P. 2001. The S-layer of *Lactobacillus acidophilus* ATCC 4356; Identification and Characterization of Domains Responsible for S-protein Assembly and Cell Wall Binding. J. Mol. Biol. 305, 245-257.

32.- Sun J., Le G., Shi Y. and Su G. 2006. Factors involved in binding of *Lb. plantarum* Lp6 to rat small intestine mucus. Lett. Appl. Microbiol. Journal compilation, 1-7.

33.- Styriak I., Nemkova R., Chang Y., and Ljunhg A. 2003. Binding of extracellular matrix molecules by probiotic bacteria. Lett. Appl. Microbiol. 37, 329-333.

34.- Tatay S. 2005. Gram-positive Adhesins. Contrib. Microbiol. 12, 90-113.

35.- Tuomola E., Ouwehand A.,and Salminen S. 2000. Chemical, physical and enzymatic pre-treatments of probiotic lactobacilli alter their adhesion to human intestinal mucus glycoproteins. Int. J. Food Microbiol. 60, 75-81.

36.- Van de Broeck W., Coxx E., Oudega B. and Goddeeris B.M. 2000. The F4 fimbrial antigen of E.coli and its receptors. Vet Microbiol. 71, 223-244.

Figure Legends

Figure 1. Adhesion of *Lb. salivarius* strains to intestinal mucins from adult pigs and piglets by dot blot assay.

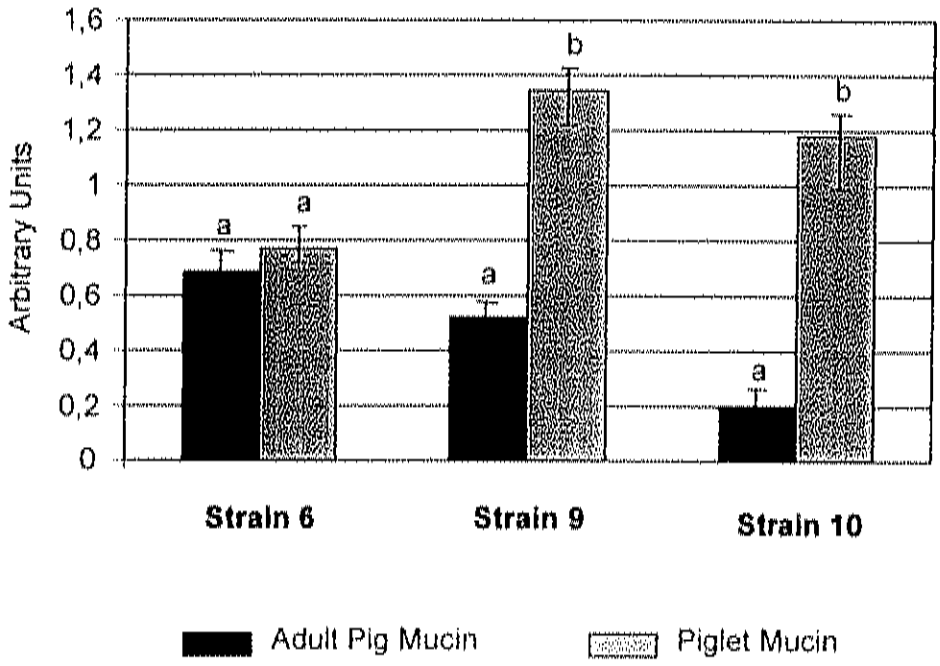
Figure 2. (a) SDS-PAGE Non Reducing gel of piglet mucins, Comassie blue stained, (b) Western blot analysis showing bacterial interaction with proteins of the piglet mucins, (c) SDS- PAGE Non Reducing gel of adult pig mucins, Comassie Blue stained, (d) Western blot analysis showing bacterial interaction with proteins of the adult pig mucins.
50 μg of mucins per lane were loaded in the gels and transferred to a nitrocellulose membrane.
* = Molecular weight marker, D= Duodenum, J= Jejunum and I= Ileum mucins.

Figure 3. Adhesion of *Lb. salivarius* to neoglycoproteins and glycoproteins by dot blot assay.
S6= Strain 6, S9= Strain 9, S10= Strain 10 and NC= Negative control.

Figure 4. Inhibition of adhesion of *Lb. salivarius* strains to 5 μg/mL of intestinal piglet mucins by glycoprotein or carbohydrates.
PGM= Pig Gastric Mucin Gal= Galactose and Man= Mannose.
[a] Values with different superscript differs significantly ($p<0.05$).

Figure 5. Enzymatic and chemical treatment of *Lb. salivarius* strains to diminish their adhesion to 5 μg/ml of intestinal piglet mucins.
[a] Values with different superscript differs significantly ($p<0.05$).

Figure 1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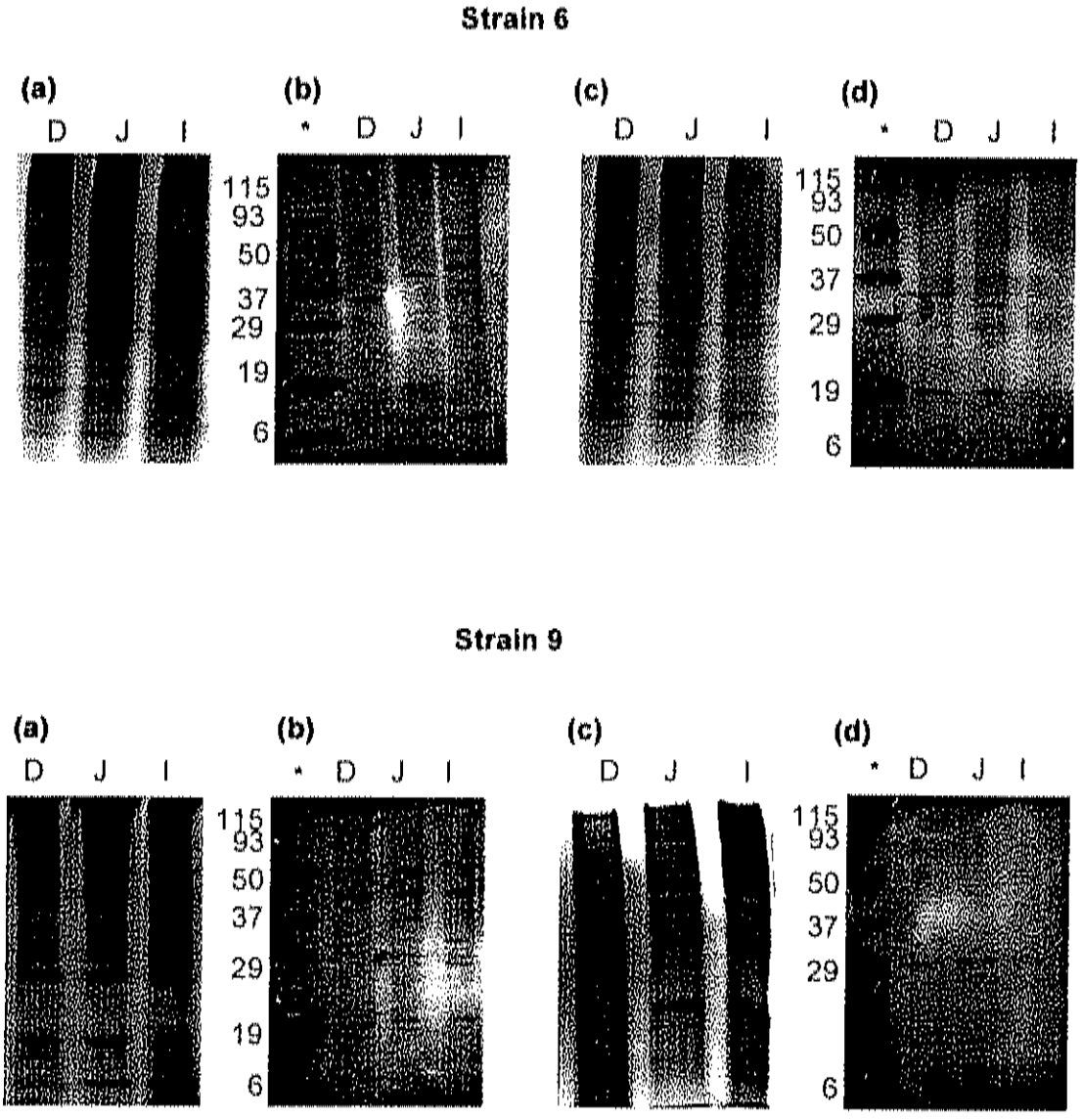
Strain 9
Strain 10
Adult Pig Mucin
Piglet Mucin

Figure 2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(a)
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(b)
* D J I
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50
37
29
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6
(c)
D J I
(d)
* D J I
115
93
50
37
29
19
6
Strain 9
(a)
D J I
(b)
* D J I
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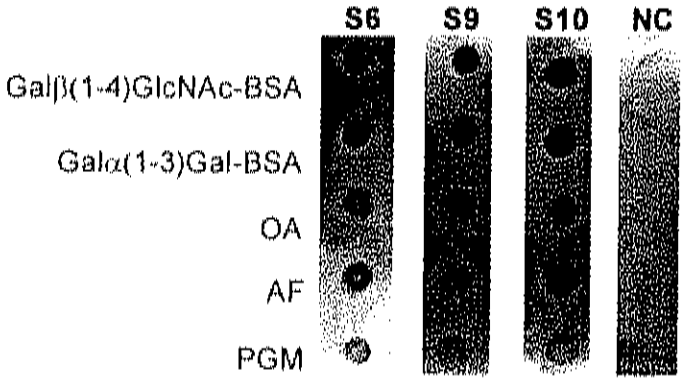
Figure 3



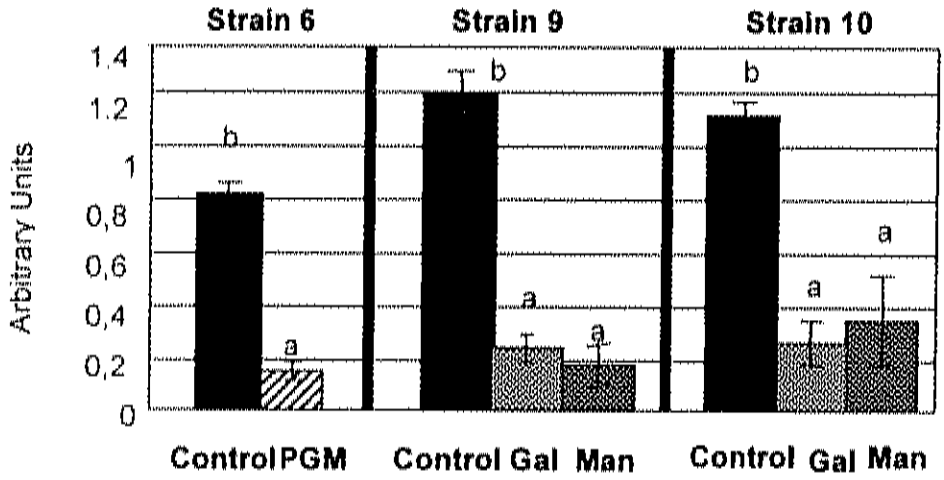
	Strain 6	Strain 9	Strain 10
Galβ(1-4)GlcNAc-BSA	1.33±0.16[b] AU	1.25±0.08[b] AU	1.35±0.19[b] AU
Galα(1-3)Gal-BSA	1.42±0.22[b] AU	1.2±0.18[b] AU	1.55±0.12[b] AU
Ovoalbumin (OA)	0.35±0.14[a] AU	0.26±0.11[a] AU	0.47±0.09[a] AU
Asialofetuina (AF)	0.62±0.26[a] AU	0.48±0.29[a] AU	0.6±0.23[a] AU
Pig gastric mucin (PGM)	0.46±0.18[a] AU	0.22±0.13[a] AU	0.38±0.09[a] AU

[a] Values in the same column with different superscript differs significantly ($p<0.05$).

AU= Arbitrary Units

± Standard Deviation

Figure 4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Strain 9
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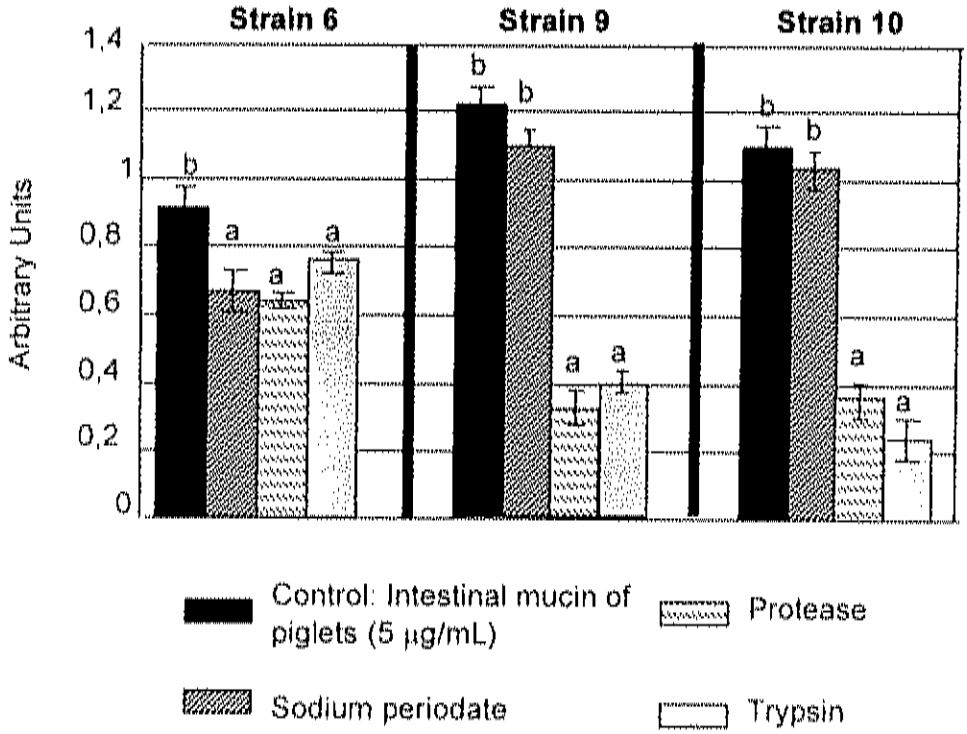
Control PGM
Control Gal Man
Control Gal Man

Figure 5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0,8
0,6
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a
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b
a
a
b
b
a
a
Control: Intestinal mucin of piglets (5 µg/mL)
Protease
Sodium periodate
Trypsin

ARTÍCULO 3

Evaluation of Adherence of *Lactobacillus* Strains to Cell Membranes by Blot Analysis and Optical Tweezers

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Abstract

Bacterial adhesion to intestinal mucosa is one of the most important criteria for selection of any potentially probiotic strain. Specific molecules on the bacterial cell surface mediate their adhesion to different surfaces. Little information is available on the effect that milk fat globule membrane (MFGM) and intestinal mucins components have on the adhesion of probiotic bacteria. We have combined two techniques, dot blot analysis and optical tweezers to characterize the binding properties of three *Lactobacillus* strains. Dot blot analysis has been proven to be helpful in the determination of the affinity of bacteria to specific components in milk or the surface of bacteria. Optical tweezers have many applications in measuring forces in biological systems due to their ability to measure piconewton (pN) scale forces and to manipulate biological particles with minimal damage. This work describes the construction and calibration of a dual-beam optical tweezers laser trap apparatus and the use of this trap to measure the adhesion force of probiotic strains to intestinal mucosa and milk fat globule membrane.

Key Words: *Lactobacillus*, adhesion, glycoprotein, optical tweezer

Introduction

Live microorganisms with documented health benefits to humans; mostly come from the *Lactobacillus* and *Bifidobacterium* genera. Adhesion to the intestinal mucosal layer is an important factor to determine the degree of effectiveness of lactic acid bacteria (LAB) as probiotic. Therefore it is important to consider the role that mucins have in the whole phenomena of adhesion of LAB and biological surfaces. Mucins are proteins heavily glycosilated that cover the intestinal epithelial cells and that are also present in the milk fat globule membranes (MFGM) (Mather, 2000). Certain strains have already been shown to bind to mucosa layer which suggests that carbohydrate moieties of glycoconjugates play a key part in host-probiotic interaction (Buck *et al.*, 2005). Bacterial adhesion has been studied, among other techniques, using western blots and ELISA (Mukai *et al.*, 2002; Buck *et al.*, 2005). We present a method for directly and objectively measuring the force of binding between bacteria and given surface.

Optical tweezers consist of an optical microscope in which the objective is used to focus a laser beam to a diffraction-limited spot in the image plane. A dielectric, nonabsorbing particle such as a glass bead or a bacterium in that plane will experience a force that tends to move it to the region of highest light intensity, namely the focal point of the laser (Figure 1A). Objects smaller than the wavelength of the laser light develop an electric dipole in response to the electric field associated with that light beam, and that dipole is drawn up intensity gradients towards the focus. Larger objects act as lenses, refracting the rays of light and redirecting the momentum of their photons. The resulting recoil draws them toward the higher flux of photons near the focus. This recoil is imperceptible for macroscopic lenses but can have substantial influence on mesoscopic objects. Moreover, a small scattering force, the magnitude of which is directly proportional to the light intensity, pushes the particle beyond the focal point of the laser.

Such laser tweezers can trap objects as small as 5 nm and exert forces exceeding 100 picoNewtons (pN). Fast photodiodes allow investigators to

detect the position of such objects at rates exceeding 10 kHz. By combining those analytic capabilities with particle-tracking techniques, they can measure displacements below 1 nm and forces as small as femtoNewtons (fN) (Fällman *et al.*, 2004).

The laser tweezers exert a force proportional to the deflection, and, therefore, the setup allows investigators to determine the force generated by the molecular machine. Moreover, when force is applied to the object in the trap, it is possible to measure the elastic response of a polymer to externally applied forces (Maier, 2006). While this technique has been used for over 20 years to manipulate and study the properties of micron-sized dielectric particles, it is only recently that his precise force measurement instrument has been applied to the study of biological systems (Williams, 2004).

Materials and Methods

Bacterial strains and culture conditions

Three strains were used in this experiment from the Cal Poly DPTC Collection: *Lb. salivarius* (named 10, directly isolated from piglet intestine), *Lb. acidophilus* (NCFM from ATCC catalog) and *Lb. gallinarum* (33199 from ATCC catalog). Each one was used to inoculate MRS broth with cysteine hydrochloride 0.5 gr/L (Difco) and incubated at 37° C for 18-24 h in 5% CO_2. From the resulting bacterial growth the cells were diluted and used freshly in our experiments.

Protein preparation

Intestinal mucin. Mucins were isolated from small intestine from healthy piglets. Extraction and dual ethanol precipitation as described by Tuomola *et al.* (2000) was conducted.

Milk Fat Globule Membrane Proteins. A solution with raw milk and sucrose at 5% concentration was made. Then PBS pH 7.0 was placed carefully. The solution was centrifuged at 1500 x *g* (GS-6R Beckman, USA) for 20 min at 25°C. The cream was resuspended in PBS until to obtain a mixture about

40% fat and then shacked for 5 minutes. The samples were warmed to 40°C and centrifuged 2800 x *g*. The aqueous phase (buttermilk) was recovered and sodium citrate (2% w/v) was added and stored overnight at 4°C. Buttermilks were centrifuged at 50000 x *g* (Beckman TLC1000, USA) at 4°C for 2 h. The pellet was collected and rinsed with deionized water using a bench top homogenizer (Polytron PT 3100, Brinkman, NY, USA). The solution was stored at -50°C (Corredig and Dalgleish 1997). Protein concentration was quantified by BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

Dot blot Assays

Bacteria were washed twice with PBS buffer pH 7.2 (0.02M) and adjusted to an OD_{460} of 1.0 with the same buffer. The cells were Sulfo-*N*-hydrosuccinimide (NHS)-biotin labeled (Pierce Rockford IL, USA) for 2 min at room temperature as previously described Mukai *et al.* (2002).

Mucins and proteins from MFGM were diluted, by serial dilutions, at 40 µg/mL concentration in PBS buffer. 15 µl of protein were immobilized in nitrocellulose membranes (BioRad, Hercules CA, USA).and the membranes were blocked with 2% bovine serum albumin (Sigma, St Louis MO, USA) in PBS buffer for 2 h at room temperature, prior to the addition of biotinylated bacteria. Following overnight incubation at 4° C, membranes were washed five times with PBS buffer to remove unbound bacteria and developed with Avidin-Peroxidase and 3,3'-diaminobenzidine (Sigma, St Louis MO, USA) to detect adhesion (Ruhl *et al* 1996). Intensity of the dots was measured with a GS-700 Imaging Densitometer & Molecular Analyst Software Ver 2 (BioRad Hercules CA, USA). Experiment was performed by triplicate.

Separation of Glycoproteins from Milk Fat Globule Membrane Proteins

We obtained the proteins from milk fat globule membranes as described above. A column 1x4 cm was packed with boronate gel (Pierce, Rocford, IL, USA) as described in the technical data sheet and used to obtain only the glycoproteins $\geq$ 50kDa.

The gel was equilibrated with 5 volumes of 0.1M HEPES (Sigma St. Louis MO, USA) pH 8.5 and then 300 µl of sample were loaded. The column was washed with 5 volumes of starting buffer at a flow rate of 1 ml/min. The proteins were eluted with a solution of 0.5M sorbitol (Sigma St. Louis MO, USA).

Proteins coupling to carboxylated microspheres

10µm carboxylated microspheres (Polysciences Inc., Warrington PA, USA) were used to couple proteins as described in the technical data sheet 238C. The concentration was estimated at 89 µg/mL on the spheres coated with intestinal mucins and the coated ones with MFGM protein at concentration of 150 µg/mL. The amount of protein concentration used for coating the microspheres with glycoproteins obtained from MFGM extract was 32 µg/mL. The measurement of proteins on the surface of the microspheres was made after through rinsing and is assumed uniform coating of the surfaces.

Optical tweezer (OT) system

The coated microspheres were incubated with *Lactobacillus* strains (OD_{450} of 0.02 with PBS pH 7.0) for at least 30 minutes at 37° C (500 µl of bacterial suspension with 25 µl of coated microspheres). The tweezers system at Cal Poly was built around a Zeiss Axiovert 200 microscope and uses a 1064 nm Nd:YVO laser. The system has two optical traps and one of the traps is steerable using a motorized gimbal mount.

The laser is strongly focused through a lens with a very short focal length (numerical aperture microscope objective =1.25). The optical system is schematized in figure 1B. A bacterial cell was trapped and pushed against the surface of a sphere for several seconds. The trap was then moved and the power adjusted until the bacterium could just be pulled from the sphere (Fällman *et al.*, 2004, Simpson *et al.*, 2004).

Stokes drag $F=3\pi\eta\delta\upsilon\kappa$ was used to get force measurements (F= drag force, η= medium viscosity, δ= cell diameter, υ= velocity of stage movement and κ= correction factor) (Simpson *et al.*, 2002).

Statistical Analysis

ANOVA and Tukey-Kramer test were used for the mean comparisons ($p<0.05$) of the dot blot assays, with the statistical package NCSS 6.0 (Hintze, 1997).

Results and Discussion

Dot blot assays showed that the three strains had greater affinity to MFGM proteins than intestinal mucin ($p<0.05$). This could be explained by the large amount of glycoproteins in the MFGM extract compared to the intestinal mucin we observed in our preparation (data not shown). The MFGM contains proteins like MUC1, MUC15 and PAS 6/7 which are highly glycosilated. Therefore, it is possible that the interaction of the bacteria with the carbohydrate moieties of these proteins increased the binding since they are more exposed on the MFGM (Mather, 2000). Also, higher bacterial adhesion to intestinal mucins of piglets was observed for strain 10 in respect to strains NCFM and 33199, whereas with MFGM there were no differences (Figure 2). It could be because strain 10 was originally isolated from piglet intestine and the other strains from dairy product (NCFM) and hen intestine (33199) respectively

From a large number of trials, no sticking events were seen with the intestinal mucin coated spheres for NCFM and 33199 strains. This is in agreement with the dot blot assays results. Strain 10 had such a strong binding force that it could not be removed in many attempts using the highest setting in the OT. Just in one instance, the particle was removed with a force of 10 pN (Table 1). This inconsistence is perhaps due to the small size of the cell and the difficulty of trapping the particle in a consistent way with the OT.

On the other hand, we pushed NCFM and 33199 strains against a 10 μm polystyrene sphere coated with MFGM (Figure 3). With some approximation and calculations, we were able to measure, with significant confidence the binding force. We were able to estimate the detachment force in a range from 12 to > 50 pN for NCFM and <6 to 10 pN for 33199 strain (Table 2). Previous variability is comparable to the forces measured by Simpson *et al.* (2002, 2003 and 2004) using *Staphylococcus aureus* or *S. epidermis* and fibronectin like receptor. That variation could be related to the number of bonds formed at the binding point (Bjorham *et al.*, 2005). From about 10 trials with strain 10, once the bacterium was adhered to the spheres, it could not be removed, even at the highest laser power.

Although dot blot assays did not show differences between strains in adhesion to MFGM, our results with OT indicate that the strain 33199 does not bind as strongly as the NCFM strain, deduced from the fact that we never saw permanent attachment of the strain 33199. When strain 10 was attached to the sphere, it appeared impossible for the sphere not to interfere with the trapping beam. This effect could be due to the small size of that bacterium (Simpson *et al*; 2002), since it was not observed with the other two longer strains.

Trying to prove that the carbohydrates play an important role in the bind of our *Lactobacillus* strains to MFGM proteins, we obtained a fraction rich in glycoproteins from MFGM using a boronate gel. Boronate is known to form reversible complexes with sugars (Lodoño and Bendayan, 2001). In that manner, we were able to separate the MFGM glycoproteins with molecular mass greater than 50 kDa (Figure 4). This MFGM glycoprotein extract was coated to the microspheres and used to confirm that the glycoproteins were involved in the adhesion. We could confirm that the glycoproteins are involved in the adhesion. For example, the force needed to detach the NCFM strain from microspheres was generally >50pNwith the purified MFGM glycoproteins extract (Table 3) while with the more complex MFGM extract (containing less glycoproteins) weaker interactions were observed: from no-binding to 12 -25 pN (Table 2). A similar trend was observed with the 33199

strain (Table 3). We observed again that the NCFM strain had stronger adhesion to glycoproteins than 33199 (Table 3). In a previous work using OT, *H. pylori* showed similar binding force (25 pN) to its fucosylated receptor (Bjorham *et al.*, 2005). Liang *et al*; (2000) quantified the adhesion of E. coli pili to carbohydrate-coated surfaces corresponding to a force of about 1.7 pN (very small compare to our results). Based on those observations we think that we quantified specific interactions because the nonspecific ones are on the order of nanoNewtons (nN). This is several orders in magnitude greater than the forces we can generate with OT that are best applied to study smaller specific binding forces (Simpson *et al.*, 2003).

Regardless of the technical details and the fact that we have a non optimized procedure, this work presents a proof of concept and some valuable valid data that indicates the importance of the carbohydrates in the process of adhesion. We also showed a clear difference in the binding force between strains of LAB and the usefulness of the OT as a tool for these measurements. Future developments will indeed help us to understand the binding phenomena between LAB and biological surfaces.

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References

1.- Bjorham O., Fallman E., Axner O., Ohlsson J., Nilsson U., Boren T. and Schedin S. 2005. Measurements of the binding force between the *Helicobacter pylori* adhesin BabA and the Lewis b blood group antigen using optical tweezers. J Medical Optics. 10, 44124-44132.

2.- Buck B., Altermann E. Svingerud T. and Klaenhammer T. 2005. Functional Analysis of putative adhesion factors in *Lb. acidophilus* NCFM. Appl. Environ. Microbiol. 71, 8344-8351.

3.- Corredig M. and Dalgleish D. 1997. Isolates from industrial buttermilk: Emulsifying properties of material derivated from the milk fat globule membrane. J. Agr. Food Chem. 45, 4595-4600.

4.- Fällman E, Schedin S, Jass J, Andersson M, Uhlin B & Axnir O. 2004. Optical Tweezers Based Force Measurement System for Quantitating Binding Interactions: System Design and Application for the Study of Bacterial Adhesion. Biosensors and Bioelectronics. 19, 1429-1437.

5.- Hintze, J.L. 1997. NCSS 97 *user's guide-II [Computer software manual.]* Kaysville, UT: Number Cruncher Statistical Systems.

6.- Liang M., Smith S., Metallo S., Choi I., Prentiss M. and Whitesides G. 2000. Measuring the forces involved in polyvalent adhesion or uropathogenic E. coli to mannose-presenting surfaces. Proc. Natl. Acad. Sci. 97:13092-13096.

7.- Lodoño I., and Bendayan M. 2001. Temporary effects of circulating Amadouri products on glomerular filtration properties in the normal mouse. Am. J. Physiol. Renal. Physiol. 280: 103-111.

8.- Maier B. 2006. Manipulation of single molecules in living bacteria. Microbe. 1: 330-335.

9.- Mather I. 2000. A review and proposed nomenclature for major proteins of the milk-fat globule membranes. J. Dairy Sci. 83: 203-247.

10.- Mukai T., Asasaka T., Sato E., Mori K., Matsumoto M. and Ohori H. 2002. Inhibition of binding of *Helicobacter pylori* to the glycolipid receptors by probiotic *Lactobacillus reuteri*. Immun. Medical Microbiol. 32: 105-110.

11.- Ruhl S., Sandberg A., Cole M. and Cisar J. 1996. Recognition of Immunoglobulin A1 by Oral Actinomyces and Streptococcal Lectins. Infect. Immun. 64: 5421-5424.

12.- Simpson KH, Bowden MG,, Höök M and Anvari B. 2002. Measurement of Adhesive Forces Between *S. epidermis* and Fibronectin-Coated Surfaces Using Optical Tweezers. Lasers in Surgery and Medicine.52: 31-45.

13.- Simpson K., Bowden G., Hook M. and Anvari B. 2003.Measurement of adhesive forces between individual Staphylococcus aureus MSCRAMMs and protein coated surfaces by use of optical tweezers. J Bacteriol. 185: 2031-2035.

14.- Simpson K, Bowden MG, Peacock J, Arya M, Höök M and Anvari B. (2004). Adherence of *Staphylococcus aureus* fibronectin binding protein A mutants: an investigation using optical tweezers. Biomol Engineering. 21: 105-111.

15.- Tuomola E., Ouwehand A. and Salminen S. 2000. Chemical, physical and enzymatic pre-treatments of probiotic lactobacilli alter their adhesion to human intestinal mucus glycoproteins. Int J Food Microbiol. 60: 75-81.

16.- Williams M. 2004. Optical Tweezers: Measuring piconewton forces. Biophysics J. 83: 1932-1945.

Figure Legends

Figure 1. a) Basic principles for the trapping of a bacterium with an optical tweezer system (Picture from Maier, 2006). **b)** Schematic view of optical tweezer system.

Figure 2. Evaluation of adhesion of *Lactobacillus* strains to intestinal mucins and MFGM proteins by dot blot analysis

[a] Values with different superscript differs significantly ($p<0.05$)

Figure 3. a) MFGM coated sphere and 33199 strain in the trap. The bacteria, which are long, tend to point straight up in the traps when unattached and appear circular. **b)** Bacterium just as we push it against the sphere. **c)** Bacterium being pulled with one end of it attached to the sphere. **d)** Bacterium has just detached.

Figure 4. SDS-PAGE of the Separation of Glycoproteins present in MFGM using a boronate gel.

Lane a = Molecular weight marker, **Lane b**= MFGM proteins **Lane c**= Glycoproteins obtained by elution with 0.5M sorbitol, **Lane d**= PAS stain to confirm presence of glycoproteins in the elution with sorbitol.

Figure 1.

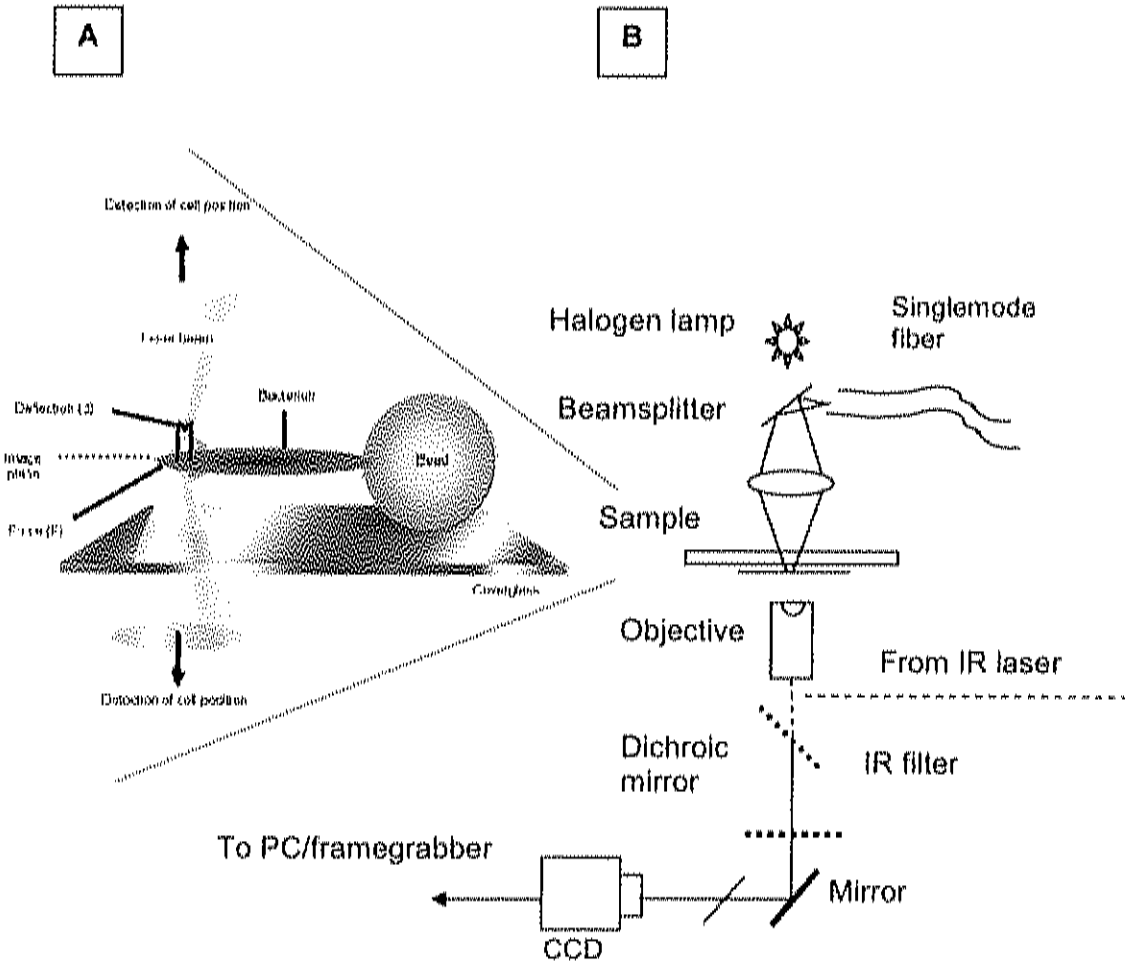
A
B
Halogen lamp
Singlemode fiber
Beamsplitter
Sample
Objective
From IR laser
Dichroic mirror
IR filter
To PC/framegrabber
Mirror
CCD

Figure 2.

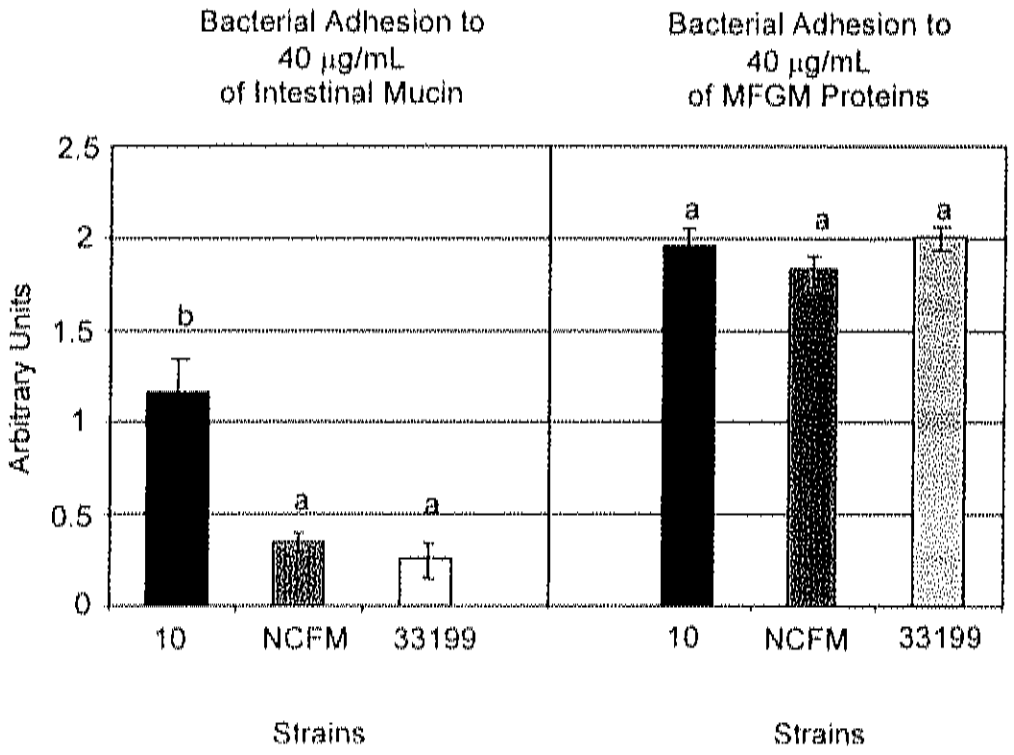
Bacterial Adhesion to
40 µg/mL
of Intestinal Mucin
Bacterial Adhesion to
40 µg/mL
of MFGM Proteins
Arbitrary Units
2.5
2
1.5
1
0.5
0
b
a
a
a
a
a
10
NCFM
33199
10
NCFM
33199
Strains
Strains

Figure 3.

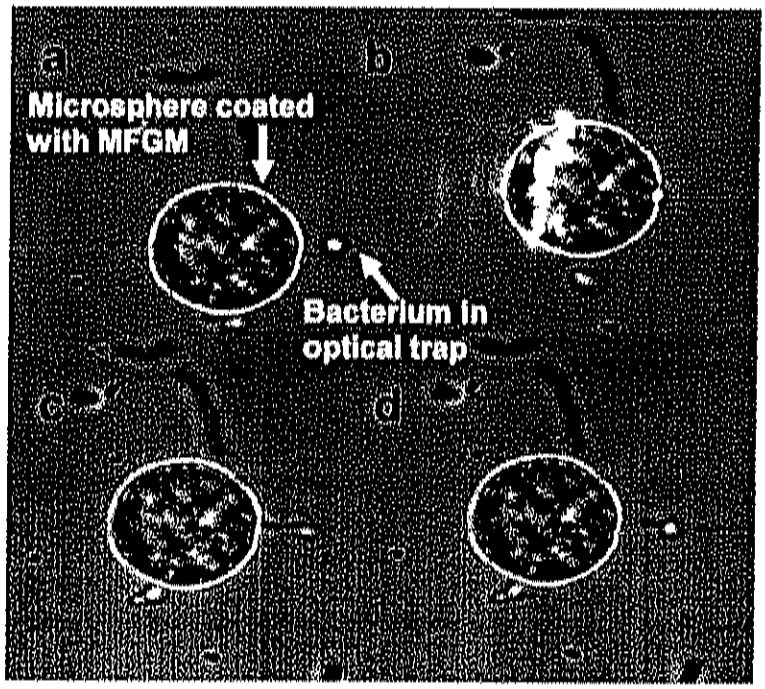
a
b
Microsphere coated with MFGM
Bacterium in optical trap
c
d

Table 1.

Interaction forces between *Lactobacillus salivarius* (strain 10) and microspheres coated with intestinal mucins from piglets as measured by OT

Repetitions	Force for Removal if Applicable (pN)
34	Could not be detached
1	10 pN

Table 2.

Interaction forces between *Lb. acidophilus* (NCFM) or *Lb. gallinarum* (33199) with microspheres coated with MFGM proteins as measured with an OT

NCFM strain

Repetitions	Force for Removal if Applicable (pN)
23	Could not be detached (>50 pN)
2	12 pN
2	15 pN
4	25 pN
11	No bind to the microspheres

33199 strain

Repetitions	Force for Removal if Applicable (pN)
12	Easily removed <6 pN
2	10 pN
2	8 pN
5	6 pN
21	No bind to the microspheres

* Weakest binding that could be measured with the Strokes Drag

Figure 4.

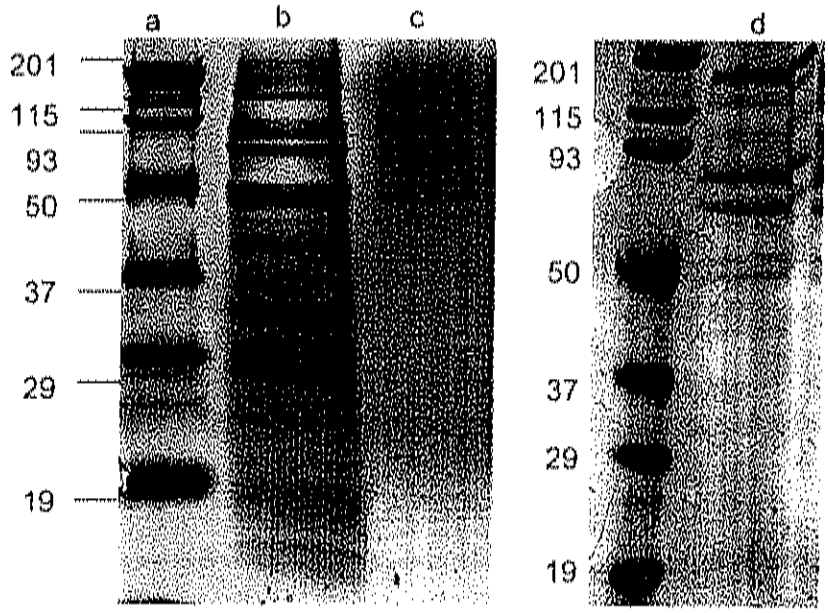
a
b
c
d
201
115
93
50
37
29
19
201
115
93
50
37
29
19
Gel Code
Blue Stain
PAS Stain for
Glycoproteins

Table 3.

Interaction forces between *Lb. acidophilus* (NCFM) or *Lb. gallinarum* (33199) with microspheres coated with purified MFGM glycoproteins as measured with an OT

NCFM strain

Repetitions	Force for Removal if Applicable (pN)
40	Could not be detached
2	25 pN

33199 strain

Repetitions	Force for Removal if Applicable (pN)
6	10 pN
12	12 pN
8	15 pN
1	25 pN
15	No bind to the microspheres