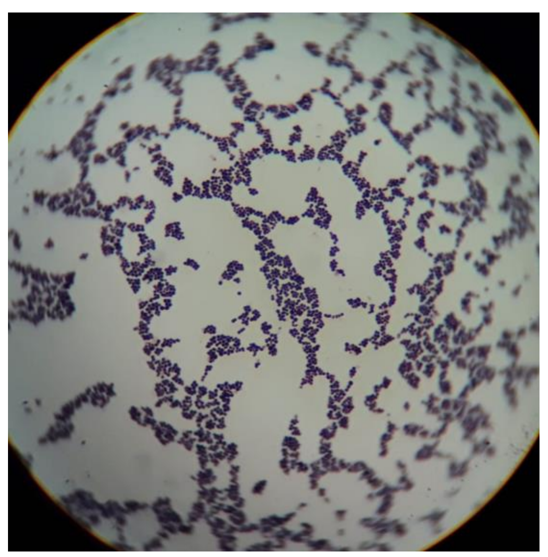
Búsqueda y selección de bacterias probiótica desde trucha común (*Salmo trutta*), trucha arcoíris (*Oncorhynchus mykiss*) y esturión siberiano (*Acipenser baerii)* para su potencial aplicación en salud humana y animal.

Trabajo Final de Máster

Máster de Investigación en Ciencias de la Salud

Universidad Pública de Navarra



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HACE CONSTAR

Que el Trabajo Final de Máster "**Búsqueda y selección de bacterias probiótica desde trucha común (*Salmo trutta*), trucha arcoíris (*Oncorhynchus mykiss*) y esturión siberiano (*Acipenser baerii)* para su potencial aplicación en salud humana y animal**" PRESENTADO POR Jose Emilio Martínez Rodríguez, estudiante del Máster Universitario de Investigación en Ciencias de la Salud en el curso 2020-2021 ha sido realizado bajo mi dirección y cuenta con mi visto bueno para su defensa a 7 de septiembre de 2021.

RESUMEN

El uso desproporcionado de antibióticos en la explotación animal ha tenido como resultado la aparición de bacterias resistentes a éstos, trayendo consigo efectos adversos tanto en estos animales como en las personas que constituyen el consumidor final. Como consecuencia, se ha estado estudiando el uso de probióticos como alternativa a los antibióticos. El propósito de este trabajo es la búsqueda de posibles probióticos, obtenidos a partir de un cepario de bacterias provenientes de diferentes tejidos corporales de los peces de agua dulce *Salmo trutta, Oncorhynchus mykiss* y *Acipenser baerii.* Para poder determinar su posible efecto probiótico, estas bacterias fueron aisladas y posteriormente enfrentadas en ensayos antagonistas a patógenos frecuentes en peces. Además de valorar su efecto probiótico, las bacterias también fueron identificadas mediante la secuenciación del gen 16S rRNA. En conclusión, de este trabajo se obtienen 3 especies de bacterias que son candidatas a probióticas, y que podrían ser usadas para salud animal y humana.

PALABRAS CLAVE

Acuicultura, Bacteria, Esturión Siberiano, Probiótico, Trucha Arcoíris, Trucha Común.

ABSTRACT

The disproportionate use of antibiotics in animal husbandry has resulted in the emergence of antibiotic-resistant bacteria, with adverse effects on both animals and people who consume these animals. Therefore, the use of probiotics as an alternative to antibiotics has been studied. The purpose of this work is to search for possible probiotics, obtained from a strain of bacteria from different body tissues of the freshwater fish *Salmo trutta, Oncorhynchus mykiss* and *Acipenser baerii*. To determine their possible probiotic effect, these bacteria were isolated, continuously, they were tested in antagonistic assays against certain fish pathogens. In addition to testing their probiotic effect, the bacteria were also identified by 16S rRNA gene sequencing. To conclude, from this study it had been obtained 3 bacteria species which are probiotic candidate, and they could be used in human and animal health.

KEY WORDS

Aquaculture, Bacteria, Brown Trout, Probiotic, Rainbow Trout, Siberian Sturgeon.

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GLOSARIO DE ABREVIATURAS

APROMAR: Asociación Empresarial de Productores de Cultivos Marinos

DO: Densidad óptica

EARS-Net: European Antimicrobial Resistance Surveillance Network

EMA: Agencia Europea de Medicina

FAO: Organización de las Naciones Unidas para la Alimentación y la Agricultura

FDA: Administración de Medicamentos y Alimentos de los EE. UU.

L: Marcador de peso molecular

LAB: Bacterias ácido-lácticas

LMR: Límites Máximos de Residuos

MAPA: Ministerio de Agricultura, Pesca y Alimentación

MRS: Man, Rogosa, Sharper

MX: Maximum Likelihood

NJ: Neighboor joining

OIE: Organización Mundial de Sanidad Animal

OMS: Organización Mundial de la Salud OMS

p/v: peso/volumen

PBS: Tampón fosfato salino

PCR: Reacción en cadena de la polimerasa

RAM: Resistencia a los antimicrobianos

TSB: Caldo Soja Tripticaseína

UFC: Unidades formadoras de colonias

1 INTRODUCCIÓN

1.1 ACUICULTURA

1.1.1 Acuicultura mundial

Según la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO, de sus siglas en inglés *Food and Agriculture Organization*), la acuicultura se define como la cría de organismos acuáticos, comprendidos los peces, moluscos, crustáceos y plantas. Además de la cría, otra de las características de la acuicultura es que los humanos intervienen para aumentar la producción de estos organismos (FAO, 2020).

Un informe de la FAO en 2020 resumió la producción total de la pesca y la acuicultura en el mundo en el año 2018, resultando en 179 millones de toneladas de peso vivo entre ambas. De estas 179 millones de toneladas, la producción acuícola aportaba un total de 82 millones de toneladas, de las cuales 54 millones eran peces de aleta, los casi 30 millones restantes eran crustáceos, moluscos y otros animales acuáticos. Por otro lado, si sumásemos la cifra de algas acuáticas producidas en acuicultura llegaríamos casi a las 120 millones de toneladas de producción en total.(FAO, 2020).

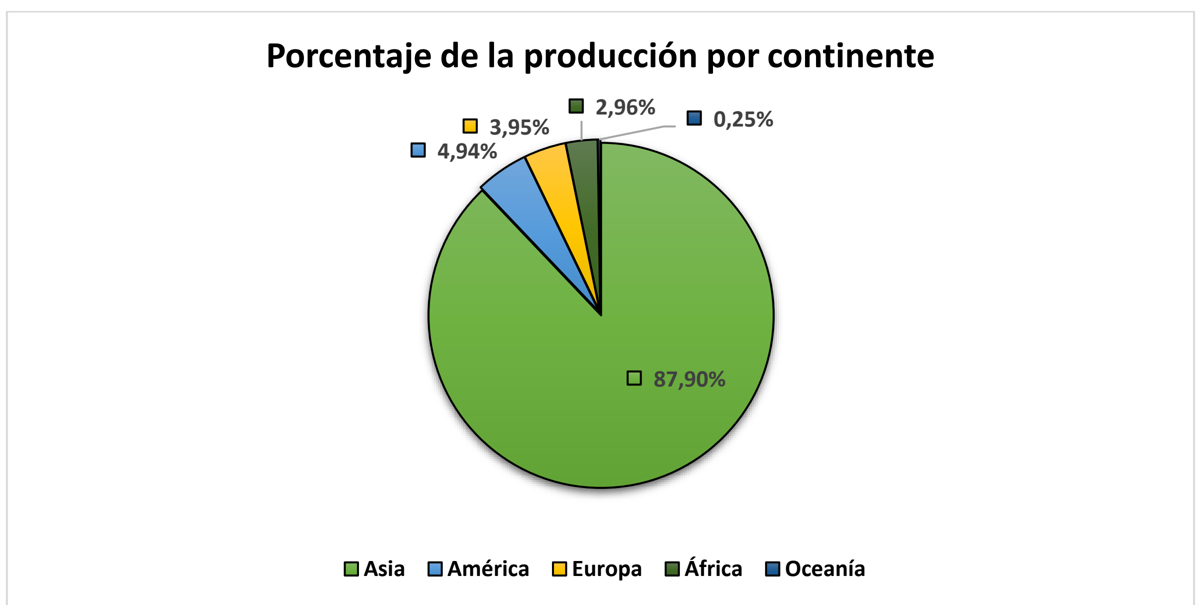


Figura 1: Porcentajes de la producción acuícola mundial dividida por continentes.

A nivel mundial, Asia es el continente con la mayor producción acuícola (89%), siendo China el principal productor de esta región (57%), seguido de la India (8%). A la región de Asia, le sigue América (5%), siendo Chile el mayor productor de la región. Tras América se sitúa Europa (4%), con Noruega liderando la producción europea (44%), y África (3%), en donde Egipto encabeza la producción (70%). En último lugar de producción se encuentra Oceanía que únicamente aporta

un 0,25% (Figura 1) (FAO, 2020). En el caso de las importaciones de productos pesqueros, el país que encabeza la lista es Estados Unidos (14%), seguido de Japón (9%), China (9%) y España (5%).

En términos globales de producción, el mayor porcentaje corresponde a peces de aleta, donde la principal especie producida es la carpa herbívora, con casi 6 millones de toneladas producidas (10,5%). En segundo lugar, en términos globales se encuentran las especies del género *Crassostrea,* una familia de moluscos bivalvos con un total de 5 millones de toneladas anuales producidas en todo el mundo. La trucha arcoíris se encuentra en el top 15 mundial del total de producción acuícola, con 848.000 toneladas producidas, es decir, un 1,6% del total de peces de aletas (FAO, 2020).

1.1.2 Acuicultura en España

En el 2019 según el Ministerio de Agricultura, Pesca y Alimentación (MAPA), y la Asociación Empresarial de Productores de Cultivos Marinos (APROMAR), la acuicultura en España produjo un total de aproximadamente 340.000 toneladas de peso vivo, alcanzando un valor en el mercado de 585 millones de euros. El año anterior, 2018, España produjo más toneladas, 350.000, pero el valor obtenido fue menor, unos 570 millones de euros, según MAPA (APROMAR, 2018.; APROMAR, 2020; Producción, 2019; Valor, 2019).

En comparación con la media mundial, España se diferencia en que su producto mayoritario es el mejillón, el cual suma un total de 261.000 toneladas en 2019. Seguido del mejillón, se encuentra la lubina con 24.000 toneladas, la trucha arcoíris con un total de 13.760 toneladas, y la dorada con 12.445 toneladas. Las toneladas de trucha común y esturión siberiano producidas en España, ascienden a 3,88 y a 1,49 respectivamente (Figura 2) (Producción, 2019).

De entre las comunidades autónomas de España, Galicia encabeza la lista tanto de producción como la de valor, con un total de 269.731 toneladas de peso vivo y 220 millones de euros (cifras del año 2019). Del total de toneladas producidas casi el 95% corresponde a mejillones, convirtiendo a Galicia en la principal productora de esta especie. La producción de trucha arcoíris la encabeza Castilla y León (2.983 toneladas), mientras que la de trucha común la lidera Navarra (2,93 toneladas). Por otro lado, la mayor parte de la producción de esturión siberiano se encuentra en la comunidad de Cataluña (1,49 toneladas) (Producción, 2019).

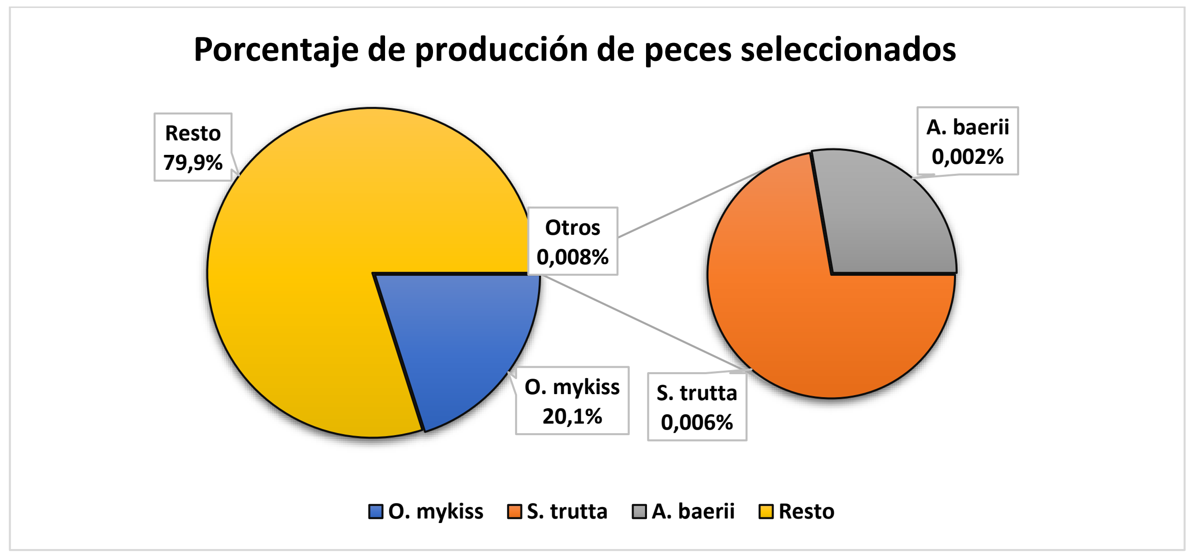


Figura 2: Porcentajes de la producción acuícola en España, y porcentajes de la producción de los peces seleccionados en el trabajo.

1.2 ANTIBIÓTICOS

Según la Organización Mundial de la Salud (OMS) los antibióticos son medicamentos que se utilizan para prevenir y tratar infecciones en los seres humanos, los animales y las plantas. A lo largo de los años el uso de antibióticos ha aumentado considerablemente favoreciendo a uno de los grandes problemas de la salud pública, que es la resistencia a los antimicrobianos (RAM), cuyo problema es que no existen tratamientos disponibles para esas infecciones. Por ejemplo, en Europa, según el *'European Antimicrobial Resitance Surveillance Network'* (EARS-Net), casi 700.000 infecciones en humanos son causadas al año por bacterias resistentes a antibióticos, y aproximadamente 33.000 personas mueren al año como consecuencia directa de estas infecciones ("Antimicrobial resistance in the EU/EEA (EARS-Net) - Annual Epidemiological Report for 2019," 2020).

El uso de antibióticos también se extiende en el mundo de la producción animal, cuyo principal objetivo es estimular el crecimiento de éstos. La OMS afirma que en ciertos países cerca de un 80% del consumo de antibióticos ocurre en el sector animal ("OMS, 2017," n.d.). A consecuencia de esto la Organización Mundial de Sanidad Animal (OIE) desarrolló un plan de acción global para el uso responsable y prudente de estos medicamentos, de manera que se pueda preservar la eficacia de éstos a largo plazo.

En el año 2018 en la Unión Europea se cambió la legislación y se creó un nuevo reglamento sobre el uso de medicamentos veterinarios, el cual engloba al reglamento relativo a la fabricación, la comercialización y el uso de piensos medicamentosos, y, por otro lado, al reglamento por el que se establecen procedimientos para la autorización y el control de los medicamentes de uso

humano y veterinario (*Reglamento (UE) 2019/6 del Parlamento Europeo y del Consejo, de 11 de diciembre de 2018, sobre medicamentos veterinarios y por el que se deroga la Directiva 2001/82/CE (Texto pertinente a efectos del EEE)*, 2019). Además, la Agencia Europea de Medicina (EMA) publica un escrito en el cual aparece una clasificación de antibióticos hecha por el Grupo de Expertos *Ad Hoc* en Asesoramiento Antimicrobiano (AMEG), la cual categoriza a los antibióticos en base a las posibles consecuencias para la salud pública en el caso de que aumentase las resistencias a los antimicrobianos. Esta nueva legislación autoriza el uso de los siguientes antibióticos: animopenicilinas sin inhibidores de la beta-lactamasa, tetraciclinas, penicilinas naturales de espectro reducido, sulfonamidas, bacitracina, ácido fusídico, metronidazol, derivados de nitrofurano, espectinomicina y penicilinas antiestafilocócicas ("Categorisation of antibiotics in the European Union EMA/CVMP/CHMP/682198/2017," n.d.). Estos medicamentos y la legislación pueden variar según la región, por ejemplo, en la Administración de Medicamentos y Alimentos de los EE. UU. (FDA) se permite el uso de otros medicamentos.

Otro aspecto para tener en cuenta es la eliminación de residuos de medicamentos veterinarios, que en ningún caso el nivel de estos debe afectar a la salud de los consumidores. Para esto la EMA fija unos Límites Máximos de Residuos (LMR), que tienen en cuenta diferentes factores, como el metabolismo y la eliminación de las sustancias, el tipo de residuos y la cantidad que puede ser ingerida por una persona sin conllevar un riesgo a su salud ("Aesan - Agencia Española de Seguridad Alimentaria y Nutrición," n.d.).

No obstante, aún con el gran uso de antibióticos, las enfermedades infecciosas siguen siendo uno de los principales problemas en el sector de la acuicultura. Además, ahora que la regulación en el uso de los antibióticos es más estricta, es necesario buscar y desarrollar nuevas alternativas más seguras, tanto con la salud animal como con la salud pública, y que a su vez también sean alternativas económicas para el control de estas enfermedades. Por ello, una alternativa que está en auge y que tiene gran importancia es el uso de estrategias que permitan su reducción e incluso eliminación completa de antibióticos, entre las que destaca el uso los probióticos (Hai, 2015).

1.3 PROBIÓTICOS

El término 'probiótico' ha ido evolucionando durante los últimos 50 años, una de las primeras definiciones fue dada por Fuller en 1989, el cual los define como 'suplemento alimenticio microbiano vivo el cual afecta de manera beneficiosa al animal hospedador mejorando su microbiota intestinal (Fuller, 1989). Años más tarde la FAO y la OMS han descrito a los

probióticos como microorganismos vivos que cuando son administrados en una cantidad adecuada confieren una beneficio para la salud del hospedador (Hill et al., 2014).

Los probióticos han sido ampliamente utilizados en acuicultura y en los últimos años se han convertido una parte fundamental para mejorar el crecimiento de la producción acuícola, así como para contrarrestar las resistencias a antibióticos. Si comparamos los probióticos utilizados en agricultura y/o ganadería con los utilizados en acuicultura, el rango de probióticos utilizados en este último sector es mucho mayor (Nayak, 2010).

Los probióticos más ampliamente usados en acuicultura pertenecen a los géneros *Lactobacillus, Lactococcus, Leuconostoc, Enterococcus, Carnobacterium, Shewanella, Aeronomonas, Pseudomonas, Clostridium, Vibrio, Enterobacter* y además las levaduras del género *Saccharomyces* (Kuebutornye et al., 2019; Nayak, 2010)**.** Para que una cepa pueda ser considerada probiótica debe satisfacer una serie de requisitos. Entre dichos requisitos, resulta fundamental que la cepa no resulta patógena para las especies hospedadoras, otras especies acuáticas y los humanos..Además, los microorganismos candidatos deben estar libres de plásmidos que contengan genes de resistencia a antibióticos, así como ser resistentes a pH ácidos y a las sales biliares (condiciones gastrointestinales) (Merrifield et al., 2010). Estas cepas también deben estar registradas para su uso como suplementos alimenticios y presentar actividad antagonistas hacia ciertos patógenos.

Existen ciertas características, como por ejemplo la capacidad de adherirse y/o crecer en el mucus intestinal y la capacidad de colonizar la superficie del epitelio intestinal, que no son estrictamente necesarias pero pueden favorecer el potencial probiótico de la cepa candidata (Gómez and Balcázar, 2008).

Los lactobacilos constituyen un grupo heterogéneo de bacterias pertenecientes a las llamadas bacterias ácido-lácticas (LAB), las cuales generan principalmente ácido láctico como producto final durante la fermentación de los carbohidratos. Además, otros rasgos que las caracterizan es que son bacterias gram-positivas, no esporuladas, catalasa negativas, carecen de citocromos, y son organismos aerotolerantes o anaerobios facultativos. Pueden tener forma tanto de coco como de bacilo, y además habitar diferentes nichos como el suelo, el agua, estar presentes en animales e incluso en el tracto digestivo humano (García-Hernández et al., 2016; Holzapfel et al., 2001).

1.3.1 Probióticos como tratamiento

Con más de 220 especies reconocidas como probióticas, el género *Lactobacillus* es el principal y el más diverso dentro de las LAB. Dentro de este género una de las especies más importante por ser extensamente utilizada es *Lactobacillus plantarum*. Esta bacteria puede ser suministrada en la dieta y ha demostrado tener un papel importante en la salud humana para el tratamiento de distintas patologías, como es el caso de la prevención de casos de diarrea, en el descenso del colesterol y en la reducción de los síntomas del síndrome del intestino irritable (Seddik et al., 2017). Además, en acuicultura se ha demostrado que, si la dieta es suplementada con *L. plantarum*, existe una mejora tanto en el crecimiento como en la respuesta inmunitaria del esturión siberiano (Pourgholam et al., 2016).

En el caso de la trucha común, se ha demostrado una mejora de los niveles de lisozima y una mayor estimulación de la respuesta inmune humoral, cuando la dieta es suplementada con las especies *Leuconostoc mesenteroides* y *L. sakei* (Balcázar et al., 2007a; Pourgholam et al., 2016). En otro estudio realizado por Balcázar et al. (2009), se demostró que el tratamiento probiótico con *L. mesenteroides* puede ser una vía alternativa al uso de antibióticos frente a la infección producida por un patógeno común de trucha común *A. salmonicida*, donde la mortalidad provocada por este patógeno disminuye significativamente.

Robertson et al. (1999) consiguieron aislar una cepa de *Carnobacterium* sp. desde el intestino de *Salmo salar*, y demostraron su eficacia *in vitro* e *in vivo* frente a diferentes enfermedades causadas por diversos patógenos de peces. Uno de los principales patógenos de trucha arcoíris es *Aeromonas salmonicida* subsp. *salmonicida*, causante de la forunculosis, y se ha demostrado que *Carnobacterium* es efectiva en el control de esta enfermedad. Existen otros patógenos frente a los cuales ciertas cepas de esta bacteria son efectivas, como es el caso de *Vibrio ordalii* o *Yersinia ruckeri*, siendo esta última el agente etiológico de una de las enfermedades más comunes en piscifactorías de agua dulce, la enfermedad de la boca roja (Marana et al., 2019; Martínez Cruz et al., 2012).

El género *Carnobacterium* contiene un total de 9 especies, sin embargo, las especies habitualmente aisladas, tanto en la naturaleza como en peces, son únicamente dos, *Carnobacterium maltaromaticum* y *Carnobacterium divergens*. Ambas son capaces de producir bacteriocinas, y además su uso como probiótico puede aumentar la supervivencia de ciertos peces, como en el caso de la ya mencionada trucha arcoíris. En otros estudios con este animal también se ha visto una mejora en los niveles de lisozimas y en el sistema inmune humoral, cuando las truchas son alimentadas con suplementos de estas bacterias. No obstante, ciertas

cepas de *C. maltaromaticum* han demostrado tener un efecto patógeno sobre estos peces, aunque solo se ha visto este efecto en situaciones de estrés severo (Leisner et al., 2007).

1.3.2 Mecanismos de acción de los probióticos

Al ser un grupo tan heterogéneo, los mecanismos por los que pueden actuar los probióticos una vez administrados son muy variados. Uno de los mecanismos más estudiados es la posibilidad de que los probióticos actúen modulando el sistema inmunitario a través de la inducción de los linfocitos T reguladores, que regulan e inhiben a las células efectoras de la inflamación, disminuyendo así la inflamación de la mucosa intestinal en la enfermedad inflamatoria intestinal (Boirivant and Strober, 2007).

La producción de diferentes metabolitos como los ácidos grados de cadena corta, el peróxido de hidrógeno, bacteriocinas y el ácido láctico, entre otros, también es un método por el cual impiden la colonización de patógenos (Gupta and Garg, 2009). De estos compuestos las bacteriocinas se encuentran entre los principales compuestos antimicrobianos producidos por los microorganismos, y se pueden clasificar en 5 subcategorías, de acuerdo a sus características bioquímicas y genéticas (Kemperman et al., 2003):

- Clase I Lantibióticos: péptidos pequeños policíclicos con aminoácidos modificados y con poca estabilidad al calor.
- Clase II No Lantibióticos: péptidos no modificados lineales y de pequeño tamaño, en este caso son termoestables.
- Clase IIc: péptidos pequeños, termoestables, que son transportados por péptidos líder y además no son modificados.
- Clase III: este grupo está compuesto por péptidos de elevado peso molecular, y además termolábiles.
- Clase IV: se tratan de glucoproteínas, es decir, bacteriocinas constituidas por una parte proteica y una o más partes lipídicas o glucídicas.

El peróxido de hidrógeno es producido por las bacterias mediante peroxidasas, este compuesto es liberado al medio, y puede actuar de diferentes formas, por un lado es tóxico para otras bacterias que comparten el hábitat, como puede ser el tracto digestivo, y además puede destruir las bacterias mediante la inactivación de enzimas (Pridmore et al., 2008).

La acidificación del pH intestinal es otro mecanismo por el cual se favorece la colonización de bacterias beneficiosas para la microbiota intestinal, compitiendo de esta manera con posibles patógenos (Balcázar et al., 2006).

Los probióticos también son capaces de modular la permeabilidad intestinal (Figura 3), además de intervenir en la actividad y expresión de diferentes citoquinas relacionadas con células epiteliales y al tejido linfoide asociado al intestino. El estudio llevado a cabo por Madsen et al (2001) demostró que cepas de *Lactobacillus* spp. eran capaces de disminuir la producción de IFN-γ y TNF-α, favoreciendo el tratamiento de la colitis (Madsen et al., 2001).

También se ha podido comprobar que los probióticos compiten por los recursos energéticos, como, por ejemplo, por el hierro. Muchos patógenos necesitan hierro para su crecimiento, y para conseguirlo secretan agentes conocidos como sideróforos, que actúan uniendo al hierro. De igual manera los probióticos secretan también este agente, compitiendo por el hierro y provocando que éste sea un elemento limitante para el crecimiento de los patógenos (Kesarcodi-Watson et al., 2008). Además de competir por los recursos energéticos, los probióticos compiten por los lugares de fijación, previniendo la colonización de ciertas zonas corporales, como pueden ser las paredes intestinales, por patógenos.

Este mecanismo es la principal y primera barrera de defensa contra las bacterias patógenas, debido a que de esta forma inhiben la colonización por parte de estas. De no poder adherirse a las superficies intestinales, no podrían ejercer su función como probióticos, ya que se desecharían por las heces (Bermudez-Brito et al., 2012).

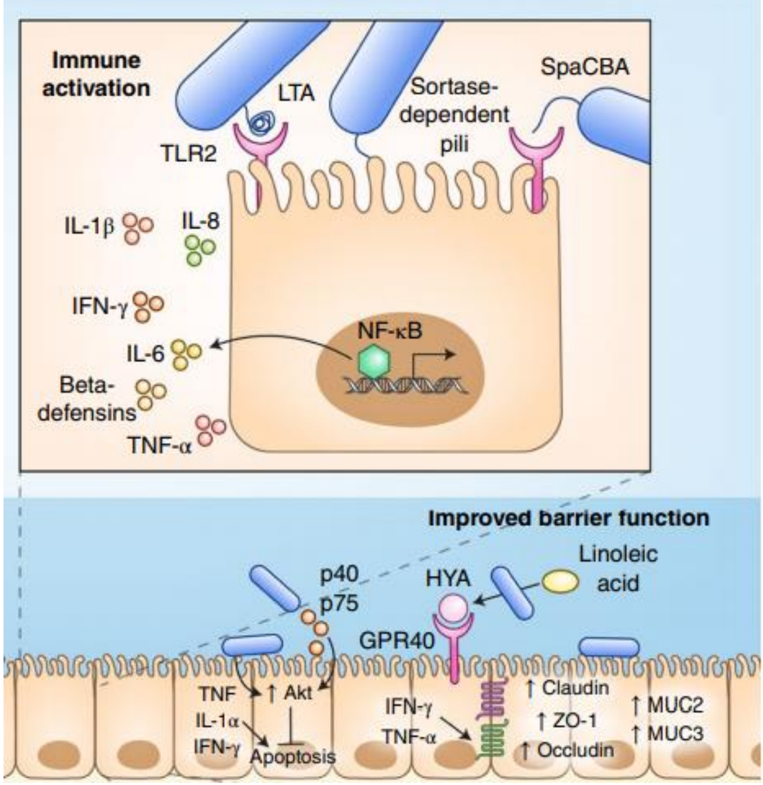


Figura 3: Mecanismo de modulación de la permeabilidad epitelial de los probióticos mediante la inmunomodulación (imagen extraída de (Suez et al., 2019)).

1.3.3 Probióticos en humanos

En el caso de su uso en humanos el número de géneros más utilizados como probióticos disminuye a un total de tres, donde se encuentran los géneros de bacteria *Lactobacillus* y *Bifidobacterium*, además de la especie de levadura *Saccharomyces boulardii*. Dentro de los géneros de bacteria destacan algunas especies, donde las más comúnmente usadas dentro de los *Lactobacillus* son *L. rhamnosus, L. bulgaricus, L. casei, L. reuteri* y *L. acidophillus.* En el caso de *Bifidobacterium,* también hay especies más ampliamente usadas, que son *B. animalis, B. infanti, B. lactis* y *B. longum* (Islam, 2016; Williams, 2010).

Su uso en humanos está ampliamente extendido, y se categorizaron en dos subdivisiones, las cuales son nutribióticos y farmabióticos. La primera categoría corresponde a los probióticos administrados en forma de comida o como suplemento alimenticio, los cuales tienen un papel beneficioso en el mantenimiento de la salud humana. La segunda categoría hace referencia a los probióticos que pueden tener un papel farmacológico en el desarrollo de algunas enfermedades. (Lee et al., 2018).

En relación con los probióticos que pueden tener un papel en la salud humana, existen diferentes enfermedades y trastornos los cuales pueden ser tratados en cierta manera con probióticos, dentro de estos trastornos se encuentran:

- Trastornos relacionados con el aparato digestivo.
- Alergia.
- Enfermedades cardiovasculares.
- Trastornos del aparato urogenital.
- Enfermedades de la piel.
- Trastornos causados por otras bacterias patógenas.

Además de tener efectos sobre estos trastornos, los probióticos también pueden modular el sistema inmune del huésped, y mantener la salud y bienestar del humano, reduciendo el riesgo de padecer alguna de las enfermedades ya mencionadas (*Probióticos en los alimentos*, 2006; Williams, 2010).

Otros trastornos en los cuales los probióticos pueden causar un efecto beneficioso son por ejemplo en el síndrome del intestino irritable, donde se ha visto que el tratamiento con *Lactobacillus plantarum* disminuía los síntomas causados por esta patología. En otros pacientes los cuales padecían de diarrea asociada a *Clostridium difficile*, o que padecían un gran riesgo de infección por esta bacteria, también se pudo comprobar que *L. plantarum* reducía los síntomas

además de reducir la incidencia por infección del patógeno. *L. plantarum* también tiene un rol en la reducción del colesterol en personas con el colesterol alto (Seddik et al., 2017).

En otro estudio se ha demostrado el efecto probiótico de *Lactobacillus plantarum* y *Leuconostoc mesenteroides* frente al virus de la gripe en humanos, tanto en personas de todas las edades, como en pacientes inmunodeprimidos (Bae et al., 2018). Además, se ha demostrado la capacidad de *L. mesenteroides* para metabolizar el ácido linoleico y así reducir la grasa abdominal inducida por una dieta alta en grasas (Pham et al., 2020).

1.4 POSTBIÓTICOS

Los postbióticos son definidos como compuestos bioactivos producidos durante procesos de fermentación que favorecen el bienestar y salud, estos compuestos son producidos por probióticos (Malagón-Rojas et al., 2020). Estos compuestos además pueden ser suministrados de dos modos diferentes, ya que pueden ser secretados por los propios probióticos vivos, o pueden ser liberados una vez se haya producido la lisis del probióticos, de modo que no es necesario que este sea suministrado vivo (Teame et al., 2020).

Postbióticos obtenidos de dos especies de LAB pertenecientes a los géneros *Lactobacillus* y *Leuconostoc*, aisladas de trucha arcoíris, han demostrado tener la capacidad de mejorar la microbiota intestinal del pez, y además tener efecto antagonista contra *Lactococcus garvieae* (Pérez-Sánchez et al., 2020). Esto demuestra que no es necesario suministrar la bacteria viva, sino que obteniendo ciertos productos de ella se puede ejercer un efecto beneficioso sobre el hospedador.

2 JUSTIFICACIÓN DEL TRABAJO

Tal y como se ha mencionado anteriormente, desde los años 90 el incremento de la producción acuícola ha sido exponencial (FAO, 2020). Dicho incremento ha supuesto también un aumento del uso de antibióticos en la acuicultura, favoreciendo así la aparición, selección y diseminación de diferentes resistencias bacterianas. En el caso de la acuicultura, las bacterias presentes en el agua afectadas por los antibióticos usados en la producción acuícola pueden adquirir resistencias y ello puede afectar de manera muy grave a la salud animal, la salud humana e incluso al medio ambiente (Cabello et al., 2013). Por lo tanto, se trata de un problema que perjudica a la salud y bienestar global y requiere atención urgente.

Con el paso de los años y debido a las limitaciones en el uso de antibióticos, los probióticos se han presentado como una alternativa al uso de estos últimos en la acuicultura (Hai, 2015). Por ello, el objetivo del presente trabajo es la identificación y selección de bacterias procedente de peces de producción acuícola con potencial probiótico que puedan ser utilizadas para el control de enfermedades causadas por infecciones bacterianas que afecten a la salud de los peces y también a la salud humana. Las muestras de bacterias fueron aisladas de diferentes tejidos, branquias, intestino, bazo, vejiga, boca, y piel, de tres especies diferentes de peces: trucha común (*S. trutta*), trucha arcoíris (*O. mykiss*) y esturión siberiano (*A. baerii*), que fueron criadas en piscifactorías de diferentes regiones de España.

3 HIPÓTESIS Y OBJETIVOS

Hipótesis: Es posible aislar e identificar potenciales bacterias probióticas desde peces de producción acuícola y que tengan aplicación en el tratamiento de enfermedades de interés en salud humana y animal.

Los objetivos del presente trabajo son los siguientes:

- **Objetivo general**: Seleccionar y evaluar bacterias candidatas probióticas obtenidas desde mucus intestinal, piel, cavidad oral, branquias, vejiga y bazo de los peces de agua dulce *O. mykiss* y *S. trutta* y *A. baerii* para realizar ensayos antagonistas contra una bacteria de potencial zoonótico.
- **Objetivos específicos:**
    1. Selección de bacterias candidatas probióticas aisladas desde *Oncorhynchus mykiss Salmo trutta*, y *Acipenser baerii* usando el medio selectivo Man, Rogosa y Sharper (MRS), tinción de Gram, amplificación y secuenciación del gen 16SrDNA.
    2. Evaluación del efecto antagonista de las bacterias candidatas, contra la bacteria con potencial zoonótico *Lactococcus garviea*.

4 MATERIALES Y MÉTODOS

4.1 AISLAMIENTO Y SELECCIÓN INICIAL DE BACTERIAS CANDIDATAS

Las bacterias candidatas se obtuvieron desde muestras de mucus intestinal, piel, branquias, cavidad oral, bazo y vejiga de ejemplares de *Acipenser baerii, Salmo trutta* y desde *Oncorhynchus mykiss* de granjas elegidas en base a las condiciones sanitarias y de calidad de agua (granjas libres de patógenos y sin tratamientos antibióticos). Una vez obtenidas estas muestras se siguió el esquema mostrado en la Figura 4.

Las muestras de mucus intestinal, cutáneo, branquial, oral, de vejiga y de bazo (Figura 5) fueron tomadas con hisopo, y se sembraron directamente en placas de agar MRS, que fue preparado según las instrucciones del fabricante (Scharlab, Barcelona) y se incubaron en estufa a 22°C de 12 a 48 h.

Una vez crecidas las muestras, se picaron con asa estéril colonias aisladas, de distinta forma y coloración y se resembraron en medio líquido de MRS por entre 12 a 48 h (Figura 6). A continuación, con una nueva asa estéril se tomó una gota, con la horquilla del asa, desde estos cultivos líquidos y se realizaron nuevas siembras por agotamiento en agar MRS, las placas se incubaron por otras 12 a 48 h.

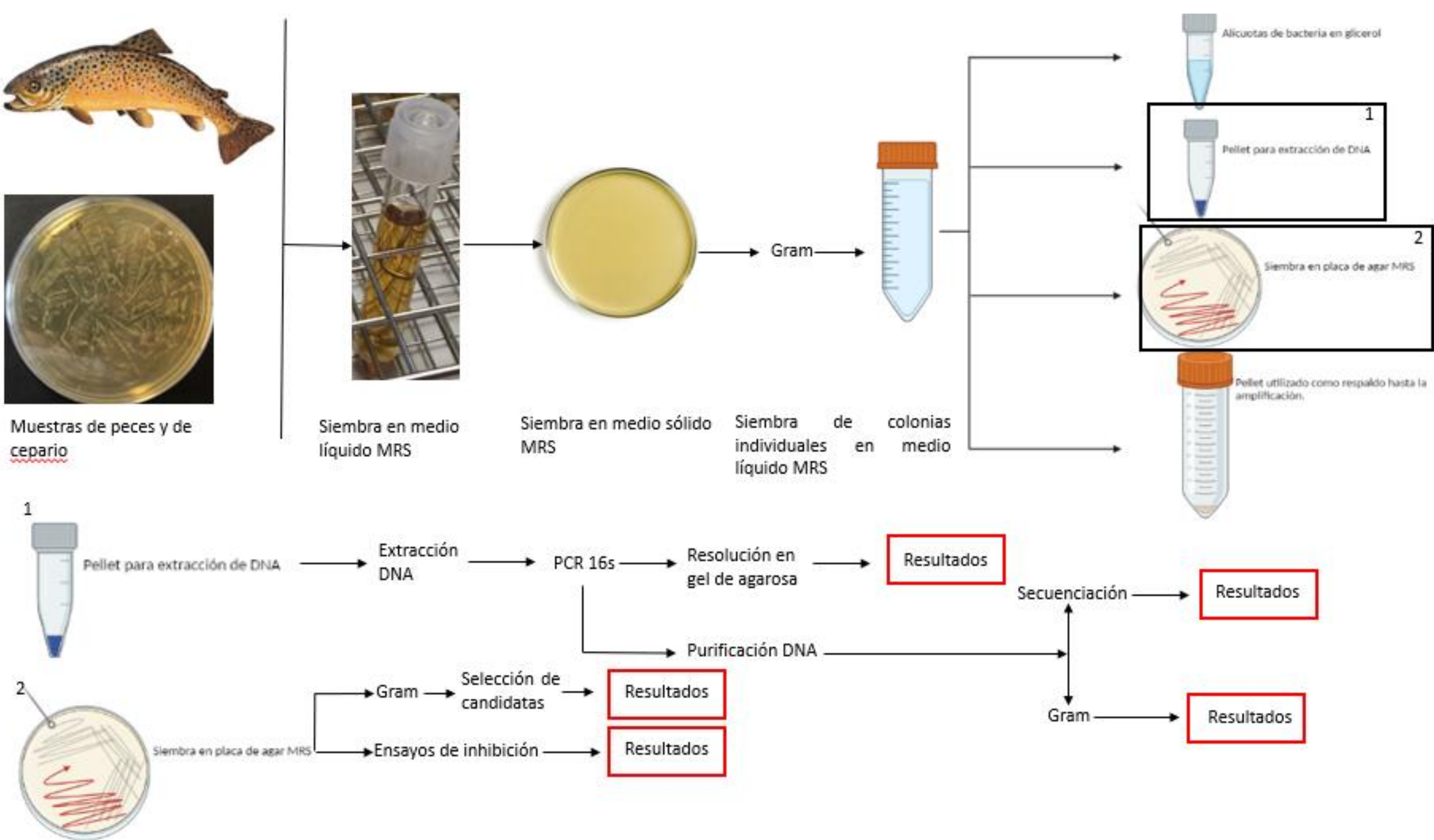


Figura 4: Esquema del procedimiento seguido durante el trabajo.

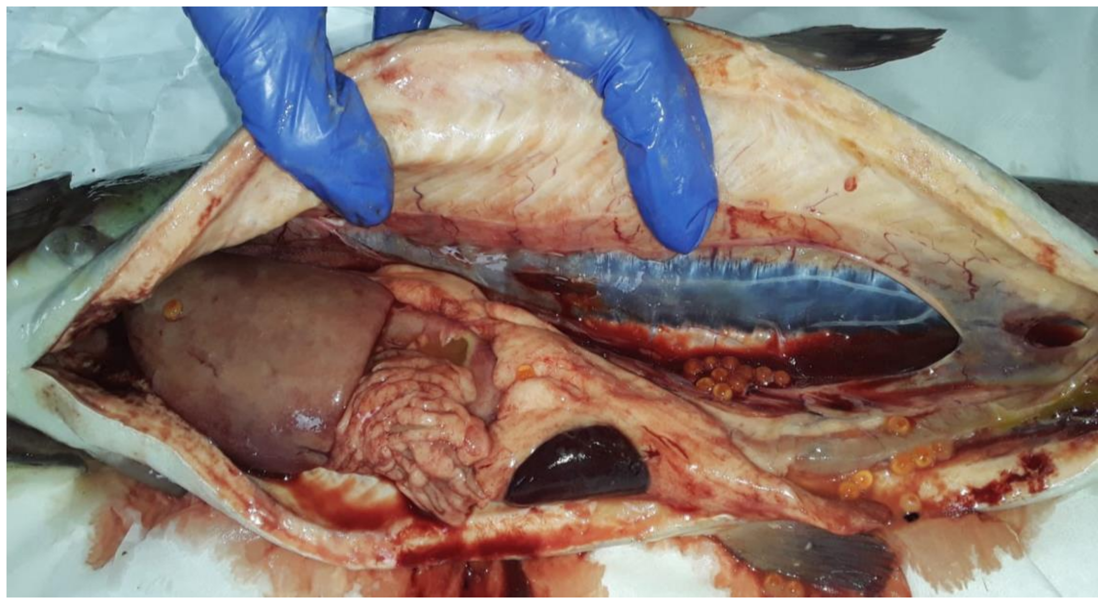


Figura 5: Disección de *Onchorhyncus mykiss*.

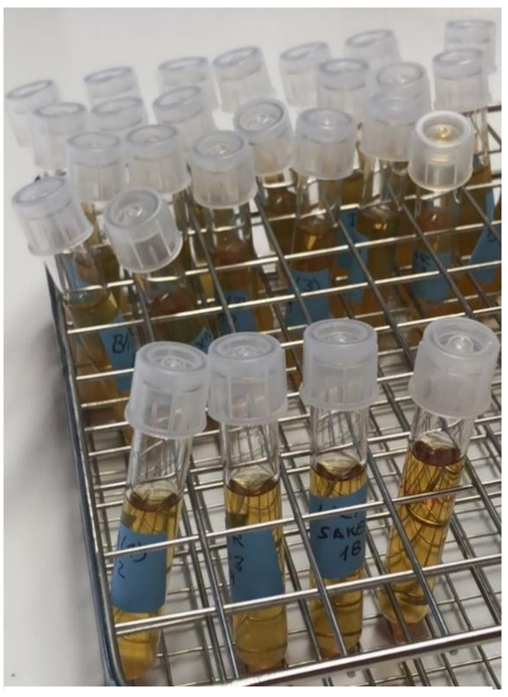


Figura 6: Muestras resembradas en medio líquido MRS.

Paralelamente se reactivaron bacterias almacenadas a -80°C en glicerol stock (glicerol al 30% v/v) de muestreos anteriores y tomando una muestra con un asa se realizó siembra por agotamiento en agar MRS. A partir de estas muestras y de otros cultivos en agar procedentes de colonias obtenidas del cepario de muestreos anteriores, se realizó tinción de Gram para descartar levaduras y realizar un rastreo en busca de cocos, bacilos y/o cocobacilos Gram positivos. Para la tinción de Gram, se tomaron colonias aisladas desde las placas de agar MRS con un asa, y se fijaron mediante mechero en un portaobjetos. Una vez fijadas, se tiñeron con

cristal violeta que se dejó actuar durante 2 minutos. Se lavó el cristal violeta con agua, y se añadió lugol a la muestra. Pasado 1 minuto se decantó el lugol en papel secante, y se lavó, esta vez con una mezcla decolarante de etanol y acetona. Se volvió a lavar con agua, y se le añadió el colorante fucsina, que se dejó actuar también por 1 minuto. Al transcurrir el tiempo se lavó una última vez con agua y se dejó secar. Las muestras fueron observadas al microscopio, utilizando aceite de inmersión en los casos que fuese necesario.

A continuación, se procedió a hacer una resiembra de colonias individuales a partir de las placas de agar MRS. Para la resiembra se usaron tubos de centrífuga con 10 ml de medio liquido MRS, y se incubaron en estufa a 22ºC entre 24 a 48h. Cuando el medio líquido, en comparación a un medio líquido MRS control, presentó turbidez, los 10 ml de medio de cada tubo Falcon se utilizaron para los siguientes 4 análisis que se detallan a continuación (Figura 7):

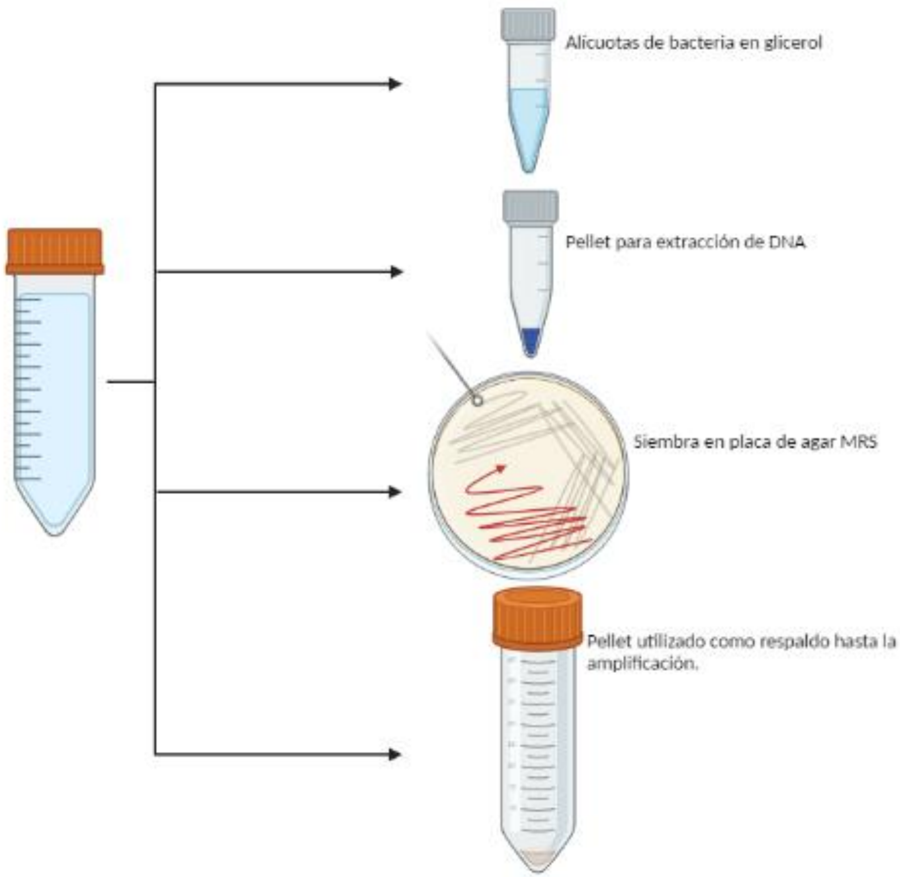


Figura 7: Esquema de la metodología desarrollada a partir del cultivo inicial de bacterias.

- Generar 3 alícuotas de 1 ml de bacterias en glicerol stock al 30% v/v (Figura 8A). Se preparó una solución de glicerol al 30% utilizando 30 ml de glicerol puro y 70 ml de agua mili Q, esta mezcla fue esterilizada en un autoclave. Luego se tomaron 500 µL de medio de cultivo

bacteriano y 500 µL de este glicerol al 30%, se mezcló con vórtex y se almacenaron las 3 alícuotas a -80°C.

- Obtener pellet bacteriano para realizar extracción de ADN. Para lo cual se tomaron dos alícuotas de 1 ml de medio bacteriano en dos tubos de microcentrífuga de 1,5ml (Figura 8B). Las alícuotas fueron centrifugadas a 13000rpm por 1 minuto y se descartó el sobrenadante. El pellet se lavó con tampón fosfato salino (PBS) 1X para eliminar los restos de medio. Una vez lavado, fue nuevamente centrifugado para eliminar el PBS, y quedarnos únicamente con el pellet. Los 2 tubos de pellet se almacenaron a -20°C para la posterior extracción de ADN bacteriano.
- Sembrar en placa de agar MRS por agotamiento a partir del cultivo líquido inicial y se dejó crecer durante 24h-48h a 22°C.
- El remanente de medio líquido que quedaba en el tubo inicial se centrifugó a 4000rpm por 10 minutos. Se eliminó el sobrenadante y se lavó el pellet con PBS 1X, una vez lavado se retiró el PBS y se almacenaron los tubos con el pellet hasta la amplificación del gen 16S ribosomal (Figura 8C).

Las placas de agar MRS sembradas (Figura 8D) fueron revisadas una vez más para comprobar la pureza del cultivo líquido y de las alícuotas de glicerol stock almacenadas. Para esto se realizó nuevamente la tinción de Gram a colonias individuales y estas fueron comparadas con los resultados procedentes de la tinción de Gram de la etapa anterior. Como resultado se obtuvieron 40 bacterias candidatas para las siguientes etapas de análisis (Tabla 5).

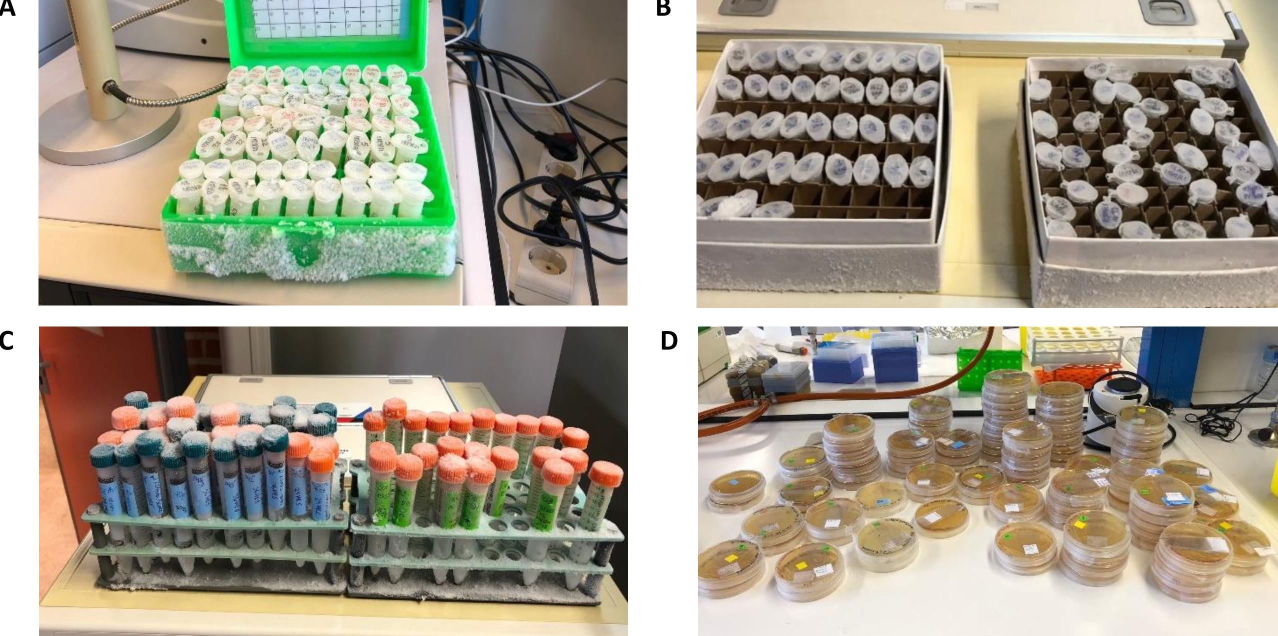


Figura 8: (A) Alícuotas de muestras en glicerol. (B) Alícuotas con el pellet bacteriano usado para la extracción de ADN. (C) Tubos con pellet bacteriano. (D) Placas de agar MRS con las muestras que fueron sometidas a la tinción de Gram

4.2 EXTRACCIÓN DE ADN, AMPLIFICACIÓN Y SECUENCIACIÓN DEL GEN 16S RIBOSOMAL DE EUBACTERIAS

Se realizó la extracción de ADN total utilizando Qiagen DNA mini kit. Para ello el pellet bacteriano fue descongelado y resuspendido en 180 µL de una solución de lisozima con una concentración de 20 mg/mL, previamente preparada con 980 µL de Tris-HCl EDTA pH 8,0 + 20 µL de TRITON X100 + 20 mg de lisozima. El pellet con la solución de lisozima se dejó incubar por 1 hora en un baño termorregulado (Lan Technichs Biosan) a 37°C en agitación continua a 1000 rpm. Luego se añadió Proteinasa K y 200 µL de Buffer AL del kit de extracción y se incubó a 56°C por 30 minutos, también con agitación continua a 1000 rpm. A continuación, se incubó a 95°C por 15 minutos, se centrifugó a 7500 rpm x 10 segundos y se agregaron 200 µL de etanol 96% frío, que se mezcló por vórtex durante 15 segundos. Se centrifugó brevemente a 13000rpm y se agregó toda la mezcla a la columna de extracción del kit, y se procedió según el manual del fabricante. El ADN obtenido fue cuantificado, también fueron medidos los parámetros de absorbancia tanto en el ratio 260/280 como en el ratio 260/230, ambos indicadores de contaminación, de proteínas y de EDTA o etanol, respectivamente. En ambos parámetros ratios ≥1,80 significa que las muestras de DNA son puras (Tabla 1). Las mediciones se hicieron con el NanoDrop One (Thermo Fisher Scientific) (Figura 9) y las muestras se almacenaron a -80°C.

Figura 9: Ejemplo de valores obtenidos en el NanoDrop One.

El gen 16S rRNA fue amplificado mediante la reacción en cadena de la polimerasa (PCR). Para ellos se utilizaron los partidores 27 F (5´-AGA GTT TGATCC TGG CTC AG-3´) y 1492 R (5´-ACG GCT ACC TTG TTA CGA CTT-3´) (Weisburg 1991) en un PTC 200 Peltier Thermal Cycler (Bio-Rad MJ Research) para obtener un amplicón de una longitud de 1500 pb aprox. La amplificación se llevó a cabo usando 12,5 µL de 2X KAPAG 2G Fast Hotstart Ready Mix (Molecular BioProducts, Sigma-

Aldrich, Madrid), 1,25 µL de cada partidor a una concentración inicial de 10 µM, 8 µL de agua libre de nucleasas y 2 µL de templado, que consistió en una dilución 1:100 del ADN bacteriano extraído en el paso anterior. Para esta dilución se tomaron 2 µL del ADN bacteriano que fue disuelto en 198 µL de agua libre de nucleasas.

El perfil térmico durante la PCR fue el siguiente: desnaturalización inicial a 95°C por 3 minutos, 40 ciclos de desnaturalización a 95°C por 15 segundos, anillamiento a 45°C por 15 segundos y extensión a 72°C por 15 segundos, además de una extensión final de 2 minutos a 72°C.

Para verificar que los productos de PCR habían amplificado de manera correcta, fueron resueltos mediante electroforesis en gel de agarosa al 1,5% peso/volumen (p/v) (Figura 10). Se utilizó GelRed 1000X (Biotium, Barcelona) como colorante del ADN en el gel de agarosa. Además, como estándar de peso molecular se usó el FastGene 1 kb DNA marker plus. En el gel se cargaron 2 µL de producto de PCR, a voltaje constante de 100 V durante 1 hora. Las bandas se pudieron visualizar bajo luz UV en cámara oscura con un sistema de imagen para geles (SYNGENE PX*i*).

Antes de su secuenciación, los productos de PCR fueron purificados utilizando el kit de Macherey-Nagel de purificación de DNA, RNA y proteínas (Macherey-Nagel, Madrid). Previamente se probó el protocolo de geles de agarosa y el de productos de PCR, y finalmente se decidió utilizar el protocolo de productos de PCR. Para ello se midió la concentración de los productos de PCR con el NanoDrop One y se prepararon alícuotas de 55 ng/µL de los productos de PCR purificados (Figura 11) que fueron almacenadas a -20ºC hasta su envío a Macrogen España (Madrid) empresa que se encargó de la secuenciación. Las alícuotas de concentración 55 ng/µL se consiguieron mediante el uso de la siguiente fórmula C1*V1=C2*V2, donde C1 es la concentración de ADN que obtenemos de una segunda medición de los productos de PCR (Tabla 2). V1 es el volumen de producto de PCR que debemos utilizar para generar la nueva alícuota. C2 es la concentración de 55 ng/µL y V2 es el volumen final de la alícuota (en este caso Macrogen exigen un volumen de 22 µL). Para llegar a dicho volumen, se utilizó agua libre de nucleasas. Los datos de los cálculos realizados se muestran en la Tabla 3. Estas alícuotas fueron guardadas a -20ºC hasta su envío

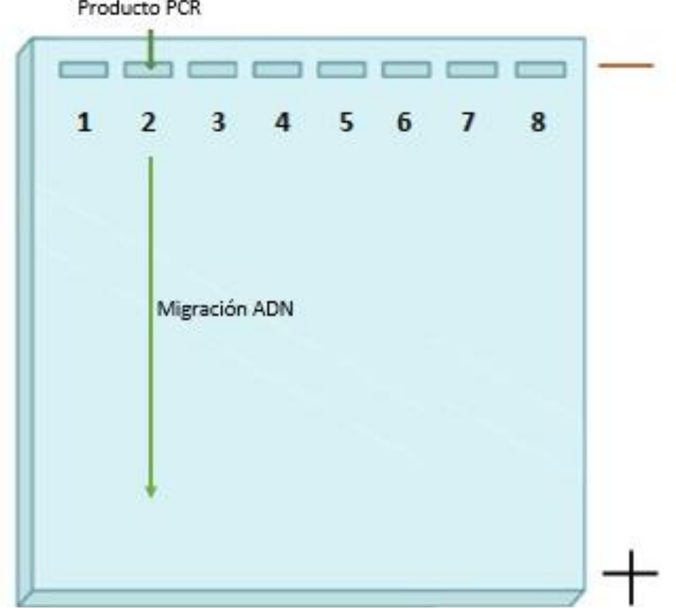


Figura 10: Esquema del gel de agarosa utilizado para la resolución de los productos de PCR. Pocillo 1 y 8: Estándar de peso molecular. Pocillos 2-7: Productos de PCR.

Figura 11: Alícuotas con la concentración indicada de 55 ng/µL para su posterior secuenciación.

Por otro lado, además de la purificación y la preparación para su posterior secuenciación, a estas muestras se les hizo una última tinción de Gram para verificar que las muestras enviadas eran puras (Figura 12). Para esta última tinción, se utilizaron las bacterias previamente guardadas en glicerol, las cuales fueron descongeladas, y con un asa de siembra se sembraron en agar MRS nuevamente. Se dejaron crecer durante 24h a 22ºC, y se procedió a hacerles la tinción a las colonias individuales de cada placa.

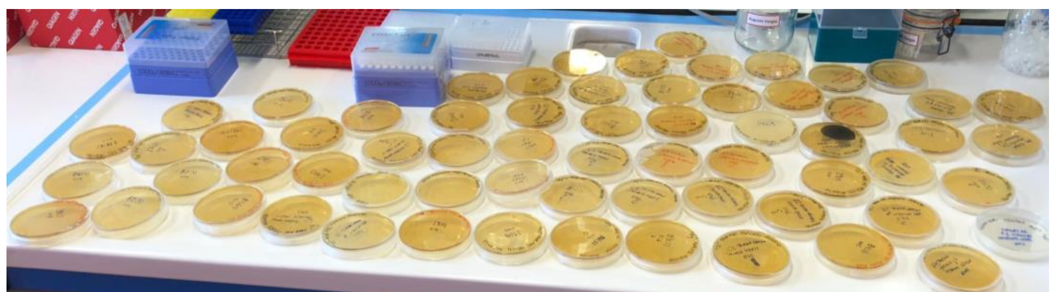


Figura 12: Placas de agar MRS con las muestras a las cuales se les hizo Gram para comprobar su pureza.

4.3 EVALUACIÓN DEL EFECTO ANTAGONISTA DE LAS BACTERIAS CANDIDATAS

Tres cepas patógenas fueron usadas para estos ensayos, las cepas fueron *Lactococcus garvieae, Yersinia ruckerii* y *Vagococcus salmoninarum.* Fueron aisladas de un brote natural y se utilizaron para evaluar el efecto antagonista de las bacterias candidatas probióticas. Para ello se tomó con

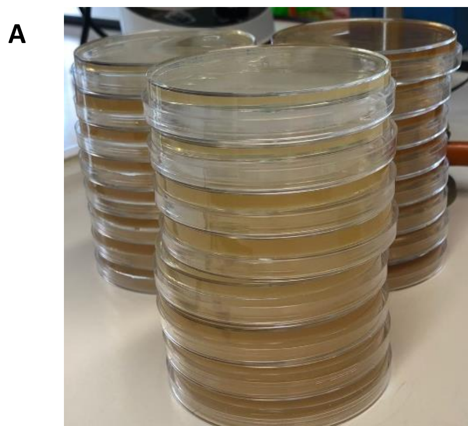
una asa cada uno de los patógenos desde un criovial y se hizo crecer en 10ml de MRS y Caldo Soja Tripticaseína (TSB) (Scharlab, Barcelona) *overnight* a 22ºC.

Al día siguiente se tomaron 100 µL de cada medio y fueron resembradas nuevamente en 10 ml de MRS y TSB para alcanzar una densidad óptica (DO) determinada, en este caso de 0,125. Para alcanzar esta DO se dejó crecer el patógeno por algunas horas, luego se tomó un 1 ml de cada medio en 2 tubos de 2 ml y se centrifugó a 13000 rpm x 1 minuto. Se lavó el pellet con PBS 1X, y se volvió a centrifugar a 13000rpm x 1 min, se descartó el sobrenadante y el pellet fue resuspendido, una vez más, en 1 ml de PBS. Se midió la DO a 600 nm en el NanoDrop One.

La obtención de la DO 0,125 se consiguió mediante dilución de la bacteria en suspensión usando PBS. Una vez se consiguió, se realizaron diluciones seriadas en base 10 hasta la dilución 10^{-8}, y se procedió a sembrar 0,1 ml de patógeno en placas de agar MRS (Figura 13A) y TSA. Se incubaron las placas por 24 h para hacer recuento de las colonias y comprobar la cantidad de unidades formadoras de colonias (UFC)/ml (Tabla 4). El recuento de las UFC se hizo únicamente de las placas de MRS, ya que en las de TSA no crecieron todos los patógenos.

Respecto a las bacterias con potencial probiótico, se tomaron colonias individuales a partir de las placas de agar MRS previamente sembradas, y se siguió el mismo procedimiento que con la bacteria patógeno. En este caso las muestras se sembraron en medio MRS agar y líquido, y se descartaron los medios TSB y TSA porque en ellos no crecen las bacterias probióticas (Figura 13B).

A



B

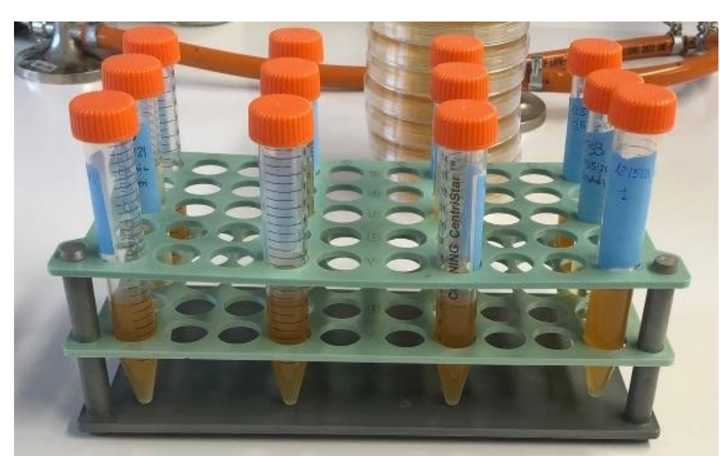


Figura 13: (A) Muestras de las 3 bacterias patógenas sembradas en medio agar MRS. (B) Muestras de bacterias potenciales probióticas en medio líquido MRS, usadas para los ensayos antagonistas.

Con las concentraciones estandarizadas se realizaron tres tipos de ensayos antagonistas, en los dos primeros se utilizaron las 3 bacterias patógenas, mientras que en el último únicamente se usó *Lactococcus garvieae*.

Se llevó a cabo el siguiente procedimiento

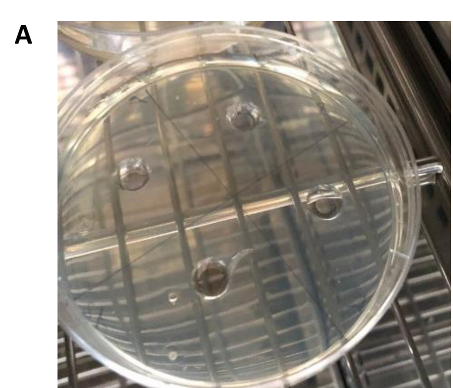
1. Se sembraron por extensión placas de agar MRS y de TSA cada una con un patógeno y las placas fueron divididas en cuatro. En cada cuarto de placa se creó un pocillo en el cual se inocularon 50 µL de la candidata probiótica (Figura 14A). El pocillo se consiguió usando la parte trasera de una punta de pipeta de 1000 µL.

2. Se tomaron varias colonias de la candidata con un asa desde una placa de cultivo. Las colonias fueron resembradas en la superficie de una placa en la cual se había sembrado previamente el patógeno, e igualmente que en el ensayo anterior las placas donde se había sembrado el patógeno estaban divididas en 4 (Figura 14B).

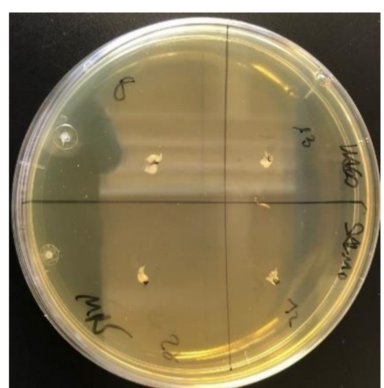
En ambos ensayos se dejaron crecer las placas durante 16h a 22ºC y se observó si se habían formado halos de inhibición.

3. Se procedió a tomar una muestra de patógeno, en este caso *L. garvieae*, y de bacteria candidata, con una DO 0,1 en ambas muestras. La muestra de patógeno fue centrifugada a 13000rpm x 1 min, se descartó el sobrenadante y el pellet fue resuspendido con 200 µL de la muestra de candidata. El mix de candidata y patógeno se incubó durante 1h a 22ºC. Al cabo de 1h la muestra fue centrifugada a 13000rpm x 1 min, se descartó el sobrenadante y el pellet se resuspendió en 200 µL de PBS. Se hicieron diluciones seriadas en base 10 hasta la dilución 10^{-8}, y se procedió a sembrar por extensión 10 µL en placa MRS (Figura 14C) y por duplicado, además de sembrar una muestra control de patógeno y un control de candidata. Las muestras se dejaron crecer en la estufa durante 24h a 22ºC, y una vez crecidas se contaron las colonias.

A



B



C

Figura 14: (A) Muestra de la placa del primer ensayo. (B) Muestra de la placa del segundo ensayo. (C) Muestra de la placa del tercer ensayo.

4.4 DATOS OBTENIDOS EN LAS ETAPAS DE ANÁLISIS

En este apartado se muestran los datos y valores obtenidos durante los experimentos detallados en los apartados anteriores. Dichos valores no se consideran resultados propiamente dicho, ya que son datos generados durante las fases de prueba que fueron necesarios para la toma de decisiones durante todo el trabajo experimental.

Tabla 1 Muestras, concentraciones de ADN y ratios 260/280, 260/230

Muestra	Concentración (ng/µL)	260/280	260/230
1	20,8	2,34	2,00
2	31,8	2,26	2,72
4	32,7	1,93	1,64
5	106,2	2,10	2,37
6	59,6	2,11	3,54
7	51,2	2,06	2,26
8	57,1	2,09	2,29
9	104,5	2,15	2,70
11	62,2	2,10	2,42
12	39,4	2,05	5,94
13	13,5	1,92	0,90
14	5,6	1,98	2,00
15	32,3	2,25	2,42
16	58	2,07	2,18
18	66,2	2,11	2,46
19	40,8	2,05	2,14
21	46,5	2,09	4,11
22	83,7	2,17	2,70
23	53,5	2,06	2,11
24	50,2	2,11	2,29
25	42,8	2,08	4,60
27	25,5	2,01	5,49
28	13,7	1,88	0,82
41	37,1	2,11	1,95
44	30,1	2,20	1,57
47	36,5	2,17	2,07
48	95,1	2,19	2,50
49	35,9	2,18	2,20
50	34	2,07	1,77
10g	91,3	2,12	2,56
10p	110,5	2,10	2,61
20g	35,6	2,06	4,54
26g	45,9	1,96	2,05
26p	39,4	2,10	6,03
33C	38,1	2,10	4,11
36C1	32,7	2,03	3,49
36C2	29,4	1,99	6,94
40gC	30,8	2,23	10,03
45C	70,7	2,19	3,73
46C	46,4	2,13	5,30

Tabla 1: Datos obtenidos durante la extracción inicial de ADN.

Tabla 2 Concentraciones de ADN medidas en dos días consecutivos

Muestra	Concentración (ng/μL)	Concentración cálculo (ng/μL)
1	123	110
2	191	152
4	164	153
5	172	159
6	151	161
7	176	134
8	194	136
9	176	148
11	214	152
12	248	144
13	75	86
14	186	144
15	171	140
16	155	132
18	185	138
19	162	108
21	195	124
22	175	180
23	168	85
24	171	142
25	173	122
27	129	136
28	147	132
41	157	148
44	169	167
47	163	153
48	155	161
49	210	171
50	149	157
10g	254	167
10p	202	218
20g*	73	53
26g	180	142
26p	205	160
33c	159	158
36c1	173	169
36c2	236	165
40gC	186	168
45c	183	191
46C	174	176

Tabla 2: Columna 2: Concentraciones de ADN de los productos de PCR. Columna 3: Concentraciones de ADN usadas para obtener la concentración final de 55 ng/µL.

Tabla 3 Datos utilizados para la obtención de la concentración usada en la secuenciación

Muestra	Ci(ng/µL)	Cf(ng/µL)	ViprodPCR(µL)	V H2O	Vf muestra(µL)
1	110	55	11,00	11,00	22
2	152	55	7,96	14,04	22
4	153	55	7,91	14,09	22
5	159	55	7,61	14,39	22
6	161	55	7,52	14,48	22
7	134	55	9,03	12,97	22
8	136	55	8,90	13,10	22
9	148	55	8,18	13,82	22
11	152	55	7,96	14,04	22
12	144	55	8,40	13,60	22
13	86	55	14,07	7,93	22
14	144	55	8,40	13,60	22
15	140	55	8,64	13,36	22
16	132	55	9,17	12,83	22
18	138	55	8,77	13,23	22
19	108	55	11,20	10,80	22
21	124	55	9,76	12,24	22
22	180	55	6,72	15,28	22
24	142	55	8,52	13,48	22
25	122	55	9,92	12,08	22
27	136	55	8,90	13,10	22
28	132	55	9,17	12,83	22
41	148	55	8,18	13,82	22
44	167	55	7,25	14,75	22
47	153	55	7,91	14,09	22
48	161	55	7,52	14,48	22
49	171	55	7,08	14,92	22
50	157	55	7,71	14,29	22
10g	167	55	7,25	14,75	22
10p	218	55	5,55	16,45	22
20g*	53	55		22,00	22
26g	142	55	8,52	13,48	22
26p	160	55	7,56	14,44	22
33c	158	55	7,66	14,34	22
36c1	169	55	7,16	14,84	22
36c2	165	55	7,33	14,67	22
40gC	168	55	7,20	14,80	22
45c	191	55	6,34	15,66	22
46C	176	55	6,88	15,13	22

Tabla 3: Volúmenes de producto de PCR y de H_2O libre de nucleasas, utilizados para conseguir la concentración final de 55 µL, exigida para la secuenciación.

Tabla 4 Patógenos, UFC y concentración de bacterias patógenas en las placas de agar MRS

Patógeno	Dilución	UFC	Concentración final (UFC/ml)
Y. ruckerii	10^-1	Incontable	
Y. ruckerii	10^-2	Incontable	
Y. ruckerii	10^-3	Incontable	
Y. ruckerii	10^-4	Incontable	
Y. ruckerii	10^-5	68	68*10^6
Y. ruckerii*	10^-6	7	7*10^7
Y. ruckerii	10^-7	0	0
Y. ruckerii	10^-8	0	0
V. salmoninarum	10^-1	Incontable	
V. salmoninarum	10^-2	Incontable	
V. salmoninarum	10^-3	Incontable	
V. salmoninarum	10^-4	Incontable	
V. salmoninarum	10^-5	72	72*10^6
V. salmoninarum*	10^-6	7	7*10^7
V. salmoninarum	10^-7	0	0
V. salmoninarum	10^-8	0	0
L. garvieae	10^-1	Incontable	
L. garvieae	10^-2	Incontable	
L. garvieae	10^-3	Incontable	
L. garvieae	10^-4	Incontable	
L. garvieae	10^-5	60	60*10^6
L. garvieae*	10^-6	7	7*10^7
L. garvieae	10^-7	0	0
L. garvieae	10^-8	0	0

Tabla 4: *Concentraciones usadas para los ensayos de inhibición.

5 RESULTADOS

Tras el procesado del contenido procedente de branquias, intestino, boca, bazo, vejiga y piel de todos los peces de las 3 especies de peces analizadas, trucha común, trucha arcoíris y esturión siberianos, obtuvimos un total de 56 cepas diferentes, de las cuales, se preseleccionaron un total de 40 cepas que se correspondían con cocos, cocobacilos y bacilos Gram positivos. De estas 40 cepas preseleccionadas, 19 cepas fueron secuenciadas, que se dividían en 6 aisladas de trucha común, 9 de trucha arcoíris y 4 de esturión siberiano.

Por otro lado, en los ensayos de inhibición, se utilizó como patógeno *Lactococcus garvieae*, y como posibles bacterias probióticas se utilizaron 8 cepas diferentes, procedentes de las 40 anteriormente seleccionadas.

5.1 SELECCIÓN DE BACTERIA CANDIDATAS

De la primera etapa de ‘Materiales y métodos’ donde las bacterias pasaron por un proceso de selección, 40 bacterias fueron elegidas. De estas 40 bacterias seleccionadas, todas resultaron ser Gram positivas. Además, presentaban diferentes morfologías, habiendo:

- Bacilos: 9 cepas
- Cocos: 18 cepas
- Cocobacilos: 13 cepas

En la muestra nº28 observamos, en primer lugar, el método de sembrado utilizado. Se puede ver el crecimiento de colonias puras sin ningún tipo de contaminación (Figura 15A). En la segunda imagen se muestra la bacteria a nivel microscópico y se puede observar cómo se trata de una bacteria Gram positiva con morfología de coco (Figura 15B).La Tabla 5 presenta los resultados de la tinción de Gram y del examen de morfología realizado en las 40 cepas seleccionadas.

Los resultados en esta primera etapa de análisis a nivel macroscópico, es decir, en las placas donde las bacterias fueron sembradas, no se observaban ningún tipo de contaminación. Las colonias que crecían eran de un único tipo. Una vez que se corroboró esto, se procedió al Gram de las muestras.

En los resultados de la tinción de Gram se ve, en primer lugar, uniformidad en el tipo de microorganismo, es decir, en un ningún caso nos encontramos con una levadura, ni con un hongo. Por otro lado, también hay un 100% de bacterias Gram positivas, lo cual es un aspecto

fundamental para tener en cuenta durante el rastreo de las candidatas a probióticas, en el caso de encontrar algún tipo de bacteria que fuese Gram negativa, se descartaría.

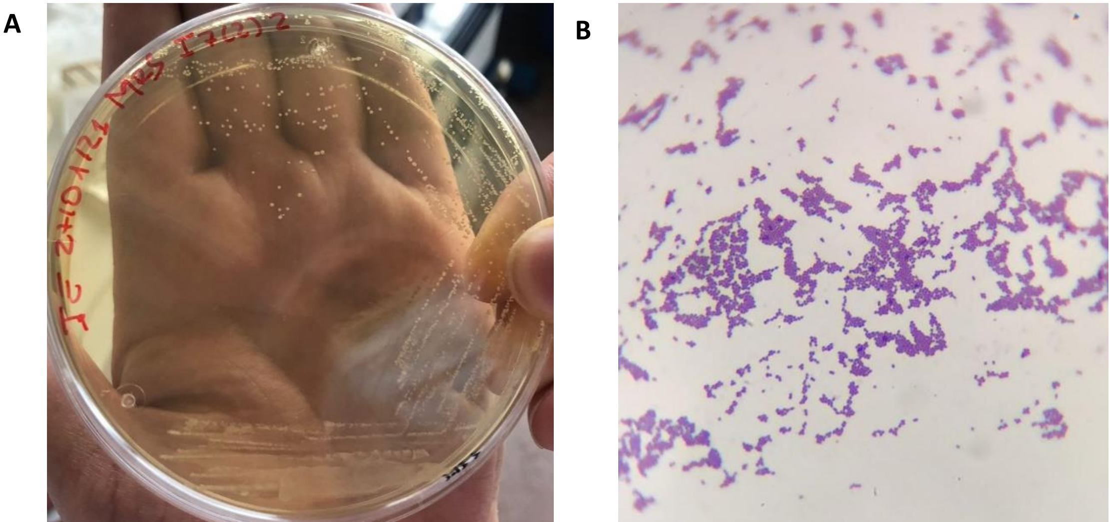


Figura 15: Muestra nº 28. (A) Bacterias sembradas en medio agar MRS. (B) vista a microscopio en aumento 40X de la tinción de Gram de la muestra crecida en medio agar MRS.

Tabla 5 Muestras con su correspondiente forma y Gram observadas al microscopio

Muestra	G(+)	G(-)	Levaduras	Forma
1	x			Coco
2	x			Bacilo
4	x			Bacilo
5	x			Coco
6	x			Cocobacilo
7	x			Cocobacilo
8	x			Bacilo
9	x			Bacilo
10g	x			Coco
10p	x			Coco
11	x			Bacilo
12	x			Cocobacilo
13	x			Cocobacilo
14	x			Cocobacilo
15	x			Bacilo
16	x			Cocobacilo
18	x			Coco
19	x			Bacilo
20g	x			Cocobacilo
21	x			Coco
22	x			Coco
23	x			Bacilo
24	x			Bacilo
25	x			Cocobacilo
26g	x			Cocobacilo
26p	x			Cocobacilo
27	x			Cocobacilo
28	x			Coco
33C	x			Coco
36C1	x			Coco
36C2	x			Coco
40gC	x			Coco
41	x			Cocobacilo
44	x			Coco
45C	x			Coco
46C	x			Coco
47	x			Cocobacilo
48	x			Coco
49	x			Coco
50	x			Coco

5.2 PCR, RESOLUCIÓN EN GEL DE AGAROSA Y GRAM

Pasada la primera etapa en donde se obtuvieron los resultados morfológicos de las bacterias, se procedió con la extracción de ADN. Una vez se consiguió extraer el ADN de todas las bacterias, se pasó a la siguiente fase, la de amplificación, en la cual se les hizo PCR siguiendo el protocolo explicado en 'Materiales y métodos'. Los productos obtenidos de la PCR se resolvieron en el gel de agarosa, utilizando un marcador de peso molecular como guía (Figura 16).

En la resolución en el gel de agarosa lo que se buscaba era ver si el producto de PCR había amplificado de manera correcta. En este caso la longitud del amplicón deseado era de unas 1500 pb, en la Figura 17 se muestran los resultados de la resolución en el gel, y se puede ver como en todos los casos la amplificación es correcta, estando la banda entre las 1500-2000 pb.

Como ya se ha descrito en la parte de ´Materiales y métodos`, además de la resolución en el gel de agarosa, se realizó la purificación del ADN a partir de los productos de PCR, para su posterior secuenciación. Durante este último proceso, se hizo una nueva tinción de Gram, y se observa como coinciden los resultados de esta última tinción, con los resultados de la primera que se hizo. Además, en este caso tampoco se observaron contaminaciones, de modo que las muestras que iban a ser enviadas a secuenciar eran puras, y no había cambios en comparación con las primeras muestras seleccionadas (Figura 18).

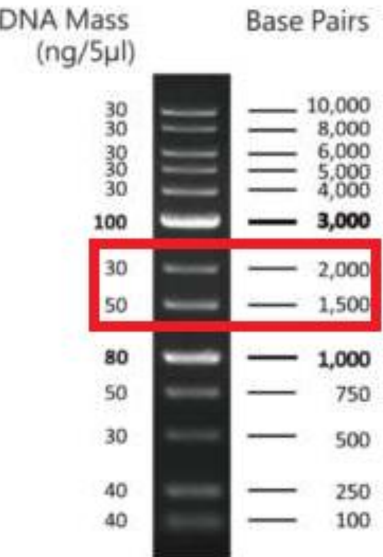


Figura 16: Esquema de las bandas de diferente peso molecular proporcionadas por el marcador (L) 'MWD1P-1kb-ladder-dna' (NipponGenetics). El cuadrado rojo muestra en donde se debería ubicar la banda de amplificación del producto de PCR.

Figura 17: Resolución de los productos de PCR**.** En rojo se muestran las bandas de los productos de PCR entre las 1500-2000 pb.

Arriba: Orden de izq. a dcha.: L, 21, 22, 23, 24, 25 26p, L.; L, 41, 44, 47, 48, 49, 50, L.; L, 27, 28, 26g, 18, 19, 20g, L.

Abajo: Orden de izq. a dcha.: L, 11, 12, 13, 14, 15, 16, 8; L, 46C, 40gC, 45C, 33C, 36C1, 36C2, L; L, 1, 2, 4, 5, 6, 7, L.

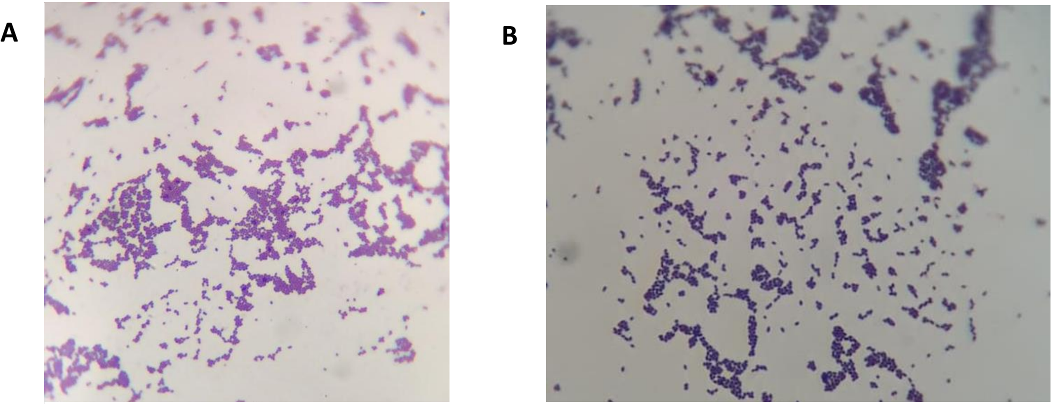


Figura 18: Muestra nº28: Vista a microscopio en aumento 40X, de bacteria Gram positiva con forma de coco. (A) Resultados de la 1ª tinción de Gram. (B) Resultado de la 2ª tinción de Gram, previo a su secuenciación.

5.3 SECUENCIACIÓN

De las 40 muestras enviadas a secuenciar obtenemos 19 secuencias de 19 bacterias. En la siguiente tabla (Tabla 6) se pueden observar las muestras de bacterias que fueron secuenciadas junto al tejido, al pez del que proceden y a la especie bacteriana que se obtiene una vez se han secuenciado.

Tabla 6 Procedencia y especie obtenida a partir de la secuenciación

Muestra	Tejido	Procedencia	Especie
2	Branquias	*S. trutta*	*Carnobacterium maltaromaticum*
9	Branquias	*S. trutta*	*Carnobacterium maltaromaticum*
13	Branquias	*O. mykiss*	*Lactobacillus plantarum*
16	Intestino	*O. mykiss*	*Lactobacillus plantarum*
24	Branquias	*S. trutta*	*Carnobacterium maltaromaticum*
25	Intestino	*O. mykiss*	*Lactobacillus plantarum*
26P	Branquias	*S. trutta*	*Carnobacterium maltaromaticum*
28	Intestino	*O. mykiss*	*Leuconostoc mesenteroides*
33C	Intestino	*S. trutta*	*Carnobacterium maltaromaticum*
36C1	Boca	*O. mykiss*	*Carnobacterium maltaromaticum*
36C2	Boca	*O. mykiss*	*Carnobacterium maltaromaticum*
40gC	Intestino	*A. baerii*	*Carnobacterium maltaromaticum*
41	Bazo	*S. trutta*	*Carnobacterium maltaromaticum*
44	Bazo	*O. mykiss*	*Vagococcus salmoninarum*
45C	Intestino	*A. baerii*	*Carnobacterium maltaromaticum*
46C	Intestino	*A. baerii*	*Carnobacterium maltaromaticum*
48	Vejiga	*O. mykiss*	*Leuconostoc mesenteroides*
49	Vejiga	*O. mykiss*	*Leuconostoc mesenteroides*
50	Intestino	*A. baerii*	*Carnobacterium maltaromaticum*

Los resultados de la secuenciación llevada a cabo por Magrogen revelaron que las muestras analizadas comprendían un total de 4 especies diferentes: *Carnobacterium maltaromaticum, Leuconostoc mesenteroides, Lactobacillus plantarum y Vagococcus salmoninarum*. Las tres primeras especies fueron contempladas como posibles candidatas para proceder a los siguientes análisis. Por el contrario, *Vagococcus salmoninarum* fue descartada al tratarse de un patógeno de peces.

De cada muestra analizada recibimos dos secuencias, correspondientes a 27f y 1492r. Para cada secuencia disponemos de 3 documentos diferentes:

1. Documento PDF de la cromatografía con los picos de color por cada nucleótido (Figura 19).
2. Documento .txt, en donde se muestra la secuencia nucleotídica completa.

3. Documento ab.1, este archivo permite el uso de softwares para el análisis de genética molecular evolutiva. En este caso se usó el software MEGA X.

La secuencia de nucleótidos (documento .txt) fue utilizada para la identificación de la bacteria mediante la herramienta bioinformática de acceso libre Blast (NCBI) (Figura 20). Una vez identificada, utilizando la herramienta Aligner de JustBio, se alinearon la secuencia obtenida en el trabajo (facilitada por Macrogen) y la secuencia obtenida en el Blast.

Además, gracias a las secuencias se pueden formar diferentes árboles filogenéticos para ver las posibles relaciones filogenéticas, en este caso creamos dos árboles, 'Maximum likelihood (MX)' y 'Neighboor joining (NJ)' (Figura 21-22).

1. Cromatografía:

Figura 19: Archivo PDF de la muestra 28, secuencia 27f, con los picos de cada nucleótido (MACROGEN).

2. Secuencia nucleotídica 27f de la muestra 28:

CGSRAKGGMCGCTGYCYWWWMMTGTYAAGTYCGTAMSMASATTMGMMRKA
YWGCTTGCACCTTTCAAGTGAGTGGCGAACGGGTGAGTAACACGTGGACA
ACCTGCCTCAAGGCTGGGGATAACATTTGGAAACAGATGCTAATACCGAA
TAAAACTTAGTGTCGCATGACACAAAGTTAAAAGGCGCTTCGGCGTCACC
TAGAGATGGATCCGCGGTGCATTAGTTAGTTGGTGGGGTAAAGGCCTACC
AAGACAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATTGGGAC
TGAGACACGGCCCAAACTCCTACGGGAGGCTGCAGTAGGGAATCTTCCAC
AATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGCTTTC
GGGTCGTAAAGCACTGTTGTATGGGAAGAACAGCTAGAATAGGAAATGAT
TTTAGTTTGACGGTACCATACCAGAAAGGGACGGCTAAATACGTGCCAGC
AGCCGCGGTAATACGTATGTCCCGAGCGTTATCCGGATTTATTGGGCGTA
AAGCGAGCGCAGACGGTTTATTAAGTCTGATGTGAAAGCCCGGAGCTCAA
CTCCGGAATGGCATTGGAAACTGGTTAACTTGAGTGCAGTAGAGGTAAGT
GGAACTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAG
TGGCGAAGGCGGCTTACTGGACTGCAACTGACGTTGAGGCTCGAAAGTGT
GGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACACCGTAAACGATGA
ACACTAGGTGTTAGGAGGTTTCCGCCTCTTAGTGCCGAAGCTAACGCATT
AAGTGTTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATT
GACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACG
CGAAGAACCTTACCAGGTCTTGACATCCTTTGAAGCTTTAGAAGATAGAA
GTGTTCTCTTCGGAGACAAAGTGACAGGTGGTGCATGGTCGTCGTCAGCT
CGTGTCGTGAGAAGGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATT
GTTAGTTGCCAGCATTCAGATGGCACTCTAGCGAGACTGCCGTGACAAAC
CGGAGGAGGGCGGGGACGACGTCAGATCATCATGCCCCTTATGACTGGGC
TACACCGGCTACAAGGCGTAAAAACAATGCAACCCGCCGAGGGGAGCAAT
CCTTAAATACCCCCCCTTCCGAATGATTTGCACCCACTCCTGAATCGGAA
CCCTTTAATCGGAAAAACCCCCCGGGAAAATTTCCGCCTTTTCACGCGGC
TCCCCCCCGGGATTTTATCCCACCCGGGCCCTTTAAAGCTAGAAGAGGGG
GAGGAGAATACAAAAAAAAAAAAACTACCACTCCACCCCCCCCCTTAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGAAATAA
A

3. Identificación de la bacteria mediante BLAST:

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Leuconostoc mesenteroides strain MR7 16S ribosomal RNA gene, partial sequence	Leuconostoc mesenter...	2124	2124	85%	0.0	96.33%	1347	MK680149.1
☑	Leuconostoc mesenteroides subsp. dextranicum strain M-25 16S ribosomal RNA gene, partial sequence	Leuconostoc mesenter...	2121	2121	80%	0.0	98.20%	1288	MT555755.1
☑	Leuconostoc mesenteroides strain 4622 16S ribosomal RNA gene, partial sequence	Leuconostoc mesenter...	2121	2121	80%	0.0	98.20%	1445	MT545113.1
☑	Leuconostoc mesenteroides strain 4605 16S ribosomal RNA gene, partial sequence	Leuconostoc mesenter...	2121	2121	80%	0.0	98.20%	1455	MT545101.1
☑	Leuconostoc mesenteroides strain 4595 16S ribosomal RNA gene, partial sequence	Leuconostoc mesenter...	2121	2121	80%	0.0	98.20%	1453	MT545093.1
☑	Leuconostoc mesenteroides strain 4576 16S ribosomal RNA gene, partial sequence	Leuconostoc mesenter...	2121	2121	80%	0.0	98.20%	1444	MT545083.1
☑	Leuconostoc mesenteroides strain 4560 16S ribosomal RNA gene, partial sequence	Leuconostoc mesenter...	2121	2121	80%	0.0	98.20%	1446	MT545072.1
☑	Leuconostoc mesenteroides strain 4526 16S ribosomal RNA gene, partial sequence	Leuconostoc mesenter...	2121	2121	80%	0.0	98.20%	1449	MT545042.1
☑	Leuconostoc mesenteroides strain 4491 16S ribosomal RNA gene, partial sequence	Leuconostoc mesenter...	2121	2121	80%	0.0	98.20%	1449	MT545016.1
☑	Leuconostoc mesenteroides strain 4490 16S ribosomal RNA gene, partial sequence	Leuconostoc mesenter...	2121	2121	80%	0.0	98.20%	1438	MT545015.1
☑	Leuconostoc mesenteroides strain 4489 16S ribosomal RNA gene, partial sequence	Leuconostoc mesenter...	2121	2121	80%	0.0	98.20%	1447	MT545014.1
☑	Leuconostoc mesenteroides strain 4488 16S ribosomal RNA gene, partial sequence	Leuconostoc mesenter...	2121	2121	80%	0.0	98.20%	1442	MT545013.1
☑	Leuconostoc mesenteroides strain 4486 16S ribosomal RNA gene, partial sequence	Leuconostoc mesenter...	2121	2121	80%	0.0	98.20%	1445	MT545011.1
☑	Leuconostoc mesenteroides strain 4485 16S ribosomal RNA gene, partial sequence	Leuconostoc mesenter...	2121	2121	80%	0.0	98.20%	1444	MT545010.1
☑	Leuconostoc mesenteroides strain 4484 16S ribosomal RNA gene, partial sequence	Leuconostoc mesenter...	2121	2121	80%	0.0	98.20%	1448	MT545009.1

Figura 20: Secuencias de bacterias que coinciden con la secuencia nucleotídica obtenida de la secuenciación.

4. Alineamiento de la secuencia obtenida con la secuencia obtenida en BLAST**:**

- Seq F: secuencia obtenida de la muestra
- Seq Car: secuencia obtenida de BLAST

```
seq_F          CGSRAKGGMC-----GCTGYCYWWWMMTGTYAAGTYCGTAMSMASATTMGMMRKAYWGCT
seq_Leu        GGGAATGGGCGGGGTGCTATACATGCAAGTC--GAACGCAC------AGCGAAAGGTGCT
                *. *.** *     ***. .      :**   *: ** *       :      .  ***

seq_F          TGCACCTTTCAAGTGAGTGGCGAACGGGTGAGTAACACGTGGACAACCTGCCTCAAGGCT
seq_Leu        TGCACCTTTCAAGTGAGTGGCGAACGGGTGAGTAACACGTGGACAACCTGCCTCAAGGCT
               ************************************************************

seq_F          GGGGATAACATTTGGAAACAGATGCTAATACCGAATAAAACTTAGTGTCGCATGACACAA
seq_Leu        GGGGATAACATTTGGAAACAGATGCTAATACCGAATAAAACTTAGTGTCGCATGACAAAA
               *********************************************************.**

seq_F          AGTTAAAAGGCGCTTCGGCGTCACCTAGAGATGGATCCGCGGTGCATTAGTTAGTTGGTG
seq_Leu        AGTTAAAAGGCGCTTCGGCGTCACCTAGAGATGGATCCGCGGTGCATTAGTTAGTTGGTG
               ************************************************************

seq_F          GGGTAAAGGCCTACCAAGACAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATT
seq_Leu        GGGTAAAGGCCTACCAAGACAATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACATT
               ************************************************************

seq_F          GGGACTGAGACACGGCCCAAACTCCTACGGGAGGCTGCAGTAGGGAATCTTCCACAATGG
seq_Leu        GGGACTGAGACACGGCCCAAACTCCTACGGGAGGCTGCAGTAGGGAATCTTCCACAATGG
               ************************************************************

seq_F          GCGAAAGCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGCTTTCGGGTCGTAAAGCACT
seq_Leu        GCGAAAGCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGCTTTCGGGTCGTAAAGCACT
               ************************************************************

seq_F          GTTGTATGGGAAGAACAGCTAGAATAGGAAATGATTTTAGTTTGACGGTACCATACCAGA
seq_Leu        GTTGTATGGGAAGAACAGCTAGAATAGGAAATGATTTTAGTTTGACGGTACCATACCAGA
               ************************************************************

seq_F          AAGGGACGGCTAAATACGTGCCAGCAGCCGCGGTAATACGTATGTCCCGAGCGTTATCCG
seq_Leu        AAGGGACGGCTAAATACGTGCCAGCAGCCGCGGTAATACGTATGTCCCGAGCGTTATCCG
               ************************************************************

seq_F          GATTTATTGGGCGTAAAGCGAGCGCAGACGGTTTATTAAGTCTGATGTGAAAGCCCGGAG
seq_Leu        GATTTATTGGGCGTAAAGCGAGCGCAGACGGTTTATTAAGTCTGATGTGAAAGCCCGGAG
               ************************************************************

seq_F          CTCAACTCCGGAATGGCATTGGAAACTGGTTAACTTGAGTGCAGTAGAGGTAAGTGGAAC
seq_Leu        CTCAACTCCGGAATGGCATTGGAAACTGGTTAACTTGAGTGCAGTAGAGGTAAGTGGAAC
               ************************************************************

seq_F          TCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTT
seq_Leu        TCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTT
               ************************************************************

seq_F          ACTGGACTGCAACTGACGTTGAGGCTCGAAAGTGTGGGTAGCAAACAGGATTAGATACCC
seq_Leu        ACTGGACTGCAACTGACGTTGAGGCTCGAAAGTGTGGGTAGCAAACAGGATTAGATACCC
               ************************************************************

seq_F          TGGTAGTCCACACCGTAAACGATGAACACTAGGTGTTAGGAGGTTTCCGCCTCTTAGTGC
seq_Leu        TGGTAGTCCACACCGTAAACGATGAACACTAGGTGTTAGGAGGTTTCCGCCTCTTAGTGC
               ************************************************************

seq_F          CGAAGCTAACGCATTAAGTGTTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAG
seq_Leu        CGAAGCTAACGCATTAAGTGTTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAG
               ************************************************************
```

```
seq_F           GAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG
seq_Leu         GAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG
                ************************************************************

seq_F           AACCTTACCAGGTCTTGACATCCTTTGAAGCTTTAGAAGATAGAAGTGTTCTCTTCGGAG
seq_Leu         AACCTTACCAGGTCTTGACATCCTTTGAAGCTTTTAGAGATAGAAGTGTTCTCTTCGGAG
                **********************************:..***********************

seq_F           ACAAAGTGACAGGTGGTGCATGGTCGTCGTCAGCTCGTGTCGTGAGAAGGTTGGGTTAAG
seq_Leu         ACAAAGTGACAGGTGGTGCATGGTCGTCGTCAGCTCGTGTCGTGAGATG-TTGGGTTAAG
                ***********************************************:* **********

seq_F           TCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCAGCATTCAGAT-GGCACTCTAGCGA
seq_Leu         TCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCAGCATTCAGATGGGCACTCTAGCGA
                ********************************************** *************

seq_F           GACTGCCG-TGACAAACCGGAGGAGGGCGGGGACGACGTCAGATCATCATGCCCCTTATG
seq_Leu         GACTGCCGGTGACAAACCGGAGGAAGGCGGGGACGACGTCAGATCATCATGCCCCTTATG
                ******** ***************.***********************************

seq_F           AC-TGGGCTACACC--GGCTACA-AGGCGTAAAAACA----ATGCAACCCGCCGAGGGGA
seq_Leu         ACCTGGGCTACACACGTGCTACAATGGCGTATACAACGAGTTGCCAACCCGCGAGGGTGA
                ** **********.   ****** :******:*.*..    :  ******** ..** **

seq_F           GCAATCCTTAA---ATACCCCCCCTTCCGAATGATTTGCACCCACTCC------TG-AAT
seq_Leu         GCTAATCTCTTAAAGTACGTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGT
                **:*: ** ::   .***  * *. * **.** .*:  *: *.****       ** *.*

seq_F           CGGAACC-CTT-TA--ATCGGAAAAACCCCCCGGGAAAATTT-----CCG---CCTTTTC
seq_Leu         CGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGTCTTGTAC
                ***** * **: **  . ****:.*.*.* *** *.:.*:*:     ***   * * *:*

seq_F           ACG-----CGGCTCCCCCCCGGGATTTTATCCCACCCGG-------GCCC-----TT---
seq_Leu         ACACCGCCCGTCACAC-CATGGGAGTTTGTAATGCCCAAAGCCGGTGGCCTAACCTTTTA
                **.     ** *:*.* *. **** ***.*.. .***..       * **     **

seq_F           -------------TAAAGCTAGAAGAGGGGGAGGAGAATACAAAAAAAAAAAAACTACCA
seq_Leu         GGAAGGAGCCGTCTAAGGCAAGACAAGAATGGG---------------------------
                             ***.**:***..**.. *.*

seq_F           CTCCACCCCCCCCTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
seq_Leu         ------------------------------------------------------------


seq_F           AAAGAAATAAA
seq_Leu         -----------
```

Árbol MX

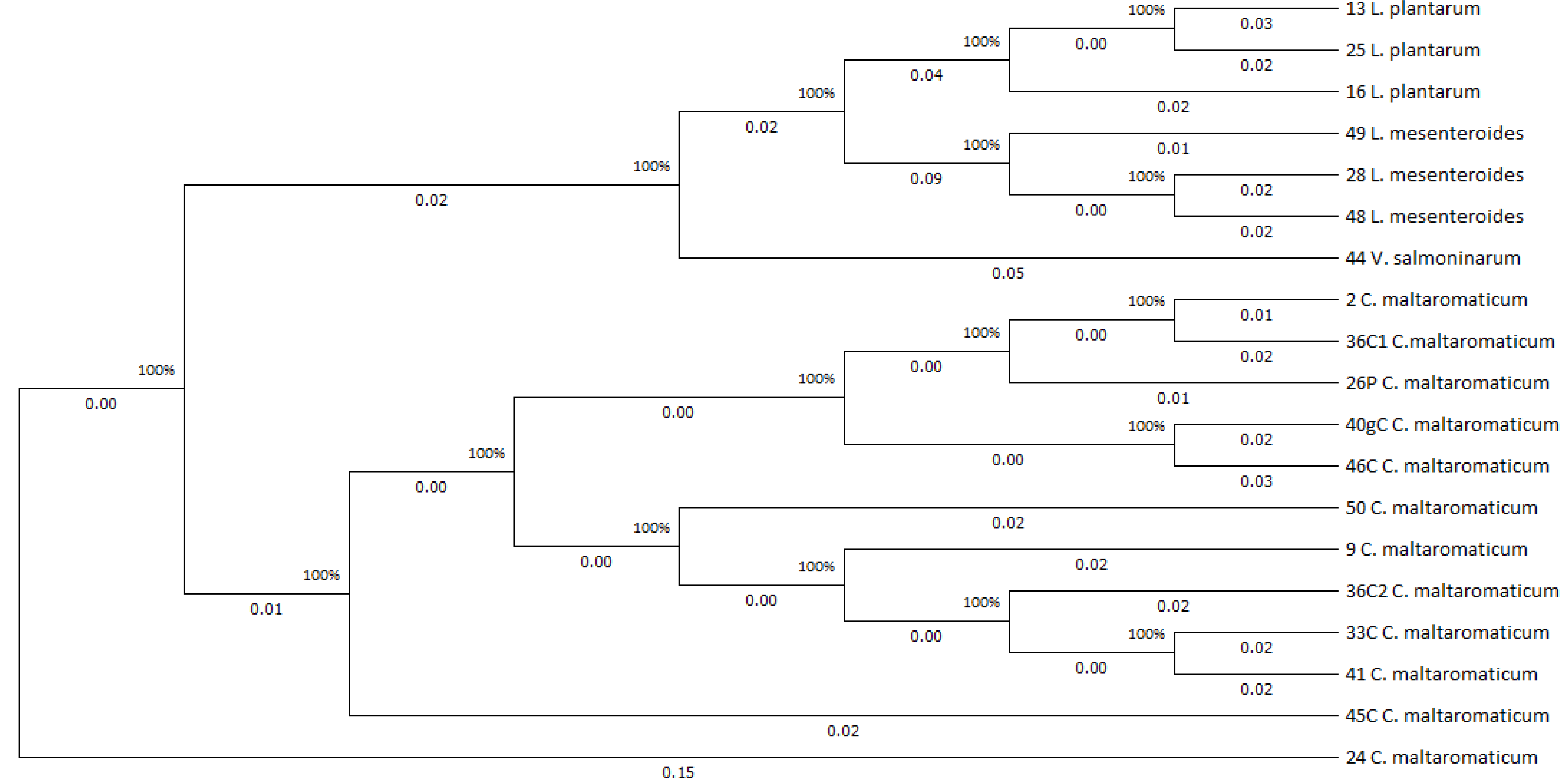


Figura 21: Este árbol se dedujo mediante el método de Máxima Verosimilitud usando 19 secuencias nucleotídicas, en las cuales se eliminaron los lugares en donde había espacios vacíos, de este modo se muestra el árbol con la mayor probabilidad logarítmica. Los porcentajes que se muestran junto a las ramas son los árboles en los que los taxones asociados se agrupan. Los números en las ramas muestran la distancias genéticas en sustituciones nucleotídicas por sitio. Este análisis se consiguió usando MEGA X.

Árbol NJ

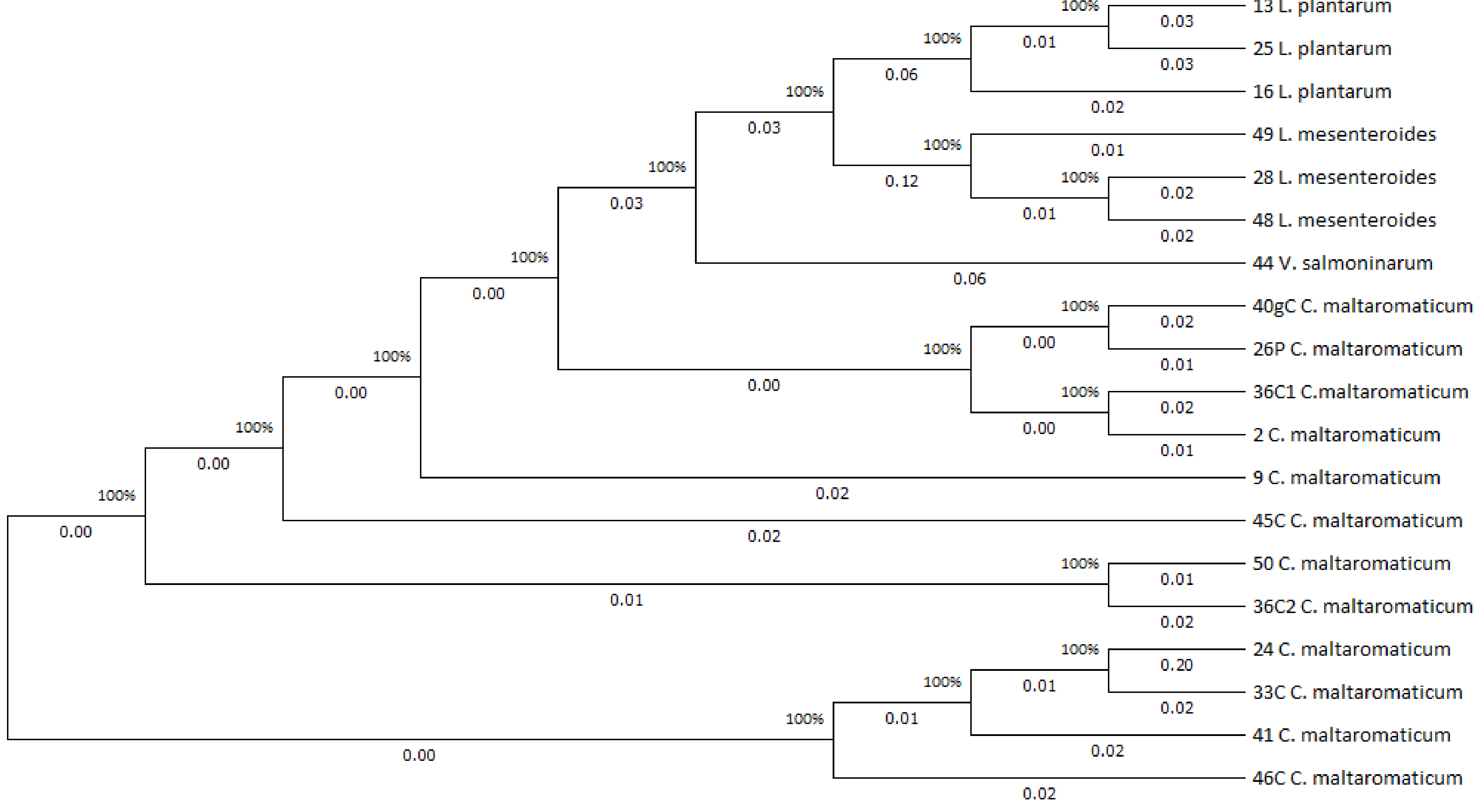


Figura 22: Este árbol se dedujo usando el método de NJ, usando 19 secuencias nucleotídicas, en las cuales se eliminaron los lugares en donde había espacios vacíos. Junto a las ramas se muestra el porcentaje de árboles replicados en los que los taxones asociados se agruparon en la prueba bootstrap, esta prueba consta de 1000 réplicas. Además, la distancia evolutiva se calcula mediante el método de distancia p. Este análisis se consiguió usando MEGA X.

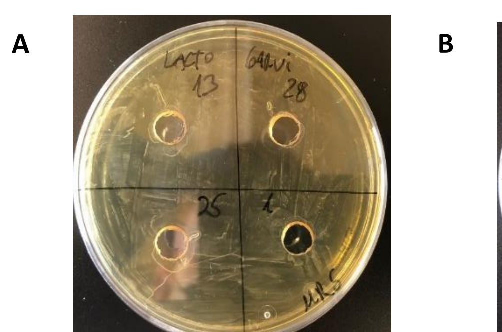
5.4 ENSAYOS DE INHIBICIÓN

Los resultado de los ensayos de inhibición 1 y 2 indican que las bacterias candidatas no son sensibles al patógeno, ya que no se observaron halos de inhibición en ninguno de los cuadrantes (Figura 23A-23B).

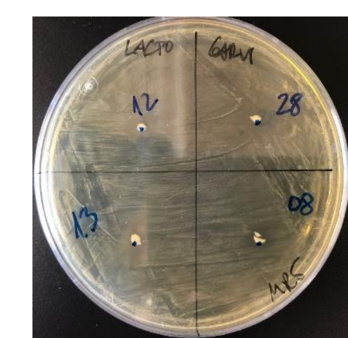
En los ensayos 1 y 2 los resultados esperados serían los halos de inhibición formados por las bacterias candidatas que se sembraron junto con las bacterias patógenas. En ambos ensayos los resultados no fueron concluyentes ya que no se formaron los halos de inhibición deseados.

En el ensayo número 3 (Figura 23C), en cambio, se observó cierta inhibición del patógeno *L. garvieae* frente a las muestras 13 y 16 (Tabla 7). No obstante, al tratarse de un número reducido de muestras se trata de un resultado preliminar y sería necesario repetir el experimento para confirmar la inhibición.

A



B



C

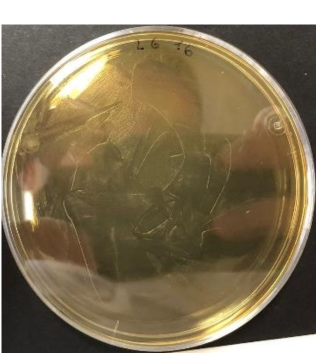


Figura 23: (A) Resultados de ensayo 1. (B) Resultados de ensayo 2. (C) Resultados de ensayo 3.

- Patógeno: (A, B, C) L. garvieae.
- Muestras: (A) 1, 13, 25, 28. (B) 8, 12, 13, 28. (C) 16

Tabla 7 Resultados obtenidos del ensayo nº3

Muestra	**Dilución**	**UFC/ml**	**Total**	**Escala Log**
***L. garvieae* control**	10^-6	10*10^8UFC/ml	1000000000	9,0
***L. garvieae* control**	10^-5	101*10^7UFC/ml	1010000000	9,0
***L. garvieae* 16**	10^-6	No crecimiento	1	0,0
***L. garvieae* 16**	10^-5	6*10^7UFC/ml	60000000	7,8
***L. garvieae* 13**	10^-6	7*10^8UFC/ml	700000000	8,8
***L. garvieae* 13**	10^-5	74*10^7UFC/ml	740000000	8,9

6 DISCUSIÓN

La evaluación y rastreo de probióticos es un área de extensa investigación en acuicultura desde que se ha demostrado que tienen la capacidad de ser una alternativa al uso de antibióticos, que en los últimos años su uso ha sido restringido debido a las posibles resistencias que pueden adquirir las bacterias patógenas (Hjelm et al., 2004). Además en acuicultura los probióticos pueden mejorar muchos aspectos de la producción, al conferir resistencias a enfermedades, mejorar las tasas de crecimiento, su inmunidad o la captación de nutrientes por medio de la estimulación de las enzimas digestivas (Assan et al., 2022; El-Saadony et al., 2021).

Un aspecto interesante del uso de probióticos en acuicultura es el hecho de utilizar bacterias provenientes del propio medio (medio acuático) puede incrementar notablemente las propiedades beneficiosas de las cepas y ofrecer ventajas frente a microorganismos procedentes de otro ambiente, como por ejemplo el medio terrestre (Wanka et al., 2018). En particular, en el presente estudio se aislaron bacterias de tres especies de peces que se cultivan en la Península Ibérica (trucha común, trucha arcoíris y esturión siberiano). Este primer rastreo de cepas probióticas se realizó mediante el análisis morfológico de las cepas aisladas y su posterior secuenciación, además de comprobar su capacidad para inhibir diferentes tipos de patógenos de peces *in vitro*. Para ello se utilizaron los patógenos *L. garvieae, Y ruckerii* y *V.salmoninarum,* las cuales son bacterias que producen enfermedades infecciosas en peces, y permitieron evaluar la capacidad inhibitoria de las cepas aisladas (Meyburgh et al., 2017; Torres-Corral and Santos, 2019; Wrobel et al., 2019).

Una vez realizada la tinción de Gram y concluida la primera etapa de análisis se pudo determinar que las 40 bacterias seleccionadas eran bacilos, cocos y cocobacilos Gram positivos (Tabla 5). Este hallazgo era previsible ya que este tipo o de morfología es frecuente en el grupo de probióticos usados en acuicultura, donde los géneros *Lactobacillus, Carnobacterium* y *Leuconostoc* son predominantes, aunque existen otros géneros que también son ampliamente utilizados (Nayak, 2010).

El gen 16s rRNA es un gen ribosomal y es de gran importancia ya que ha sido aceptado como diana para la identificación de bacterias y el análisis filogenético de bacterias. Por ello su amplificación es un paso clave para su posterior secuenciación. En este trabajo el gen amplificó de manera correcta, obteniendo un amplicón de una longitud aproximada de 1500 pb (Amann et al., 1995; Kwon et al., 2004), que fue resuelto en gel de agarosa. Tras la purificación de los productos de PCR, estos fueron secuenciados y se identificaron 4 bacterias diferentes, *Carnobacterium maltaromaticum* (aislada de trucha común, trucha arcoíris y esturión siberiano)

Lactobacillus plantarum (aislada de trucha arcoíris)*, Leuconostoc mesenteroides* (aislada de trucha arcoíris) y *Vagococcus salmoninarum* (aislada de trucha arcoíris). Las tres primeras son pertenecientes al grupo de bacterias ácido-lácticas, mientras que la última se trata de un patógeno.

Carnobacterium maltaromaticum ha sido previamente aislado del intestino de los tres peces del trabajo (González et al., 2000; Józefiak et al., 2019; Kim and Austin, 2006). Esta bacteria aun no siendo de las más abundantes en el intestino de estos peces, sí que es una bacteria que forma parte de la microbiota intestinal tanto de trucha común como de trucha arcoíris (Kim and Austin, 2006).

También se ha podido comprobar que la suplementación con insectos en la dieta de *A. baerii* aumenta la cantidad de esta bacteria en el intestino, contribuyendo a la modulación del intestino y mejorando la salud intestinal (Józefiak et al., 2019). Otra investigación indicó que la administración de esta especie de bacterias en la dieta es capaz de mejorar la inmunidad intestinal en trucha arcoiris (Kim and Austin, 2008). Por otro lado, se ha visto que la suplementación con *C. maltaromaticum* confiere protección frente a uno de los patógenos utilizados en este trabajo, *Y. ruckeri* (Vendrell et al., 2008).

A pesar de estos hallazgos y de que *C. maltaromaticum* sea considerada una especie con propiedades probióticas, se ha descrito que ciertas cepas podrían ser perjudiciales y causar causar infecciones en peces e incluso la muerte. En este contexto, como precaución, es necesario examinar en profundidad la cepa hallada en nuestro laboratorio antes de considerarla como posible cepa probióticas (Roh et al., 2020).

En este estudio también se consigue aislar e identificar *Lactobacillus plantarum* y *Leuconostoc mesenteroides*, ambas bacterias tienen una alta capacidad probiótica al poder adherirse y sobrevivir en el mucus intestinal (Vendrell et al., 2008). Los resultados, aunque necesiten más número de pruebas y ensayos debido al corto periodo del estudio, muestran que *L. plantarum* es capaz de reducir el crecimiento del patógeno *in vitro* (Tabla 7), esto concuerda con los resultados de otro estudio en el que se demuestra la capacidad de *L. plantarum* para reducir la muerte en los peces producida por la infección de *L. garvieae* (Vendrell et al., 2008).

En otro estudio, en el cual también se aisló *L. plantarum* desde el tracto digestivo de trucha arcoíris, se pudo comprobar que la dieta suplementada con esta bacteria incrementaba el crecimiento y mejoraba la respuesta inmunitaria no solo en la propia trucha arcoíris, sino en diferentes especies de peces, contra el patógeno *Y. ruckeri* (Soltani et al., 2019).

Estudios anteriores con *L. mesenteroides* demostraron que la administración de cepas de esta especie junto a aditivos alimentarios en trucha arcoíris resultó beneficiosa y confirió resistencia contra enfermedades como la furunculosis causada por *A. salmonicida* (Balcázar et al., 2007b). En el caso de humanos, un estudio ha demostrado que el consumo de *L. mesenteroides* puede resultar beneficioso en el tratamiento contra el cáncer de colon, debido a la capacidad de esta especie para activar la ruta que induce la apoptosis de las células cancerígenas (Zununi Vahed et al., 2017).

Otro tipo de cáncer en el cual se ha demostrado que el uso de probióticos, en este caso de *L. plantarum*, es beneficioso ha sido en el melanoma humano maligno. En esta patología se ha visto que *L. plantarum* reduce la capacidad migratoria de las células cancerígenas, además de inducir la propia apoptosis de estas células (Park et al., 2020). Esto hace pensar que las bacterias aisladas en este trabajo podrían ser usadas tanto en animales como en personas, abriendo un frente para más ensayos.

El aislamiento de *V. salmoninarum* es un hecho importante, aunque para nuestro trabajo no sea significativo ya que quiere decir que la trucha de la cual se aisló esta bacteria estaba infectada. Esta bacteria causa una enfermedad conocida como 'streptococcosis' de agua fría, y no es la primera vez que se ha aislado. Esta enfermedad ocurre en aguas en la cual la temperatura ronda los 10-12ºC y los casos se dan en el estrés post-desove (Ruiz-Zarzuela et al., 2005). Además, se trata de una enfermedad muy poco común, y que puede causar ratios de mortalidad mayores al 50%. Es interesante comentar que una de las bacterias con potencial probiótico de nuestro trabajo, *Carnobacterium maltaromaticum*, puede causar una patología similar a la de *V. salmoninarum*, siendo también una 'streptococcosis' de agua fría, en situaciones de estrés excesivo (Standish et al., 2020).

Estas bacterias no solo tienen efecto probiótico cuando son suministradas vivas, sino que postbióticos producidos por ellas también ejercen efectos beneficiosos para la salud. Ciertos metabolitos postbióticos aislados de cepas de *Lactobacillus plantarum*, las bacteriocinas I-UL4, TL1, RS5, RI11, RG11 y RG14, han mostrado tener efecto citotóxico sobre diferentes líneas celulares tumorales, sin causar este mismo efecto sobre células normales (Chuah et al., 2019). Estas mismas bacteriocinas han mostrado tener efectos inhibitorios en la proliferación de diferentes patógenos, como son *E. coli* y *Salmonella entérica* (Kareem et al., 2014).

Otras dos bacteriocinas, en este caso producidas por *C. maltaromaticum*, Cbn BM1 y Cbn B2, también tienen la capacidad de inhibir el crecimiento de ciertas especies de patógenos de los géneros *Listeria* sp., *Enterococcus* sp. y *Carnobacterium* sp. (Jasniewski et al., 2009). De modo

que las bacterias aisladas en este trabajo, no solo se pueden usar vivas, sino que también se podrían utilizar los metabolitos producidos por ellas, para su posible uso como terapia.

En conclusión, las bacterias aisladas e identificadas en este trabajo se presentan como buenas candidatas para su posible utilización futuro como bacterias probióticas, y así es respaldado por la literatura disponible. Además de su posible uso como probióticos, sus metabolitos también podrían ser una buena opción para la generación de compuestos postbióticos. Sin embargo, este trabajo es un primer cribado y se necesitarían más ensayos para valorar la efectividad de las cepas aisladas.

7 CONCLUSIÓN

Las principales conclusiones que se derivan del presente trabajo de investigación son:

1. La secuenciación de las diferentes bacterias aisladas nos ha permitido obtener cuatro especies diferentes, tres de las cuales de ellas son bacterias con potencial probiótico.
2. Aunque se observan cambios en los ensayos de inhibición realizados, éstos no han resultado ser significativos. Sería necesario llevar a cabo un mayor número de pruebas para confirmar los indicios observados.
3. Las bacterias obtenidas tienen la capacidad de actuar frente a diferentes patógenos como son *Yersinia ruckeri* y *Aeromonas salmonicida*.
4. La metodología utilizada nos permite explorar y obtener de manera rápida microorganismos que tienen potencial probiótico tanto para el organismo hospedador como para el ser humano.

8 LIMITACIONES Y FORTALEZAS

8.1 PERSPECTIVAS

En base a las conclusiones obtenidas, existe la posibilidad de desarrollar aún más el trabajo, siendo las siguientes perspectivas las opciones para continuar con el trabajo en el futuro:

1. Realizar análisis bioquímicos, de pH, de hidrofobicidad, de crecimiento, de tolerancia a sales biliares y de sensibilidad a antibióticos.
2. Llevar a cabo ensayos de inhibición y la caracterización de sustancias inhibitorias de las bacterias probióticas.
3. Probar las bacterias candidatas en modelos *in vivo* en peces.
4. Probar las bacterias candidatas en modelos *in vitro* para posible uso en humanos.

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ANEXO I

Publicación en MDPI 'nutrients': Human Microbiota Network: Unveiling Potential Crosstalk between the Different Microbiota Ecosystems and Their Role in Health and Disease.

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Review

Human Microbiota Network: Unveiling Potential Crosstalk between the Different Microbiota Ecosystems and Their Role in Health and Disease

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Abstract: The human body is host to a large number of microorganisms which conform the human microbiota, that is known to play an important role in health and disease. Although most of the microorganisms that coexist with us are located in the gut, microbial cells present in other locations (like skin, respiratory tract, genitourinary tract, and the vaginal zone in women) also play a significant role regulating host health. The fact that there are different kinds of microbiota in different body areas does not mean they are independent. It is plausible that connection exist, and different studies have shown that the microbiota present in different zones of the human body has the capability of communicating through secondary metabolites. In this sense, dysbiosis in one body compartment may negatively affect distal areas and contribute to the development of diseases. Accordingly, it could be hypothesized that the whole set of microbial cells that inhabit the human body form a system, and the dialogue between the different host microbiotas may be a contributing factor for the susceptibility to developing diseased states. For this reason, the present review aims to integrate the available literature on the relationship between the different human microbiotas and understand how changes in the microbiota in one body region can influence other microbiota communities in a bidirectional process. The findings suggest that the different microbiotas may act in a coordinated way to decisively influence human well-being. This new integrative paradigm opens new insights in the microbiota field of research and its relationship with human health that should be taken into account in future studies.

Keywords: microbiota; dysbiosis; crosstalk; metabolites; human diseases

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1. Introduction

Evolution has been involved in the development of all microorganisms, and during this evolutionary process, many of them have co-evolved with humans, inhabiting different parts of the body and forming what is known as human microbiota [1]. Lederberg and McCray first introduced the concept of "microbiota" in 2001, referring to it as "the ecological community of commensal, symbiotic and pathogenic microorganisms that literally share our body space and have been all but ignored determinants of health and disease" [2]. Although they are predominantly anaerobic bacteria [3], we can also find viruses, fungi, archaea, and even protists [4]. According to the literature, the microbiota has a ratio of about 1:1 when compared to the number of human cells, meaning that a reference person hosts about 4×10^{13} bacteria [5,6]. Interestingly, most of these microorganisms are found in the digestive tract, which harbors between 150 and 400 different bacteria species [7]. Firmicutes and Bacteroidetes are the most predominant phylum followed by Actinobacteria, Proteobacteria, and Synergistetes [8,9].

In addition, the microbiota presents a reservoir of genes (microbiome) that is way larger than the human genome [10,11]. Specifically, the colon is the most colonized section with a mass of microorganisms close to 1.5 kg of weight [12]. Nevertheless, this is not the only area where microorganisms are hosted, as they also inhabit the skin, the vagina, the genitourinary tract, the respiratory tract, and the oral cavity [13].

Besides the great controversy around the topic, most scientific works support the hypothesis that humans are born sterile and have their first contact with exogenous microorganisms at the time of birth. Depending on the type of delivery, the newborn has the first contact with their mother's birth canal (vaginal microbiota) or belly skin (skin microbiota) in the case of caesarean deliveries [14,15]. Besides the process of colonization lasting approximately three years [16], the type of delivery has a profound impact on the child's microbiota colonization and immune system programming, and influences the risk of suffering many diseases in adulthood [17–19]. Besides the delivery mode, other factors such as breastfeeding [17] and adulthood diet [20], exposition to agents such as drugs [21] or antibiotics [22,23], physical activity [24,25], psychological stress [26], sleep quality [27], and host's circadian clock [28], among others, collectively contribute to the microbiota composition and activity.

The microorganisms that currently conform the mammalian microbiota have gone through selection pressure, and they survived due to the advantageous role they play in the host homeostasis [29]. As mentioned above, an enormous diversity of bacteria have been found in the different body compartments [30], and this specific bacteria species create a particular ecosystem whose equilibrium importantly affects the proper functioning of this body compartment. This compartmentalization may be explained by the divergent characteristics of each organ and tissue [31]. Most of this knowledge comes from large research projects, and special attention must be paid to the Human Microbiome Project (HMP), an ambitious project aimed to make it visible the power of the microbiota in health and disease [32]. During the first phase (HMP1), 250 subjects were sampled and screened for their microbial genomes in five body sites which are considered significant: the mouth, the nasal cavity, the vagina, the gastrointestinal track and the skin (for details see [32]). The HMP1 contributed with invaluable findings on the subject and led to several publications [33,34], as the same time that new gaps were identified. During the second phase, The Integrative Human Microbiome Project (HMP2), which specifically focused on some physiological conditions (pregnancy, irritable bowel syndrome, and prediabetic state), efforts were coordinated to explore the function of the intestinal microbes [35]. The combination of metagenomics with other -omic platforms (such as metabolomics, metaproteomics, and metabolomics) will provide an overview of the current activity of the microbiota [36,37], the HMP2 highlighted interesting findings on the interplay between host and its resident gut microorganisms [35].

As any biological system, the microbiota is dynamic and fluctuates. A healthy (eubiotic) microbiota is resilient and can restore equilibrium when it undergoes oscillations [38,39]. On the contrary, in other cases, host and environmental factors restrain the microbiota from compensating the alterations, and dysbiosis occurs. This is important since dysbiosis has been related to multiple pathologies in humans, including metabolic disorders such as diabetes mellitus [40], obesity [41], and non-alcoholic fatty liver disease (NAFLD) [42], inflammatory diseases like inflammatory bowel disease or asthma [43], or cognitive dysfunctions like Alzheimer's disease [44] and autism spectrum disorder [45]. Good evidence for this can be found in the list of ongoing research projects within the Horizon 2020 program on the role of microbiota in specific conditions such as cardiometabolic diseases (MetaCardis), chronic inflammatory diseases (SISCID), cancer (GOMS), chronic liver disease (MICROB-PREDICT), or autism (Gemma).

The main objective of this article is to summarize the available evidence on the possible communications between the different microbiota niches in the human body. Besides, we will also refer to interactions between microbiota communities and different organs. In the following sections, we will go deeper into the main commensal microbiota communities

that shape the human body, define the dominant microorganisms that reside in them, and establish possible connections between the different microbiota niches and between these microbiotas and specific diseases or disorders. We focused on the major body sites investigated within the HMP1 (skin, oral, vaginal, and gut) [32]. In addition, for a more inclusive understanding, we decided to include the respiratory tract microbiota, that has recently attracted much attention in the current situation with the COVID-19 pandemic disease [46,47], as well as the urinary [48] and the penile microbiota [49].

2. Main Microbial Communities in the Human Body

2.1. Skin Microbiota

The skin is a complex organ which provides the first mechanical and biological barrier between the environment and the human cells. It is divided into two main layers: the epidermis and the dermis. Most bacteria species in the skin microbiota belong to Actinobacteria, Firmicutes, and Proteobacteria phyla [50], and four main genera, *Corynebacterium*, *Propionibacterium*, *Staphylococcus*, and *Streptococcus* [50,51].

They are located according to their environmental requirements so that anaerobic microorganisms like *Propionibacterium* spp. are placed in sites with more anaerobic conditions such as the sebaceous glands, and other more tolerant bacteria, like *Corynebacterium* spp., are distributed along the whole skin site [51]. Besides it is one of the largest organs of the human body, the skin ranked fourth place in the human body's part with the highest number of bacteria [50,52].

In utero, the baby's skin is sterile, therefore the skin microbiota is established few moments after birth. From this point, the microbes colonize the skin until it reaches an equilibrium [53]. The type of delivery is crucial for the configuration of the skin microbiota in the baby. In this way, children born in a natural way (birth canal) present bacterial communities similar to that in the mother's vaginal microbiota, mainly *Lactobacillus* and *Prevotella* spp. On the other hand, those children born by C-section have microorganisms from the mother's skin microbiota, predominantly *Propiniobacterium*, *Corynebacterium*, and *Staphylococcus* [15].

Interestingly, the distribution of the microbial communities on the skin surface is not homogeneous. One study reported that the front part of the body is more colonized, and is represented by *Propionibacterium*, *Corynebacterium*, and *Proteobacteria*, while the rear is represented by *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* [50]. Data indicates that differences exist between female and male skin microbiota. Such discrepancies are driven by important dissimilarities in factors such as hormone production, sebum production or make-up use, that dramatically influence the environmental conditions of the skin [54]. Besides sex, aspects such as personal hygiene, immune status or the presence of skin diseases influence the structural composition of the skin microbiota [50]. In addition, another study indicated that environmental factors such as lifestyle can also impact skin microbiota since significant differences were identified between US residents, that live a Western lifestyle, and Amerindians from the Amazonas [55].

Alterations in the skin microbiota are related to some skin disorders. To illustrate, acne is triggered by bacteria overgrowth of *Propionibacterium* spp. and particularly *Propionibacterium acnes* [50,56]. In addition, *S. aureus* spp. (*S. aureus* and *S. epidermidis*) and *Malassezia* spp. fungi were identified in most cases of atopic dermatitis [51,57], and some *Corynebacterium* spp. were related to the onset of AD [58]. Besides, people with hidradenitis suppurativa, an inflammatory skin disease, present an enrichment in *Corynebacterium*, *Porphyromonas*, and *Peptoniphillus* spps. [59]. Bacteria are not the only microbial cells that can promote skin problems, since a mite (Demodex) and a fungi (Malassezia) were shown to be involved in the development of Rosacea and Seborrheic dermatitis, respectively [50].

2.2. Oral Microbiota

The oral microbiota is an important part of the human microbiota and has been described to harbor more than 700 different microbial species. The fact that it is close to many

other anatomic regions makes the oral microbiota the second most complex microbiota niche in the human body after the gut [60,61]. There are discrepancies between studies regarding the main component of the oral microbiota, and while some authors suggest that the most important phyla in the oral microbiota are Actinobacteria, Bacteroidetes, Firmicutes, Proteobacterias, and Synergistetes, others pointed to other phyla such as Fusobacterias and Spirochaetes [62].

Within the oral microbiota, certain species like Fusobacterium, Gemmela, Veillonella, Streptococcus, or Granulicatella are ubiquitous, while others like Bacteroidetes, Pasterutella, Prevotella, Neisseria, and Corynebacterium are associated to some particular regions [63].

The oral cavity comprises many different surfaces including saliva, soft tissues (cheek, palate, and tongue), and hard tissues (tooth), where bacteria and other microorganisms could potentially colonize and predominate [60]. For instance, saliva is predominated by *Streptococcus*, *Veillonella*, and *Prevotella*, meanwhile, the surface of soft tissues are colonized by *Streptococcus salivarus*, *Rothia*, and *Eubacterium.* The teeth are also home to microorganisms. Members from the *Corynebacterium*, and *Actinomyces* genera normally colonize the supragingival region, while the subgingival area is characterized by anaerobic species from the *Spirochaetes*, *Fusobacteria*, *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes*, genera [60].

Oral dysbiosis has been related to certain diseases. For example, oral candidiasis, that was linked to caries, is thought to be caused by a dysbiotic oral microbiota characterized by increased levels of *Streptococcus* and *Lactobacillus* in the oral cavity [64]. Another study described a different oral microbiota in HIV-infected subjects, characterized by a lower microbial diversity, and enrichment in *Veillonella*, *Rothia*, and *Streptococcus* spp. [65].

2.3. Respiratory Tract Microbiota

In the past, it was the assumption that lungs were sterile; however, huge advances in culturing techniques demonstrated that the microbial colonization of the respiratory tract begins in utero. After birth, the respiratory tract is colonized with the mother's microbiota. In a similar manner to the skin microbiota, the diversity of the respiratory microbiota highly depends on the mode of delivery. Natural delivery prompts the colonization of microbes from the mother's vaginal and gut microbiota, while C-section newborns are colonized by the mother's skin microbiota [66,67]. Studies on the respiratory microbiota have highlighted the limitations in the determination of a core respiratory microbiota, due to the great interpersonal variability. Nevertheless, data indicates that certain bacteria general such as *Streptococcus*, *Haemophilus*, *Moraxella*, *Staphylococcus*, and *Veillonella* are commonly presented in samples of the respiratory microbiota [68].

The respiratory tract can be divided into two parts, the upper and the lower respiratory tract. They both are attached to each other, however, they present different environmental conditions (pH, temperature, PCO_2, and PO_2 conditions) [69], and also harbor different bacterial communities [70]. The upper respiratory tract, which can be divided into nasal cavity, nasopharynx, and oropharynx, contains most of the bacteria, that are predominatly *Staphylococcus*, *Propionibacterium*, *Corynebacterium*, *Streptococuus*, *Moraxella*, *Haemophillus*, *Prevotella*, and *Veillonella* [69]. The lower respiratory tract includes the trachea and lung's bronchial trees, and is mostly represented by *Prevotella*, *Veillonella*, *Streptococcus*, and *Tropheryma* [69]. The bacteria density decreases as we descend in the tract, being the lungs the location with the lowest bacterial count [68,69].

2.4. Gut Microbiota

The gut microbiota is by far the most studied of the microbiota niches in the human body, and this is because it contains around 70% of the human microbiota [71]. The microbiota in this area is not evenly divided, and the microbial composition and relative abundance change according to the section of the digestive tract [10]. This can partially be explained by the chemical, nutritional, and immunological gradient along the digestive tract [71]. As with other microbiota communities, the gut has first contact with microorganisms after delivery, and is deeply influenced by environmental determinants such as

early life events [19] such as the delivery mode [15], or breastfeeding [17]. Normally, it is first colonized by facultative anaerobes, and there is a gradual shift towards anaerobes species [72].

Although the microbiota is present all along the gastrointestinal tract, the greatest number of bacteria is concentrated in the large intestine, specifically in the colon [73], where bacteria of the phylum Bacteroidetes and Firmicutes predominate, representing 90% of the gut microbiota [10]. Members of the *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, *Clostridium*, *Escherichia*, *Streptococcus*, and *Ruminococcus* genera are among the most representative intestinal microbes [74].

There is a long list of factors that influence the gut microbiota composition. Some notable examples are the host genome [12,30], geography [75], adulthood diet [20], physical activity [27], host's circadian clock [28], and psychological stress [26]. Nevertheless, the dietary factors are probably the strongest and more powerful determinants shaping the gut microbiota. The characteristics of the diet have a profound effect on the gut microbiota profile, affecting both composition and diversity [72,76]. The dietary pattern, the contribution of the macronutrients, the presence of bioactive components or functional food, or the use of nutraceuticals such as probiotics and prebiotics can effectively alter the microbiota composition and confer health benefits to the host [74,77].

The relationship between gut and brain has been extensively studied as well. This gut–brain axis is very important due to the role of gut's microbiota has on behavior and development of the brain [78]. However, the absence of microbiota in the brain means that the hypothesis presented in this review does not fit on this axis, since the relationship between the brain and the intestine occurs through metabolites that are capable of crossing the blood–brain barrier. This evidence has been highlighted in different studies, in which a relationship was found between an altered intestinal microbiota and an affected brain [79]. It is crucial to mention the relationship established between the main neurodegenerative disorders, Parkinson disease (PD) and Alzheimer's disease (AD), and a gut's microbiota dysbiosis. In the first example, it seems that an overgrowth of *Helicobacter pylori* on the GI tract is linked to a severe form of the PD. In addition, an increase of pro-inflammatory bacteria is linked to PD, these bacteria are *Proteobacteria*, *Enterococcus*, and *Enterobacteriaceae*. Similar results have been reported in AD, where a decrease of *Eubacteria* (*E. rectale*), which is anti-inflammatory, and an overgrowth of *Escherichia* and *Shigella*, pro-inflammatory, lead to an aggravation of the disorder [80]. The communication between both systems can be divided into five pathways, neuroanatomical pathway, neuroendocrine mediated by hypothalamic–pituitary–adrenal axis, gut immune system, neural regulators synthesized by gut bacteria and intestinal and blood–brain barrier [78].

2.5. Genital Microbiota

The vaginal microbiota is simpler than other microbiota niches, for instance, the gut microbiota, and presents lower alpha and beta diversity [81]. It is governed by *Lactobacillus* spp., mostly *L. crispatus*, *L. iners*, *L. gasseri*, and *L. jensenii*, which exert an important defensive function, and other species from the genera *Atopobium*, *Dialister*, *Gardnerella*, *Megasphaera*, *Prevotella*, *Peptoniphilus*, *Veinovella*, *Lachnospiraceae*, *Streptococcus*, *Staphylococcus*, and *Gemella*, among others [82,83]. The stability of the human female microbiota is known to fluctuate during lifespan. Indeed, due to the great endogenous and exogenous fluctuations during the menstrual cycle, the vaginal microbiota, and particularly lactobacilli, also fluctuate during the period [84]. During menopause, however, the drop in estrogen levels has been associated with a decline in *Lactobacillus* spp. and genitourinary complications such as urinary tract infections [85]. In addition, during pregnancy the hormonal changes and the many physiological and structural alterations influence the vaginal microbiota. According to cross-sectional studies, it exhibits important changes including a decline in alpha diversity, increased number of *Lactobacillus* spp., particularly *L. iners*, *L. crispatus*, *L. jensenii*, and *L. johnsonii*, increased abundance of Clostridiales, Bacteriodales, and Actinomycetales, as well as changes in the profile of microbial metabolites produced

by the vagina microbiota [86,87]. Interestingly, microbiota variations during pregnancy are not restricted to the vaginal tract since compositional changes have also been reported in the oral and gut microbiota of pregnant women [81,86,88]. To illustrate, it has particular relevance the greatest rate of the periodontal disease reported in pregnant women that is itself linked to preterm birth [81,88].

The vaginal microbiota, and particularly *Lactobacilli* spp., plays a vital role in the female reproductive fitness and pregnancy outcome. To illustrate this, *Gardnerella vaginalis* and *Atopobium vaginae* have been associated with a poor pregnancy rate [88]. The studies on the vaginal microbiota vastly outnumbering the number of studies on the penis microbiota, and many of them are related to bacterial vaginosis (BV). This is the most common genital tract infection in women and is characterized by greater bacterial diversity, an enrichment in anaerobic and facultative bacteria species from the genera *Atopobium*, *Gardnerella*, *Mycoplasma*, *Prevotella*, *Bifidobacterium*, *Megasphaera*, *Leptotrichia*, *Sneathia*, *Dialister*, or *Clostridium*, as well as a reduced number of *Lactobacilli* normally found in healthy women [83,89–92]. The BV has been extensively studied, and findings from a large number of human studies concluded that, in the majority of the studied population, a set of women had a microbiota enriched with *L. iners* or *L. crispatus*, and that women in the second group presented protection against developing a vaginal microbiota prone to BV than those in the first group [83]. Two particular bacteria, *G. vaginalis* and *A. vaginae*, have gained increasing attention and are among the main bacteria involved in BV. Their pathogenicity seems to be related to their ability to establish microbial biofilms with other species [89,91,93]. Certain probiotic strains (*L. reuteri* RC-14 and *L. rhamnosus* GR-1) have been demonstrated to impair those biofilms and showed promise as potential therapeutic agents for the restoration of the normal vaginal microbiota in women with BV [89].

In the same line, vaginal dysbiosis can also be associated with vulvovaginal candidiasis, which is the overgrowth of *Candida* spp. *Candida albicans* is the most frequent species; however, other *Candida* spps. like *C. tropicalis*, *C. glabrata*, *C. krusei*, *C. dubliniensis*, and *C. parapsilosis* have been identified [94]. *C. albicans* is a commensal fungi that is naturally present in the oral, gut, and vaginal microbiota; however, when there are imbalances in the microbiota composition and there is a drop in certain bacteria groups, *Candida* spps. take advantage, expand, and behave has a pathobiont causing oral, vaginal, or intestinal inflammation and candidiasis [95]. The risk factors for developing vaginal candidiasis are many, including hormonal environment, personal hygiene, exposition to antibiotics or antifungal agents [94]. On the other hand, the presence of certain *Lactobacillus* spp., such as *L. crispatus*, has been negatively associated to vaginal candidiasis and showed a protective for BV and STIs too [94].

A dysbiotic vaginal microbiota has been associated with infections by human papillomavirus or human immunodeficiency virus, risk of suffering BV and STIs, infertility, and also female reproductive health complications such as septic postpartum, neonatal infections, or miscarriage [83,87,88]. Moreover, the vaginal microbiome affects the success rates of in vitro fertilization, and the characteristic of the microbial communities in the placenta and the amniotic liquid importantly affect the pregnancy and reproductive outcome [88]. Therefore, research effort should concentrate to improve understanding of the conditioning factors of the female microbiota and the consequences of its perturbations.

Published studies on the penis microbiota are still relatively limited in number; however, it has potential health implications. The composition of the penile microbiota skin is dramatically affected by circumcision, including a decrease in anaerobic bacteria counts, and such changes seem to have a protective effect against STIs like human papillomavirus and human immunodeficiency virus [96]. One study in Black South African observed that the penis microbiota was dominated by *Corynebacteriaceae*, *Prevotellaceae*, *Clostridiales*, *Porphyromonadaceae*, and *Staphylococcaceae* families. Most subjects presented a microbiota enriched in *Corynebacterium* spp. [96]. Results from another study indicate that those men with a high presence of anaerobic bacteria in the penis have a greater risk for acquiring HIV as compared to those men with a healthy microbiota [97]. On top of that, data suggests that

men and women share genital microbiota during heterosexual intercourse [88], and thus the penile microbiota may also have a key effect on women's urogenital impact. Moreover, an interesting study indicated that the profile of the penile microbiota could predict the risk for BV in women [49].

2.6. Urinary Microbiota

In contrast to other microbiota reservoirs, the female urine microbiota has been poorly investigated. Hopefully, in the last years, there has been a rapid rise in interest in describing its composition. According to one clinical trial, the healthy female urinary microbiota can be categorized into urotypes according to the relative abundance of *Lactobacillus*, *Gardnerella*, *Sneathia*, *Staphylococcus*, and *Enterobacteriacae* members [98]. Frequently, *Lactobacillus* is the dominant genus [48]. In an American multi-ethnic population of women aged 35–75 years, the urinary microbiome of women with urgency urinary incontinence differed from that in controls. The results indicate that the microbiota analyzed was poor in *Lactobacillus* spp., which are of great importance for the bladder health [99], and enriched in members of the genera *Actinobaculum*, *Actinomyces*, *Aerococcus*, *Arthrobacter*, *Corynebacterium*, *Gardnerella*, *Oligella*, *Staphylococcus*, and *Streptococcus*, and certain bacteria species (*Actinobaculum schaalii*, *Actinomyces neuii*, *Aerococcus urinae*, *Arthrobacter cum- minsii*, *Corynebacterium coyleae*, *Gardnerella vaginalis*, *Oligella urethralis*, and *Streptococcus anginosus*), some of which are uropathogens [98]. Besides, both groups presented a distinct profile of Lactobacillus spp., being that *L. gassesi* was more characteristic in cases, while *L. crispatus* was more represented in controls [98].

As in the case of the female urinary microbiota, the male urinary microbiota has been hardly investigated. Besides there was a great intra-subject variability, one study in a sample of sexually active men indicated that the male urinary microbiome (urobiome) was mostly represented by Firmicutes, followed by other phyla such as Actinobacteria, Fusobacteria, Proteobacteria, and Bacteroidetes, and underrepresented by Tenericutes and TM7 [100]. Interestingly, the analysis revealed that the majority of the identified microbial groups matched to species from the female urogenital tract, and that, to some extent, the composition resembles that in other body regions such as the skin or the colon [100]. The analysis also indicated that sexually transmitted infections (STIs) by pathogens such as *C. trachomatis* and *N. gonorrhoeae*, are associated with a urine microbiota poor in terms of genera diversity. It was mostly represented by *Lactobacillus*, *Corynebacterium*, *Streptoccus* and *Sneathia* spp., and other taxa like *Aerococcus*, *Anaerococcus*, *Prevotella*, *Gemella*, *Veillonella*, and *Sneathia* spp. were less representative. This dysbiotic microbiota was also linked to a greater risk for STI or bacterial vaginosis in women [100]. These authors have also suggested that urine samples offer a good representation of the male urinary microbial community, particularly the urethral epithelium, and therefore show promise for the diagnosis of sexually transmitted infections (STI) in male subjects [101]. Though current knowledge is limited, it may be possible to screen the risk for STIs using microbiota samples in a near future. This is important since it has been established an association between the dysbiotic microbiota in STIs and BV and HIV [101,102]. Besides that, the urinary microbiota is suspected to relate to prostate cancer. A detailed study showed a greater prevalence of pro-inflammatory bacteria and uropathogens in urinary samples from men with prostate cancer [103].

As with women, men's reproduction could be also influenced by the microbiome. One pilot study reported that seminal microbiota from infertile men has a greater α-diversity and differs from rectal samples in terms of β-diversity, is enriched in *Aerococcus* and poor in *Collinsella* [104]. Besides, some bacteria genera were linked to features of sperm quality such as sperm concentration or total motile sperm count [104]. On top of that, men's infertility seems to influence other microbiota niches, and the rectum microbiota in this population had a drop in Anaerococcus and enrichment in *Lachnospiraceae*, *Collinsella*, and *Coprococcus*, while the urinary microbiota was rich in *Anaerococcus* members [104].

The main information of this section has been summarized in Table 1.

Table 1. Summary of the main microbiota niches in the human body and potential connection with other microbiotas and body sites.

Microbiota Niche	Predominant Taxonomic Groups	Associated-Diseases or Conditions and Characteristic Microbiota Composition (If Available)	Potential Communication with Other Microbiota Niches/Organs
Skin microbiota	Actinobacteria, Firmicutes, and Proteobacteria phyla [50]. *Corynebacterium*, *Propionibacterium*, *Staphylococcus*, and *Streptococcus* genera [50,51].	• Natural delivery: *Lactobacillus* and *Prevotella* spp. [15]. • C-section delivery: *Propiniobacterium*, *Corynebacterium*, and *Staphylococcus.* • Acne: ↑ *Propionibacterium* spp. (*Propionibacterium acnes*) [50,56]. • Atopic dermatitis: *S. aureus* spp. (*S. aureus* and *S. epidermidis*) and *Malassezia* spp. fungi frequently found in cases [51,57], • Hidradenitis suppurativa: ↑ *Corynebacterium*, *Porphyromonas*, and *Peptoniphillus* spp. [59]. • Seborrheic dermatitis: *Malassezia* spp. [50]. • Rosacea: *Demodex* mite [50].	• Gut–skin: ↓ intestinal microbial diversity in atopoic dermatitis [105] and psoriasis [106].
Oral microbiota	Actinobacteria, Bacteroidetes, Firmicutes, Proteobacterias, and Synergistetes phyla [62] *Fusobacterium*, *Gemmela*, *Veillonella*, *Streptococcus* and *Granulicatella* genera [63] Saliva: *Streptococcus*, *Veillonella*, and *Prevotella* spp. [60] Soft tissues: *Streptococcus salivarus*, *Rothia*, and *Eubacterium* spp. [60]. Tooth: *Corynebacterium*, *Actinomyces*, *Spirochaetes*, *Fusobacteria*, *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes* spp. [60]	• Caries: ↑ *Streptococcus* and *Lactobacillus* spp., associated with oral candidiasis [64]. • Rheumatoid arthritis: ↑ diversity and differential microbiota composition (for details see [58]). • Osteoartritis: ↑ diversity and differential microbiota composition (for details see [58]). • Systemic lupus erythematosus: ↓ diversity, ↑ *Lactobacillaceae*, *Veillonellaceae*, and *Moraxellaceae* families [107]. • HIV: ↓ diversity, ↑ *Veillonella*, *Rothia*, and *Streptococcus* spp. [65]. • BV: ↑ bacteria members associated with periodontal disease [92].	• Oral cavity–gut–vagina: compositional changes in the oral and gut microbiota of pregnant women [81,86,88]. • Oral cavity–vaginal: ↑ Rates of periodontal disease in pregnant women, associated to preterm birth [81,88]; identification of *G. vaginalis* in the oral cavity was associated with a greater risk for BV [90]. • Oral cavity–gut: presence of periodontal pathogens (*Poryphyromonas*, *Fusobacterium*, *Oscillibacter*, *Peptostreptococcus*, *Roseburia*, and *Ruminococcus* spp.) in intestinal samples from colorectal cancer [61]; presence of members of the oral microbiota in gut samples of patients with liver cirrhosis [108].

Table 1. *Cont.*

Microbiota Niche	Predominant Taxonomic Groups	Associated-Diseases or Conditions and Characteristic Microbiota Composition (If Available)	Potential Communication with Other Microbiota Niches/Organs
Respiratory tract microbiota	Bacteroidetes, Actinobacteria, and Firmicutes [109] *Streptococcus*, *Haemophilus*, *Moraxella*, *Staphylococcus*, and *Veillonella* spp. [68]. Upper respiratory tract (nasal cavity, nasopharynx, and oropharynx): *Staphylococcus*, *Propionibacterium*, *Corynebacterium*, *Streptococuus*, *Moraxella*, *Haemophillus*, *Prevotella*, and *Veillonella* spp. [68]. Lower respiratory tract (trachea and lung's bronchial trees): *Prevotella*, *Veillonella*, *Streptococcus*, and *Tropheryma* spp. [68].	• COVID-19: ↑ *Klebsiella oxytoca*, *Faecalibacterium prausnitzii* and *Rothia mucilaginosa* [110].	• Gut–lung: COVID-19 associated with ↑ *Coprobacillus*, *Clostridium ramnosum*, and *Clostridium hathewayi*, ↓ *Faecalibacterium prausnitzii* in faecal samples [110,111]; asthmatic presented different compositional characteristic in the gut microbiota [109,112].
Gut microbiota	Bacteroidetes and Firmicutes phyla [10]. *Bifidobacterium*, *Lactobacillus*, *Bacteroides*, *Clostridium*, *Escherichia*, *Streptococcus* and *Ruminococcus* spp. [74].	• Alzheimer's disease: ↓ *E. rectale*, ↑ *Escherichia* [113,114]. • Asthma: ↑ *Bacteroides fragilis*, ↓ *Escherichia coli*, *faecalibacterium*, *Lachnispira*, *Rothia*, *Veillonella*, *Akkermansia municiphila* [113,114]. • COPD: ↑ *Enterobacter cloacae*, *Citrobacter*, *Eggerthella*, *Pseudomonas*, *Anaerococcus*, *Proteus*, *Clostridium difficile* and *Salmonella* [113,114] • Cystic fibrosis progression: ↑ *Ruminococcus gnavus*, *Enterobacteriaceae*, ↓ *Faecalibacterium prausnitizii*, *Bifidobacterium adolescentis*, *Eubacterium recatale*, *Streptococcus*, *Staphylococcus*, *Veillonela dispar*, *clostridium difficile*, *Pseudomonas aeruginosa*, *Escherichia coli* [114]. • Liver cirrhosis: different intestinal composition vs. controls [108]. • Lung cancer: ↑ *Enterococcus*, ↓ *Actinobacteria* and *Bifidobacterium* [112]. • Parkinson's disease: *Helycobacter pylori infections* and ↑ *Proteobacteriam Enterococcus* and *Enterobacteriacea* [80]. • Rheumatoid arthritis: ↑ diversity and differential microbiota composition (for details see [58]). • Osteoartritis: ↑ diversity and differential microbiota composition (for details see [58]). • Pulmonary diseases: ↑ Proteobacteria and Firmicutes [114].	• Gut–liver: SIBO was found in more than half of subjects with liver cirrhosis, and was associated to systemic endotoxemia [115]. • Gut–vagina: identification of *Gardnerella vaginalis* and *Leptotrichia/Sneathia* spp. in rectal microbiota samples was associated with greater risk for BV [90].

Table 1. *Cont.*

Microbiota Niche	Predominant Taxonomic Groups	Associated-Diseases or Conditions and Characteristic Microbiota Composition (If Available)	Potential Communication with Other Microbiota Niches/Organs
Vaginal microbiota	*Lactobacillus* spp., (*L. crispatus*, *L. iners*, *L. gasseri*, and *L. jensenii*), *Atopobium*, *Dialister*, *Gardnerella*, *Megasphaera*, *Prevotella*, *Peptoniphilus*, *Veinovella*, *Lachnospiraceae*, *Streptococcus*, *Staphylococcus* and *Gemella* [82,83].	• BV: ↑ bacterial diversity, ↑ anaerobic and facultative bacteria species from *Atopobium* (*A. vaginae*), *Gardnerella* (*G. vaginalis*), *Mycoplasma*, *Prevotella*, *Bifidobacterium*, *Megasphaera*, *Leptotrichia*, *Sneathia*, *Dialister*, *Clostridium* spp.), *Lactobacilli* spp. [83,89–93]. • Placenta and the amniotic liquid microbiota influences Pregnancy: ↓ α-diversity, ↑ *Lactobacillus* spp. (*L. iners*, *L. crispatus*, *L. jensenii*, and *L. johnsonii*), *Clostridiales*, *Bacteriodales*, and *Actinomycetales*, differential profile of microbial metabolites [86,87] • Pregnancy and reproductive outcome [88]. • Poor pregnancy rates: *Gardnerella vaginalis* and *Atopobium vaginae* [88]. • *Lactobacillus crispatus* gives protections against BV and STIs [83] • Lactobacillus *iners* is associated to a greater risk for bacterial vaginosis [83]. • Vaginal dysbiosis associated to septic postpartum, miscarriage or neonatal infections [83,91]. • Vaginal dysbiosis associated to infertility [88].	• Vagina–oral cavity: compositional changes in the oral and gut microbiota of pregnant women [81,86,88]; oral dysbiosis in women with BV [92]. • Vagina–bladder: presence of urinary and genital microorganism in urinary samples [116]; similar composition in urinary and vaginal microbiota samples [117].
Penile microbiota	*Corynebacteriaceae*, *Prevotellaceae*, *Clostridiales*, *Porphyromonadaceae*, and *Staphylococcaceae* families [96]. *Corynebacterium* spp. [96]	• Circumcision: ↓ anaerobic bacteria and protective effect against STIs (HPV and HIV) [96,97]. • Greater risk for HIV: ↑ anaerobic bacteria [97]. • Infertility: ↑ α-diversity, ↑ *Aerococcus* spp., ↓ *Collinsella* spp. [104]. • Some bacteria correlates to sperm quality [104].	• Penis–vagina: penile microbiota (*Parvimonas*, *Lactobacillus iners*, *Fastidiosipila*, *Negativicoccus*, *L. crispatus*, *Dialister*, *Sneathia sanguinegens*, *Gardnerella vaginalis*, *Prevotella corporis*, and *Corynebacterium*) can predict the risk for BV in women [49]. • Semen–urine–rectum: ↓ *Anaerococcus*, ↑ *Lachnospiraceae*, *Collinsella*, and *Coprococcus* spp. in rectum microbiota in infertile men [104].

Table 1. *Cont.*

Microbiota Niche	Predominant Taxonomic Groups	Associated-Diseases or Conditions and Characteristic Microbiota Composition (If Available)	Potential Communication with Other Microbiota Niches/Organs
Female urinary microbiota	*Lactobacillus* spp. [48].	• Urgency urinary incontinence: ↓ *Lactobacillus* spp. (*L. crispatus*) [98,99], ↑ *Actinobaculum*, *Actinomyces*, *Aerococcus*, *Arthrobacter*, *Corynebacterium*, *Gardnerella*, *Oligella*, *Staphylococcus*, and *Streptococcus genera*, ↑ *Actinobaculum schaalii*, *Actinomyces neuii*, *Aerococcus urinae*, *Arthrobacter cumminsii*, *Corynebacterium coyleae*, *Gardnerella vaginalis*, *Oligella urethralis*, and *Streptococcus anginosus* [98].	• Bladder–vagina: different urinary microbiota in women with BV [116]; presence of bacteria genera natural from the bladder in ephisodes of BV [83].
Male urinary microbiota	Firmicutes, Actinobacteria, Fusobacteria, Proteobacteria, and Bacteroidetes [100]. *Lactobacillus* (*L. iners*), *Aerococcus*, *Anaerococcus*, *Prevotella*, *Gemella*, *Veillonella*, *Sneathia*, *Corynebacterium*, *Staphylococcus* and *Streptococcus* spp. [100,103].	• STIs (*Chlamydia trachomatis* and *Neisseria gonorrhoeae*); ↓ diversity at genus level. • Prostate cancer: ↑ pro-inflammatory bacteria and uropathogens [103]. • *Gardnerella vaginalis* was associated with chronic inflammation in prostate biopsies [103].	• Urine(bladder)–skin–colon–vagina: the composition of male urine resembles that in the skin, colon, and vagina [100]. • Urine–vagina: male urinary microbiota of subjects infected with STIs is linked to a greater risk for STI or bacterial vaginosis in women [100].

BV: bacterial vaginosis; COVID-19: coronavirus disease 2019; HIV: human immunodeficiency virus; HPV: human papillomavirus; SIBO: small intestinal bacteria overgrowth; STIs: sexually transmitted infections.

3. The Interplay between the Different Microbiotas

Just as there is communication between the human cells, bacteria also communicate between the different niches where they are established [118], as well as with the human cells [119,120]. As expected, since its harbors the vast majority of the microorganisms, the gut microbiota is the main core of communication, and it seems plausible that the main interplay is established between gut microbiota and the others through the well-described gut-liver axis [121], gut–brain axis [122], gut–skin axis [123], and oral–gut axis [124]. On the other hand, other authors have shown crosstalk between different regions in which the gut microbiota is not involved, such as the oral–pulmonary axis [69].

When speaking about microbiota communication, secondary metabolites deserve special attention. They represent a way of communication between bacteria but also have a key role in the regulation of the host's immune system [114,125]. Metabolites may distribute to distant sites of the organism by entering the circulation [113,126], traveling throughout the blood, and finally could accumulate in other regions, perturbing the health of the target zone. The short-chain fatty acids (SCFAs), such as butyrate, propionate, acetate, or lactate are the best studied and the most prominent immunomodulatory metabolites [127]. They can exert pleiotropic effects on several body sites, influencing the normal functionality of the liver, gut, or pancreas [127]. SCFAs are a by-product of fiber fermentation by certain intestinal microorganisms, being *Roseburia intestinalis*, *Faecalibacterium prausnitzii*, *Eubacterium hallii*, *Bacteroides uniforms*, *Prevotella copri*, *Akkermansia muciniphila*, *Bifidobacterium* spp., and *Lactobacillus* spp. the most important SCFAs producers [113,126]. There are other metabolites produced by the gut microbes that are considered biomarkers of a disturbed gut, such as free phenol and p-cresol [126,128]. As well as metabolites, previous evidence suggests that bacteria themselves also could enter the circulation due to a disturbed intestinal barrier function, causing, once again, damage into the zone where they move [128]. As was mentioned above, the different microbiotas which inhabit the human body may create various axes forming a net with a cross-talk between all of them, mainly through microbial-derived metabolites. Nevertheless, it is a hypothesis, and more evidence is needed to clarify the underlying mechanisms of microbiota communication [61].

Previous studies have demonstrated the importance of the gut microbiome over skin health, and some of them suggest that imbalances in gut–skin axis could lead to inflammatory skin diseases like atopic dermatitis (AD) [126]. In addition, it was reported a link between low intestinal microbial diversity and AD, that was attributed to a reduced and abnormal immune maturation in childhood [105]. In the same way, the reduction of the gut microbiota diversity is also present in skin disease as psoriasis [106].

Another axis that has been studied extensively is the oral–gut axis, and available evidence suggests that the oral microbiota has a great influence on the intestinal one. A possible explanation is that the oral microbiota can affect the gut by the dissemination of some bacteria, such as *Poryphyromonas*, *Fusobacterium*, *Oscillibacter*, *Peptostreptococcus*, *Roseburia*, and *Ruminococcus*, which are periodontal pathogens and have also been found in samples from patients with colorectal cancer (CRC) [61]. Other members of the oral microbiota (*Veillonella* and *Streptococcus*) are thought to be involved in the development of liver cirrhosis, a disease related to intestinal dysbiosis, demonstrating that oral microbiota may also affect intestinal microbiota and ultimately the liver [108].

Further evidence supporting the link between different microbiotas is the case of rheumatoid arthritis, where both oral and gut microbiota are disturbed and seem to be contributing factors in the disease development [58]. The similar has been described for the SARS-CoV-2. A recent study concluded that the virus could promote an oral dysbiosis, probably because the oral cavity serves as SARS-CoV-2 reservoir [129]. Previous reports have indicated that the infection also promotes a proinflammatory status in the lungs and has an impact on the lung microbiota, that present greater levels of *Klesiella oxytoca*, *Faecalibacterium prausnitzii*, and *Rothia mucilaginosa* [110]. This oral dysbiosis could lead to the translocation from the oral cavity to the digestive tract, resulting in gut inflammation and dysbiosis, both of with are frequently observed in subjects presenting COVID-19 [46,130].

COVID-19′s severity was associated to a characteristic gut microbiota profile with higher levels of *Coprobacillus*, *Clostridium ramosum*, *and Clostridium hatheway* and lower number of *Faecalibacterium prausnitzii* [110,111]. Moreover, the composition of the gut microbiota changed during the progression of the disease, and some of these alterations remain after the resolution of infection [131]. It should be noted that bacterial co-infection occurred in 7% of hospitalized COVID-19 patients. Compared with patients in mixed wards/intensive care unit (ICU) settings, ICU COVID-19 patients have a higher proportion of bacterial infections [132]. These studies suggested that high vigilance should be stablished against infections derived from the oral microbiome during infection by respiratory viruses such as SARS-CoV-2. Uncovered risk factors such as increased inhalation, poor oral hygiene, and viral infection have been related to the occurrence of respiratory infection [133,134]. The mechanisms by which the oral microbiome can influence respiratory disease such as COVID-19 is complicated and multifactorial, simultaneously affected by environmental, host, and microbial factors [135,136].

There are other examples that support the hypothesis of the gut–lung axis, since asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis, and lung cancer have been associated with important alterations in the gut microbiota composition [109,112–114]. Interestingly, the oral dysbiosis may also affect distant sites and produce systemic complications, such as the case of systemic lupus erythematosus, where the diversity of oral microbiota is compromised, and membres of *Lactobacillaceae*, *Veillonellaceae*, and *Moraxellaceae* families are increased [107].

Previous studies have suggested a cross-talk between gut and lung microbiota, and that a previous gut disturbance may be responsible for subsequent lung diseases [113,114]. As in the case of another microbiota axis, the gut microbiota has a relevant role in this communication, while the contribution of the lung microbiota remains to be elucidated [137]. For instance, it has been demonstrated that gut dysbiosis is accompanied by the secretion of SCFAs from the gut microbiota to the lungs, which causes lung inflammation and a major susceptibility to allergens [114].

Two body locations that also are in close contact are the oral cavity and the lung. Both the oral and the lung microbiota present some similarities that could be explained by their communication through the respiratory tract. Indeed, considering that, it seems plausible that the lung microbiota has origin in the oral one [138]. For example, it has been associated with poor oral health could contribute to asthma or pneumonia [129,139], and changes in the oral microbiota were reported in HIV-infected subjects [65]. Previous studies have suggested a relationship between oral dysbiosis and lung disease; however, the mechanisms involved are still not fully understood. Further findings suggest that oral bacteria may communicate the lung through by inflammatory proteins; however, this issue remains unclear [124].

Previous studies also support the idea that there exists a gut–lung axis that allows for the exchange of molecules (microbial metabolites, hormones, toxins, proteins) between the gut and the lung, mainly through the systemic circulation [114]. Compelling evidence suggest that such interactions, that importantly influences the immune and inflammatory states, are implicated in different lung diseases including infections (tuberculosis, pneumonia), genetic diseases (cystic fibrosis), inflammatory diseases (asthma, COPD), and cancer (lung cancer) [113,114]. Even though each disease was associated to different disturbances in the intestinal microbiota (for details see [114]), it was observed an overgrowth of Proteobacteria and Firmicutes taxa in these cases. To illustrate, several studies have linked gut dysbiosis in early life to asthma, condition in which the genus *Faecalibacterium* and *Roseburia* are present in lower proportion, and other bacteria genera are enriched as compared to healthy individuals [109]. In addition, gut microbiota has been associated with the development of lung cancer, with some studies indicating that the use of antibiotics before and during the therapy can decrease the efficiency of the antitumor drugs due to the interaction between antibiotics and gut microbiota, that is strongly affected by xenobiotics [112]. Although the causality remains to be clarified, the available information

strongly suggests that the gut microbes play a critical role in lung health, and therefore should be contemplated in lung disease's prevention and treatment.

It has also been investigated the communication between the gut and the liver by means of the gut–liver axis. As example, SIBO was found in more than half of subjects with liver cirrhosis, and was associated to systemic endotoxemia [28]. Besides, another group identified a different microbiota profile in controls and patients with liver cirrhosis, which was characterized by members from oral origin [108]. Indeed, these authors developed a discrimination index with gene markers from the intestinal microbiota.

Due to their proximity within the human body, it is plausible that the urinary microbiota affects the genital one. One study hypothesized that there may be a urogenital microbiome that comprises microorganisms from both the urinary and the vaginal tracts [98], and another study also confirmed the presence of both urinary and genital microorganisms in the urine [116]. A further cross-sectional study on more than 200 women corroborated that urinary and vaginal microbiome share more than half of the most abundant operational taxonomic units. Both microbiota niches were dominated by *Lactobacillus*, especially the vaginal niche, and presented varying levels of *Gardenerella*, *Prevotella*, and *Ureaplasma* [117]. In the same line, it has been reported that the urinary microbiota from women suffering BV clustered differently to that of healthy women, and differences persisted following the antibiotic treatment [116]. A previous study pointed that bacteria genera frequently identified in episodes of BV are also naturally found in the bladder of healthy women, suggesting the transference of microorganisms from the urinary to the genital tract [83].

Interestingly, other microbiota niches could be involved in the development of BV and dysbiotic vaginal microbiota. A relevant publication reporting data on a prospective cohort study of young women who reported sex with other women suggested that women presenting certain bacteria in the oral cavity (*G. vaginalis*) or anal samples (*G. vaginalis* and *Leptotrichia/Sneathia* spp.) are more likely to suffer from BV [90]. In the same line, a recent report on young South African females with a high prevalence of BV indicated that dysbiosis in the oral and vaginal microbiota are frequently concurrent and that the oral cavity of women presenting vaginal dysbiosis was enriched in bacteria members linked to periodontal disease [92]. Another relevant study corroborated the correspondence between the oral, vaginal, and rectum microbiota [140]. The information above offers novel potential targets to restore vaginal dysbiosis and therefore decrease the risk for adverse life events previously mentioned.

Human microbiome analysis has been largely based on observation, with associations of disease phenotypes with particular microbiota constituents. However, one of the most controversial points in the study of the human microbiota is to establish whether the presence of a certain population of microorganisms is a cause or effect of the underlying disease and how this change can affect other niches where a specific microbiota resides. Different mechanisms can explain this connection, from metabolites (such as SCFA) to part of bacteria (such as extracellular bacterial vesicles) that migrate from different parts of the human body, to even the bacteria themselves that can cross epithelial barriers (such as the intestine epithelial cells) that lose their integrity in disease conditions (such as obesity). Extracellular bacterial vesicles have caught the attention of researchers [121,122] as one of the mechanisms by which distant microorganisms could communicate, as it has been shown to occur with exosomes as an intercellular communication system in multicellular organisms.

Changes in the local microbiota occur in close contact with nearby cells, both host cells (with which there is a symbiotic or commensal relationship) and with nearby microorganisms with which they compete for the location and the nutrients in their environment. In this sense, the equilibrium that occurs is dynamic depending on multiple factors, both intrinsic (metabolism of the microorganisms present) and extrinsic (nutrients, pH conditions, oxygen pressure) that ultimately modulate the local microbiota present in a certain organ. In turn, the host cells are also influenced by the presence of a certain microbiota

and respond to it by adapting in a truly dynamic equilibrium that, when disrupted, is responsible for the development of a disease.

The main information of this section has been summarized in Figure 1.

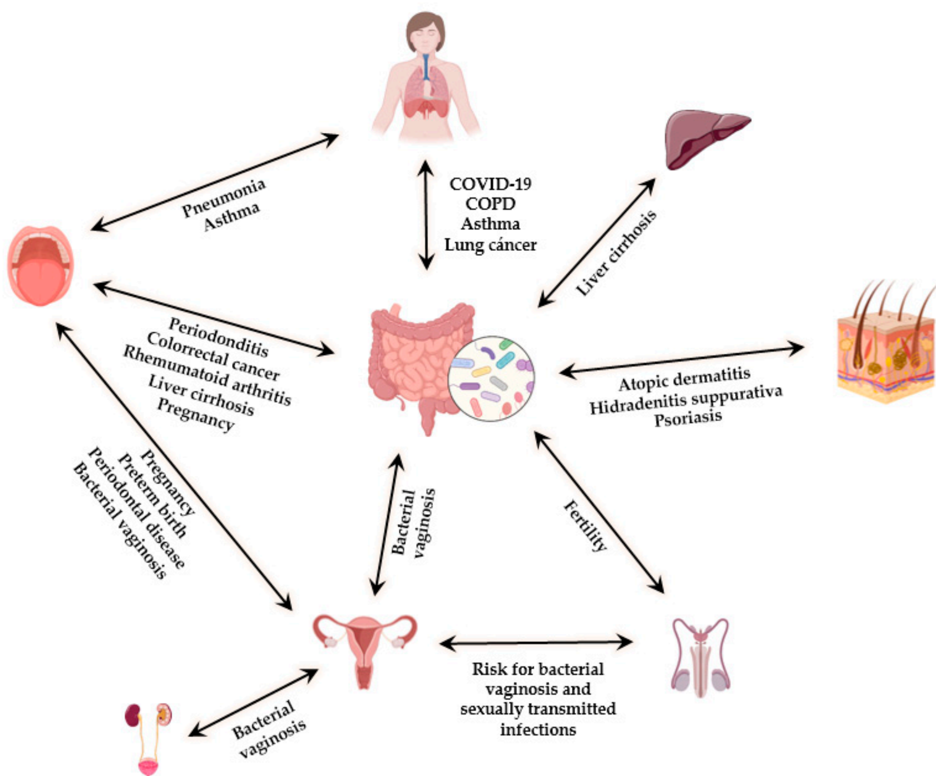

Figure 1. Schematic illustration representing the main microbiota niches in the human body, possible interaction between them, and related diseases.

4. Conclusions

In conclusion, although there is a lack of evidence in the field of microbiota communication, several studies have emphasized the influence of the gut microbiota on microbiota located in other parts of the body. Regarding this aspect, there are theoretical grounds for believing that the gut microbiota plays a more active role in the host phenotype. As with any cell in a biological system, it could be that the microbiota is a well-organized and structured network in which the intestinal microbiota behaves as a central regulator that integrates peripheral microbiota. Because of the foregoing, the different microbiotas become potential approaches to investigate, so that restoring a particular microbiota system may indirectly lead to improvements in a distant microbiota and thus confer health improvements to the host. This new approach would provide new therapeutic strategies.

Nevertheless, we are aware that to date, the intestinal microbiota has been one of the most widely researched, and at present, there is insufficient research on other microbiota regions to prove this hypothesis. In addition, mechanistic studies are lacking and the resources required for these experiments have not been well established. This is a compelling area for future research, and to achieve this objective microbiota research should focus on a much more integrative model that takes into account the target microbiota but also other supposedly unrelated microbiotas. For that purpose, multi-omics approaches and appropriate bioinformatics analysis appear indispensable.

To the best of our knowledge, this is the first report to hypothesize the potential interplay and crosstalk between the different human microbiotas. We would be pleased if our contribution opened a new door to a better understanding of the relationship between host health and microbiota.

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Article 1

Screening and selection of probiotic bacteria from Brown trout (*Salmo trutta*), Rainbow trout (*Oncorhynchus mykiss*) and Siberian sturgeon (*Acipenser baerii*) for potential application in human and animal health

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Simple Summary: The improper and overuse of antibiotics have played a major role in the emergence of antibiotic-resistant bacteria and increased levels of antibiotic residues in animal products, which have disastrous effects on the health of both animals and humans. To counter this situation, several alternatives have been studied, nevertheless, probiotics are the most studied and safety alternative to reduce the use of antibiotics.

Abstract: The disproportionate use of antibiotics in animal husbandry has resulted in the emergence of antibiotic-resistant bacteria, with adverse effects on both animals and people who consume these animals. Therefore, the use of probiotics as an alternative to antibiotics has been studied. The purpose of this work is to search for possible probiotics, obtained from a strain of bacteria from different body tissues of the freshwater fish *Salmo trutta, Oncorhynchus mykiss* and *Acipenser baerii*. To determine their possible probiotic effect, these bacteria were isolated, and once isolated, they were tested in antagonistic assays against certain fish pathogens. In addition to testing their probiotic effect, the bacteria were also identified by 16S rRNA gene sequencing. Conclusively, our results provide a beginning for an extensive study where bacteria obtained would be characterized and their effects in vivo would be studied, both in human and fish.

Keywords: probiotic, aquaculture, rainbow trout, brown trout, siberian sturgeon.

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1. Introduction

Aquaculture is defined by the Food and Agriculture Organization (FAO) as the farming of aquatic organism with human intervention [1]. On this industry the use of antibiotics was excessive, causing one of the biggest issues in public health, which is the antimicrobial resistance [2]. Due to this issue a new law in Europe was created, where only a few antibiotics are allowed for their use on animal [3].

An alternative for the use of antibiotics was studied, and probiotics, which been defined as live microorganism which when administered in adequate amounts confer a health benefit on the host [4], are the chosen one, due to their characteristics and their effects. Probiotics must have a number of characteristics to be considered as such: they must not be pathogenic, neither host species, other aquatic animals and humans. Also they must be free from antibiotic resistance plasmids, and the last characteristic, they must resist acid pH and bile salts [5]. Nevertheless, there are some others characteristics that also are important but not essential, such as the capacity to colonized the gastrointestinal tract, or the inhibitory effects against pathogens [6].

Acid lactic bacteria (LAB) are a heterogenous group of Gram-positive, rod- and coccus-shaped organisms, that are non-spore forming, catalase-negative, facultative anaerobe or aerotolerant and lastly, they produce lactic acid as the main product during carbohydrate fermentation [7,8]. The major group of LAB are the species corresponding to the genus of *Lactobacillus*, where one of the most important and use specie is *Lactobacillus plantarum*. This specie is used either in human health and aquaculture, being important in the treatment against diarrhea and promoting the growth in fish [9,10].

Another important specie is *Leuconostoc mesenteroides*, used for the humoral immune response stimulation on fishes, moreover, an increase on lisozima's levels are seen with the supplementation with this bacteria [7]. One study carried by Balcázar [11] demonstrated the potential use of this probiotic against *Aeromona salmonicida,* a pathogen of brown trout.

The genus *Carnobacterium* have 9 species, however, only two of them had been isolated from fish in nature, these are *Carnobacterium divergens* and *Carnobacterium maltaromaticum*. Both have the capacity to produce bacteriocins, therefore they have effects on the control of some pathologies, such as furunculosis [12]. However, it has been demonstrated that some strains of *Carnobacterium maltaromaticum* could have pathogenic effects in hard stress situations [13].

Supplied as live bacteria, probiotics could exert their effect in a varied way. One of the most studied mechanism of action is the regulation by the probiotics of the immune system, inducing regulatory T cells, which ameliorate gut inflammation [14]. Production of metabolites, like short chain fatty acids (SCFAs), hydrogen peroxide, bacteriocins and acid lactic, are other mechanism in which probiotics inhibit the growth of pathogens. In the same way, the acidification of the gut promotes the colonization of the gut by probiotic, and competing with non-probiotic bacteria [15,16].

Energy resources are a limited factor in the growth of pathogens; thus, probiotics can inhibit their growth by taking these resources. For instance, iron is a crucial element for the development of bacteria, therefore probiotics secret agents that could join to the iron, inhibit the join of it to pathogen [17]. Not only they compete for energy resources, but adhesion to intestinal mucosal inhibit the development of pathogen, by preventing colonization by these organisms [18]

Probiotics are not only used as the live bacteria, but some bioactive compounds from bacteria could be used, these compound are known as postbiotics [19]. There are some postbiotics coming from *Lactobacillus* and *Leuconostoc* that have demonstrate an effect agains *Lactococcus garvieae*, a fish's pathogen [20]. Moreover, probiotics could be administrated as live bacteria, and once inside the organism the lysis of probiotic promote the release of these compounds [21].

In conclusion, there are two main reasons for the alternative and increased use of probiotics, the largest aquaculture production and the restriction in the use of antibiotics [22]. Therefore, the principle aim of the present study is the research and the selection of bacteria from fish with potential use as probiotic. Our results showed bacteria that could be used as probiotics, nevertheless, more essays are needed due to this work is a first step on the research of potential probiotic and the time was limited.

2. Materials and Methods

2.1 Isolation and initial screening

Candidate bacteria were obtained from intestinal, skin, gill, oral, bleed and bladder mucus samples of three wild specimens of *Acipenser baerii, Salmo trutta* and *Oncorhynchus mykiss*. They were obtained from farms chosen by their health requirements.

Samples were taken with swab, and then were sown directly in Man Rogosa Sharpe (MRS) agar plates, which it was prepared by according to the manufacturer's instructions (Scharlab, Barcelona) and incubated in an oven at 22°C for 12 to 48 hours.

Once samples were grown, colonies with different color and shape were taken with sterile handle, and they were re-sown in MRS liquid, 12 to 48 hours. Continuously, a drop of this culture was unleashed again in MRS agar, and once again they were incubated 12-48 hours at 22ºC.

In parallel, bacteria stored at -80°C in glycerol stock (30% v/v glycerol) from previous samples were reactivated and a sample was taken with a loop and sieved by depletion on MRS agar. From these samples and other cultures which proceed from colonies took on previous sampling, Gram stain was performed to rule out yeasts and to screen for Gram-positive coccus, rod and/or coccus-rod.

Individual colonies were then reseeded in Falcon tubes containing 10 ml of MRS broth from the MRS agar plates. Tubes were incubated in an oven at 22°C for 24-48h. When the liquid medium, compared to a control MRS liquid medium, showed turbidity, the 10 ml of medium from each Falcon tube was used for the following stages of analyses.

2.2 DNA extraction and genotypic identification

Genomic DNA of each isolate was extracted following the method described by Qiagen protocol, using Qiagen DNA mini kit. Following DNA extraction, the 16S rRNA gene was amplified using primers 27 F (5′-AGA GTT TGATCC TGG CTC AG-3′) y 1492 R (5′-ACG GCT ACC TTG TTA CGA CTT-3′) (Weisburg 1991) in a PTC 200 Peltier Thermal Cycler (Bio-Rad MJ Research). Amplification reactions were performed in a total volume of 25 μL containing 12,5 μL de 2X KAPAG 2G Fast Hotstart Ready Mix (Molecular BioProducts, Sigma-Aldrich, Madrid), 1,25 μL of each primer 8 μL of H_2O nuclease free and 2 μL of DNA template. The PCR temperature profile consisted of an initial denaturation at 95 ºC for 3 min followed by 40 cycles of 15 s at 95 ºC, 15 s at 45 ºC, 15 s at 72 ºC and a final extension for 2 min at 72 ºC. The genetic fingerprints were resolved in a 1.5% agarose gel.

PCR products were purified using Macherey-Nagel kit-RNA, DNA, and protein purification (Macherey-Nagel, Madrid) and were sent to Macrogen Spain (Madrid) for their sequencing. Besides purification, Gram staining were done to isolates, to verify that the samples submitted were pure.

2.3 Antagonist assays

The pathogen *Lactococcus garvieae* was grown in 10 mL MRS broth overnight at 22ºC. 100 μL was taken and were re-seed in 10 mL MRS broth. 1 mL of the bacterial suspension was centrifugated on a centrifuge tube (13000 rpm, 1 min). Supernatant was discarded and pellet was washed in PBS, and was centrifugated once (13000 rpm, 1 min). Supernatant was discarded and pellet was once again washed in PBS, to adjust the suspension to an optical density (OD) 0,125. After adjust OD 0,125, serial dilutions were made using PBS. Dilutions were seed in agar MRS plates and were incubated 24 h at 22ºC. On the other hand, this process was the same for the probiotic bacteria.

Once concentrations were standardized.

To evaluate a direct antimicrobial effect of probiotic bacteria strains against *L. garvieae*, 'inhibition' experiments were carried out incubating pathogenic bacteria and probiotic bacteria in ratio1:1. In details, 200 μL of *L. garvieae* solution with an OD 0.1 were pelleted and the supernatant was discarded. Afterwards, 200 μL of the candidate bacteria was added and the mixes were incubated for 60 min at 22°C. At the end of the incubation, the tubes were centrifuged at 13000 rpm for 1 min. Pellets were re-suspended in 200 μL of PBS, serially-diluted, and 10 μL of the suspensions were seeded on MRS agar, and incubated 24 h at 22 °C. Each experiment was conducted in duplicate, and a control of pathogen and candidate were included.

3. Results

After processing the gill, intestine, mouth, spleen, bladder and skin content from 3 fishes species analyzed, brown trout, rainbow trout and Siberian sturgeon, 56 different strains were obtained. From these 40 strains were shortlisted, all of them were Gram-positive, the morphology were coccus, rod and coccus-rod. 19 of these 40 strains were

sequenced, which were divided into 6 isolates from brown trout, 9 from rainbow trout, and 4 from Siberian sturgeon. On the other hand, on inhibition essays, *Lactococcus garvieae* were used as pathogen, and as potential probiotic, 8 different strains were chosen from the initial 40.

3.1. Selection of candidates bacteria

From the first step of 'Material and methods', where bacteria went trough a selection process, 40 were chosen. From these 40 all are Gram-positive, moreover their shape is varied (table 1), and is divided into:

- Rod: 9 strains
- Coccus: 18 strains
- Coccus-rod: 13 strains

Table 1 Gram and shape of selected bacteria.

Sample	G(+)	G(-)	Yeast	Shape
1	x			Coco
2	x			Bacilo
4	x			Bacilo
5	x			Coco
6	x			Cocobacilo
7	x			Cocobacilo
8	x			Bacilo
9	x			Bacilo
10g	x			Coco
10p	x			Coco
11	x			Bacilo
12	x			Cocobacilo
13	x			Cocobacilo
14	x			Cocobacilo
15	x			Bacilo
16	x			Cocobacilo
18	x			Coco
19	x			Bacilo
20g	x			Cocobacilo
21	x			Coco
22	x			Coco
23	x			Bacilo
24	x			Bacilo
25	x			Cocobacilo
26g	x			Cocobacilo
26p	x			Cocobacilo

Sample	G(+)	G(-)	Yeast	Shape
27	x			Cocobacilo
28	x			Coco
33C	x			Coco
36C1	x			Coco
36C2	x			Coco
40gC	x			Coco
41	x			Cocobacilo
44	x			Coco
45C	x			Coco
46C	x			Coco
47	x			Cocobacilo
48	x			Coco
49	x			Coco
50	x			Coco

Results of this first analysis step show that there is any type of contamination in our samples, thus our plates, where bacteria were grown, showed only one type of bacterial colony, this is also seen during Gram stain (Figure 1). Besides, results show a 100% of Gram-positive bacteria, which is an essential aspect on the research of probiotics, in case bacteria were Gram negative, they were rejected.

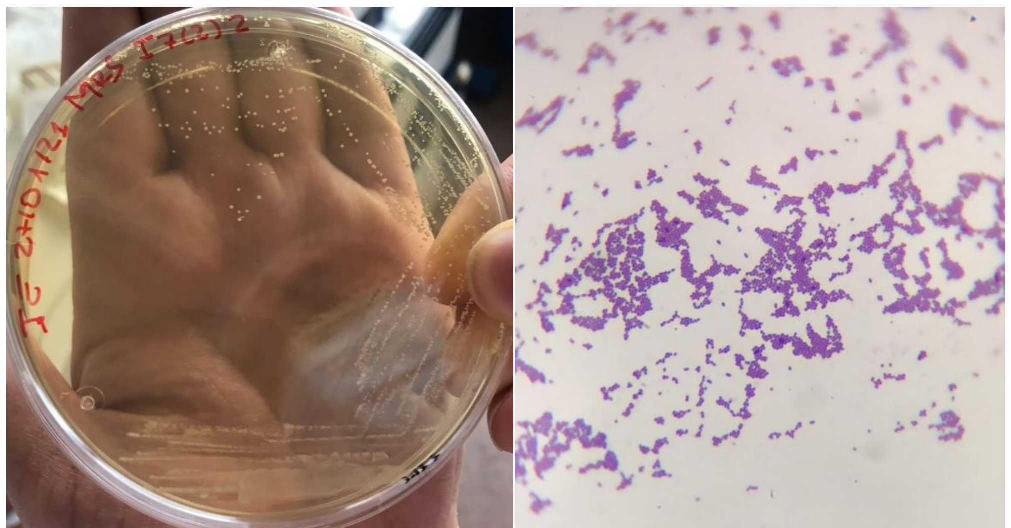

Figure 1. Sample nº28: Left: Bacteria seeded in Agar MRS plate. Right: Microscope view of the Gram stain of the sample grown on MRS agar medium

3.2. DNA extraction and genotypic identification

After the first stage, where morphology results were obtained, it proceeds with DNA extraction. Once DNA were extracted of all bacteria, the next stage would be the DNA amplification by the polymerase reaction chain (PCR). Products obtained during PCR were resolved on agarose gel (Figure 2). As a result, products are expected to amplify over 1500 base pairs (bp). To verify the correct amplification, a molecular ladder was used as guide.

Besides, once resolve was finished, DNA products from PCR were purified, a previous stage to DNA sequencing. To verify that there is no contamination on products that will be sequenced, new Gram stain was made. Data shows that, firstly, there is no contamination and, secondly, samples have no changes compared to first samples (Figure 3)

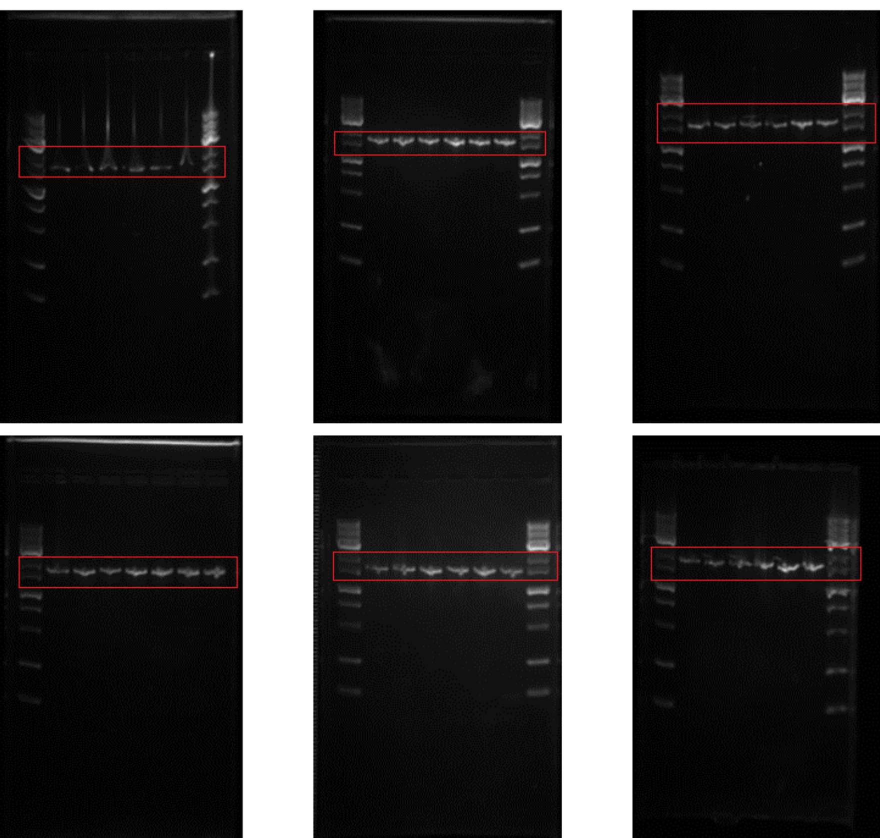

Figure 2. PCR products resolved. Red squares show stripes where products amplify between 1500-2000 bp.

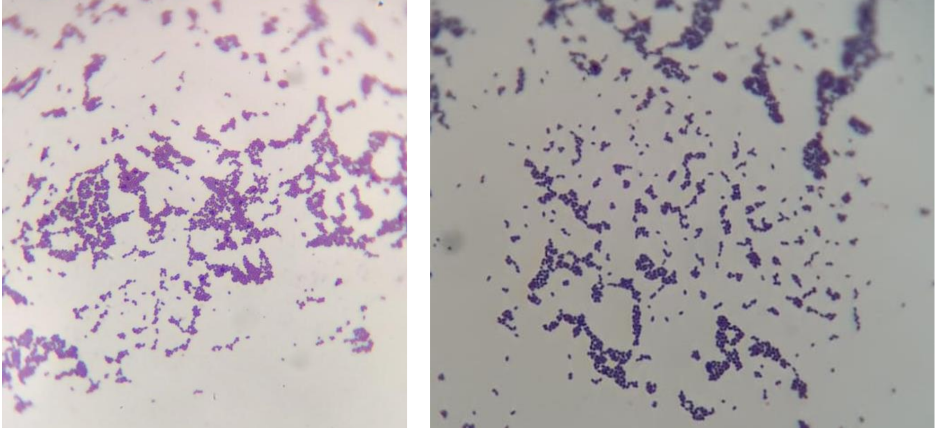

Figure 3. Sample nº28, microscope view. Left: results from the first Gram stain. Right: Results from the second Gram stain Previous sequencing.

3.3. Sequencing

19 sequences are obtained from the initial 40 samples. Sequences were identified by BLAST analysis. Data shown in table 2 bacteria obtained and their source, both tissue and fish.

Table 2 Species obtained from sequencing.

Sample	Tissue	Origin	Specie
2	Branquias	*S. trutta*	*Carnobacterium maltaromaticum*
9	Branquias	*S. trutta*	*Carnobacterium maltaromaticum*
13	Branquias	*O. mykiss*	*Lactobacillus plantarum*
16	Intestino	*O. mykiss*	*Lactobacillus plantarum*
24	Branquias	*S. trutta*	*Carnobacterium maltaromaticum*
25	Intestino	*O. mykiss*	*Lactobacillus plantarum*
26P	Branquias	*S. trutta*	*Carnobacterium maltaromaticum*
28	Intestino	*O. mykiss*	*Leuconostoc mesenteroides*
33C	Intestino	*S. trutta*	*Carnobacterium maltaromaticum*
36C1	Boca	*O. mykiss*	*Carnobacterium maltaromaticum*
36C2	Boca	*O. mykiss*	*Carnobacterium maltaromaticum*
40gC	Intestino	*A. baerii*	*Carnobacterium maltaromaticum*
41	Bazo	*S. trutta*	*Carnobacterium maltaromaticum*
44	Bazo	*O. mykiss*	*Vagococcus salmoninarum*
45C	Intestino	*A. baerii*	*Carnobacterium maltaromaticum*
46C	Intestino	*A. baerii*	*Carnobacterium maltaromaticum*
48	Vejiga	*O. mykiss*	*Leuconostoc mesenteroides*
49	Vejiga	*O. mykiss*	*Leuconostoc mesenteroides*
50	Intestino	*A. baerii*	*Carnobacterium maltaromaticum*

From sequencing it was revealed 4 different species, 3 of them were considered as potential probiotic: *Carnobacterium maltaromaticum, Leuconostoc mesenteroides* and *Lactobacillus plantarum.* Meanwhile, *Vagococcus salmoninarum* was discarded due to it is a fish pathogen. Apart of sequences, other files were received, corresponding to chromatography, and a file with whom a phylogenetic tree was made (Figure 4)

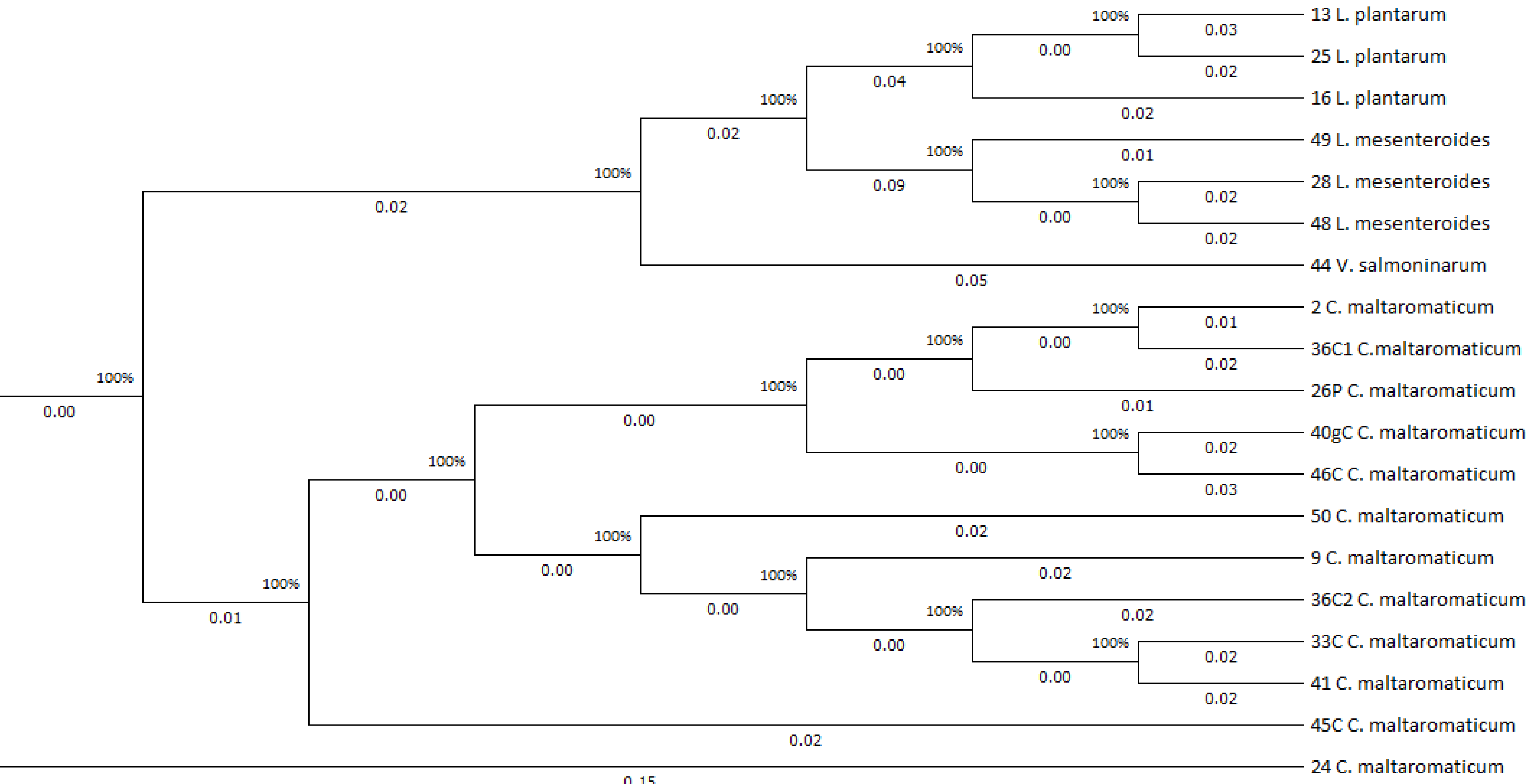

Figure 4: This tree was deduced by the Maximum Likelihood method using 19 nucleotide sequences, where gaps were removed, thus showing the tree with the highest log-likelihood. The percentages shown next to the branches are the trees in which the associated taxa cluster. The numbers on the branches show the genetic distances in nucleotide substitutions per site. This analysis was achieved using MEGA X.

3.4 Inhibition assays

Inhibition assays were not significant, it was observed some inhibition from sample 13 and 16 of *L. garvieae.* However, as the number of samples is reduce, this is a preliminary result and the experiment would need to be repeated to confirm the inhibition..

4. Discussion

Evaluation and screening of probiotics is an area of extensive research in aquaculture since they have been shown to have the potential to be an alternative to the use of antibiotics, which in recent years have been restricted due to the potential resistance that pathogenic bacteria can acquire [23].Furthermore, in aquaculture, probiotics can improve many aspects of production by conferring disease resistance, improving growth rates, immunity or nutrient uptake by stimulating digestive enzymes [24,25]

An interesting aspect of the use of probiotics in aquaculture is the fact that using bacteria from the environment itself (aquatic environment) can significantly increase the beneficial properties of the strains and offer advantages over microorganisms from another environment, such as the terrestrial environment [26]. On this study, bacteria were isolated from three fish species farmed in the Iberian Peninsula (brown trout, rainbow trout and Siberian sturgeon). This first screening of probiotic strains was carried out by morphological analysis of the isolated strains and their posterior sequencing. Moreover, to testing their ability to inhibit different types of fish pathogens in vitro. The pathogen *L. garvieae*, which is a bacteria that cause infectious diseases in fish, was used to evaluate the inhibitory capacity of the isolated strains [27].

Once the Gram stain was performed and the first stage of analysis was completed, it was determined that the 40 selected bacteria were Gram-positive rod, coccus and coccorod (Table 1). This finding was predictable since this morphology frequently appears in the group of probiotics used in aquaculture, where the genera Lactobacillus, Carnobacterium and Leuconostoc are predominant, although there are other genera that are also widely used [28].

The 16s rRNA gene is a relevant ribosomal gene as it has been accepted as a target for bacterial identification and phylogenetic analysis of bacteria. Therefore, its amplification is a key step for its subsequent sequencing. On this work, the gene amplified correctly, obtaining an amplicon of approximately 1500 bp in length [29,30]. After purification of the PCR products, they were sequenced and 4 different bacteria were identified, *Carnobacterium maltaromaticum* (isolated from brown trout, rainbow trout and Siberian sturgeon), *Lactobacillus plantarum* (isolated from rainbow trout), *Leuconostoc mesenteroides* (isolated from rainbow trout) and *Vagococcus salmoninarum* (isolated from rainbow trout). The first three belong to the lactic acid bacteria group, while the last one is a pathogen.

Carnobacterium maltaromaticum has been previously isolated from gut of the three fishes previously mentioned [31–33]. Although this bacterium is not one of the most abundant in the gut of these fishes, it is a bacterium that belongs to the gut's microbiota of both brown and rainbow trout [33].

It has also been shown that insect supplementation in *A. baerii*'s diet increases the amount of this bacterium in the gut, contributing to gut modulation and improving gut health [32]. Another research indicated that administration of this bacterial species in the diet is able to improve intestinal immunity in rainbow trout [34].

Despite these findings and the fact that *C. maltaromaticum* is considered a specie with probiotic properties, it has been described that certain strains could be harmful and cause infection and even death in fish. In this context, as a precaution, it is necessary to thoroughly examine the strain found in our laboratory before considering it as a possible probiotic strain [35].

This study also isolated *Lactobacillus plantarum* and *Leuconostoc mesenteroides*, both bacteria have a high probiotic capacity as they are able to adhere to and survive in the intestinal mucus [36]. The results, although needing more tests and trials due to the short

period of the study, show that *L. plantarum* is able to reduce the growth of the pathogen in vitro, this is in agreement with the results of another study demonstrating the ability of *L. plantarum* to reduce the death in fish caused by *L. garvieae* infection [36].

Previous studies with *L. mesenteroides* showed that administration of these strains together with food additives in rainbow trout was beneficial and conferred resistance against diseases such as furunculosis caused by *A. salmonicida* [7]. In humans, a study has shown that consumption of *L. mesenteroides* may be beneficial in the treatment of colon cancer, due to the ability of this species to activate the pathway that induces apoptosis in cancer cells [37].

In human malignant melanoma it has been demonstrated that the use of *L. plantarum* has beneficial effects. In this pathology. *L. plantarum* has been shown to reduce the migratory capacity of cancer cells, as well as inducing apoptosis of these cells [38]. This suggests that the bacteria isolated in this work could be used in both animals and humans, opening the way for further trials.

These bacteria not only have a probiotic effect when fed live, but postbiotics produced by them also exert beneficial health effects. Certain postbiotic metabolites isolated from *Lactobacillus plantarum* strains, bacteriocins I-UL4, TL1, RS5, RI11, RG11 and RG14, have been shown to have a cytotoxic effect on different tumor cell lines, without causing the same effect on normal cells [39]. These same bacteriocins have been shown to have inhibitory effects on the proliferation of different pathogens, such as *E. coli* and *Salmonella enterica.* [40].

5. Conclusions

In conclusion, bacteria isolated and identified in the present study show themselves as good candidates for possible future use as probiotic bacteria, and this is supported by the available literature. In addition to their possible use as probiotics, their metabolites could also be a good option for the generation of postbiotic compounds. However, this work is a first screening and further trials would be needed to assess the effectiveness of the isolated strains

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