Departamento de Medicina y Cirugía Animal
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"*Evaluación del efecto probiótico de las cepas Lactobacillus reuteri CECT7266 y Lactobacillus fermentum CECT7265 en perros sanos*"

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Memoria presentada para optar al Grado de Doctor
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CERTIFICA

Que la tesis titulada "**Evaluación del efecto probiótico de las cepas Lactobacillus reuteri CECT7266 y Lactobacillus fermentum CECT7265 en perros sanos** " presentada por la Sra. Núria Sánchez Alzuria para optar al grado de Doctor en Veterinaria se ha realizado bajo mi dirección y, considerando está acabada, autorizo su presentación para que sea juzgada por la comisión correspondiente.

Y para que así conste a los efectos que correspondan, firmo la presente

En Bellaterra (Cerdanyola del Vallès), el 30 de noviembre de 2015.

Teresa Rigau i Mas

Celina Torre Lloveras , directora del departamento de R&D de Affinity Petcare S.A.U.

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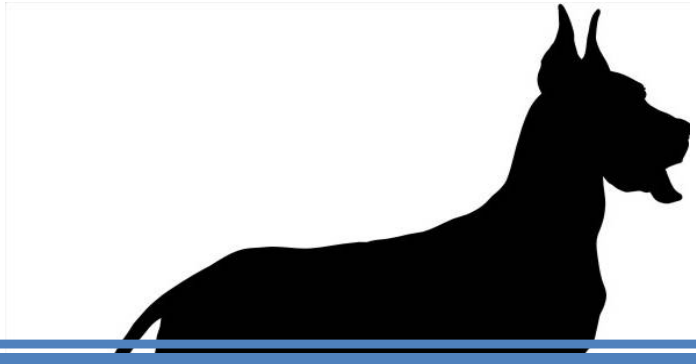


INTRODUCCION

I. INTRODUCCIÓN

Las superficies corporales, piel y mucosas, constituyen la puerta de entrada de microorganismos invasores, patógenos o no. Por esta razón, todos los seres vivos plantean a este nivel su primera línea defensiva. La microbiota de la piel y mucosas representa el sistema crítico de defensa, con múltiples intervenciones tanto directas como indirectas (Rodríguez, 2007).

"La promesa de la investigación del microbioma pasa en gran medida por el futuro de los probióticos. Con el tiempo, puede llegar a ser posible restaurar la salud de un microbioma empobrecido, simplemente tragando una cápsula repleta de miles de millones de células bacterianas, o por el consumo de yogur " (Specter, 2012).

1. PROBIÓTICO

El termino *probiótico* deriva del griego “pro” y “bios” y significa “para la vida”. Lilly y Stillwell (1965) utilizaron por primera vez esta palabra para describir “substancias secretadas por un microorganismo que estimulan el crecimiento de otros”, y esto contrasta con el término *antibiótico*. Los griegos sabían por experiencia que la leche coagulada era un buen remedio contra los males estomacales, de hígado o de enfermedades respiratorias. Plinio (siglo I a.C), historiador romano recomendaba la ingestión de leche fermentada para tratar gastroenteritis (Schrezenmeir y Vrese, 2001). Su uso se remonta al Antiguo Testamento donde se dice que Abraham atribuye su longevidad al consumo de leche (Vasiljevic y Shah, 2008).

La definición actual propuesta por la FAO (Food and Agricultura Organization) y por la WHO (World Health Organization) es de “microorganismos vivos, que cuando se administran en cantidades

adecuadas, confieren un beneficio de salud al huésped" o "microorganismos vivos, que cuando se ingieren (incluidos en la comida o en fórmula de cápsula), se aplican localmente (en tracto urogenital) o en modo aerosol (en tracto respiratorio superior), dan al consumidor uno o varios beneficios probados para la salud".

El primero en observar que existían microorganismos vivos beneficiosos para la salud fue el microbiólogo ucraniano Élie Metchnikoff, que consiguió el Nobel de Medicina en 1908 por descubrir el mecanismo de acción de las leches fermentadas (yogures, leche búlgara) y sus efectos beneficiosos para la salud humana gracias a los cambios que producían en el balance de la microflora intestinal. El consumo de yogur que contenía bacterias productoras de ácido láctico provocaba la reducción de las bacterias productores de toxinas en el intestino y esto se relacionó con el incremento de la longevidad de estas personas. Fue también el primero en dar nombre al *Lactobacillus bulgaricus*, en honor a los lácticos que consumían los búlgaros (Mackowiak, 2013).
Estos microorganismos además de producir efectos beneficiosos por sí mismos, mejoran las propiedades de la flora autóctona (Tijdschr Tandheelkd, 1992).

Los requisitos que ha de cumplir un microorganismo para ser considerado probiótico son los siguientes:

- formar parte de la microbiota del intestino
- no ser patógeno ni toxicogénico
- mantenerse viable en medio ácido del estómago y en contacto con la bilis del duodeno
- poseer capacidad de adhesión a las células epiteliales intestinales
- adaptarse a la microbiota intestinal sin desplazar a la ya nativa o existente

- producir sustancias antimicrobianas
- tener capacidad para aumentar de forma positiva las funciones inmunes y las capacidades metabólicas (West et al. 2009)

1.1 Tipos de bacterias probióticas

Las bacterias probióticas más comúnmente usadas son los Lactobacilos (o bacterias ácido lácticas) y las Bifidobacterias (*Turroni et al*., 2014). Aunque hay un tipo de levaduras que pueden considerarse probioticas. Entre ellas se encuentran las siguientes: Saccharomyces boulardii (reconocida como líder), Saccharomyces cerevisiae, Kluyveromyces lactis y Kluyveromyces fragilis (Morales, 2004; Bekatorou *et al.*, 2004; Coenen *et al.*, 2000; Kumura *et al.,* 2004).

Las bacterias ácido-lácticas (BAL) constituyen un grupo de bacterias Gram-positivas anaerobias estrictas o aerotolerantes, generalmente inmóviles, no esporuladas ni pigmentada, denominadas así debido a que producen ácido láctico durante la fermentación de carbohidratos, provocando la acidificación del medio. Dentro de las BAL se encuentran en los géneros *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcu, Tetragenococcus, Vagococcus* y *Weissell* (Salminen *et al.*, 2004). Desde el punto de vista filogenético, sin embargo, *Propionibacterium* y *Bifidobacterium* no están emparentadas con las BAL. (Scheilfer *et al.*, 1995). Aunque las *Bifidobacterium*, se consideran que actúan en el mismo contexto que las bacterias acido lácticas, comparten características típicas y poseen un modo único de fermentación de los azúcares (Klaenhammer y Muller, 1999).
Los géneros más importantes y las características más significativas de los Lactobacilos y las Bifidobacterias son:

Los lactobacilos son bacilos regulares Gram-positivos, no esporulados, capaces de colonizar hábitats tan diversos como el material vegetal, los productos lácteos y la piel y las mucosas del hombre y los animales (Klander y Weiss, 1986). El pH óptimo de crecimiento oscila entre 5,5 y 6,2. En cuanto a la temperatura pueden ser mesófilas o termófilas. En el género se incluyen más de 100 especies con propiedades muy heterogéneas. Esta diversidad se ve reflejada genotípicamente, ya que la proporción de G+C de las distintas especies varía entre un 32% y un 52%. Las especies se clasifican como mesófilas o termófilas, de acuerdo a su temperatura óptima de crecimiento, y en homofermentadoras, heterofermentadoras facultativas o heterofermentadoras estrictas, dependiendo de sus características fermentativas.

Clasificación de los *Lactobacillus*:

Grupo I. Especies homofermentadoras estrictas. Comprende el grupo acidophilus formado por las especies: *Lactobacillus acidophilus*, *L. amilovorus*, *L. crispatus*, *L. gallinarum*, *L. gasseri* y *L. johnsonii*. También se incluyen en este grupo otras especies como *L. delbrueckii*, *L. helveticus*, *L. jensenii*, *L. leichmanii* y *L. salivarius*.

Grupo II. Especies heterofermentadoras facultativas. Las especies principales de este grupo son *L. casei*, *L. curvatus*, *L. plantarum*, *L. pentosus* y *L. sakei*.

Grupo III. Especies heterofermentadoras estrictas. Entre otras, el grupo incluye las especies *L. brevis*, *L. buchneri*, *L. cellobiosus*, *L. fermentum*, *L. reuteri* y *L. viridescens*.

2.- Bifidobacterium. Las bifidobacterias son microorganismos Gram-positivos, anaerobios estrictos, inmóviles, no esporulados y, en su mayoría, catalasa negativos. Su pH óptimo de crecimiento se sitúa entre 6,0 y 7,0 y su temperatura (de crecimiento) alrededor de 37ºC.

Presentan una gran variedad de formas (cocoide, con bifurcaciones, con cadenas estrelladas o extremos espatulados). Su nombre hace referencia a las formas con dos ramas en Y o V que muestran en ocasiones. El género está compuesto por 29 especies, de las que unas 12 se aíslan (se han aislado) del tracto gastrointestinal humano y el resto proceden de ambientes tan diversos como alimentos, cavidad oral, (y) tracto gastrointestinal animal, intestino de insectos y aguas residuales (Tannock, 2005). Las bifidobacterias se distinguen de las BAL por su alta proporción de G+C (>50%). También es característica del género la presencia del enzima fructosa-6-fosfato fosfocetolasa, que conduce a la formación de ácido láctico y ácido acético en una proporción 2:3 sin generación de gas.

Estudios realizados en diversas especies, incluida la humana, demuestran que Lactobacilos y Bifidobacterias inhiben una gran variedad de patógenos, incluyendo *E. coli, Salmonella, Helicobacter pylori, Listeria monocytogenes y Rotavirus* (Chenoll *et al.,* 2011; Sgouras *et al.*, 2004; Todoriki *et al.*, 2001; Chu *el al.*, 2005; Makras *et al.*, 2006; Muñoz *et al.*, 2011; Nakamura *et al.,* 2012). Para ganar ventaja competitiva, las bacterias también pueden modificar su entorno y hacerlo menos apropiado para los competidores. La producción de substancias antimicrobianas, como ácido láctico y acético, es un ejemplo de esta modificación del entorno (Schiffrin y Blum, 2002).

1.2 Mecanismos de acción de los probióticos

Los probióticos son cruciales para el bienestar y la salud del hospedador. Estas bacterias representan una herramienta efectiva en el control y prevención de enfermedades (Kim et al. 2006), ya que son capaces de interferir en el crecimiento y la virulencia de patógenos (Basu et al. 2007; Lee et al. 2005)

Los principales mecanismos de acción de los probióticos incluyen la mejora de la barrera epidérmica y el incremento de la adhesión a la mucosa intestinal junto con el efecto de inhibición de la adhesión de patógenos. Además realizan una exclusión competitiva con los microorganismos patógenos, producen substancias anti-microbianas y tienen la capacidad para modular el sistema inmunitario (Figura 1) (Bermúdez-Brito *et al.*, 2012).

Las bacterias probióticas son capaces de tratar y prevenir desórdenes intestinales, reduciendo el pH, potenciando la reparación de la mucosa (Hickson *et al.* 2007) y aumentando la proliferación de anaerobios (Apostolou *et al.* 2001).

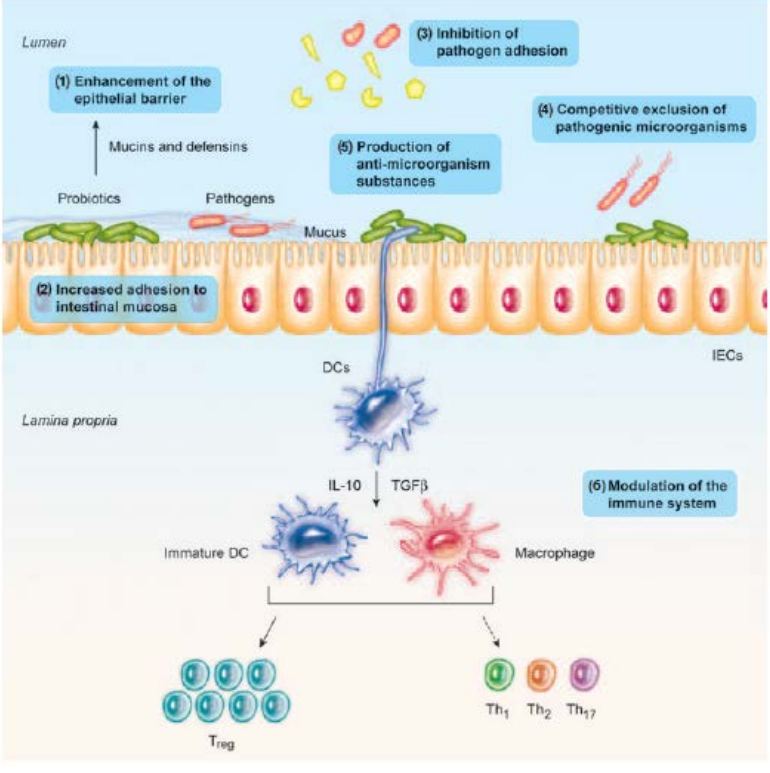


Figura 1. Representación esquemática de los mecanismos de acción de los probióticos (Bermúdez-Brito *et al.*, 2012).

1.2.1 Adhesión a la mucosa intestinal

Un requisito indispensable para que se produzca la colonización de las células epiteliales gastrointestinales por parte de los probióticos es la adhesión intestinal. Esta adhesión se relaciona con los efectos beneficiosos de las bacterias probioticas (Castagliuolo *et al.,* 2005) y además es necesaria para que se produzca la modulación del sistema inmunitario (Schiffrin *et al.,* 1997; Perdigon *et al.,* 2002) y la competición contra los patógenos (Hirano *et al.,* 2003).

Las células intestinales secretan moco, el cual recubre las vellosidades del epitelio del tracto digestivo y tiene muchas funciones, entre ellas, proteger al huésped contra la colonización bacteriana mediante la modificación o la inhibición de la asociación bacteriana con la superficie de la mucosa, pudiendo prevenir la adhesión de bacterias patógenas (Collado *et al.,* 2005; González-Rodríguez *et al.,* 2012; Juntunen *et al.,* 2001). Así mismo, el moco puede proporcionar un hábitat idóneo para algunas bacterias y es posible que sirva de nicho ecológico tanto para flora comensal beneficiosa o para microorganismos potencialmente patógenos (Freter, 1984; Neutra and Forstner, 1988).

Las bacterias acido-lácticas muestran varios determinantes de superficie (proteínas de superficie) que permiten la interacción con las células epiteliales intestinales y con el moco intestinal. Esta interacción específica podría explicar la competencia que se produce hacia bacterias patógenas (Beachey, 1981; Wadström *et al.*, 1987; Conway *et al.*, 1989; Henriksson *et al*., 1991; Coconnier *et al.,* 1992; Schneitz *et al.,* 1993; Rojas and Conway, 1996; Schiffrin, 1997; Haller *et al.,* 2001; Ouwehand *et al.*, 2002a; van Tassell y Miller, 2011).

Otros investigadores demostraron la unión mediante proteínas de superficie de los Lactobacillus a líneas celulares secretoras de moco (Coconnier *et al.,* 1992) y que los lactobacilos colonizaban la capa de moco del intestino delgado de los lechones y que los compuestos proteicos estaban involucrados en la unión de *Lactobacillus fermentum*104R (Rojas and Conway, 1996).

Una de las proteínas de superficie estudiadas es la adhesina, sintetizada por diversas cepas de *Lactobacillus*., y al igual que otras proteínas de superficie, intervienen en la unión a la barrera que forma la mucosa intestinal (Buck *et al.*, 2005). La mezcla de diferentes probióticos han demostrado incrementar la síntesis de mucinas de superficie y la capacidad de modular la expresión génica de la mucina en la adhesión de células bacterianas al epitelio intestinal (Caballero-Franco *et al.*, 2007).

El ejemplo mejor estudiado de adhesión mucoso-adhesinas bacterianas y su posterior colonización en tracto gastrointestinal, es el producido por el *Lactobacillus reuteri* (Hynönen *et al.,* 2002). Uno de los mecanismos estudiados es la adhesión de las proteínas de unión al colágeno, las cuales se han aislado y purificado de la superficie de diferentes *Lactobacillus reuteri* (Roos *et al.*, 1996; Alejund *et al.*, 1991; Alejund *et al.*, 1994; Styriak *et al.*, 1999). El *L. fermentum* se une al moco intestinal gracias a la presencia de proteínas facilitadoras de unión (Henriksson *et al.*, 1991; Rojas *et al.*, 2002).

1.2.2 Exclusión competitiva de microorganismos patógenos

La exclusión competitiva de los microorganismos patógenos es el mecanismo más importante del efecto beneficioso de los probióticos (Adlerbeth *et al.,* 2000).

Los mecanismos que una especie bacteriana utiliza para excluir o reducir el crecimiento de otras especies es variado e incluye: la creación de un entorno hostil, la eliminación de puntos de unión

disponibles para las bacterias, la producción y secreción de substancias antimicrobianas y metabolitos selectivos y la competición por los nutrientes esenciales (Rolfe, 1991).

Las propiedades de adhesividad específicas relacionadas con la interacción entre proteínas de superficie y mucinas podrían inhibir la colonización de bacterias patógenas y son el resultado de la actividad antagonista de muchas cepas de probióticos contra patógenos gastrointestinales (Servin, 2004). Algunas cepas de Lactobacilos y Bifidobacterias comparten sitios de unión a carbohidratos con bacterias entero patógenas (Nesser *et al.*, 2000; Fujiwara *et al.*, 2001), lo que hace posible la competencia por los lugares de unión a las células del huésped (Mukai *et al.*, 2002).

1.2.3 Producción de substancias antimicrobianas

Otros efectos beneficios para la salud que ofrecen los probióticos incluyen, la formación de compuestos de bajo peso molecular (< 1000 Da), como los ácidos orgánicos y la producción de sustancias antibacterianas denominadas bacteriocinas (>1 1000 Da).

Los ácidos orgánicos, especialmente ácido acético y ácido láctico, provocan una disminución del pH en el tracto gastrointestinal y esto tiene un fuerte efecto inhibidor contra bacterias Gram negativas y se consideraban el compuesto antimicrobiano principal responsable de la actividad inhibidora de los probióticos contra los patógenos (Alakomi *et al.*, 2001; De Keersmaecker *et al.*, 2006; Makras *et al.*, 2006). La forma no disociada del ácido orgánico entra en la célula bacteriana y se disocia dentro de su citoplasma. La eventual disminución del pH intracelular o la acumulación intracelular de la forma ionizada del ácido orgánico puede conducir a la muerte del patógeno (Ouwehand, 1998; Russell and Diez-González, 1998).

Las bacteriocinas actúan de diferentes maneras, destruyendo las células patógenas o inhibiendo la síntesis de la pared celular (Hassan *et al.*, 2012). La producción de bacteriocinas confiere a las cepas productoras una ventaja competitiva dentro de los entornos microbianos complejos y como consecuencia una actividad antimicrobiana asociada. La producción de bacteriocinas podría permitir el establecimiento y aumentar la prevalencia de las cepas productoras, así como, permitir la inhibición directa del crecimiento de patógenos dentro del tracto gastrointestinal (O'Shea *et al.*, 2012).

Algunas cepas de bifidobacterias poseen la capacidad de estimular la producción de defensinas por parte de las células epiteliales. Estas proteínas son activas frente bacterias, hongos y virus, además de estabilizar la barrera intestinal (Furrie *et al.*, 2005).

1.2.4 Acción sobre el sistema inmunitario

El intestino constituye una pieza clave del sistema inmunitario, por este motivo el sistema inmunitario intestinal (Stockinger *et al.*, 2011) representa el mayor órgano inmunológico del cuerpo, (Artis, 2008) ya que contiene la mayor colección de células inmunocompetentes del organismo (Alverdy y Chang, 2008). Para el correcto funcionamiento del intestino, es necesario que se establezca un equilibrio entre el sistema inmunitario intestinal y la microbiota (van den Abbeele *et al.,* 2011). Así mismos en las primeras etapas de vida, la colonización de intestino por parte de la microflora es esencial para el desarrollo normal de las respuestas inmunitarias celular y humoral (Hooper y Gordon, 2001).

El sistema inmunitario intestinal está compuesto por el tejido linfoide asociado al intestino o GALT, agregados linfoides en el intestino grueso y células inmunitarias diseminadas a lo largo de la lámina propia y del epitelio del tracto gastrointestinal (Cerovic *et al.*, 2009; Aureli *et al.,* 2011), que se encuentran e contacto con el resto del

sistema inmunitario vía nódulos mesentéricos linfoides locales (Rakoff-Nahoum y Medzhitoz, 2008).

La entrada de sustancias antigénicas de mayor tamaño se produce por sitios especializados de la mucosa intestinal, las Placas de Peyer (Singh *et al*,. 2009). Estas placas están constituidas por células B, células plasmáticas productoras de inmunoglobulina (Salzman, 2011), rodeados de Células T, células dendríticas y macrófagos. (Sipos *et al.*, 2010). Estas células son esenciales para la modulación de la respuesta inmunitaria intestinal (Van Wijk & Knippels, 2007) e inflamatoria o tolerancia (Ishii et al., 2010). Su superficie está recubierta por algunas células secretoras de moco y unas células epiteliales con pocas vellosidades, las células M. (microfold) (Lotz *et al*., 2007). Las células M transportan antígenos luminales, incluyendo bacterias (Soloff y Barratt-Boyes, 2010), sin modificarlos y los liberan intactos en las placas de Peyer (Tezuka y Ohteki, 2010)

Para el correcto funcionamiento y defensa del organismo, el sistema inmuntario intestinal debe diferenciar entre patógenos y bacterias comensales (Lee y Mazmanina, 2010), activando mecanismos de defensa contra patógenos (Westendorf *et al*. 2010), mientras evita respuestas inflamatorias hacia las microbiota comensal (Coombes y Powrie, 2008).

Otra de las células importantes dentro del sistema inmunitario intestinal, son las células dendríticas. Presentan dos estadios funcionales: células inmaduras y maduras. Las células inmaduras se encuentran en tejidos periféricos, y tras detectar productos microbianos o entrar en contacto con citoquinas pro-inflamatorias, se transforman en células maduras. En este momento se reduce la captura de antígenos pero aumenta la capacidad de estimulación de células T (López *et al*,. 2010; Macsonald *et al*., 2011).

El intestino contiene un gran número de linfocitos, incluso en ausencia de inflamación (Atarashi *et al*., 2011). Existen diferentes poblaciones

de linfocitos T, los linfocitos T citotóxicos o $CD8^+$ y los $CD4^+$. Estos últimos se dividen en T cooperadoras o helper y las T reguladoras. Dentro de las T reguladoras, las más conocidas son las Th1, son células inflamatorias, que liberan interferón gamma y el factor de necrosis tumoral alfa (TNF-α), dando como resultado la activación de macrófagos y producción de IgG (Jutel y Akdis, 2011). Otras son las Th2 que producen IL-4, IL-6 e IL-13, citoquinas asociadas a la producción de inmunoglobulinas y eosinofilia.

Y finalmente las Th17, que juegan un papel importante en la defensa frente a infecciones producidas por hongos o bacterias (Dubin y Kolls, 2008). Las Th17 producen IL-17, IL-21, Il-22 y TNF-α, que aumentan las respuesta de neutrófilos y la defensa de mucosas aumentando la expresión de péptidos antimicrobianos (Westendorf *et al*., 2010).

Las células dendríticas son las únicas capaces de determinar el tipo de respuesta: tolerancia, inflamatoria o regulatoria (Ng *et al*., 2010).

Las bacterias, virus, protozoos y hongos inician la respuesta inmunitaria innata, la cual induce la activación de neutrófilos, monocitos y macrófagos, células dendríticas, células NK y sistema de complemento (Neish, 2009). Los receptores reconocedores de patrones (PRR) son clave en el reconocimiento y control de patógenos (Willin *et al.,* 2010). Estos receptores se expresan en células inmunitarias innatas como las células dendríticas, macrófagos y neutrófilos (Takeichu y Akira, 2010) y se definen por reconocer específicamente ligandos de macromoléculas microbianas y se unen a ellos. Estos ligandos son característicos de cada cepa bacteriana que se denominan patrones moleculares asociados a patógenos (PAMP) (Kumar *et al.*, 2011). Aunque las bacterias sean comensales no patogénicas, contienen componentes que son reconocidos por el sistema inmunitario de la mucosa. Existen varios tipos de PRR, y los más conocidos son la familia de los receptores análogos a *Toll* (*Toll-like receptors*, TLR). Hasta la fecha se han identificado quince TLR en

mamíferos (Schreibelt *et al.,* 2010). Los TLR 1, 2, 4, 5, 6, 10 y 11 se expresan en la superficie celular y reconocen PAMP derivados de bacterias, como componentes de la pared bacteriana. Los TLR 3, 7, 8 y 9 se expresan en vesículas intracelulares y reconocen ácidos nucléicos de bacterias (Kumar *et al.*, 2011). La activación de los TLR en las células dendríticas induce su maduración, acompañado de cambios en la expresión de receptores de quimioquinas que van a favorecer su movilización a los ganglios linfáticos. Las células dendríticas maduras poseen mayor capacidad de estimular linfocitos T, por lo tanto la activación de los TLR sirve de enlace entre la inmunidad innata y adaptativa (Schreibelt *et al.,* 2010).

Las células dendríticas intestinales trabajan junto a las células M (Strober *et al.,* 2009) para translocar antígenos luminales a través de la barrera epitelial y se los entrega a las células dendríticas subyacentes residentes en las Placas de Peyer y folículos linfoides aislados. Desde donde junto a células plasmáticas podrán viajar por el torrente circulatorio y linfático y serán reclutadas en diversos puntos efectores como glándulas salivales, lacrimales, tracto respiratorio y los sitios efectores en la lámina propia del tracto gastrointestinal (Delcenserie *et al.,* 2008).

Las cepas probióticas de *Lactobacillus* son conocidas por su capacidad de prevención de enfermedades y sus propiedades terapéuticas (Yan y Polk, 2011). Los probióticos regulan la respuesta inmune innata y adaptativa mediante la modulación de las funciones de las células dendríticas, macrófagos y linfocitos T y B (Vanderpool *et al.*, 2008; Yan y Polk, 2010).

Aunque los probióticos ejercen sus efectos biológicos de diferentes maneras y los mecanismos de estos efectos beneficiosos no están del todo aclarados, se considera que la inmunorregulación es uno de los mecanismos más importantes (López *et al.*, 2010). Los efectos inmunoreguladores de los *Lactobacillus* son específicos de cada cepa.

En consecuencia, en los últimos años, se han llevado a cabo muchos estudios para detectar los probióticos con efectos inmunomoduladores (Dongarra *et al.*, 2013; Tsai *et al.,* 2010; Ku *et al.*, 2014).

La activación de los Toll-like receptors es uno de los mecanismos por los que los probióticos regulan diferentes funciones inmunomoduladoras, como la señalización TLR9, esencial para conseguir los efectos antiinflamatorios. Los probióticos pueden disminuir la inflamación intestinal a través de: la regulación a la baja de la expresión de TLR; la secreción de metabolitos que pueden inhibir el TNF-α; y la inhibición de la señalización del NF- κβ en los enterocitos (Goméz-Lorente *et al.*, 2010). El TLR2 reconoce peptidoglicanos, que son el componente principal de las bacterias Gram-positivas, incluyendo el género *Lactobacillus*. Varios estudios han demostrado que el TLR2 es necesario para que algunas cepas de *Lactobacillus* puedan ejercer sus efectos inmunomoduladores (Vinderola *et al.*, 2005).

Los lactobacilos y las bifidobacterias probióticas inducen distintas respuestas innatas y perfiles de citoquinas que median posteriormente la respuesta celular T-helper (Chen *et al.*, 2003; Konieczna *et al.*, 2012; Mileti *et al.*, 2009 Schwarser *et al.*, 2013; Schabussova *et al.*, 2012).

1.3 Probióticos en la práctica veterinaria

Los probióticos se han consolidado como una alternativa al uso de antibióticos en la prevención o tratamiento de desórdenes gastrointestinales (Ouwehand et al. 2004). Además los medicamentos pueden producir efectos adversos como toxicidad o resistencia (O'Flaherty et al. 2005). Numerosos estudios muestran la gran utilidad de las bacterias ácido-lácticas como probióticos en animales.

Sin embargo, no se puede asumir que todas las bacterias ácido-lácticas posean propiedades beneficiosas. Además, cuando se describe un efecto en una cepa concreta, no se puede extrapolar a las restantes cepas de la misma especie (Gareau et al. 2010).

A continuación se enumeraran las indicaciones clínicas más importantes en veterinaria.

- **diarrea**: el tratamiento con probióticos puede reducir la infección por Clostridium difficile, Salmonella spp y Campylobacter spp.(Vahjen etb al., 2003)

- **inmunidad**: los lactobacilos pueden alertar el sistema inmunitario y favorecer el rechazo de microorganismos infecciosos por medio de la modificación de parámetros inmunológicos como son la producción de inmunoglobulinas especificas de tipo A (para defensa de las mucosas), concentración de macrófagos, producción de interferón y otras citoquinas o en la activación de la fagocitosis. (Blazer,2001). Un estudio demostró que *Lactobacillus murinus* incremento significativamente los niveles de IgA fecales, demostrando los efectos potenciales inmunomoduladores (Delucchi et al., 2014).

- **Enfermedad renal crónica:** aunque la mayoría de los estudios se han llevado a cabo en ratas, el uso se podría extrapolar a perros. Las especies de probióticos productoras de ureasa, pueden hidrolizar la urea para crear un gradiente de difusión positivo desde la sangre hacia el tracto gastrointestinal. 63. (Ranganathan et al., 2005)

-**Enfermedad urinaria**: se ha estudiado in vitro el uso de *Lactobacillus acidophilus*, reduciendo significativamente la concentración de oxalato. (Cho, et al., 2015) e in vivo se ha demostrado el uso de *Lactobacillus animalis* (Murphy et al., 2009)

- **Dermatitis atópica**: Kim et al (2015), demostró el uso de Lactobacillus sakei para reducir significativamente la severidad de las lesiones producidas por la dermatitis atopica canina.

- **Barrera gastrointestinal y pancreatitis**: Xu et al. (2006) demostraron que la suplementación con probióticos redujo significativamente la actividad en suero de la amilasa, la alanina aminotrsanferasa, y las concentraciones de endotoxinas. Los cambios histiopatologicos en ileon y páncreas se eliminaron significativamente. El grado de translocación de bacterias se redujo significativamente, lo que sugiere que se mejora la barrera gastrointestinal .
Investigadores en otros estuidos obtubieron resulatdos similares.Quin et al., 2002)

1. 2 MICROBIOTA GASTROINTESTINAL

Una correcta salud intestinal es crucial para el bienestar el huésped, de lo que se asume que si se produce una alteración de la microbiota intestinal, tanto en composición como en actividad, puede aparecer diversas enfermedades, tanto en animales como en personas (Harris *et al.*, 2010; Lee and Hase, 2014; Summa *et al.*, 2012). La microbiota gastrointestinal es una colección compleja de microorganismos, entre los que se encuentran bacterias, hongos, protozoos, virus y levaduras (Sommer y Bäckhed, 2013).

El epitelio intestinal constituye la barrera más importante de separación entre el medio interno y el externo, creando una fuerte resistencia a la difusión libre de solutos y entrada de patógenos y antígenos dañinos. Para garantizar el correcto funcionamiento de la barrera, los espacios intracelulares deben estar correctamente sellados mediante las uniones celulares. En esta región se han identificado diversas proteínas de unión a *Lactobacillus* (Suchodolski 2010).

Estudios filogenéticos basados en el análisis del 16S rRNA estiman que el intestino de los mamíferos contiene aproximadamente 10^{10} a 10^{14} microorganismos, alrededor del 10 veces más que el número de

células que contiene el cuerpo completo (Frank *et al.*, 2007; Suchodolski *et al.*, 2009; Handl *et al.*, 2011).

En perros, las diferentes regiones anatómicas contienen diferentes cantidades de microorganismos. En el estómago varía entre 10^1-10^6 ufc/g o ml (Benno *et al.*, 1992). En el duodeno y yeyuno se producen grandes variaciones entre individuos, los contajes pueden ser bajos (<10^3 ufc/g o ml) o alcanzar los 10^9 ufc/g o ml en algunos perros (Johnston *et al.*, 1993; German *et al*, 2003). En el íleo y ciego se encuentran de 10^7-10^8 ufc/g y finalmente en el colon y recto se encuentran de 10^{10}-10^{11} ufc/g (Benno *et al.*, 1992).

Dentro de cada región anatómica encontramos diferentes grupos mayoritarios (Figura 2). Los microorganismos predominantes son *Bacteroiedes, Clostridium, Lactobacillus, Bifidofacterium* spp y *Enterobacteriaceae* (Grze-skowiak *et al.*, 2015).

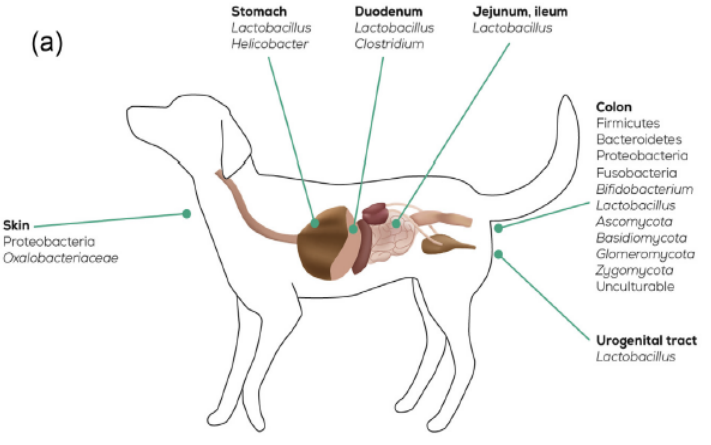


Fig. 2. Microorganismos mayoritarios del tracto gastrointestinal canino (Grze-skowiak *et al.*, 2015).

1.3 MICROBIOTA VAGINAL DE LA PERRA

La mucosa vaginal está también colonizada por una microbiota que

vive en un estado de equilibrio con el hospedador y que parece jugar un papel importante en la resistencia a cualquier tipo de infección o alteración (Bjurström y Linde-Forsberg, 1992; Noguchi *et al*., 2003).

Los microorganismos diagnosticados en la microbiota vaginal de perras sanas son muy variados y es frecuente aislar microorganismos aerobicos como *Escherichia coli, Streptococcus* sp. (α i β-hemolíticos)*, Staphylococcus* sp*., Pasteurella multocida, Proteus mirabilis, Bacillus* sp*., Micoplasma* sp*.,* (Hirsh y Wiger, 1977; Olson y Mather, 1978; Allen y Dagnall, 1982; Baba *et al.,* 1983; Duijkeren, 1992; Stornelli *et al.*, 2000; Root Kustritz, 2006; Maksimović *et al*., 2012) y en menor frecuencia encontramos también cultivos mixtos con *Corynebacterium sp., Pseudomones sp., Micrococcus sp., Nisseria sp., Klebsiella sp.* y *Moraxella sp.* (Hirsh y Wiger, 1977; Olson y Mather, 1978; Allen y Dagnall, 1982; Gunay *et al*., 2010). Aunque algunos de estos microorganismos citados son potencialmente patógenos no se han encontrado diferencias entre los microorganismos aislados en perras sanas y perras infértiles (Olson y Mather, 1978; Root Kustritz, 2006). Se consideran bacterias oportunistas que pueden causar problemas si por algún motivo se producen lesiones, alteraciones de pH vaginal, inmunodeficiencia general, cambios hormonales, tratamientos con antibióticos, entre otros (Osset Lladonosa, 2003; Rodríguez, 2007; Delucchi et al. 2008).

En la mayoría de las citologías vaginales de perras en proestro y estro es fácil identificar bacterias vaginales (Groppetti *et al.*, 2012). En el celo y estimulado por los estrógenos, el epitelio de esta mucosa, compuesto por células ricas en glucógeno, se exfolia y subministra un substrato idóneo para los lactobacilos que forman parte de la microbiota local. Estas bacterias se multiplican activamente y producen gran cantidad de ácido láctico que acidifica el medio hasta un pH de 4,0 y peróxido de hidrógeno (H_20_2) con capacidad

bacteriostática o bactericida, ejerciendo de este modo un mecanismo antimicrobiano muy eficaz. Además, los lactobacilos se adhieren a receptores de las células epiteliales vaginales previniendo la adherencia de patógenos potenciales (Rodríguez, 2007; Deluchi *et al*., 2008; Reid *et al.*, 2009). Un mecanismo inmunitario importante en les superficies mucosas, y en todas las secreciones, en este caso urogenitales y orina, son las inmunoglobulinas A (IgA) que també previenen la colonización de posibles patógenos al bloquear las adhesinas bacterianas o víricas de les células epiteliales (Rodríguez, 2007).

Münnich y Küchenmeister (2014) ponen de manifiesto los posibles efectos negativos de las bacterias que habitualmente encontramos en la mucosa vaginal de una perra sana, sobre la misma hembra y sus neonatos, los cuales, se contaminan durante su paso por el canal del parto. Estos autores concluyen que, dentro de las causas infecciosas, las infecciones bacterianas son la causa más común de mortalidad en neonatos, y que les principales bacterias que se aíslan en cultivos de estos neonatos, son las mismas que podemos encontrar como microbiota normal en la mucosa vaginal.

1.4 MICROBIOTA DE LA LECHE

Hasta hace poco se pensaba que la colonización del intestino de los recién nacidos empezaba gracias a la contaminación con la flora vaginal y fecal de la madre en el momento del parto vaginal (Mackie *et al.*, 1999; Tannock, 1995)

El dogma que el intestino es estéril al nacer, ha sido rechazado ya que muestras de meconio (Jiménez *et al.*, 2008) de sangre de cordón umbilical (Jiménez *et al.*, 2005), de placenta (Satokati *et al.,* 2005) y de líquido amniótico (Romero *et al.*, 1993) evidencian la presencia de bacterias.

En la especie humana es donde encontramos más estudios que demuestran la estabilidad y diversidad de la microbiota de la leche (Backhed *et al.*, 2005; Dethlefsen e*t al.*, 2006; Eckburg *et al.*, 2005; Egert *et al.*, 2006). En el hombre, el consumo de 800 ml/día supone una ingesta de 10^5-10^7 de bacterias comensales (Martín *et al*., 2003) y se considera la flora fecal un reflejo de la composición bacteriana de la leche materna (Heikkilä y Saris, 2003). La leche maternal constituye una fuente de probióticos que pueden colonizar el tracto intestinal de los bebes y modular su función (Lara-Villoslada *et al.*, 2007a; Lara-Villoslada *et al.*, 2007b; Martín *et al.*, 2004; Martín *et al.*, 2003).

La leche materna incluye diversas especies predominantes, como *Staphylococci, Streptococci, Micrococci, Lactobacilli, Enterococci, Lactococci y Bifidobacteria* (Gueimonde *et al.*, 2007; Martín *et al.*, 2009; Martín *et al.*, 2004; Martín *et al.*, 2003; Pérez-Cano *et al.*, 2010; Solis *et al.,* 2010). Se considera que el origen de estas bacterias es endógeno, desde el intestino materno a la glándula mamaria tras la translocación a través del epitelio intestinal. La translocación de bacterias viables y muertas, desde el intestino hasta tejidos fuera del intestino, especialmente tejido linfoide asociado a intestino (GALT), es normal y fisiológicamente beneficioso para la estimulación inmunitaria. (Guamer y Malagelada, 2003; MacFie, 2002; Wiest y Rath, 2003). Las bacterias se transfieren desde el intestino a la glándula mamaria durante las últimas etapas de la gestación y durante la lactación, a través de la vía entero-mamaria (Martín *et al.*, 2004; Pérez *et al.,* 2007). Esta via endógena utiliza células epiteliales denominadas M (micropliegues, microfolds). Tal como se há citado anteriormente, las células M son capaces de absorber y transportar las bacterias, para que sean procesadas y presentadas a las células linfoides subepiteliales (Man *et al.*, 2008; Owen, 1999) y a las células dendríticas y macrófagos (Martín *et al.*, 2004; Pérez *et al.,* 2007).

Aunque no hemos encontrado estudios en perros, asumimos que al tratarse de mamíferos entre estos se comparten patrones fisiológicos,

por lo que lo anteriormente descrito se podría extrapolar a perros. En el estudio de Martín *et al.* (2010) se aislaron *Lactobacillus* de la leche de perras lactantes y se evaluó el potencial probiótico de diferentes cepas de *Lactobacillus*. En este estudio se aislaron dos cepas: *Lactobacillus reuteri* y *Lactobacillus fermentum,* las cuales poseían potencial probiótico ya que exhibían una alta capacidad antimicrobiana, altos ratios de supervivencia, actividades enzimáticas deseables como la producción de α-glucosidasa. Además, estas dos cepas no degradaron mucina y el MICs de diversos antibióticos se mostraba dentro de los valores recomendados por EFSA (Martín *et al.* (2010).

Considerando la importancia de la lactación y los mecanismos por los cuales la leche es una fuente de bacterias acido lácticas, a partir de estas dos cepas aisladas, *Lactobacillus reuteri* y *Lactobacillus fermentum,* se realizó la siguiente tesis doctoral.

OBJETIVOS

OBJETIVOS

El presente trabajo de investigación tiene como objetivo general estudiar el efecto probiótico de las cepas *Lactobacillus reuteri* CECT7266 y *Lactobacillus fermentum* CECT7265 obtenidas de leche de perra.
El objetivo general se subdivide en los siguientes objetivos específicos:

1. Estudiar el efecto de la suplementación oral de las cepas sobre la evolución de la flora fecal y de marcadores del sistema inmunitario, en cachorros.

2. Investigar la transferencia de las cepas administradas oralmente a la leche de hembras lactantes y a sus cachorros.

3. Evaluación de la colonización vaginal de las dos cepas estudiadas tras la suplementación oral en hembras sanas.

III. CAPITULO 1
EFFECT OF FOOD SUPPLEMENTATION WITH LACTOBACILLUS REUTERI CECT7266 AND LACTOBACILLUS FERMENTUM CECT7265 IN HEALTHY PUPPIES.

1. EFFECT OF FOOD SUPPLEMENTATION WITH *Lactobacillus reuteri* CECT7266 AND *Lactobacillus fermentum* CECT7265 IN HEALTHY PUPPIES.

1.1 INTRODUCTION

The vertebrate gastrointestinal tract is home to a vast collection of microbial, mostly bacterial, species, which is referred to as the gut microbiota. Comparisons of the characteristics of germ-free animals and those of conventional animals have clearly demonstrated that the gut microbiota has considerable influence on host biochemistry, physiology, immunology, and high-level resistance to gut infections (1, 2, 3). The use of probiotic strains (particularly lactobacilli and bifidobacteria) is being promoted in humans as a mean to balance the gut microbiota and to exert preventive and therapeutical effects. Beneficial effects on gastrointestinal disorders, infections, chronic inflammation or allergic diseases have been attributed to probiotic treatments in humans (4).

Similarly to what has been observed in humans, composition of dog intestinal microflora also shows a large individual variability and may also depend on diet composition. Diets rich in fermentable carbohydrates lead to a higher number of lactic acid bacteria and bifidobacteria (5). Nevertheless, the direct administration of probiotics has been shown to be more effective than the use of diet manipulation or prebiotic administration in the rapid modulation of the intestinal microbiota. In this sense, probiotics are now also being used in domestic animals in order to improve the animal health. Different authors have proposed that native probiotic strains could be well adapted to the target ecological niche (6), however the commercial probiotic products for dogs are not usually of canine origin. In a previous study, it was shown that *Lactobacillus rhamnosus* GG, a

human isolate, survived gastrointestinal transit in dogs but fecal colonization was less efficient than in humans (7).

In order to use probiotic bacteria of canine origin, different bacterial strains have been isolated from canine feces (8, 9). Although, species such as *L. reuteri, L. fermentum* or *L. animalis* have already been tested as canine probiotics (7, 10, 5), their efficacy and commercialization has been limited due to technical issues. For this reason most of the probiotics used for dogs contain the more resistant specie *Enterococcus faecium* which has been shown to produce a beneficial effect and stability (11). However, the safety of this specie in humans has been questioned and in fact *Enterococcus* spp. have not been added to the QPS list (Quality Presumption of Safety) published by EFSA (12).

Human milk has been demonstrated to be a source of lactic acid bacteria with excellent probiotic potential (13). Recently, it has been demonstrated that canine milk also contains lactic acid bacteria with probiotic potential that could be used in canine application (14). Several *Lactobacillus* species (*L. reuteri, L. fermentum, L. animalis, L. murinus, L. johnsoni*) were identified in that study. Lactobacilli belonging to the same or closely related species have been frequently isolated or detected in canine feces (15, 16) and, interestingly, seem to be dominant in suckling puppies, even 1 day after birth (17). The lactobacilli pattern of canine milk seems to be host-specific, a finding that has also been reported for human milk (18), canine feces (19, 20) and feces of lactating piglets (21). In addition, this pattern seems to be restricted to a low number of *Lactobacillus* species.

Two strains isolated from canine milk, *L. fermentum* CECT7265 and *L. reuteri* CECT7266, were selected based on their higher probiotic potential. These strains are endowed of properties such as mucin adherence and resistance to gastrointestinal conditions

which are prerequisite to be considered probiotic strains. In addition to this, both strains displayed high antimicrobial activities and imunomodulatory properties in *in vitro* assays that could results in beneficial effects on canine health (14).

In this context we have carried out a study in healthy dogs with the aim of evaluating the effects of the inclusion of both probiotic strains, *L. fermentum* CECT7265 and *L. reuteri* CECT7266, in diet.

1.2 MATERIAL AND METHODS

1.2.1 Animals and diet.

Thirteen Beagle puppies (14 weeks of age) were included in the study. Puppies were purchased from the Service for the Animal Science of Córdoba (University of Córdoba, Spain) and housed in the Service for the Animal Science of Granada (University of Granada, Spain). Guidelines for the care and use of animals were followed as described (Institute of Laboratory Animal Resource Commission of Life Sciences, 1996) and approved by the ethical committee of the Granada University.

The study design was a blind (for the investigators), cross-over study. Puppies were randomly distributed into two groups: group A with 3 male and 3 female dogs (n=6) and group B with 4 male and 3 female dogs (n=7). Puppies were housed in three subgroups of 3 animals and one subgroup of 4 animals in indoor/outdoor kennel runs. Each subgroup consisted of puppies in the same treatment group and same gender. Housing in groups provided the puppies with social interaction. Five days at week, each group of puppies was taken for a walk for 20 minutes. Their health status, growth and behavior were weekly evaluated by a veterinarian. Puppies consumed fresh water and food ad libitum. The consumption of food was recorded each two days and body weight recorded every week. The control group

received a commercial extruded dry dog food (Advance medium puppy, Affinity Petcare). The probiotic group received the same dry dog food supplemented with *L. reuteri* CECT7266 and *L. fermentum* CECT7265 at a dose of $1x10^9$cfu/kg of each strain. These diets were also prepared by Affinity Petcare in their installations following identical industrial process. Dogs in group A started the study with the diet supplemented with the *Lactobacillus* strain and group B received a control diet. After 4 weeks of treatment and two weeks of washout with normal diet, group B changed to the supplemented diet and group A received the control diet during another 4 weeks. At the beginning and at the end of each period of treatment, feces and blood samples were collected.

1.2.2 Collection of blood and fecal samples.

After an overnight fast lasting at least 10 hours, blood samples were taken from dogs just before and after the treatment period and analyzed for complete blood count or immune markers. Complete hemogram and biochemistry were carried out in serum samples. Major leukocyte subset phenotypes were counted in EDTA-treated whole-blood samples via flow cytometry on a FACScalibur (Becton Dickinson, Oxford, United Kingdom) by using the corresponding fluorochrome-conjugated monoclonal antibodies supplied by ABD serotec. The results were expressed as the percentage of mononuclear cells that stained positively.

Fresh fecal samples were collected at the same time as blood samples and placed into pre-weighted bottles. Samples were homogenized in a peptone-saline solution (100mg/ml) within 12 h.

1.2.3 Counts of fecal bacterial groups

To estimate the concentration of selected bacterial groups, appropriate dilutions were spread in quadruplicate onto plates of MRS

agar for lactobacilli, MRS agar supplemented with 0.5mg/L dicloxacilin, 1g/L LiCl and 0.5g/L L-cysteine hydrochloride for bifidobacteria, reinforced clostridial agar containing 10 □g/mL of polimixin and bile aesculin agar for bacteroides. All media were obtained from Oxoid (Basingstoke, UK) whereas antibiotics and other supplements were obtained from Sigma Chemical Co. (St Louis, MO). Culture plates were incubated in absence of oxygen at 37°C for 24 to 48 hours. Similarly, 1mL of suitable dilutions was spread onto specific count plates petrifilm (3M St Paul, MN) for total aerobes and for enterobacteria enumeration. Plates were incubated at 37 °C for 24 hours. After the incubation, the specific colonies grown on the selective culture media were counted and the number of viable microorganism per gram of feces (cfu/g) was calculated. The mean and standard error per group were calculated from the log values of the cfu/g. The remaining supernatants were stored at -80ºC to measure fecal IgA concentration.

1.2.4 Quantification of short chain fatty acids

The concentration of short chain fatty acids (SCFAs) in the fecal samples was quantified according to the method described by Rodriguez-Cabezas et al. (2002).(22) In short, fecal samples were homogenized with 150 mM NaHCO3 (pH 7.8) (1: 5, wt/v) in an argon atmosphere. Samples were incubated for 24 h at 37ºC and stored at 80ºC until the extraction. To extract the SCFAs, 50 AL of 100 mM 2-methylvaleric acid (internal standard), 10 AL of sulfuric acid and 0.3 ml of ethyl acetate were added to 1 ml of the homogenate. The mix was centrifuged at 10,000 g for 5 min at 4ºC. The supernatants were dehydrated with sodium sulphate (anhydrous) and centrifugated 10,000 g for 5 min at 4ºC. Later, the sample (0.5 ml) was splitless inoculated into a gas chromatograph (mod. CP-3800, Varian, Lake Forest, CA) equipped with an ID (CPWAX 52CB 60 m0.25 mm), and connected to a FID detector (Varian). Helium was used as the carrier

and the makeup gas, with a flow rate of 1.5 ml/min. The injection temperature was 250ºC. Acetate, propionate and butyrate concentrations were automatically calculated from the areas of the resulting peaks using the Star Chromatography WorkStation program (version 5.5), which was connected on-line to the FID detector. The supernatants obtained after the homogenization of the fecal samples were used to measure the fecal pH and the ammonium concentration in a pH-meter (mod. GLP21-21, Crison, Barcelona, Spain) equipped with electrodes for pH and ammonium (mV) measurements. The water content of the feces was calculated by the difference between fresh and dried samples. Fecal enzymatic activities were determined using the APIzym strip system (BioMerieux, Lyon, France).

1.2.5 Quantification of water content

Fecal samples with a known weight were introduced into a heater for 72 hours at 60ºC to eliminate the remaining water. Percentages of water content of the samples were calculated.

1.2.6 Inhibition of pathogen adhesion to mucins by fecal water samples.

The adhesion of bacteria was determined according to the method described by Cohen and Laux (23) with some modifications. In short, 100 □l of a solution (1m
MO, USA) in HEPES-buffered Hanks salt solution (HH) was immobilized in polystyrene microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) after overnight incubation at 4ºC. The wells were washed twice with 250 □l of HH. In a pa
Salmonella cholerasuis CECT4155 were grown overnight at 37ºC in Lysogeny broth (Oxoid, Basingstoke, UK) and the bacterial pellets from 1 ml fractions were obtained by centrifugation and washed with HH. Then, 10 □l of 10 mM carbo
the pellets and the bacterial suspensions were incubated for 20 min at

37ºC. Subsequently, the bacterial cells were washed 3 times with HH and, finally, re-suspended in 1 ml of HH. Then, a suspension of 50 □l of the fluorescent-labelled bacteria (~ $5x10^7$cfu/ml) and 50 □l fecal supernatant (100mg/ml) was added to each well. After incubation for 1 h at 37ºC, the plates were washed twice with 250 □l of HH to remove unattached cells, and incubated for 1 h at 60ºC in the presence of 50 □l of 1% sodium dodecyl sulphate (SDS)-NaOH (0.1 mol/liter) to release and lyse bound microorganisms. Fluorescence was measured in a fluorescence microplate reader (Tecan Austria GMBH, Salzburg, Austria). Adhesion was assessed as the percentage of the fluorescence retained in the wells after the washing steps by comparing it to that present in the labelled bacterial aliquots originally added to the wells.

1.2.7 Total immunoglobulin measurements

Total IgA, IgG and IgE concentrations in plasma and total IgA concentration in feces were measured by ELISA quantitation kits (Bethyl, Montgomery, TX) following manufacturer's recommendations in both cases.

1.3 RESULTS

1.3.1 Tolerance and Clinical observations

During the experimental protocol, no noticeable activity or behavioral changes were observed in the animals. The health status of the puppies throughout the study was normal. Blood cell count, hemogram and serum biochemistry parameters were within the normal ranges. Food intake and body weight did not differ between the two groups during the trial (Table 1). In the second part of the cross-over

the animals reached the adult weight so their body weight did not increase as much as during the first part of the cross-over.

1.3.2 Intestinal parameters.

The main bacterial groups in feces were analyzed and results of concentrations are included in Table 2. The consumption of the supplemented diet for 4 weeks led to a significant increase in the number of fecal lactobacilli. However, a significant decrease was observed in the control group. The probiotic treatment also induced a significant decrease in the amount of the enterobacteria. Other components of the microbiota, such as bifidobacteria, bacteroides and clostridia were not significantly altered.

The ability of the fecal microbiota to produce SCFAs such as butyric, propionic and acetic acids was measured. A significant increase was observed in the capability to produce butyric acid at the end of the treatment with the diet supplemented with the lactobacillus strains (Table 3).

Regarding the consistency of feces, a significant decrease was observed in the water content in feces after the 4 weeks of treatment. However, differences were not statistically significant with respect to the control group (Table 3). No symptoms of constipation or soft stools were detected during the study.

The capability of fecal water to inhibit the adhesion of an intestinal pathogen (*Salmonella*) to mucins was tested in vitro. The consumption of the diet containing probiotic strains induced a significant increase in the in vitro capability of fecal water of dogs to inhibit the adhesion of the pathogen bacteria to mucins (Figure 1).

1.3.3 Immunological parameters.

All the leukocyte populations analyzed by flow cytometry showed values within the normal ranges. A significant increase in the proportion of T and B lymphocytes after the probiotic treatment was observed. However, a trend was also observed in the control group ($p=0.07$). No significant differences were observed between control and probiotic group in the proportion of the rest of the population measured: total lymphocytes, B lymphocytes, T-helper lymphocytes, Cytotoxic T lymphocytes and Natural Killer cells (Table 4).

The percentages of mononuclear cells of blood samples showing phagocytic activity in vitro increased significantly after 4 weeks of probiotic treatment (Figure 2).

The humoral response was also evaluated through measurement of immunoglobulin production. Data showed a significant increase in the plasma concentrations of IgG after probiotic treatment and in comparison with the control group. The concentration of fecal IgA was also measured in fecal supernatants but no differences were detected between times and treatments (Table 5).

1.4 DISCUSSION

L. fermentum CECT7265 and *L. reuteri* CECT7266 are two strains isolated from canine milk. In order to exert health-promoting probiotic effects, it is considered important for probiotic strains to survive the environment in the animal´s gastrointestinal tract. Previous characterization of the probiotic potential of both strains showed that both strains are able to resist low pH values and bile salt concentration and are also able to adhere to intestinal mucins. The oral administration of both probiotic strains to healthy dogs during four weeks induced a significant increase of lactobacilli that could be

directly related with the presence of the bacteria in the feces of the animals. These results contrast with a decrease in this bacterial group during the control periods.

An increase in lactobacilli in microbiota can also affect other bacterial groups due to changes in pH of the enviroment, the production of antibacterial substances or prebiotic substances, competition for nutrients or adhesion etc. These phenomena can both promote or interfere in the colonization of other bacterial groups. The diet containing the probiotic strains also induced a significant decrease in enterobacteria, improving the proportion of beneficial bacteria in microbiota which could help to maintain a healthier intestinal enviroment.

These changes in the compostion of the microbiota were accompanied by changes in the concentration of SCFAs and intestinal environment. These fatty acids are produced by fermentation of carbohydrates by bacteria in the intestinal lumen and perform important functions such as carbohydrate and lipids metabolism, control of the colonic pH, maintenance of the integrity of the colonic mucosa, intestinal motility or absortion (24, 25, 26). Butyrate is the main energy source of colonocytes essential for a correct intestinal function. Although lactobacilli does not directly produce butyrate, an increase has been previously reported in this SCFA after Lactobacillus consumption (27) that could promote the proliferation of producer bacterial groups, and metabolites produced by lactobacilli can even be used by other bacteria to produce butyrate (27, 28) Also, a higher concentration in SCFAs could induce a decrease in pH that would favour the presence of lactic acid bacteria and would interfere with the growth of potential pathogens.

Probiotic bacteria protect the gut from bacterial infection through different mechanisms including the production of antibacterial substances and competing for adhesion to intestinal mucosa. The first

step in the intestinal infection by pathogen bacteria is the adhesion to the mucus. The preventive effect of probiotics against pathogen adhesion involves not only competition for adhesion but also the production of compounds that affect the pathogen interfering with its adhesion. *L. fermentum* CECT7265 and *L. reuteri* CECT7266 previously showed high antibacterial activity in *in vitro* assays (14). In this study the fecal water of animals after probiotic administration also inhibited the in vitro adhesion of *Salmonella* to mucins, demonstrating their antibacterial capabilities and suggesting the presence of antibacterial substances in fecal water promoted by the probiotic strains. The normal composition of dog intestinal microbiota can be altered by stressful conditions, such as weaning, dietary changes or antibiotics administration. These changes can induce gastrointestinal disorders or intestinal infections. The oral administration of *L. fermentum* CECT7265 and *L. reuteri* CECT7266 to healthy dogs improves the composition and other intestinal parameters that may be a valuable tool to improve the animal health and prevent gastrointestinal disorders.

The probiotic treatment seems to reinforce the adaptive immune system maturation that occurs during the normal growth of the animals, increasing the proportion of T lymphocytes after the probiotic treatment. However, a trend is also observed in the control group, suggesting that the increase can not only be attributed to the probiotic treatment.

The percentages of mononuclear cells of blood samples showing phagocytic activity in vitro increased significantly after 4 weeks of probiotic treatment, suggesting an improvement in the innate immune response.

The effect of probiotic strains on the immune system has been extensively studied in humans given its importance in the modulation

and even maturation of the immune response (29, 30, 31). This immunological effect has been related with the preventive effect of probiotics against infections or their effect on allergy and inflammatory diseases (32, 33, 34, 35) However, only a few studies have been carried out in dogs. In this study, the analysis of immunological parameters showed a significant improvement in both innate and specific immune response. The phagocytic activity of monocytes was significantly higher after probiotic treatment. This effect has been previously observed for other probiotic strains in humans (31, 36). In dogs, a preparation of *Enterococcus faecalis* also induced the activation of phagocytic activity of monocytes (37). However, the bacteria were administered dead, so the effect could differ from that provoked by the live bacteria. The role of the activation of antigen presenting cells in the immunomodulatory effect of probiotics has been extensively studied (38). Monocytes and macrophages, together with dendritic cells, play a crucial role in the innate immune response against microbial antigens, which in turn leads to activation of the adaptive immune system (39). These cells recognize conserved molecular patterns of bacterial components through Toll-like receptors (TLR) leading to the activation of a variety of transcription factors which triggers the production of cytokines (40). Although the increase in phagocytic function was induced by non-pathogen bacteria, a pathogen could be recognized by these cells in a higher number and more effective way. These results agree with the in vitro capability of both strains to activate macrophages, inducing the release of cytokines involved in the triggering of the immune response in human (41).

With regard to the adaptative or adquired immune response, a significant increase in the plasma IgG after probiotic diet was observed. IgG, the most prevalent class of antibody, is produced in greater amounts when a particular antigen is encountered again, called the secondary immune response. The secondary immune

response is faster and the antibodies produced, mainly IgG, are more effective. IgG protects against bacteria, viruses, fungi, and toxic substances. The improvement in the humoral response induced by probiotic treatment has been related with the protector effect of probiotics against infectious diseases (32, 33). Moreover, it has been suggested that some probiotic strains with this immunoenhancing capability could be used as coadjuvants in the vaccination process (31, 42). Similarly, a strain of *Enterococcus faecium* induced an increase of distemper virus vaccine specific IgG in previously vaccinated dogs (11).

The normal microbiota constitutes the first line of defense against pathogenic microorganisms and, although the underlying mechanisms are far from totally elucidated, it seems that the production of a physiologically restrictive environment, production of antimicrobials, competition for the same substrates, and/or for adhesion to mucin, may play an important role in this protective effect (43). Moreover, the interactions that occur in the gastrointestinal tract between microbiota, epithelial cells and immune system reinforce the host defense system against pathogens (44, 45). In fact, there is increasing evidence that some probiotic strains exert a positive effect in the prevention and/or treatment of a variety of infectious and inflammatory gastrointestinal diseases (46, 47, 48, 49). In this report we have demonstrated that the administration of two *Lactobacillus* strains isolated from canine milk improves both intestinal and immune system. Although more studies must be carried out, these results suggest that the administration of *L. reuteri* CECT7266 and *L. fermentum* CECT7265 may be a valuable tool to improve the animal health.

Table 1: Weight gain of growing and food intake.

	Weight increase (kg)[1]	**Diet consumption (g/day)**
Control B	1.5±0.6	282.1
Probiotic A	1.8±0.4	281.2
Control A	0.9±0.5	265.7
Probiotic B	0.9±0.5	255.8

[1] Values are mean ± SD

Table 2: Fecal microbiota of puppies after control and probiotic periods at the beginning and at the end of the treatment 4 weeks after[1].

	Control	**Probiotic**

Bacterial group (log cfu/g feces)	**Initial count**	**Final count**	**Initial count**	**Final count**
Lactobacilli	7.6±0.1	6.8±0.3*	7.3±0.2	7.7±0.5*#
Bifidobacteria	7.5±0.1	7.0±0.5	7.2±0.2	7.4±0.2
Enterobacteria	6.5±0.6	6.1±0.3	6.8±0.5	5.5±0.2** #
Clostridia	5.5±0.3	6.1±0.5	6.2±0.2	6.1±0.2
Bacteroidaceae	6.6±0.2	6.8±0.3	6.8±0.2	6.4±0.2

[1] Values are mean ± SEM of log cfu/g feces.
*****statistically significant difference, $p<0.05$ with respect to initial count;
** statistically significant difference, $p<0.01$ respect to initial count
# statistically significant difference, $p<0.05$ with respect to control group;

Table 3: Effects of probiotic treatment on fecal concentration of short chain fatty acids (SCFA) [1].

	Control		Probiotic	
	T 0	**T 4 weeks**	**T 0**	**T 4 weeks**
Short Chain FattyAcids (mg/L)				
Acetic acid	1.26±0.2	1.28±0.0	1.05±0.7	1.17±0.0.
Propionic acid	0.60±0.0	0.64±0.0	0.56±0.0	0.57±0.0
Butiric acid	0.50±0.0	0.54±0.0	0.46±0.0	0.52±0.0*
Water content (%)	68.3±1.5	66.5±1.8	66.6±2.4	62.31±2.4 *

[1] Values are mean ± SEM

*****statistically significant difference, $p<0.05$ with respect to initial value

Table 4: Effects of probiotic treatment on leukocytes proportion in dog's blood [1].

	Control		Probiotic	
Percentage of lymphocyte	**Initial count**	**Final**	**Initial count**	**Final count**

subsets	count			
T lymphocytes	46.5±4.5	52.0±2.3	46.0±3.6	54.6±1.8*
B lymphocytes	22.7±1.5	25.7±1.5*	20.4±1.4	24.1±1.3*
T helper lymphocytes	30.82±3.0	31.78±2.20	32.24±2.4	29.79±2.1
T cytotoxic lymphocytes	12.25±1.3	11.67±0.8	11.23±0.9	11.72±0.9
Natural Killer	5.15±0.6	4.67±0.6	4.48±0.4	4.14±0.5

[1] Values are mean ± SEM

*****statistically significant difference, $p<0.05$ with respect to initial value

Table 5: Effect of probiotic treatment on plasma concentration and fecal concentration of immunoglobulins [1].

	Control		**Probiotic**	
Immunoglobulins	**T0**	**T 4 weeks**	**T0**	**T 4 weeks**

Plasmatic (mg/mL)	***IgG***	15.8±2.0	15.3±1.3	16.8±1.3	19.3±1.3*#
Plasmatic (ng/mL)	***IgE***	347.4±15.6	375.6±19.7	359.3±15.0	362.6±10.5
Plasmatic (ng/mL)	***IgA***	41.6 ±3.1	40.7±4.5	42.1±2.4	43.6±3.1
Fecal IgA (mg/g)		4.21 ±1.0	2.93 ±0.8	4.05 ±0.6	3.30 ±0.6

[1] Values are mean ± SEM

*****statistically significant difference, $p<0.05$ with respect to initial count;

statistically significant difference, $p<0.05$ with respect to control group;

Figure Legends:

Figure 1: Effect of fecal water of dogs on the adhesion of fluorescent *S. cholerasuis* on hog mucin under conditions of competition. Results are expressed as the mean (±SEM) of the percentage of fluorescence

recovered from the wells in absence of fecal water with respect to that found in the lactobacilli-containing wells after incubation. Probiotic group (grey bars) and control group (white bars). *statistically significant difference, $p<0.05$ with respect to initial value; # statistically significant difference, $p<0.05$ with respect to control group.

Figure 2: Monocytes and granulocytes cells were differentiated by size and complexity by flow cytometer. Phagocytic activity of monocytes before and after 4 weeks of treatment is expressed as the mean (±SEM) of the percentage of leukocytes cells containing fluoresceinated E. coli after in vitro incubation of the bacteria with fresh blood. Probiotic group (grey bars) and control group (white bars). *statistically significant difference, $p<0.05$ with respect to initial value; # statistically significant difference, $p<0.05$ with respect to control group.

Figure 1 Test of in vitro capability of fecal water to inhibit the adhesion of *Salmonella* to mucins.

Probiotic Control

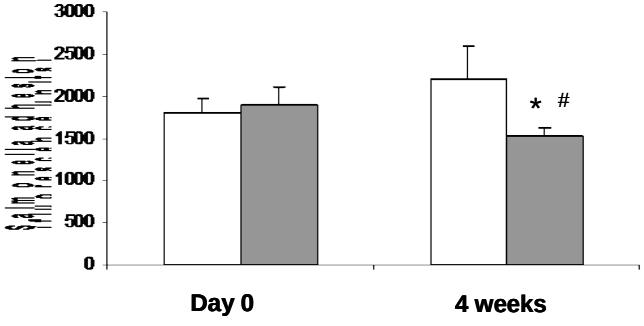
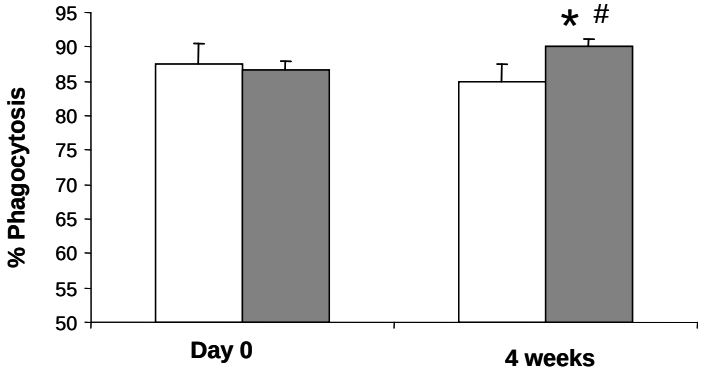


Figure 2. Percentage of mononuclear cells of blood samples between Probiotic and Control group and between time 0 and time 4 weeks



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III. CAPÍTULO 2
LACTOBACILLUS TRANSFER TO DOG'S MILK THROUGH ORAL INTAKE

2. LACTOBACILLUS TRANSFER TO DOG'S MILK THROUGH ORAL INTAKE

2.1 INTRODUCTION

The importance of bitch milk in the development of puppies is unquestionable. Dog's milk provides all the essential nutrients needed to support life and growth of newborn puppies. Bitches produce a concentrated milk containing 16-21% total solids, 8-12% fat and 7-10% protein (1, 2, 3). In addition, the newborn also receives nucleotides, immunoglobulins, cytokines, immune cells, lysozyme and other immuno-modulatory factors (4, 5, 6, 7, 8).

Intestines are highly permeable for the first 24 to 48 hours, allowing the absorbtion of intact milk constituents into the blood stream (9). The immune status of the newborn puppy depends entirely on colostrum ingestion (10), 90–95% of all circulating immunoglobulins after closure of the intestinal barrier, originate from the colostrum (11). Deprived colostrum intake leads to a deficit in the transfer of passive immunity, associated with higher mortality and morbidity rates in puppies (12).

Mother's milk is also a source of bacteria. Bacteria commonly isolated from breast milk of healthy women have included Staphylococci, Streptococci, Lactobacilli, Bifidobacteria and Enterococci (13, 14, 15, 16, 17, 18). This agrees with works which indicated that commensal staphylococci and streptococci were the predominant bacterial species in breast milk (19, 20) The bacterial composition of the infant fecal flora reflects the bacterial composition of breast milk (14, 16, 18).

Human breast milk has been shown to be a source of probiotic lactic acid bacteria to the infant gut (21, 22, 23, 24, 25). In humans, it has been estimated that an infant consuming 800 mL milk per day will ingest about 1×10^5–1×10^7 commensal bacteria (21). How milk intake (approximately 81±13 ml/day in a beagle puppy (26) affects the microbiota of the puppies has not been studied. But the presence of lactobacilli in canine milk has been described (26) in a concentration of $1.3\text{-}6.1 \times 10^2$.

The objective of the current study was to investigate the transfer of an oral probiotic containing *Lactobacillus reuteri* CECT7266 and *Lactobacillus fermentum* CECT7265 to dog's milk (27). The probiotic was orally administered during the second half of gestation and throughout lactation. The hypothesis was that the same probiotic strain administered orally could be isolated from bitch's milk.

2.2 MATERIAL AND METHODS

2.2.1 Animals and diet.

Six pregnant Jack Russell dogs (5 years mean age) were included in the study. Animals were randomly distributed into two groups. The probiotic group (P) (n=3) daily consumed during the last month of pregnancy a capsule with 200 mg of a freeze-dried probiotic mix containing $2x10^8$cfu of *L. fermentum* CECT7265 and $2x10^8$cfu of *L. reuteri* CECT7266 in a matrix of maltodextrin. Both strains were originally isolated from the teat milk of healthy dogs (37). The entire process to obtain the probiotic strains and to prepare the capsules was performed in the industrial probiotic plant of Bioserch Life S.A. (Granada. Spain). The capsules were kept at 4°C throughout the study. The control group (C) (n=3) consumed a capsule containing the maltodextrin matrix. The treatments were continued until weaning.

Diet used in the study was a super premium puppy diet (Advance puppy protect. Affinity Petcare (Barcelona)).

2.2.2 Samples.

After birth, milk samples were obtained from each of the dogs, from colostrums, and then weekly until the third week of lactation (colostrum, day 7, day 14, day 21) To collect the milk samples, teats were cleaned with soap and sterile water, and then clorhexidine was applied to minimize contamination with skin microbiota. The milk sample was collected in a sterile tube after manual expression by

using sterile gloves, and doing a pool from different mammary glands. The first drops were discarded to avoid chlorhexidine contamination.

Samples of feces were collected from puppies every week, from the first day (meconium) until 1 month of age (day 7, day 14, day 21, day 31).

2.2.3 Count of lactic acid bacteria in the milk and feces samples.

Fresh milk samples were spread onto agar plates of MRS agar (Oxoid Basingstoke. UK) for the isolation of lactobacilli. All of the plates were incubated for 48 h at 37°C anaerobically. In parallel, to evaluate potential fecal contamination of the milk, the samples were also cultured on Violet Red Bile Agar (VRBA; Difco; a selective medium for the isolation of enterobacteria) agar plates which were aerobically incubated at 37ºC for 24h. In both growth media, the lower limit of detection was 50 cfu / mL.

Fecal samples were homogenized individually in a peptone-saline solution (100 mg/mL). To estimate the concentration. appropriate dilutions were spread in quadruplicate onto plates of MRS agar for lactic acid bacteria. After incubation. colonies grown on the selective culture media were counted and the logarithms of cfu per gram of feces (cfu/g) were calculated and represented as the average ± standard error of the mean.

2.2.4 Identification of probiotic strains administered.

Colonies grown from milk samples in selective culture media were subjected to RAPD (random amplified Polymorphism of DNA) using the primer 5´- ACGCAGGCAC-3´ and the method described by Rodas et al (*29*)

Genomic DNA of colonies with similar pattern to those obtained for *L. fermentum* CECT 7265 and *L. reuteri* CECT7266 was isolated from 10 ml of overnight MRS cultures using the DNeasy tissue

kit (Qiagen, Hilden, Germany). To corroborate the homology, bacteria isolated were identified by PCR amplification of a section 16S-23S intergenic spacer region from each isolate by using primers 16-1A 5´-GAATCGCTAGTAATCG-3´ and 23-1B. 5´-GGGTTCCCCCAGGCGGA-3´. Amplified fragments were purified using a commercial kit of purification (QIA, Quick gel extraction kit, Quiagen, Hilden, Germany) and sequenced by Sistemas Genomicos (Valencia, Spain). The sequences were compared with those deposited in the EMBL database using BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST).

Colonies grown from puppies' feces in selective culture media were subjected to a sequencing of 16S rDNA using primers pb116 (5'-AGAGTTTGATCCTGGCTCAG-3') and ymlb16 (5'-GGCTGCTGGCACGTAGTTAG-3') (28). PCR conditions were: 96ºC during 30 s, 50ºC during 30 s, and 72ºC during 45s (35 cycles) and a final extension at 72ºC during 4 minutes. The amplified fragments were purified using the NucleoSpin Extract II Kit (Macherey- Nagel Gmb; Duren. Germany) and sequenced with the primers listed above in an ABI 377A (Applied Biosystems. Foster City. USA). The sequences were compared with those deposited in the EMBL database using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST). Then colonies belonging to *L.reuteri* or *L. fermentum* species were subjected to RAPD, using primers ArgDei (5'-ACCYTRGAAGGYGGYGATGTB-3') and OPL5 (5'-ACGCAGGCAC-3') (29). as described by Ruiz-Barba et al. (30).

2.3 RESULTS

In this study, samples of bitch milk were collected from 6 bitches in four periods of time by manual extraction. Feces samples were collected from puppies meconium, and every week until 1 month of age. It was impossible to collect milk samples from Bitch 3, on day 7 and day 14 and from Bitch E on day 21.

From 24 milk samples. 21 (87.5%) resulted in *Lactobacillus* growth in agar plates of MRS agar. A variable number of colonies were isolated, ranging from 40 to 2000 cfu/mL of milk (Table 1a). In P group the mean of Lactobacilli expressed as cfu/mL was higher at day 7 and 21 and lower at day 14 compared with C group (Table 1b).

442 representative colonies of the isolates were submitted to Random Amplified Polymorphic DNA (RAPD) technique to compare these isolates with the orally administered *Lactobacillus reuteri* (CECT7266) and *Lactobacillus fermentum* (CECT7265), and the ones with the same profile have been selected. The gel with RAPD corresponding to 16 isolates from colostrum bitch of P group is shown in Figure 1. Of the 16 colonies we can see that number 2 has a profile equal to that presented by *Lactobacillus reuteri* CECT7266.

Table 2 shows positive match colonies with regard to total analyzed colonies using RAPD technique as previously explained. 12 positive isolates with the same profile as CECT7266 were obtained from milk of bitches from the P group, (8 obtained 100% of homology. 1 obtained 99% homology and 3 obtained 98% homology). The DNA homologies with the other strains of Lactobacillus are less than 90%. so 98% is considered positive. Bitch 3 was always negative. No isolate matched with the *Lactobacillus fermentum* CECT7265 given orally.

No isolate from milk from C group matched using RAPD technique with the profile of the *Lactobacillus reuteri* CECT7266 or *Lactobacillus fermentum* CECT7265 given orally.

In P group, 13 puppies were born (4 from bitch A, 3 from bitch B and 6 from bitch C). In C group, 14 puppies were born (4 from bitch D, 7 from bitch E and 3 from bitch F). Unfortunately, not enough fecal samples were obtained from some puppies. Results of fecal MRS cultures are shown in Table 3. It is noteworthy that, 4 samples from meconium were processed and they evidenced presence of Lactobacilli. When the isolates were analyzed to detect positive matches, only the puppy C.1 from P group showed positive homology

results to *Lactobacillus reuteri* CECT7266 (2 of the 34 colonies analyzed).Table 4 shows the mean±SD of log cfu/g puppies feces at Day 7 and Day 31 between P and C group.

2.4 DISCUSSION

This study shows that bitch milk contains lactobacilli and could be a source for the suckling puppy.

Milk constitutes a good source of lactobacilli (16) and is responsible for the vertical mother-to-child transmission of lactobacilli and bifidobacteria inhabiting the gut (24, 16, 31). Proposed theories for the microbiota composition of breast milk include the transfer of microorganisms directly (through nipples) from maternal skin or enteric tract, and the movement of microbiota from the maternal enteric tract to the mammary gland. Through an endogenous route involving dendritic cells and macrophages the entero-mammary pathway (32, 33); and another new possibility not yet investigated is that bacteria could reach the mammary gland via adsorption across the skin surface directly into the fatty tissue or via the blood-stream (34).

Entero-mammary bacterial circulation has been confirmed (33) and it has been demonstrated that dendritic cells can penetrate the gut epithelium to take up noninvasive bacteria directly from the gut lumen (35). Bacteria may be transported by being attached to the surface of cells instead of being internalized. (36). Once associated with gut-associated lymphoid tissue cells, live noninvasive bacteria can spread to other locations since there is a circulation of lymphocytes within the mucosal associated lymphoid system. Moreover, the mechanism by which bacteria avoid being phagocytized and killed by the host's innate immunity is yet unknown. Bacterium-stimulated cells move from the intestinal mucosa to colonize distant mucosal surfaces, such as those of the respiratory and genitourinary tracts, salivary and lachrymal

glands and, most significantly, the lactating mammary gland. In fact, up to 16 lactobacillus species have been previously isolated from the blood of healthy people (37).

In this study, two bitches that received the probiotic mixture of the two Lactobacilli strains, *Lactobacillus reuteri* CECT7266 is actively transferred, especially at the end of lactation. No transfer of *Lactobacillus fermentum* CECT7265 was detected in any of the bitches indicating that somehow the *Lactobacillus reuteri* CECT7266 more easily transferred to milk. Perhaps preferentially select this bacteria or because it loses viability well in the intestinal mucosa during the translocation process in which immune cells are directly involved or latter in other processes.

The percentage of presence of the *Lactobacillus reiteri* CECT7266 bacteria in milk goes from 5 % at the beginning of lactation up to 10-13% at the end. This represents a very significant percentage which suggests it can significantly improve the puppies' probiotic bacteria supply.

The study of Donnet-Hugues *et al* (2010) (38) suggests that the milk microbiome plays a key role in programming the neonatal immune system, but the origin of the lactobacilli that colonise the neonatal gut is a subject of debate. In the past, it was suggested that they were acquired by oral contamination with maternal lactobacilli during the transit through the birth canal; however, molecular studies have shown that human Lactobacilli colonisation is not significantly related to the delivery method (vaginal delivery or caesarean section) (16, 24, 39)

This study has evidenced the presence of Lactobacilli in first-pass meconium samples, and would suggest that fetus are not sterile and that gut colonisation of puppies could start in the placenta. There are no similar studies in dogs, but in humans Hansen *et al* (2015) (40), demonstrated that low numbers of bacteria are present in meconium

samples from healthy, vaginally-delivered infants. Also, Martin *et al* (2004) (43) isolated lactic acid bacteria and other commensal bacteria from meconium obtained from healthy neonates born either by labor or cesarean section (44).

Another issue supporting the theory that the fetus is not sterile and that the commensal bacterias could transient spread from the digestive tract to a extradigestive locations, was the isolation of bacteria in the umbilical cord blood of healthy neonates born by cesarean and the isolation in amniotic fluid of a genetically labeled *Enterococcus faecium* strain in a group of pregnant mice (33, 41, 42).

In this study, the presence of a *Lactobacillus reuteri* strain, indistinguishable from *Lactobacillus reuteri* CECT7266 administered orally to mothers, has been detected in the feces of one puppy whose mother received the probiotic orally. This may suggest a transfer from mother's milk to the puppy gut since the same strain had been isolated from the colostrum and milk of this bitch. In dogs, this was the first time that Lactobacilli had been isolated from meconium and transferred through milk to puppies.

No growth was detected on VRBA plates inoculated with the same samples. This supports the hypothesis that *L.reuteri* CECT7266 could be transferred to bitch's milk due to the entero-mammary pathway, this confirms the hygienic collection of the milk samples. No lactobacilli was isolated from mammary skin of any dog of the probiotic group (data not shown), which rules out the hypothesis of a fecal contamination of the mammary gland.

This study has practical consequences and suggests that the oral administration of probiotics in pregnant and lactating bitches could have a direct effect on the health of their litters.

Table 1a. Total Lactobacilli counts obtained from milk samples expressed in Lactobacilli cfu/mL milk

Probiotic Group	**Colostrum**	**Day 7**	**Day 14**	**Day 21**
Bitch A	390	120	200	160
Bitch B	190	1220	440	2000
Bitch C	620			3635
Control Group				
Bitch D	40	40	390	215
Bitch E	600	190	180	
Bitch F	360	60	1900	40

Table 1b. Lactobacilli expressed as mean ±SD of cfu/mL at different times between P group and C group.

GROUP	**COLOSTRUM**	**DAY 7**	**DAY 14**	**DAY 21**
PROBIOTIC	400±215.2	670±777.8	320±169.71	1931.6±1738.1
CONTROL	333±352.5	96.7±81.4	823.3±938.31	127.5±123.4

Fig. 1 RAPD profiles. Lane MW, negative control; lane PNA 1 (*L.fermentum*). Lane CECT7266(*L. reuteri*); lane 1-16, colonies obtained from the MRS media.

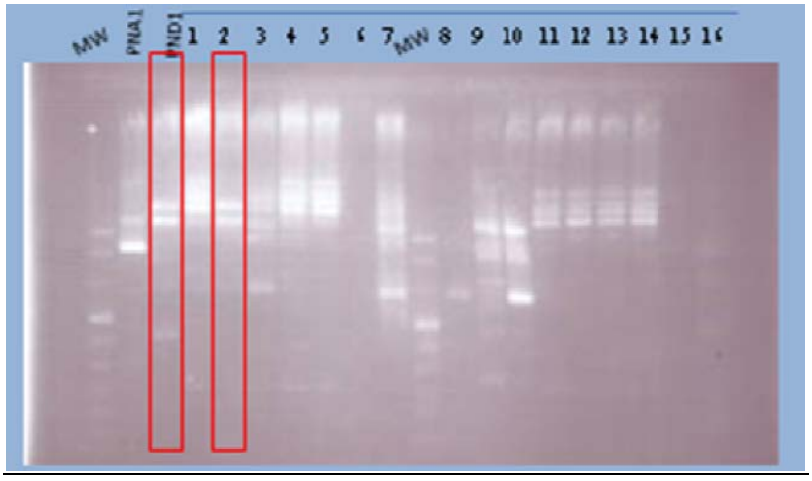

Table 2. Positive *Lactobacillus reuteri* CECT7266 match colonies with regard to total analyzed colonies.(% of CECT7266 of the total lactobacilli colonies)

		COLOSTRUM	Day 7	Day 14	Day 21
Probiotic Group	BITCH A	2/39 (5.12%)	0/12	1/20 (5%)	4/30(13.3%)
	BITCH B	1/19 (5.26%)	0/20	2/44 (4.5%)	2/20 (10%)
	BITCH C	0/20			0/30
Control Group	BITCH D	0/4	0/8	0/39	0/18
	BITCH E	0/25	0/19	0/20	
	BITCH F	0/20	0/6	0/23	0/6

Table 3. Lactobacilli colonies (cfu/g feces) growth in fecal samples of puppies in the two groups.

Probiotic Group	PUPPY	MECO-NIUM	Day 7	Day 14	Day 21	Day 30
BITCH A	A.1		8.7E+07	3.97E+08	5.45E+06	1.12E+09
	A.2					1.06E+08
	A.3		1.75E+07	5.78E+08	1.30E+07	1.87E+08
	A.4		7.17E+07	2.53E+09		
BITCH B	B.1				2.17E07	1.76E+06
	B.2			2.32E+09		2.13E+08
	B.3				1.65E+08	6.93E+06
BITCH C	C.1		2.29E+08			4.42E+09
	C.2					2.15E+09
	C.3		5.35E+08			1.14E+09
	C.4		2.00E+07	2.82E+08		2.85E+09
	C.5		2.86E+08	1.65E+06		4.73E+08
	C.6		1.19E+09	5.00E+08		5.29E+08
Control Group						
BITCH D	D.1				2.00E+07	5.30E+08
	D.2	1.27E+04	2.37E+08			2.05E+07
	D.3	1.69E+04	1.00E+06	3.06E+09	5.21E+08	1.40E+09
	D.4				1.10E+08	5.46E+08
BITCH E	E.1		4.98E+08		8.40E+07	6.26E+08
	E.2				1.24E+09	4.30E+08
	E.3		3.13E+08			1.66E+09
	E.4		4.36E+09			6.35E+08
	E.5		2.83E+08			2.08E+09
	E.6					7.97E+07
	E.7		4.02E+08			
BITCH F	F.1					2.66E+08
	F.2	1.00E+07				4.38E+08
	F.3	1.06E+08				4.14E+07

Table 4. Lactobacillus concentration, log cfu/g puppies feces (mean±SD)

	Day 7	Day 31
Probiotic Group	8.48±8.60	9.04±9.14
Control Group	8.49±9.19	8.83±8.81

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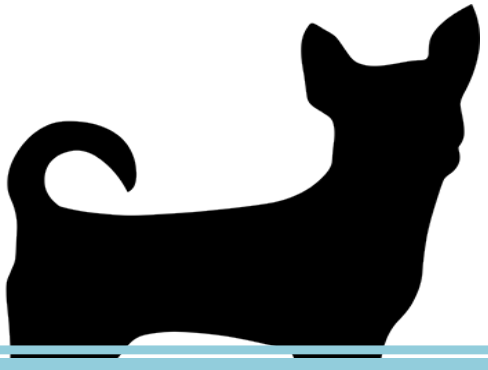


III. CAPÍTULO 3
EVALUATION OF VAGINAL BACTERIAL COLONIZATION IN HEALTHY FEMALE DOGS AFTER ORAL LACTOBACILLI ADMINISTRATION

3. EVALUATION OF VAGINAL BACTERIAL COLONIZATION IN HEALTHY FEMALE DOGS AFTER ORAL LACTOBACILLI ADMINISTRATION.

3.1 INTRODUCTION

As with other mucosal tissues in the body of dogs, the vaginal mucosa is not sterile. Studies conducted in vaginal bacteria have isolated a mixed population of aerobic and anaerobic microorganisms that include opportunistic pathogens (1, 2). Lactobacillus and Enterococcus species have been identified in the vaginal microbiota of healthy intact dogs. These vaginal lactic acid bacteria (LAB) inhibit the growth of pathogenic bacteria in vitro, including Escherichia coli, Proteus mirabilis, and Staphylococcus aureus. LAB probiotics could have health benefits (3) and they can exert this activity by competing for nutrients or interfering with adhesion to epithelial cell receptors (4).

Lactic acid producing bacteria metabolize glycogen and subsequently produce lactic acid that could decrease vaginal pH, inhibiting colonization by uropathogenic strains of bacteria (5).

Vaginal bacteria are often identified in vaginal cytology of healthy female dogs during proestrus and estrus (6). In female dogs, changes in the vaginal microbiota are associated with reproductive tract diseases, such as bacterial vaginitis, characterized by the replacement and proliferation of microorganisms that are normal components of the enteric microbiota (such as, *Escherichia coli* and *Proteus mirabilis*) (1, 7). Bjurström (1993) found that *E. coli*, beta-hemolytic streptococci, *Staphylococcus intermedius*, and *Pasteurella multocida* were the species most often isolated from bitches with pyometra, infertiliy, vaginitis and from those with dead puppies (7).

Uncomplicated urinary tract infections (UTIs) in dogs are common during their life (8, 9). Women with increased vaginal colonization with Lactobacillus species have a reduction in the episodes of recurrent UTIs (10, 11). Stapleton *et al*. (2011) and

Mastromarino *et al*. (2009) suggest that the vaginal administration of Lactobacillus might be a viable alternative or complement to the treatment with antimicrobials in human patients with recurrent UTI.(10, 11) Delucchi *et al*. (2008) also suggest that vaginal LAB might have a beneficial role within the urogenital tract of female dogs. (3)

Depletion of LAB could result in increased vaginal colonization and adherence of pathogens, with a subsequent increase in the probability of developing vaginitis, urogenital infection or in more advances stages, pyometra. This is supported by studies demonstrating the contrary hypothesis, that high level vaginal colonization with lactobacilli results in a reduction of urogenital infection in women (12, 13).

The aim of this study was to evaluate if an oral supplementation of *Lactobacillus reuteri* CECT7266 and *L fermentum* CECT7265 (25) results in colonization and persistence of lactobacillus in the vagina of healthy female dogs.

3.2 MATERIAL AND METHODS

3.2.1 Animals

Thirty-seven English Bulldog breeding bitches with no previous history of UTI, vaginitis or pyometra, from two different breeders (12 and 25 animals each), were included in the study. The median age was 3 years (2-5 years). All bitches were fed with the same dry diet (Advance medium adult, Affinity Petcare (Barcelona, Spain)). They were randomly assigned to 2 groups. The Probiotic group (P) with 17 bitches, and the control group (C) with 18 bitches. All the bitches were in the anoestrus phase of the cycle (confirmed by blood progesterone test) when starting the supplementation. Bitches each received a daily oral capsule containing either $2x10^8$CFU of *Lactobacillus reuteri* CECT 7266 and *Lactobacillus fermentum* CECT 7265 (group P) or maltodextrin (group C), for a period of 3 months.

3.2.2 Biological samples

Collection of vaginal swabs for bacterial culturing was performed at the start of the trial (T0) and 3 months later (T3). Sterile samples were obtained from the cranial aspect of the vagina of each dog. The entire area was disinfected with chlorhexidine. Sterile swabs were introduced cranial to the vestibulo-vaginal junction and were advanced as far as possible, then the swabs were rotated several times. Throughout the procedure the exposure to other areas of the genitourinary tract was minimized. The swabs were kept refrigerated until they were taken to the laboratory. Once in the lab, the biological material contained in the vaginal swabs was re-suspended in 1 ml of saline buffer (PBS).

3.2.3 Isolation and enumeration of microorganisms

Initially, proper peptone water dilutions of the samples were plated in triplicate onto Columbia Nadilixic Acid Agar (CNA, BioMerieux; a highly nutritious, general-purpose medium for the isolation and cultivation of fastidious microorganisms), Baird Parker (BP, BioMerieux; a selective medium for the isolation of staphylococci), Kanamycin-aesculin-azide (KAA, Oxoid¸ BioMerieux; a selective medium for the isolation of enterococci), Pasteurella (BioMerieux; a selective medium for the isolation of *Pasteurella* spp.), MacConkey Agar (MCK; BioMerieux; a selective medium for the isolation of enterobacteria), and Mycoplasma A7 (BioMerieux; a selective medium for the isolation of mycoplasma and ureaplasma) agar plates, which were aerobically incubated at 37ºC for up to 48 h. In the same way, the same samples were also cultured on de Man, Rogosa, and Sharpe (MRS, Oxoid, Basingstoke, UK) supplemented with L-cysteine (0.5 g/L) (MRS-Cys; a medium for the isolation of lactic acid bacteria and bifidobacteria) agar plates, which were incubated anaerobically (85% nitrogen, 10% hydrogen, 5% carbon dioxide) in an

anaerobic workstation (MINI-MACS, DW Scientific, Shipley, UK) at 37ºC for 48 h. After analyzing the first 5 samples, KAA, Pasteurella and Mycoplasma A7 agar plates were discarded due to poor/no microbial growth and/or to redundant results with other agar media. Approximately 10-25 isolates from each culture medium where growth was observed were randomly selected, grown in BHI broth and stored at –80ºC in the presence of glycerol (30%, v/v).

3.2.4 Identification of the bacterial isolates

The selected bacterial isolates were observed by optical microscopy to determine their morphology and Gram staining. Additionally, they were tested for catalase, oxidase and coagulase activities and for growth on plates of Baird-Parker (BP, BioMerieux) and Kanamycin Aesculin Azide Agar (KAA, Oxoid). Initially, most of the isolates that, on the basis of such preliminary tests, seemed to belong to the genus *Staphylococcus* were submitted to a novel multiplex PCR method based on the *dnaJ* genes. Briefly, a single colony growing on solid media was removed with a sterile plastic tip and re-suspended in 100 µl of sterile deionized water in a microcentrifuge tube. Then 100 µl of chloroform/isoamyl alcohol (24:1) was added to the suspensions, and after vortexing for 5 s the mixture was centrifuged at 16,000 × g for 5 min at 4°C. Then 5–10 µl of the upper aqueous phase was used as a source of DNA template for PCR with primers J-StGen (5'-TGGCCAAAAGAGACTATTATGA-3'), J-StAur (5'-GGATCTCTTTGTCTGCCG-3'), J-StEpi (5'-CCACCAAAGCCTTGACTT-3') and J-StHom (5'-TTGACCACTACCCTCACAC-3') in a Icycler thermocycler (Bio-Rad Laboratories, Richmond, CA). The primer pairs J-StGen/J-StAur, J-StGen/J-StEpi and J-StGen/J-StHom result in a 337 bp *S. aureus* species-specific fragment, 249 bp *S. epidermidis* species-specific fragment and a 589 bp *S. hominis* species-specific fragment,

respectively. PCR conditions were as follows: 1 cycle of 94°C for 4 min, 30 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, and a final extension of 72°C for 5 min. On the other hand, most of the isolates that seemed to belong to the genus *Enterococcus* could be identified by PCR species-specific detection of enterococcal *ddl* genes, which encode D-alanine:D-alanine ligases, following the protocol described by Dutka-Malen *et al*. (1995) (14). Confirmation of staphylococci and enterococci identification and identification of the rest of the isolates was performed by PCR sequencing of a 470 pb fragment of the 16S rRNA gene as described by Kullen *et al*. (2000) (15). The amplicons were purified using the Nucleospin® Extract II kit (Macherey-Nagel, Düren, Germany) and sequenced at the Genomics Unit of the Universidad Complutense de Madrid, Spain. The resulting sequences were used to search sequences deposited in the EMBL database using BLAST algorithm and the identity of the isolates was determined on the basis of the highest scores (>98%).

3.3.5 Genotyping of the *Lactobacillus fermentum* and *Lactobacillus reuteri* isolates by random amplification of polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE).

Later, and in order to check whether *L. fermentum* and *L. reuteri* isolates actually belonged to the strains CECT7265 and CECT7266, respectively, those lactobacilli isolates identified and members of such species were typified by RAPD. DNA was extracted from isolated colonies following the protocol of Ruiz-Barba *et al*. (2005) (15) and was used as a template to determine the RAPD profile with the primer OPL5 (5-'ACGCAGGCAC-3''). One representative of each RAPD profile was then selected and submitted to PFGE profiling. For this purpose, chromosomal DNA was extracted from the isolates and digested with the endonuclease *Sma*I (New England Biolabs, Ipswich, MA) at 25°C for 24 h. Electrophoresis was carried out in a CHEF DR II

apparatus (Bio-Rad, Birmingham, UK) in 1% (w/v) SeaKem GTG agarose (FMC, Philadelphia, PA) with 0·5× TBE buffer (45 mM Tris/HCl, 45 mM boric acid and 1 mM EDTA, pH 8·0) at 15°C. A constant voltage of 200 V was applied to the system and fragment separation was performed using a two-phase program. Electrophoretic conditions for separating the *Sma*I fragments were a pulse time from 0.5 to 5 s for 10 h and, then, another from 0.5 to 10 s for 6 h. LowRange PFG marker and MidRange PFG marker I (New England BioLabs) were used as molecular size standards. Agarose gels were stained with ethidium bromide (0.5 µg/mL) and images were digitized with a GelPrinter Plus system (TDI, Madrid, Spain).

3.4 RESULTS

Bacteria were isolated from all 37 bitches. Mixed bacterial population were common in both groups, with a mean of 3.52 log cfu/mL at T0 and with a mean of 3.66 log CFU/mL at T3

The most common bacterial species isolated at T0 in both groups were *Enterococcus canintestini (56%), Streptococcus canis (42%), Proteus mirabilis (42%)* and *Escherichia coli (31%)*. Other less frequent species were *Weissella spp. (17%), Lactobacillus rhamnosus (20%), Lactobacillus reuteri (12%)* and *Lactobacillus fermentum (5,5%)*, (Table 1 and Figure 1). Other Lactobacillus such as *L. johnsonii* and *L. murinus* appears in a very low frequency at T0 in P group (12% and 5% respectively).

The main bacterial species changed after 3 months of oral probiotic supplementation. In group P, *Enterococcus canintestini* increased to 94%, *Weissella spp.* to 39%, *Lactobacillus reuteri* to 41% and *Lactobacillus fermentum* to71% of the samples (Table 1 and Figure 1).

In P group *Lactobacillus fermentum* CECT7265 and *L. reuteri* CECT7266 average of cfu/mL increased while decreasing in C group (Table 2 and Figure 2).

By genotyping the *Lactobacillus fermentum* and *Lactobacillus reuteri* isolates from both groups, we could distinguish the ones belonging to CECT7265 and CECT7266, that were given orally. None of the *L fermentum* or *L reuteri* isolated at T0 corresponds to the CECT7265 or CECT7266, but all of the *L fermentum* and *L reuteri* at T3 in P group corresponds to the ones given orally.

Finally, the degree and sustainability of vaginal colonization by at least one *Lactobacillus* strain given orally, confirmed colonization of the vaginal epithelium in 88% of bitches who received the probiotic for three months.

3.5 DISCUSSION

Vaginal microbial species found in our study at T0 are in concordance with other studies. Delucchi et al, (2008) reported *E. canintestini* as one of the more abundant species in the canine vagina, as seen in our study (3). Also, Hutchins et al. (2014) described *Escherichia coli* and *S. pseudintermedius* as the most prevalent organisms obtained from the vaginal tract of dogs, and *Enterococcus canintestini* as the most common LAB isolated (17). These species are normal commensals of the intestinal microbiota (3) and their presence in vagina suggests a intestinal transfer of the microbiota, as described in other species such as humans (18).

In spite of this, in the present study we isolated more lactobacillus species than recorded in the bibliography for dogs (1, 3), but much less than recorded in women (5, 19). These could be explained with the difference in pH of the vagina. Vaginal pH of healthy dogs ranged from 6.0 to 7.5 (3), while in women pH is 4.5 or even lower (20).

Some of the species found in the vagina in this study are described as being associated with reproductive tract diseases and urogenital diseases. Van Duikeren (1992) described proliferation of *Escherichia coli* and *Proteus mirabilis* associated with reproductive tract diseases

(1). Windahl (2015) reported that the most prevalent bacteria in UTI in dogs were *E.coli* with a high prevalence (68,9%), *S. pseudintermedius* (9,6%) and *P. mirabilis* (8,8%) (21*)*. Though in this study these species were present in healthy bitches, and this could be associated with the low levels of cfu/ml.

As lactic acid producing bacteria, including Lactobacillus and Enterococcus species, metabolize glycogen and subsequently produce lactic acid, it is thought that the presence of these bacteria could decrease vaginal pH, inhibiting colonization by uropathogenic strains of bacteria(15).

Depletion of LAB could result in increased vaginal colonization and adherence of pathogens, with a subsequent increase in the probability of developing vaginitis, urogenital infection or in more advances stages, pyometra (24). This is supported by studies demonstrating the contrary hypothesis, that high level vaginal colonization with lactobacilli results in a reduction of urogenital infection in women (12, 13, 22).

This study is the first to show that an orally administered LAB is recovered from the vagina of healthy female dogs. In addition, the oral administration of *L. reuteri* CECT7266 and *L.fermentum* CECT7265, for a 3 months period increased the prevalence of vaginal lactobacillus. In women, Vasquez et al., (2005) demonstrated that orally administered lactobacilli can be re-isolated from the vagina (19). They postulated that presumably as a consequence of the migration from the rectum via perineum. On the contrary other recent studies were unable to recover an orally administered lactobacilli from the vagina, in human (22) or dog (17). Consistently, van de Wijgert *et al*. (2014) (23) showed that bacteria colonizing the vaginal mucosa (both commensals and vaginosis-associated microbes) have been isolated from the rectum and the mouth, suggesting that the gut and oral cavity act as extravaginal reservoirs of vaginal microbiota bacteria.

Therefore, it appears plausible that the oral administration of probiotic bacteria may potentially influence the vaginal microbiota through two possible mechanisms: 1. modification of the intestinal microbiota, by reducing potentially harmful bacteria, increasing endogenous lactobacillus and migration from the rectum (by licking); 2. direct migration to the vaginal mucosa via the gastrointestinal route.

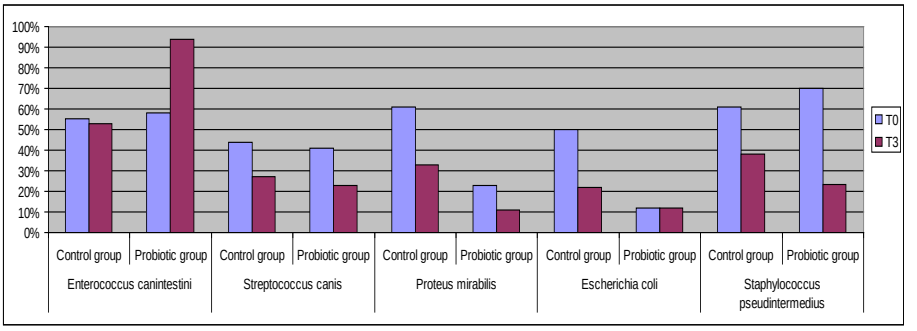
In conclusion, the oral administration of a probiotic (*Lactobacillus reuteri* CECT7266 and *Lactobacillus fermentum* CECT7265) was able to increase the prevalence of vaginal lactobacilli and this isolated *Lactobacillus reuteri* CECT7266 correspond to 99 % of the strains orally supplied.

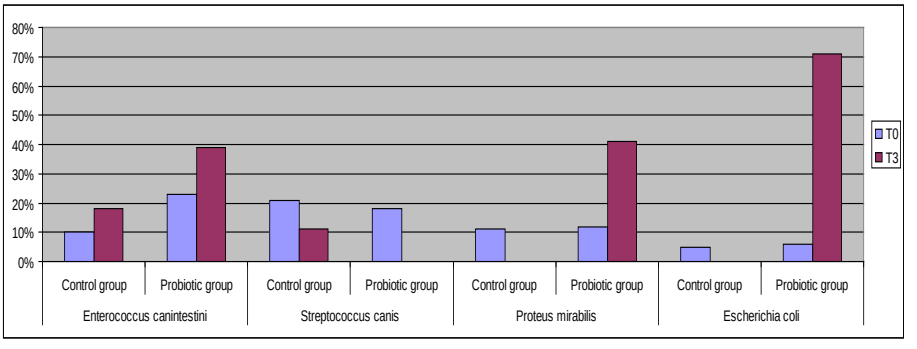
Results 1. Bacterium frequency in the vaginal microbiota of English bulldogs between control and probiotic group in a 3-month evolution

Table 1

	Group	T0	T3
Enterococcus canintestini	Control	55%	53%
	Probiotic	58%	94%
Streptococcus canis	Control	44%	27%
	Probiotic	41%	23%
Proteus mirabilis	Control	61%	33%
	Probiotic	23%	11%
Escherichia coli	Control	50%	22%
	Probiotic	12%	12%
Staphylococcus pseudintermedius	Control	61%	38%
	Probiotic	70%	24%
Weissella spp.	Control	10%	18%
	Probiotic	23%	39%
Lactobacillus rhamnosus	Control	21%	11%
	Probiotic	18%	0%
Lactobacillus reuteri	Control	11%	0%
	Probiotic	12%	41%
Lactobacillus fermentum	Control	5%	0%
	Probiotic	6%	71%

Figure 1.





Results 2. Average cfu/mL of *Lactobacillus fermentum* and *Lactobacillus reuteri* between control and probiotic group.

Table 2.

		T0	T3
Lactobacillus fermentum	Control	2,70	0,00
	Probiotic	1,70	3,70
Lactobacillus reuteri	Control	2,68	0,12
	Probiotic	2,50	3,27

Figure 2.

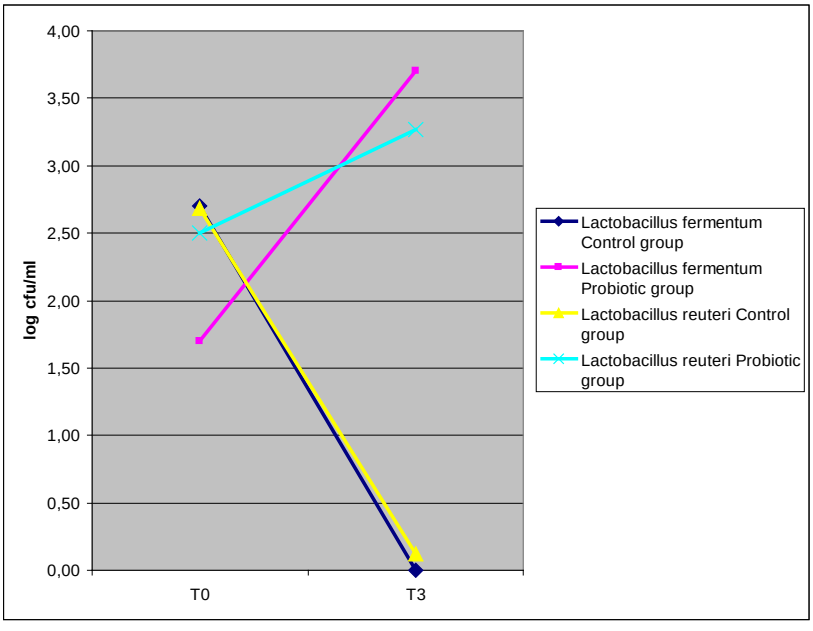


Table 3.

	Probiotic group	**Control Group**
Number bitches	18	19
% in heat	94	63
Fertility (%)	82	50
Prolificity	4,64	2,14
Cubs / female	3,61	0,79

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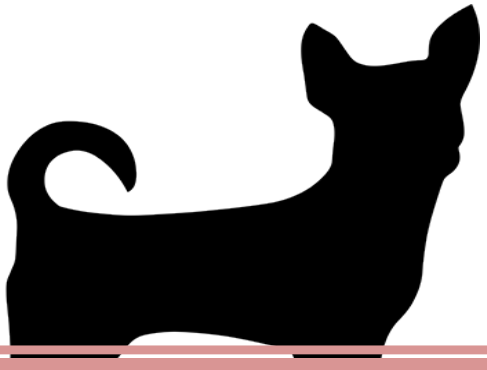

IV. DISCUSIÓN GENERAL

DISCUSIÓN

Varios autores han puesto de manifiesto que la terapia con algunas cepas probióticas puede ser efectiva en el tratamiento de patologías como diarreas (Herstad *et al.*, 2010), dermatitis atópicas (Kim *et al.*, 2015; Ohshima-Terada *et al.*, 2015; Marsella *et al.* 2013)
infecciones genitourinarias (Hutchins *et al.*, 2013; Deluchi *et al.*, 2008), o enfermedades con base inmunológica (Marsella *et al.*, 2012).

En la selección de cepas como potenciales probióticos, uno de los requerimientos básicos es la habilidad para sobrevivir en las condiciones de acidez estomacal y en los ácidos biliares en el tracto gastroinetsinal, además de la capacidad potencial de adhesión al epitelio intestinal (Dunne *et al*., 2001). Aunque uno de los criterios más efectivos para seleccionar una cepa probiótica es su habilidad para adherirse a células epiteliales y colonizar superficies mucosas del tracto gastrointestinal tanto de humanos como de animales (McNaught y MacFie, 2001; Ouwehand *et al*., 2002b). Esta adherencia y colonización se relacionan directamente con los beneficios para la salud de las cepas probióticas, como el antagonismo contra cepas patógenas, la modulación del sistema inmunitario y la reparación de la barrera intestinal (Johansson *et al*., 1993, Elliott *et al.,* 1998).

Un estudio revela que los efectos beneficiosos de los probióticos son específicos de cepa (Galdeano *et al*., 2010). Así, la combinación de diferentes cepas probióticas con funciones seleccionadas y específicas deberían ser más efectivas que una cepa única (Timmerman *et al.*, 2004). Pocos estudios se han llevado a cabo investigando el uso de probioticos multi cepa como suplementos dietéticos en estudios con animales.

El origen tradicional de las bacterias probióticas que han sido registradas para su uso en humanos o animales ha sido las heces humanas (Tulumoglu *et al.* 2013; Wang *et al.*, 2010). Recientemente

se han buscado otros orígenes más específicos para el aislamiento de cepas microbianas para su uso como probióticos, como son la leche materna (Lara-Villoslada *et al.*, 2007a; Martin *et al.*, 2005) o la vagina (McLean y Rosenstein, 2000).

En la clínica de pequeños animales, el probiótico *Enterococcus faecium*, también de origen fecal, es de momento el único probiótico disponible comercialmente para su uso en perros, y del que se han hecho las investigaciones clínicas hasta la fecha (Vahien y Männer, 2003; Marcináková *et al.*, 2006). Martin *et al.,*(2009) realizaron un estudio para determinar si la leche de perra podría ser una fuente de probióticos potencialmente útiles. De este estudio se seleccionaron dos cepas: *Lactobacillus reuteri* CECT7266 y *Lactobacillus fermentum* CECT7265. Estas dos cepas muestran un alto potencial antimicrobiano, ratios altos de supervivencia frente a ambientes adversos y actividades enzimáticas deseables como la producción de α-glucosidasa. Además estas dos cepas no degradan mucina lo que hace que se adhieran a la mucina gástrica e intestinal de manera mucho más eficiente.

Los estudios que se presentan en esta tesis son pioneros al evaluar en perros el efecto beneficioso de cepas de *Lactobacillus* obtenidas de leche de la misma especie en la que se aplican.

En primer lugar, se estudió la suplementación oral de las dos cepas en cachorros sanos. Tras la administración oral durante 4 semanas se observó un incremento significativo de los lactobacilos en heces, mientras que se observaba una reducción de los mismos en los animales que no consumían el probiótico. Además en el grupo suplementado se produjo una reducción de las enterobacterias mejorando la proporción de bacterias consideradas beneficiosas lo que podría ayudar a mantener la salud gastrointestinal. De la misma forma, en las heces de los animales suplementados con las dos cepas de *Lactobacillus* se pudo observar un incremento significativo en la concentración de butirato, este acido graso de cadena corta

constituye la principal fuente de energía de los enterocitos y colonocitos y juega un papel importante en la regulación de la proliferación y diferenciación celular, pudiendo ejercer un efecto beneficioso en la enfermedad inflamatoria intestinal (Cho y Finocchiaro, 2009). Aunque los lactobacilos no producen directamente butirato, se ha descrito anteriormente, su aumento tras del consumo de *Lactobacillus* (Olivares *et al.*, 2006), lo que indicaría una modulación de la flora intestinal con la suplementación.

El efecto preventivo de una cepa probiótica contra la adhesión de patógenos implica no sólo la competencia para la adhesión, sino también la producción de compuestos que puedan interferir en la adherencia del patógeno*. L. fermentum* CECT7265 y *L. reuteri* CECT7266 mostraron en estudios previos una alta actividad antibacteriana en ensayos in vitro (Martin *et al.*, 2010). En este estudio se pudo observar en agua fecal obtenida de los animales que ingirieron los probióticos una inhibión in vitro a la adhesión de *Salmonella* a las mucinas, lo que podría indicar un efecto protector de la suplementación a una posible proliferación de bacterias patógenas como la Salmonella.

La composición “normal” de la microbiota intestinal de perros puede alterarse en condiciones estresantes, como el destete, cambios en la dieta o la administración de antibióticos. Se ha podido demostrar que la administración oral de *L. fermentum* CECT7265 y *L. reuteri* CECT7266 a perros sanos puede modificar algunos parámetros intestinales que se pueden ser indicadores de unas condiciones que ayuden a mantener la salud intestinal, por lo que podrían ser una herramienta para ayudar en las condiciones descritas que alteran la microbiota intestinal. Aunque su efecto en condiciones patólogicas, como diarrea o enfermedad inflamatoria intestinal debera ser objeto de otro estudio.

El efecto de cepas probióticas en la modulación o incluso en la maduración del sistema inmune se ha estudiado ampliamente en

los seres humanos y en modelos animales (Isolurai et al., 2001; Gill, 1998; Olivares et al., 2006). A este efecto inmunológico observado en algunos estudios se ha atribuido el efecto en la prevención de infecciones o su efecto sobre la alergia y las enfermedades inflamatorias de algunas cepas probióticas (Kaila et al., 1992; Majamaa et al., 1995; Gionchetti et al., 2000; Isolauri et al., 2008). En este estudio, el análisis de los parámetros inmunológicos evaluados mostró una mejora significativa en marcadores de respuesta inmune innata y específica de los cachorros. Se ha podido observar un aumento significativo de la actividad fagocítica de los monocitos después de la suplementación con las cepas probióticas. Este efecto ha sido puesto de manifiesto previamente en la suplementación oral con otras cepas probióticas en los seres humanos (Olivares et al., 2006; Schiffrin et al., 1995). De la misma forma, en perros, la suplementación con una preparación de *Enterococcus faecalis* también indujo la activación de la actividad fagocítica de los monocitos (Kanasugi et al., 1997). Sin embargo, las bacterias se administraron muertas, por lo que el efecto podría ser diferente al provocado por las bacterias vivas.

En cuanto al efecto sobre la respuesta inmune adaptativa o adquirida, en este estudio se observó un aumento significativo de la Ig G plasmática después de 4 semanas de suplementación. La posible mejora en la respuesta humoral inducida por el tratamiento probiótico se ha relacionado con el efecto protector de probiótico contra las enfermedades infecciosas (Kaila et al., 1992; Majamaa et al., 1995).

En este primer trabajo se ha podido poner de manifiesto que la administración oral de dos cepas de *Lactobacillus* aislada de leche de perra a cachorros durante 4 semanas, produce efectos que se pueden considerar beneficiosos tanto a nivel intestinal como inmunológico. Estos resultados sugieren que la administración de *L. reuteri* CECT7266 y *L. fermentum* CECT7265 puede ser una herramienta útil para mejorar la salud de los animales, aunque los

efectos como ayuda en la prevención o tratamiento de situaciones patológicas requiere estudios posteriores.

Durante el segundo estudio, se administraron oralmente las dos cepas de lactobacilos y se valoró su transferencia a la leche de perras lactantes y sus cachorros. Dos perras que recibieron la mezcla probiótica demostraron una transferencia activa a la leche del *Lactobacillus reuteri* CECT7266, sobre todo al final de la lactancia. No se detectó ninguna transferencia de *Lactobacillus fermentum* CECT7265 a la leche de las perras. Esto podría ocurrir porque el *Lactobacillus reuteri* CECT7266 tenga mayor afinidad por la glandula mamaria y resista mejor a las condiciones para la transferencia que el *Lactobacillus fermentum* CECT7265 pero esto deberá ser objeto de otro estudio más detallado. .La frecuencia de presencia del Lactobacillus reuteri CECT7266 en la leche de las perras suplementadas va desde 5 % al inicio hasta 10-13% al final de la lactancia. Esta transferencia no es muy elevada pero pone de manifiesto que la suplementación oral a las hembras gestantes y lactantes es una via para suplementar a la camada mientras se alimentan de leche.

Existen diferentes vías posibles para la transferencia de bacterias a leche, movimiento de la microbiota del tracto entérico maternal a la glándula mamaria por vía externa o a través de una ruta endógena que involucra células dendríticas y los macrófagos: la vía entero-mamaria (Martin et al., 2004b; Perez et al., 2007). El poner de manifiesto la vía de transferencia a la leche de las cepas administradas oralmente no era el objetivo de este trabajo por lo que no se puede descartar una vía externa o interna o ambas.

El estudio de Donnet -Hugues et al (2010) sugiere que el microbioma de la leche juega un papel clave en la programación del sistema inmune neonatal, pero el origen de los lactobacilos que colonizan el intestino neonatal es un tema de debate. En el pasado, se sugirió que éstos son adquiridos por la contaminación oral con

lactobacilos maternos durante el tránsito por el canal del parto. Sin embargo, los estudios moleculares han demostrado que esta colonización no está significativamente relacionada con el tipo de parto: vaginal o por cesárea (Matsumiya et al., 2002; Martín et al., 2003; Ahrné et al., 2005). Otro tema de controversia actualmente es la hipótesis de que el feto no es estéril al nacer y que hay bacterias de la flora intestinal de la madre podrían translocar desde el tracto digestivo a localizaciones extradigestivas. Esta teoría se demostró cuando se consiguieron asilar bacterias en la sangre del cordón umbilical de los recién nacidos sanos nacidos por cesárea. Además en un estudio en que se administraba oralmente la cepa *Enterococcus faecium* genéticamente marcado a ratonas gestantes, se pudo observar la presencia de la misma cepa en el líquido amniótico de las ratonas (Jimenez et al., 2005; Perez et al., 2007).

En esta tesis se han podido aislar lactobacilos en las muestras de meconio recogidas justo en el momento del nacimiento, y esto podría sugerir que los fetos no son estériles y que la colonización del intestino de los cachorros podría comenzar en la placenta. No existen estudios similares en perros, pero en humanos, Hansen et al (2015), demostraron la presencia de un bajo número de bacterias en las muestras de meconio de niños sanos, nacidos por vía vaginal. Además, Martin et al (2004) pudieron aísla bacterias lácticas y otras bacterias comensales de meconio obtenidos de neonatos sanos nacidos ya sea por vía vaginal o por cesárea (Jimenez et al., 2008) .

De la misma forma que aparece en la lecha también se pudo demostrar, la presencia de la cepa *de Lactobacillus reuteri* CECT7266 administrado por vía ora a las madres se ha detectado en las heces de un cachorro cuya madre recibió la mezcla de las dos cepas de Lactobacilli por vía oral. Esto sugiera la transferencia de la leche de la madre al intestino del cachorro, ya que la misma cepa CECT7266 se había aislado del calostro y la leche de esta perra. En

perros es la primera vez que se han aislado lactobacilos de meconio y en el que se ha demostrado el paso de bacterias presentes en la leche al intestino de los cachorros amantados con esta leche.

La concentración de lactobacilos obtenida en este estudio (intervalo entre 9,04 log ufc/ ml a 8,48 log ufc / mL) es similar a los obtenidos en los cachorros de 3 a 6 meses de edad (Primer estudio de la tesis) lo que demuestra que la presencia de lactobacilos es estable durante el crecimiento de los cachorros y demuestra la solidez de los valores obtenidos en estos dos estudios.

Este estudio tiene consecuencias prácticas que sugieren que la administración oral de probióticos en perras gestantes y lactantes podría tener un efecto directo sobre la salud de la camada.

Siguiendo la línea de estudio sobre los efectos beneficiosos sobre las hembras reproductoras, se ha estudiado la flora vaginal y el posible efecto de la suplementación con lactobacillos vía oral en la modulación de la flora vaginal y por consiguiente de la salud del animal.

En el estudio de la flora vaginal normal, *Enterococcus canintestini* ha sido el aislado más abundante, lo que esta en concordancia con otros estudios anteriores (Deluchii et al., 2008; Hutchins et al., 2014). Una diferencia fue la cantidad de cepas de lactobacilos aisladas, ya que se encontraron más que en otros estudios anteriores (Van Duijkeren, 1992, Delucchi et al., 2008), aunque sorprendentemente en concentraciones más bajas que en mujeres (Reid et al., 1999, Vásquez et al., 2005). Esto se podría explicar por la diferencia en el pH vaginal entre ambas especies, siendo el pH vaginal de perros sanos entre 6.0 y 7.5 (Delucchi et al., 2008), mientras que el pH vaginal en mujeres más acido, con una media de 4,5 o incluso inferior (Reid et al., 2009). Además, algunas de las especies microbianas que aparecen en la vagina de la perras, se han asociado en algunos estudios con enfermedades del aparato reproductor y enfermedades urogenitales. Van Duikeren (1992)

describe la proliferación de *Escherichia coli* y *Proteus mirabilis* asociada con enfermedades del tracto reproductivo. Windahl (2015) manifiesta que las bacterias más prevalentes en las infecciones del tracto inferior en los perros eran producidas por *E. coli* con una alta prevalencia (68,9 %), *S. pseudintermedius* (9,6 %) y *P. mirabilis* (8,8 %). Aunque en este estudio estas especies estaban presentes en perras sanas y a una concentración baja.

Una disminución de bacterias acido lácticas podría provocar una proliferación de bacterias patógenas aumentando la probabilidad de desarrollar vaginitis, infecciones urogenitales o en casos mas graves piometra (Fieni et al., 2014). En mujeres la alta colonización vaginal con lactobacilos reduce las infecciones urogenitales, ya que ayuda a mantener un nivel bajo del pH vaginal (Stapleton et al., 2011, Barrons et al., 2008; Kullen et al., 2000)

Se ha podido demostrar que la administración oral de *Lactobacillus reuteri* CECT7266 y *Lactobacillus fermentum* CECT7265, durante un período de 3 meses aumentó la prevalencia de lactobacilos vaginales. Además recuperamos en vagina el mismo *Lactobacillus reuteri* CECT7266 que administrado vía oral. En concordancia con estos hallazgos, Vasquez et al., (2005) demostró que los lactobacilos administrados por vía oral se puede volver a aislar en la vagina de mujeres. Se postulaba que, presumiblemente como consecuencia de la migración desde el recto a través del perineo. Consistentemente, van de Wijgert et al., (2014) demostró que las bacterias que colonizan la mucosa vaginal (tanto comensales y asociados a vaginosis) se han aislado también en recto y boca, lo que sugiere que el intestino y la cavidad oral podrían actuar como reservorios extravaginales para la microbiota vaginal.

Por lo tanto, parece plausible que la administración oral de bacterias probióticas puede potencialmente influir en la microbiota vaginal a través de dos posibles mecanismos:

1. Modificación de la microbiota intestinal, mediante la reducción de bacterias potencialmente nocivas, aumentando los lactobacilos endógenas y durante la migración desde el recto (por lamido);
2. migración directa a la mucosa vaginal a través de la ruta gastrointestinal.

En la administración por vía oral de las dos cepas de lactobacilos: *Lactobacillus reuteri* CECT7266 se transfirió activamente a la leche de las hembras, y *Lactobacillus fermentum* CECT7265 colonizó la vagina, demostrando la afinidad diferencial de cada cepa.

V. CONCLUSIONES GENERALES

CONCLUSIONES GENERALES

Los resultados presentados en esta tesis nos permiten llegar a las siguientes conclusiones:

1. Tras la suplementación oral con las cepas *Lactobacillus reuteri* CECT7266 y *Lactobacillus fermentum* CECT7265 durante 4 semanas se modifica la flora intestinal de cachorros con un incremento de los lactobacillus y una reducción de las Enterobacterias excretadas en heces. Se incrementa la concentración del butirato asi como se observa una inhibición in vitro de la adhesión de Salmonella a las mucinas. A nivel plasmático se incrementa la actividad fagocítica de los monocitos y un incremento de concentración de IgG.

2. Tras la suplementación oral con *Lactobacillus reuteri* CECT7266 y *Lactobacillus fermentum* CECT7265 en hembras lactantes durante 1 mes previo al parto, se logró identificar *Lactobacillus reuteri* CECT7266 en la leche de las hembras así como en heces de su cachorro. Se han podido aislar lactobacilos en muestras de meconio.

3. Tras la suplementación oral con *Lactobacillus reuteri* CECT7266 y *Lactobacillus fermentum* CECT7265 en hembras durante 3 meses se logró aumentar la prevalencia de lactobacilos vaginales, e identificar *Lactobacillus fermentum* CECT7265 en las muestras obtenidas. Aumentó el número de hembras que salió en celo, asi como la fertilidad y la prolificidad.

VI. BIBLIOGRAFIA

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